LONG-TERM METABOLIC EFFECTS OF REPEATED NEONATAL ORAL SUCROSE TREATMENT IN MICE

by

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, a thesis entitled:

Long-term metabolic effects of repeated neonatal oral sucrose treatment in mice.

submitted by Cynthia Yamilka Ramírez Contreras in partial fulfillment of the requirements for

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in Experimental Medicine

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Abstract

Background: Preterm infants (<37 weeks of gestation) often require hospitalization in the neonatal intensive care unit and experience painful procedures due to medical care. Oral sucrose treatment for analgesia is the non-pharmacological standard of care for minor procedural pain relief. The objective of my MSc thesis research was to determine the long-term effects of repeated neonatal oral sucrose treatment on growth, adiposity, and glucose homeostasis in a mouse model.

Methodology: Neonatal female and male mice (C57BL/6J) were randomly assigned to one of four treatments (n=7-10 mice/group/sex): water, sucrose, fructose, or glucose. Pups were treated 10 times/day for the first six days of life with 0.2g/kg body weight of respective treatments (24% solution; 1-4 μL/dose) orally to model what is given to preterm infants. Mice were weaned onto a control diet and fed until age 16 weeks (adulthood). Pups were weighed daily from birth to weaning and weekly thereafter. Longitudinal growth and body composition were assessed at adulthood. Physiological assessments of glucose homeostasis (intraperitoneal glucose and insulin tolerance tests; glucose-stimulated insulin secretion test) were performed at weaning and adulthood. Insulin-like growth factor-1 (IGF-1) and liver water-soluble choline metabolites were also assessed.

Results: Female and male sucrose-treated mice gained less weight during the suckling period (p<0.01 vs other groups) and were smaller at weaning compared to the water- and glucose-treated mice (p<0.05). At age 16 weeks, female sucrose-treated mice had smaller tibias (p<0.001

vs all) and lower serum IGF-1 concentrations ($p<0.05$ vs water). This was accompanied by lower ($p<0.05$ vs water) liver free choline, phosphocholine, and glycerophosphocholine concentrations, and higher betaine ($p<0.01$ vs water) concentrations in the sucrose-treated compared to the water-treated female mice. No differences in growth or liver choline metabolites were observed in male mice. Neonatal treatments had no effect on adiposity or glucose homeostasis in female or male mice.

Conclusion: My findings suggest that repeated neonatal sucrose treatment affects growth in female mice, perhaps through an IGF-1-dependent pathway and alters liver choline metabolism. Further research is required to determine the functional consequences of these alterations.

Lay Summary

In Canada, >17,000 premature babies are born each year. These newborns are cared for in the neonatal intensive care unit, where they may undergo 10-23 painful procedures per day. One standard way to treat pain is to give liquid sugar to the baby during mildly painful procedures, such as blood tests. Depending on the number of procedures, a baby can consume a lot of sugar while in the hospital. This raises questions regarding the long-term safety of the liquid sugar. In my thesis research, I studied the long-term effects of liquid sugar during the neonatal period on growth and the metabolism of sugar using a mouse model. I gave liquid sugar to newborn mice in the same dosage that is given to premature babies and assessed growth and metabolism in adulthood. I found that female mice treated with liquid sugar had altered growth. No changes were observed in males. I concluded that female newborn mice treated with liquid sugar have growth problems and that more research is required to find how sugar affects growth.

Preface

This thesis is submitted in partial fulfillment of the requirements for the degree of Master of Science in the Department of Experimental Medicine. All experiments pertaining to this thesis were performed under the guidance and supervision of Dr. Angela Devlin. This thesis was revised and approved by Dr. Angela Devlin, Dr. Kiran K. Soma and Dr. Liisa Holsti.

The procedures and experiments presented in this thesis were conducted in Dr. Devlin's lab at BC Children's Hospital Research Institute (BCCHRI). The neonatal mouse treatments were performed in the Animal Unit of the Centre for Molecular Medicine and Therapeutics at BCCHRI by me with the help of Dr. Arya Mehran, Melody Salehzadeh and Martina Stokes. The tissue harvest was performed in the BCCHRI animal unit by me with the help of Dr. Alejandra Wiedeman and Ei-Xia Mussai. All animal work was approved by the UBC Animal Care Committee (certificate: A17-0115) and biosafety committee (certificate: B18-0029).

The liver water-soluble choline metabolites were analyzed by Roger Dyer, Senior Laboratory Technician of the BCCHRI Analytical Core for Metabolomics and Nutrition. The western blot analysis was performed by me with the guidance of Dr. Daniel Gamu, postdoctoral fellow in Dr. William Gibson's lab at BCCHRI. All other molecular experiments and the preparation of this thesis were performed by me.

My initial research findings were presented in poster format at the 79th Scientific Sessions of the American Diabetes Association in June 2019 in San Francisco, California. My abstract from this meeting is published: *Diabetes* 2019;68 (suppl 1):290-LB. Additional findings of my research were to be presented in a poster format at the Canadian Nutrition Society 2020 Annual Conference to be held in Edmonton, Alberta in May 2020; the meeting was cancelled because of

the COVID-19 pandemic. My abstract from this meeting will be published in *Appl Physiol Nutr Metab* 2020;in press. My thesis research will be submitted for publication; a manuscript is currently in preparation.

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List of Abbreviations

5'NT	5'-nucleotidase
ADP	Adenosine diphosphate
ALS	Acid-label subunit
AMP	Adenosine monophosphate
AMPD2	Adenosine monophosphate deaminase 2
ATP	Adenosine triphosphate
BCCHRI	British Columbia Children's Hospital Research Institute
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
ChREBP	Carbohydrate-responsive element binding protein
CMMT	Centre for Molecular Medicine and Therapeutics
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
F	Fructose
FGF21	Fibroblast growth factor 21
FGFR	Fibroblast growth factor receptor
G	Glucose
G3P	Glycerol-3-phosphate
G3P-O-A	Glycerol-3-phosphate acyltransferase
GA3P	Glyceraldehyde-3-phosphate
GH	Growth hormone
GHR	Growth hormone receptor
GHRH	Growth hormone releasing hormone
GLUT	Glucose transporter
GSIS	Glucose-stimulated insulin secretion test
IGF-1	Insulin-like growth factor 1
IGF-1R	Insulin-like growth factor 1 receptor
IGFBP3	Insulin-like growth factor binding protein 3
IMP	Inosine monophosphate
IP	Intraperitoneal
IPITT	Intraperitoneal insulin tolerance test
IPGTT	Intraperitoneal glucose tolerance test
JAK	Janus kinase
K^+	Potassium
KHK	Ketohexokinase-C
KOH	Potassium hydroxide

LC-MS/MS	High performance liquid chromatography-tandem mass spectrometry
mRNA	messenger RNA
Na^+	Sodium
NaCl	Sodium chloride
NICU	Neonatal intensive care unit
P	Postnatal day
PBS	Phosphate-buffered saline
PEP	Phosphoenolpyruvate
PKLR	Pyruvate kinase
PNP	Purine nucleosidase phosphorilase
PIPP	Premature infant pain profile
Pro-SI	Pro sucrase-isomaltase
RNA	Ribonucleic acid
SD	Standard deviation
SGLT	Sodium-glucose linked transporter
SI	Sucrase-isomaltase enzyme
SIF	Sucrose-isomaltase footprint
SOCS2	Suppressor of cytokine signalling 2
SREBP	Sterol regulatory element binding protein
SSB	Sugar-sweetened beverages
STAT5b	Signal transducer and activator of transcription 5b
TBST	Tris buffer saline and tween 20
TKFC	Triokinase
VLDL	Very-low-density lipoproteins
V/V	Volume to volume
W/V	Weight to volume
WHO	World Health Organization
XO	Xantine oxidase

Acknowledgements

I would like to express my sincere gratitude to my supervisor Dr. Angela Devlin for providing me this life-changing opportunity and for trusting me the development of this project. Dr. Devlin always encouraged my curiosity and supported my new ideas for the project. Under her guidance, I had the opportunity to appreciate high-quality research, learn laboratory techniques and gain an understanding of the complex interaction of nutrients in the body. I will always be grateful with Dr. Devlin for allowing me to live this experience in her lab. I would like to extend my gratitude to my supervisory committee: Dr Kiran Soma and Dr. Liisa Holsti for their guidance and proactive conversations. I always felt supported and appreciated by them.

I would like to acknowledge the Healthy Starts Catalyst Grant for the financial support for my research project and the Government of Mexico for the scholarship to pursue my master studies through the National Council of Science and Technology (Consejo Nacional de Ciencia y Tecnología, CONACYT).

I would like to thank my friend Dr. Alejandra Wiedeman, for her unconditional support and for her willingness to help. She made me feel the Latin-American warmness from back home. Special thanks to my friends –Ei-Xia Mussai and Nicha Boonpattrawong– members of the Devlin lab, for their guidance, support and for the countless moments of laughs. I felt blessed to be part of this team. I also thank Dr. Abeer Aljaadi and Amanda Henderson for their encouragement and help in several laboratory processes.

I would like to acknowledge Dr. Daniel Gamu for his amazing teaching skills that made me never give up with my western blots. Also, to Dr. Larissa Celiberto and Paula Littlejohn for their guidance, help and constant support in the analysis of jejunum samples and tibia assessments.

Thanks to my friends from back home, especially to Eyskra, Magali, Faby, and the Amai group. Finally, special recognition goes to my husband Pedro for his valuable advice, support, encouragement and for always demonstrating me his true love. Thanks to my parents and sister, I’m here following my dreams because of them.

Dedication

Para mi hermosa familia: Pit, Mamá, Papá, Pat y Jónsi. Ustedes son mi fortaleza, consuelo, y mi mayor bendición. Nada de esto sería posible sin su apoyo.

Los amo con toda mi alma.

Chapter 1: Introduction

1.1 Prevalence of Preterm Birth

Preterm birth is defined by the World Health Organization (WHO) as a live birth occurring before 37 weeks of gestation[1]. Worldwide it is estimated that 10.6% of infants are born preterm[2], and in Canada, there are approximately 17,000 preterm births annually[3].

1.2 Procedural Pain in Preterm Infants

Nearly 50% of preterm infants require hospitalization at the neonatal intensive care unit (NICU)[4] where they may experience pain due to essential procedures during their medical care. It is estimated that infants receive between 10 to 23 painful procedures per day[5, 6] and can undergo more than 400 painful procedures in total during hospitalization, depending on the gestational age at birth[7]. Early and repeated exposure to pain during critical windows of development can have negative effects on short and long-term health of the infant (summarized in Table 1-1 and 1-2). It is well-known that preterm infants are more sensitive to painful stimuli than older children[8].

Table 1-1. Examples of short-term effects of neonatal pain in preterm infants

	Author Year Country	Study design Participants	Methods	Results
Growth	Vinall et al[9] 2012 Canada	Prospective study Preterm infants (<32 weeks of gestation) n=78 Males (50%)	• Neonatal pain (total number of skin-breaking procedures. Type of procedure not specified) • Weight and head circumference (sex and age-specific percentiles)	Greater neonatal pain was associated with lower weight (Wald X^2 7.36 p=0.01) and head circumference (Wald X^2 4.36 p=0.04) percentile at 32 weeks corrected age
Brain development	Brummelte et al[10] 2012 Canada	Prospective study Preterm infants (<32 weeks of gestation) n=86 Males (48%)	• Number of skin-breaking procedures (heel lance, intravenous or central line insertion, intramuscular injection, chest tube insertion, gastrostomy tube insertion, tape removal, nasogastric tube insertion). • White matter fractional anisotropy (thickness; magnetic resonance imaging)	Increased number of skin breaking procedures was associated with reduced white matter in 7 brain regions at term corrected age (ß= -0.0002, 95% CI: -0.00045 - -0.00003; p=0.028)
	Smith et al[11] 2011 United States	Prospective study Preterm infants (<30 weeks of gestation n= 44 Males (44%)	• Cumulative stress score (Neonatal Infant Stressor Scale average daily score) • Brain regional size (thickness; magnetic resonance imaging)	Greater cumulative stress score associated with reduced bifrontal (r= -0.37; p=0.035) and biparietal (r= -0.49; p=0.002) size and white matter diameters (r= -0.38; p=0.021) at 28 days of life.

	Author Year Country	Study design Participants	Methods	Results
Neurobehavioral endpoints	Fitzgerald et al[12] 1989 United Kingdom	Randomized controlled trial comparing effects of EMLA cream in area of heal lance. Preterm infants (27-32 weeks of gestation) n=17 Sex unspecified	• Flexor reflex test on plantar surface of foot receiving a repeated painful procedure (heel lancing every 4 hours for 1-3 days) • Three groups: control (no heel-lanced); heel-lanced; heel-lanced & EMLA cream	A repeated painful procedure produces hypersensitivity and sensitization in the heal lance area compared the control group ($p<0.01$). The group that received EMLA cream presented similar values in the flexor reflex test as the control group.
	Holsti et al[13] 2006 Canada	Crossover trial Preterm infants (<32 weeks of gestation). n=43 Males (56%)	Facial response (Neonatal facial Coding System score) in infants receiving heel lancing after: • Rest or • Cluster of nursing interventions (not specified)	Infants displayed greater facial response during heel lancing after receiving a cluster of nursing interventions ($p<0.05$)
Biochemical indicators	Grunau et al[14] 2005 Canada	Crossover trial Preterm infants (<32 weeks of gestation). n=87 Males (54%)	• Number of skin-breaking procedures (heel lance, venipuncture, insertion of arterial and venous lines, lumbar puncture, chest-tube insertion) • Plasma cortisol after nursing procedures (diaper change, abdominal girth measurement, temperature, mouth care).	Greater skin-breaking procedures associated with lower plasma cortisol response to nursing procedures in very preterm infants (23-28 weeks of gestation) at corrected age 32 weeks ($r= -0.50$; $p<0.05$)

Table 1-2. Examples of long-term effects of neonatal pain in preterm infants

	Author Year Country	Study design Participants	Methods	Results
Brain development	Ranger et al[15] 2013 Canada	Prospective study Children (age 7-8 years) that were born preterm (<32 weeks of gestation). n=42 Males (38%)	• Number of skin breaking procedures (heel lance, peripheral intravenous or central line insertion, chest-tube insertion, tape removal, nasogastric tube insertion) during NICU hospitalization. • Cortical thickness (magnetic resonance imaging)	Higher number of skin breaking procedures during the neonatal period was associated with reduced cortical thickness in 21 out of 66 brain regions (p<0.05)
Neurobehavioral endpoints	Gaspardo et al[16] 2018 Brazil	Retrospective study Toddlers (age 18-36 months) that were born preterm (<34 weeks of gestation; <1,500g) n=62 Males (49%)	• Neonatal pain-related stress total index during NICU hospitalization calculated by painful procedure: extremely stressful (intubation, intravenous insertion, eye examination, chest drain); very stressful (endotracheal suctioning, heel pricks, insertion of percutaneous long line, lumbar puncture, surgery, insertion of nasal continuous positive airway pressure tube); slightly stressful (blood gases sampling) • Temperament (Early Childhood Behavior Questionnaire)	Higher neonatal pain-related stress total index was associated with lower effortful control temperament (r= -0.41; p=0.001)

	Author Year Country	Study design Participants	Methods	Results
Neurobehavior	Grunau et al[17] 2009 Canada	Prospective study Infants at 8- and 18-months corrected age that were born preterm (<32 weeks gestation) n=116 (8 months) 102 (18 months) Males 48%	• Total number of skin breaking procedures (including heel lance, intramuscular injection, chest tube insertion, central line insertion) from birth to term corrected age. • Cognitive and psychomotor development index (Bayley Scales of Infant Development)	At 8 and 18 months, higher number of skin breaking procedures was associated to lower cognitive (r=-0.41 and -0.37 $p<0.05$) and psychomotor development (r= -0.44 and -0.43 $p<0.05$) index
Biochemical indicators	Brummelte et al[18] 2015 Canada	Prospective study Children (age 7 years) born preterm (<32 weeks of gestation) n=77 Males 45%	• Total number of skin-breaking procedures (skin-breaking procedures not specified) from birth to term corrected age. • Saliva cortisol at: 30 minutes after arrival to the study site, 20 minutes after a cognitive test, and at the end of the visit.	Higher number of skin-breaking procedures in boys was associated with lower cortisol levels in saliva at all timepoints (r= -0.88; $p<0.001$)

1.2.1 Assessment and Management of Procedural Pain in the NICU

The assessment of pain in preterm infants is challenging due to the lack of verbal communication and because not a single biological marker is indicative of pain[19]. Therefore, clinicians rely on subjective pain scales that consider behavioural and physiological markers to measure and categorize acute neonatal pain[20]. Components of neonatal pain instruments include facial expression, body movements, crying time, posture and muscular tone, heart rate, blood pressure, oxygen saturation and breathing patterns[21].

According to a recent integrative review, there are 29 instruments to measure pain in infants[20]. The most studied and validated are the Neonatal Facial Coding System, the Neonatal Infant Pain Scale, the Cries Score, the Douleur Aiguë du Nouveau-né, and the Premature Infant Pain Profile (PIPP). Interestingly, none of these scales are accurate enough to be considered a gold standard for detecting pain[20]. Assessing pain in neurologically compromised and extremely preterm infants is especially challenging and these infants are at higher risk of pain mismanagement and pain underrecognition[21].

Analgesia can be provided to neonatal infants based on the threshold of pain elicited by a procedure. A recent approach to guide clinicians in pain management decision making is shown in Figure 1-1.

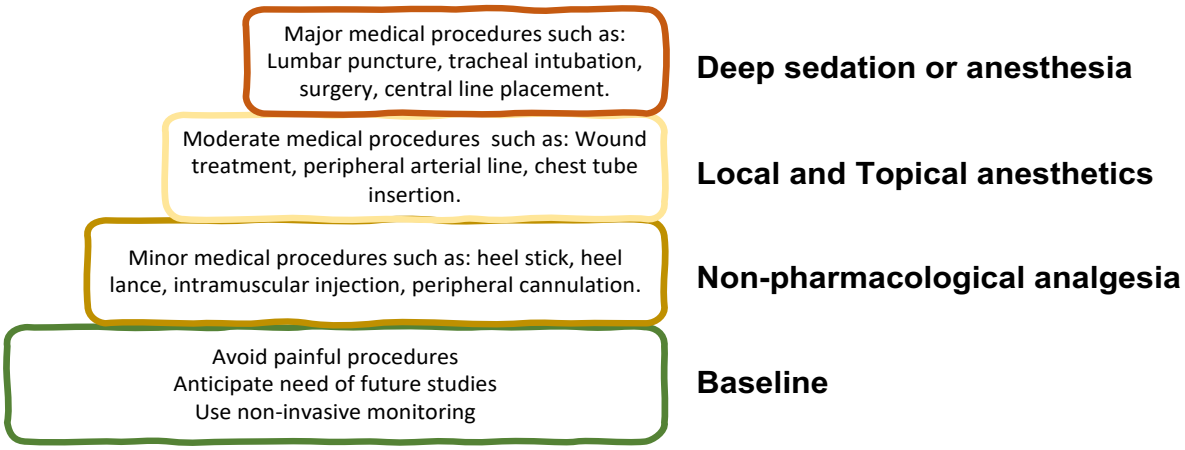

Figure 1-1. A guide to pain management
Adapted from: Witt *et al*. *Curr Emerg Hosp Med Rep*;4:1-10 (2016)

1.2.2 Non-pharmacologic Analgesia

Major medical procedures, such as surgery, mechanical ventilation and central line catheterization, justify the use of pharmacological analgesia. However, there are several minor procedures where the use of anesthetic agents is not recommended, including intramuscular injection, heel lance, and peripheral cannulation[19]. Pain from minimally invasive procedures can trigger behavioural, physiological and hormonal responses[8].

Non-pharmacological therapies are a validated alternative to relieve pain, although for some, the mechanisms of action are not fully understood[22]. These therapies include: breast feeding, oral sucrose/glucose, skin-to-skin contact, swaddling, facilitated tucking, therapeutic touch/massage, musical therapy, non-nutritive sucking, acupuncture, and therapeutic robots[23, 24]. Sensorial saturation is a recent approach to manage pain, where a combination of oral sucrose, massage therapy, and auditory stimulation is given to babies throughout the painful procedure[25]. Currently, oral sucrose is considered the clinical standard of care for minor procedural pain and it is one of the most studied non-pharmacological interventions for pain relief[26].

1.3 Oral Sucrose for Procedural Pain

Blass and colleagues were the first researchers to describe the analgesic effects of oral sucrose in an animal model[27]. In their study, heat latency (paw-lift test to a hot plate 48-49°C) was assessed in Sprague-Dawley rat pups (age 10 days, n=80, unspecified sex) treated orally with either 0.2mL of 7.5% sucrose or water, one minute before the heat test. Sucrose-treated pups increased heat latency by 3-fold compared to water-treated pups at 1 and 3 minutes post sucrose administration[27]. The same research group then demonstrated the sedative effects of oral sucrose in full-term human infants (n=54; 50% males) undergoing blood collection via heel lance. Infants received 2 mL of water or 12% sucrose, 2 minutes prior to the procedure. Crying time was assessed by researchers blinded to group assignments. Sucrose-treated infants cried for 50% less time than water-treated infants[28].

Since then, many studies have reported sedative effects of oral sucrose prior to minor medical procedures. A recent Cochrane systematic review that included 74 randomized controlled trials and 7,049 infants (sex unspecified) concluded that oral sucrose alone or combined with non-nutritive sucking is effective at reducing pain (subjectively measured by pain scales) from a single minor medical procedure, including heel lance, venipuncture and intramuscular injection, in both term and preterm infants[26]. However, there is very limited research on the effects of repeated doses of sucrose on preterm infant outcomes. Indeed, the American Academy of Pediatrics recommends further studies in the long-term effects and that sucrose have to be administered as a pharmaceutical agent so that accurate recording of dosing can be maintained[29]. Nevertheless, in Canada, 64% of NICUs have active protocols of oral sucrose treatment for analgesia for minor painful procedures[30]. The sucrose precise dose required to relieve pain in clinically stable or critical infants is not known. The sucrose doses that are used

vary by concentration (7.5%, 12%, 24%, 33%) and volume (0.05-3.00 mL)[30]. The American Academy of Pediatrics recommends an oral dose of 0.1 to 1.0 mL (or 0.2-0.5mL/kg) of 24% sucrose given 2 minutes before each minor painful procedure[29].

1.3.1 Mechanistic Pathways of Sucrose-dependent Analgesia

The mechanisms underlying the analgesic effects of sucrose are not clear, but evidence in rats indicates that they may involve release of endogenous opioids, serotonin and/or acetylcholine. Blass et al.[27] assessed the reversible antinociceptive effect of oral sucrose treatment using the opioid antagonist naltrexone. Sprague-Dawley albino rat pups (age 10 days, n=8 per group, unspecified sex) were assigned to one of three treatment groups: a) 11.5% oral sucrose and intraperitoneal injection (IP) of naltrexone (0.5 mg/kg), b) 11.5% oral sucrose and IP of isotonic saline solution and c) oral water and IP of isotonic saline solution. Sucrose solutions and water were administrated intraorally in a dosage of 0.2mL one minute before paw lift latency to heat (hot plate 48-49°C) was assessed. Sucrose-treated pups with IP of isotonic saline solution increased paw lift latency by 2.5-fold compared to water-treated pups. In contrast, sucrose antinociception was completely suppressed in sucrose-treated pups that received IP naltrexone, suggesting that the analgesic effects of sucrose involve endogenous opioids.

De Freitas et al.[31] further investigated the role of the specific μ_1-opioid receptor in sucrose antinociception using the μ_1-opioid-antagonist, naloxonazine, in adolescent rats. Male Wistar rats (age 42 days, n=8 per group) were divided in two groups and received an IP injection of: a) naloxonazine (0.25 mg/kg) or b) saline solution. Twenty-four hours post-injection, rats were then subdivided into three groups: a) IP naloxonazine + sucrose; b) IP saline + sucrose; or c) IP saline + water (control group). The water and sucrose (250 g/L) were administered

intraorally (single dose; 500 μL) and tail-flick latency was measured at 0, 5, 10, 15, 20, 25 and 30 minutes post-ingestion. The saline + sucrose group had greater tail-flick latency at 0, 5, 10, 15, and 20 minutes compared to the control group. In contrast, the naloxonazine + sucrose group had no difference in tail-flick latency at any timepoint compared to the control group. This study suggests that the μ_1-opioid receptor is required for the analgesic effects induced by sucrose.

Other mechanisms of sucrose analgesia, unrelated to the release of endogenous opioids, have also been suggested. However, these studies have been conducted in older rats, and sucrose treatment has been administered for longer periods of time[32, 33]. For example, Rebouças et al.[32] conducted a study in adult male Wistar rats (n=8/group; unspecified age) that received tap water or a sucrose solution (25g/L) *ad libitum* for 14 days. On day 15, rats in the sucrose group were subdivided and treated with one of the following IP injections: a) methysergide (3mg/kg; a serotonin antagonist); b) ketanserin (3mg/kg; a 5-HT_{2A} receptor antagonist); or c) saline solution. Latency to respond to a heat source was assessed with the tail-flick test 15 minutes post-treatment. In rats treated with IP saline, those that received sucrose had greater tail-flick latency compared to those that received water. However, in rats treated with IP methysergide or ketanserin, there were no differences in tail-flick latency between rats receiving sucrose or water. These findings suggest that sucrose analgesia involves serotonin-mediated signaling and the 5-HT_{2A} receptor and this is in agreement with other studies[34, 35].

Rada et al.[33] assessed acetylcholine release patterns in response to sucrose. Adult male Sprague-Dawley rats (n=6/group) with brain microdialysis implants in the anterior/posterior medial part of the accumbens shell, were given a 10% sucrose solution orally in three different ways: a) intermittent access (12 hour/day) for 21 days; b) access on days 2 and 21 only; c) *ad libitum* access for 21 days. Acetylcholine release to a sucrose stimulus was assessed in rats three

times (on day 1, 2 and 21). On the day of the experiment, rats underwent a 12-hours fasting and microdialysate samples were collected three times to establish baseline levels of acetylcholine before allowing them to consume a sucrose solution (10%) for 1 hour. Microdialysate samples were taken after sucrose consumption every 30 minutes for 2 hours. On day 1, all groups increased acetylcholine concentrations by 130% during the first 60 minutes of sucrose intake and decreased to baseline levels after the termination of the sucrose intake. On days 2 and 21, rats in the group with daily *ad libitum* access to sucrose and those that received sucrose twice presented the maximum peak of acetylcholine brain concentrations (~120%) sooner during the first 30 minutes of sucrose stimulus compared to rats in the group with intermittent access to sucrose that reached the maximum peak at 60 minutes (~130%). This study suggests an acute release of acetylcholine in the nucleus accumbens in response to sucrose intake, regardless of the previous frequency of sucrose consumption[33]. The contribution of each neurochemical pathway to the analgesic properties of sucrose remains unclear.

1.3.2 Detrimental Effects of Neonatal Exposure to Sucrose in Mice and Humans

The long-term neurodevelopmental and metabolic effects of repeated neonatal oral sucrose treatment are not fully understood[26]. Despite concerns about safety, 40% of hospitalized neonatal infants receive oral sucrose to relieve minor pain at some point during hospitalization[36]. Preterm infants may be exposed to high and cumulative volumes of sucrose[37]. For example, Johnston et al.[38] reported that at three Canadian level-3 NICUs, the mean doses of sucrose (0.1mL; 24%) administrated per preterm infant (<31 weeks of gestation; n=107; unspecified sex) during the first week of life was 63 doses (range 24 to 125 doses). Furthermore, depending on the medical procedure, an infant could receive up to 3 doses of sucrose per procedure[38].

There is some evidence of long-term detrimental effects of repeated neonatal oral sucrose treatment on brain size and neurodevelopment from studies in mice. Tremblay et al.[39] reported that adult C57BL/6J mice (n=109; 46% males) treated with neonatal oral sucrose had smaller brain volumes in 21 of 159 regions (measured by magnetic resonance imaging), especially in white matter, cerebellum and ventricles, independent of pain, compared to adult mice that were treated with water during the neonatal period[39]. Another study from the same research group, reported that in the absence of pain, adult C57BL/6J mice (n=160; 47% males) that received neonatal oral sucrose treatment scored lower in short-term memory tests (Morris water maze test) compared to adult mice that received oral water treatment during the neonatal period[40].

Only one study in human infants has reported on short-term neurobehavioral effects of repeated neonatal oral sucrose treatment. Johnston et al.[38] conducted a randomized controlled trial in preterm infants (<31 weeks of gestation; n=107; unspecified sex) that received either 0.1 mL of sucrose (24%) or water, two minutes before a skin-breaking (e.g. heel lance, intravenous cannulation, arterial puncture or injection) or non-skin-breaking but uncomfortable procedure (e.g. endotracheal tube suctioning, tape removal, nasogastric tube insertion). At gestational age 36 weeks (corrected for gestational age at birth), neurobehavioral development was assessed with the NAPI (Neurobehavioral Assessment of the Preterm Infant) test. The authors reported that higher doses of sucrose led to lower scores for motor development and vigor (ß= -2.158, 95% CI: -4.244, -0.072; p=0.007), and for alertness and orientation (ß= -3.819, 95% CI: -6.804, -0.834; p=0.014) in models corrected for age at birth, severity of illness, days on caffeine treatment, and number of invasive procedures.

In addition, only one study has reported on the acute adverse metabolic effects following a single dose of sucrose. Asmerom et al.[41] conducted a prospective double-blinded randomized

controlled study in preterm infants (<36.5 weeks of gestation; n=151; males 50%) undergoing heel lance for blood collection that had a central catheter in place. Infants received a dose of sucrose (24%; dose: 2mL for infants > 2 kg; 1.5mL for infants 1.5-2 kg; and 0.5mL for infants <1.5 kg) or water accompanied by a pacifier, two minutes before the heel lance. A blood sample was extracted from the central catheter before and 5 minutes after the administration of sucrose or water. Plasma hypoxanthine and uric acid concentrations, markers of hepatic fructose metabolism, increased in infants 5 minutes post-sucrose treatment, but not in those given water. These findings suggest rapid hepatic metabolism of a single dose of sucrose in preterm infants.

1.4 Sucrose Metabolism in Preterm Infants

1.4.1 Sucrose Digestion

Sucrose (beta-D-fructofuranosyl alpha-D-glucopyranoside) is a disaccharide composed of one molecule of glucose linked through an α1–4 glycosidic bond to a fructose molecule[42]. It is hydrolyzed into its monosaccharide subunits by sucrase-isomaltase (SI), a brush-border membrane enzyme located in the duodenum and jejunum of the small intestine[43] (Figure 1-2). The amino acid sequence of murine SI shares 78% homology with the human sequence[44]. Enzymatic activity of SI has been detected in human fetus at 9-10 weeks of gestation[45].

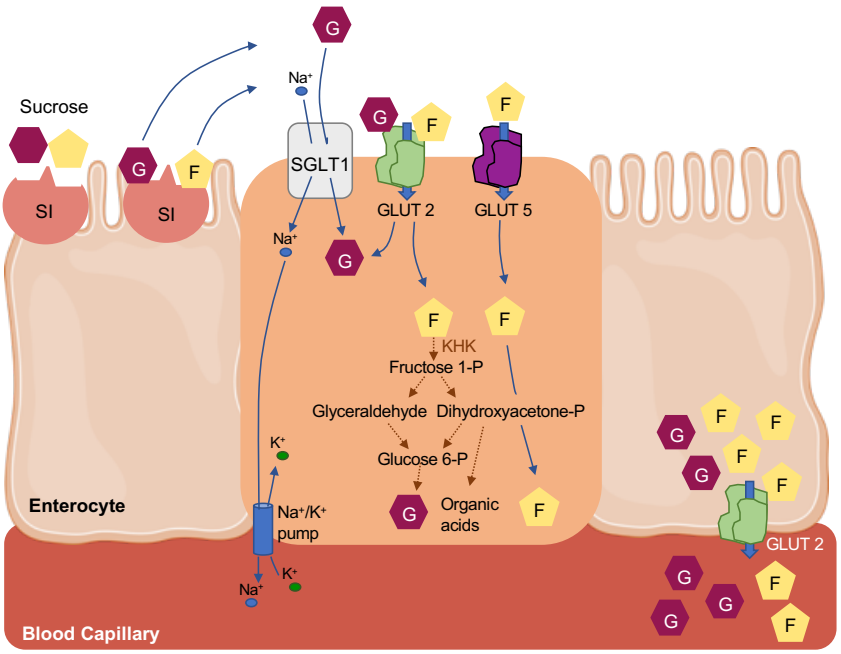

Figure 1-2. Sucrose digestion and absorption

SI: sucrase-isomaltase enzyme, G: glucose, F: fructose, SGLT1: sodium-dependent glucose transporter-1, Na^+: sodium, K^+: potassium, GLUT: glucose transporter, KHK: ketohexokinase-C isoform, P: phosphate, Adapted from: Merino *et al*. *Nutrients*;12,94:1-35 (2020). Figure created using Servier Medical Art ®

1.4.2 Fructose and Glucose Intestinal Absorption

Glucose is absorbed via the sodium-dependent glucose transporter-1 (SGLT1) and the facilitated-diffusion glucose transporter, GLUT2. Fructose is primarily absorbed via GLUT5 and to a lesser extent, by GLUT2 (Figure 1-2).

Intestinal Sodium-dependent Glucose Transporter 1 (SGLT1)

Glucose is transported across the brush border membrane via SGLT1, a Na+-dependent transporter[46] encoded by the *SLC5A1* gene. The amino acid sequence of murine Sglt1 shares 88% homology with human SGLT1[44]. The protein expression of SGLT1 has been detected in human fetal intestine at 17-20 weeks of gestation[47, 48]. The affinity of SGLT1 for glucose is high

(K_m ~0.5mM) and it is considered the principal glucose transporter in the small intestine[49]. Gorboulev et al.[50] reported a 60% reduction in plasma glucose concentrations at 10 and 15-minutes post oral gavage of D-glucose (2mg/g) in *Sglt1-/-* mice compared to *Sglt1+/+* mice. Furthermore, *Sglt1-/-* mice develop severe intestinal distension and lose weight after switching from a glucose-galactose free diet to a standard diet containing glucose. *Sglt1-/-* mice also have an 80% reduction of [^{14}C]-D-glucose in the apical brush border membrane, especially in duodenum and jejunum, and a 73% reduction in plasma glucose concentrations following oral gavage with [^{14}C]-D-glucose compared to *Sglt1+/+* mice[51]. Taken together these studies suggest a primary role for SGLT1 in intestinal glucose absorption.

Solute Carrier Family 2 Member 2

Solute carrier family 2 member 2 (GLUT2; encoded by *SLC2A2*) is a glucose facilitative transporter expressed in many tissues such as liver, intestine, kidney, pancreas and brain[52]. The murine amino acid sequence shares 82% homology with humans[44]. In fetal human intestine, *SLC2A2* mRNA has been detected at 11-15 weeks of gestation[53]. In adult mice, GLUT2 is usually located in the basolateral membrane of intestinal epithelial cells and has a higher affinity for glucosamine (Km ~0.8 mM)[54] than for glucose (Km ~17mM)[54] or fructose (Km ~66mM)[55]. However, an acute oral administration of glucose or sucrose (0.4mL of a 40% solution) induces a reversible rapid translocation (< 30 minutes) of GLUT2 to the apical membrane, and an upregulation of *Slc2a2* mRNA transcripts in mouse epithelial cells[56, 57]. Following intestinal absorption, fructose and glucose are transported from the intestinal epithelial cell into the bloodstream primarily via GLUT2 that is relocated to the basolateral membrane[58] (Figure 1-2).

In mice and humans, GLUT2 is not critical for the intestinal absorption of glucose. Stümpel et al.[59] reported similar portal glucose concentrations after an intraluminal bolus of glucose (150 mg within 1 minute) was administered to isolated perfused duodenum from *Slc2a2* -/- mice and *Slc2a2* +/+ mice[59]. Similarly, plasma glucose concentrations in subjects (n=3) with congenital GLUT2 deficiency (Fanconi-Bickel Syndrome) were not different compared to healthy subjects post oral glucose load (1g/kg)[60].

Solute Carrier Family 2 Member 5

Fructose is primarily transported from the intestinal lumen into the epithelial cell by the glucose facilitative transporter, solute carrier family 2 member 5 (GLUT5; encoded by *SLC2A5*)[61] which has high affinity for fructose (Km ~6mM)[62] (Figure 1-2). The amino acid sequence of murine Glut5 shares 82% homology with human[44]. In human adult jejunal enterocytes, GLUT5 is located along the brush border membrane[53]. Low levels of GLUT5 immunoreactivity have been detected in the immature midvillus region in fetal small intestine at 12 weeks of gestation compared to adult intestine, suggesting that GLUT5 is not an active transporter at this developmental stage[53]. Similarly, expression of *Slc2a5* mRNA in the small intestine from neonatal Wistar rat pups is 11% of levels observed in the small intestine from adult rats[63]. At weaning, *Slc2a5* mRNA increases to 22% of levels observed in adults, however, by one week post-weaning, the expression levels are similar to adult values[63].

To confirm the role of GLUT5 in the absorption of intestinal fructose, Barone et al.[61] assessed isotopic tracing of [^{14}C]-fructose in adult *Slc2a5* -/- mice. Membrane intestinal vesicles were isolated from jejunal mucosa of *Slc2a5* -/- and *Slc2a5* +/+ mice (n=4 per group) and incubated with [^{14}C]-fructose for 30 seconds and uptake indices determined. Fructose uptake by

Slc2a5 -/- intestinal vesicles were ~75% lower than in *Slc2a5* +/+ mice. Furthermore, in a separate study, *Slc2a5* -/- and *Slc2a5* +/+ mice were fed *ad libitum* with either a 60% fructose diet or an isocaloric control diet with no fructose or sucrose for 7 days, and fructose concentrations in whole blood were evaluated by fluorometry. *Slc2a5* -/- mice had ~90% lower blood fructose concentrations compared to *Slc2a5* +/+ mice[61]. Together these data indicate that GLUT5 is required for the intestinal absorption of fructose.

Fructose absorption is stimulated by co-ingesting glucose, likely due to the stimulation and faster translocation of GLUT2 to the apical membrane[64]. Furthermore, ingestion of pure fructose solutions can cause intestinal malabsorption. A crossover study in adults (age 25-51 years; n=10; 70% males) given different doses of fructose (15 g, 25 g, 37.5 g, 50 g) reported that fructose malabsorption (determined by breath hydrogen excretion test) was present in 50% of the subjects when given 25 g fructose and this increased to 80% when the subjects were given the 50g fructose dose. In contrast, there was no malabsorption when the subjects were given higher doses of sucrose (50g, 75g, 100g)[65]. Similarly, a study in Sprague-Dawley adult rats (n=8 per group, sex unspecified) reported greater fructose malabsorption (breath and flatus hydrogen excretion test) with oral fructose doses higher than 0.4g. However, when fructose was given in combination with glucose (1:1), doses greater than 0.8g did not produce malabsorption[66]. These findings suggest that, the administration of fructose as a monosaccharide may not be absorbed by the small intestine in humans or rats and that glucose promotes complete fructose absorption when given in equal amounts.

1.4.3 Fructose and Glucose Metabolism in the Liver

Glucose Metabolism

After intestinal absorption, dietary glucose is metabolized by both the liver and peripheral tissues through insulin-dependent metabolic pathways. It is estimated that the human liver extracts 15-30% of an oral glucose load (1g/kg) from the portal vein via GLUT2[67]. In hepatocytes, glucose is phosphorylated by glucokinase to glucose-6-phosphate which can be directed to three main pathways: glycolysis, glycogenesis, or the pentose phosphate pathway[68]. During the fed state, glycolysis is the principal fate of dietary glucose[68]. Phosphofructokinase-1 (PFK-1), the enzyme that catalyzes the irreversible conversion of fructose-6-phosphate to fructose-1,6-biphosphate in the glycolytic pathway, is considered the main rate-limiting step due to its high regulation by intracellular adenosine triphosphate (ATP), adenosine monophosphate (AMP), lactate, and citrate (Figure 1-3)[69]. Furthermore, fructose 2,6 bisphosphate (F2,6BP), a metabolite produced by the enzyme phosphofructokinase-2 that also utilizes fructose-6-phosphate as a substrate and is stimulated by insulin and inhibited by glucagon, is a potent allosteric activator of PFK-1[70]. Therefore, PFK-1 is tightly controlled in cells.

Pyruvate, the product of the glycolytic pathway, can be reduced to lactate or enter the mitochondria to be oxidized to Acetyl Coenzyme A (Acetyl CoA). This metabolite can enter to the citric acid cycle (TCA) pathway or can return to the cytosol to be irreversible carboxylate to malonyl-CoA by acetyl-CoA carboxylase (ACC) and activate *de novo* fatty acid synthesis. This process takes place in the cytosol and involves two key enzymes, ACC and fatty-acid synthase (FASN) that creates a molecule of palmitic acid (a 16-long carbon chain fatty acid)[71]. Fatty acids are esterified to glycerol to form triglycerides, that can be stored in the hepatocyte or assembled into very low density lipoproteins (VLDL) and transported to extra-hepatic tissues (Figure 1-3)[68].

Sterol regulatory element binding protein-1c (SREBP-1c) and Carbohydrate-responsive element-binding protein (ChREBP)[72] are two main transcriptional factors that work synergically and upregulate lipogenic genes such as stearoyl-CoA desaturase 1, ACC and FASN[73]. Glycolytic metabolites, such as glucose-6-phosphate and xylulose 5-phosphate, activate the domain glucose-response activation conserved element (GRACE) in a ChREBP region that stimulates its translocation to the nucleus[73]. The activation of SREBP-1c depends on the activation of the mammalian target of rapamycin complex 1 (mTORC1) induced by insulin; however, experimental studies in mice demonstrated that fructose consumption, which does not stimulate insulin release, also upregulates SREBP-1c[74], thus, the upregulatory mechanism of both transcriptional factor are still under investigation[75].

Fructose Metabolism

The metabolism of fructose takes place in the small intestine and liver (Figure 1-2 and 1-3). While low amounts of fructose (<0.5 g/kg) are metabolized to glucose and organic acids mostly in jejunum, higher doses (0.5-2 g/kg) are transported to the portal circulation and enter the liver[76] primarily via GLUT2[77]. One of the main differences between fructose and glucose hepatic metabolism is that fructose bypasses two glycolytic regulators: glucokinase and PFK-1. Glucokinase (GK) is the first enzyme in the glycolytic pathway and is usually sequestered in the nucleus by the glucokinase regulatory protein (GKRP) during the fasting state. In the presence of dietary glucose or fructose, GK is dissociated from GHRP and is transported to the cytosol to metabolize glucose[78]. Fructose on the other hand, is phosphorylated to fructose 1-phosphate exclusively by the enzyme fructokinase also known as ketohexokinase isoform C (KHK-C) that is located in the cytosol without any regulatory protein, hormonal/allosteric regulators or any

negative feedback system, therefore, KHK-C is able to metabolize fructose rapidly[77]. Fructose is then metabolized to dihydroxyacetone phosphate and D-glyceraldehyde by aldolase B and those metabolites enter to the triose kinase pathway and provide substrates for *de novo* lipogenesis (Figure 1-3)[79, 80]. Fructose also bypasses the principal glycolytic regulatory enzyme PFK-1, suggesting that fructose metabolism is not sensitive to the energy status of the cell[81]. Dietary fructose activates SREBP1c and ChREBP, important transcription factors that regulate lipogenic gene expression, to a greater extent than dietary glucose. Male Sprague-Dawley rats fed a high-fructose diet (60%/weight) for 2 weeks had 2.2-fold greater liver *Srebp1c* nuclear protein abundance and a 3.3-fold increase in Chrebp DNA binding compared to rats fed a high glucose diet (60% weight)[74, 82]. These studies indicate that fructose metabolism bypasses rate-limiting enzymes and rapidly upregulates lipogenic genes and provides substrates for *de novo* lipogenesis.

Another difference between fructose and glucose metabolism is the increased production of uric acid. The rapid metabolism of fructose depletes intracellular levels of ATP and increases AMP, thereby producing an upregulation of the enzyme adenosine monophosphate deaminase that ultimately generates uric acid[83]. Fructose metabolism also inhibits to a greater extent, the key transcriptional factor peroxisome proliferator-activated receptor alpha (PPARα), that upregulates several enzymes involved in ß-oxidation of fatty acids such as carnitine palmitoyltransferase I and II and acyl-CoA synthetase[84]. Roglans et al.[85] reported that adult male Sprague-Dawley rats (n=8 per group) that received a 10% fructose solution *ad libitum* for 2 weeks had lower hepatic mRNA expression of *Pparα* and increased plasma and hepatic triglyceride concentrations compared to rats that received *ad libitum* 10% glucose solution or tap water[85, 86].

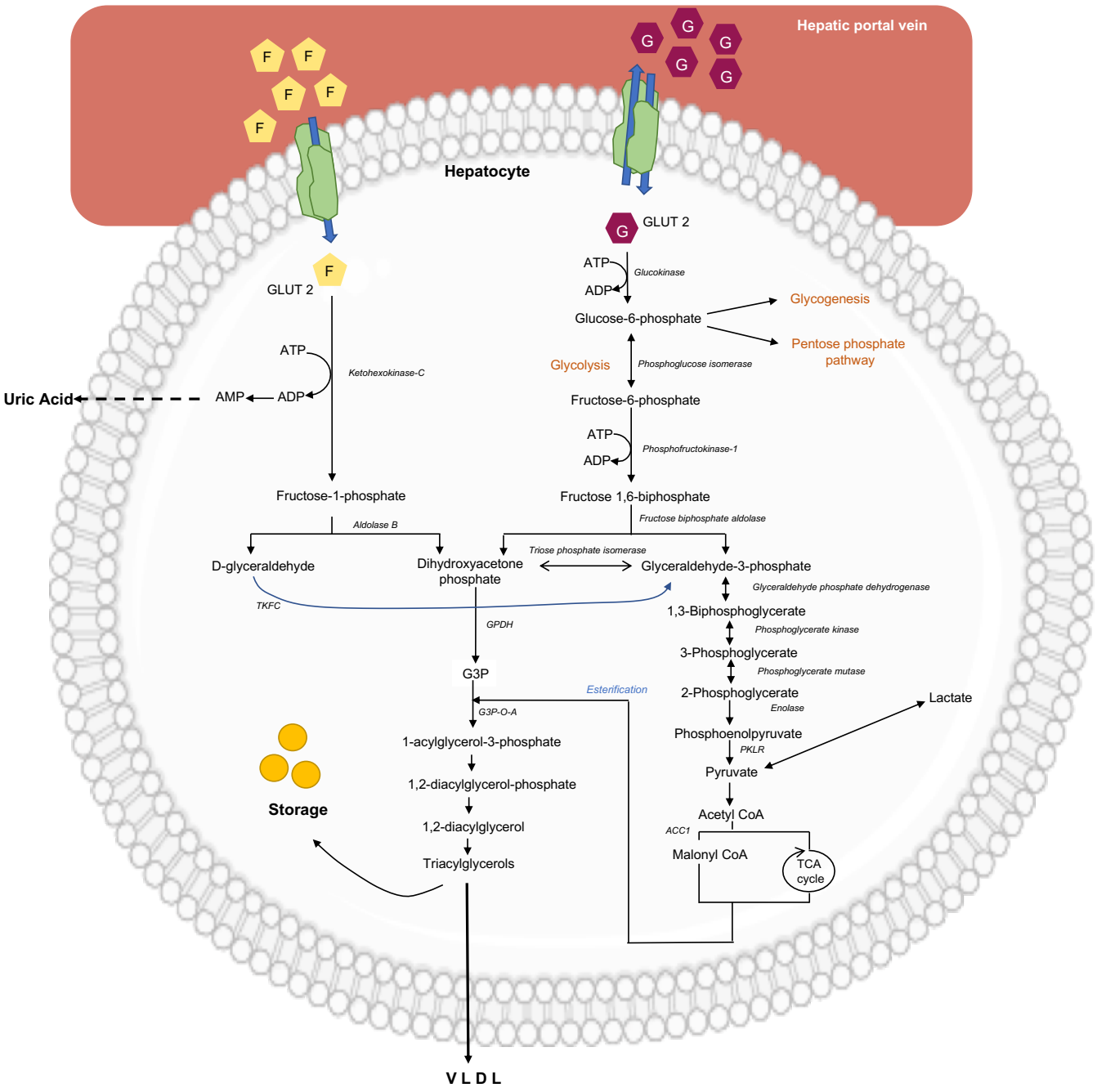

Figure 1-3. Fructose and glucose metabolism in the liver

ACC1: acetyl-CoA carboxylase, ADP: adenosine diphosphate, AMP: adenosine monophosphate, ATP: adenosine triphosphate, F: fructose, G: glucose, GPDH: glycerol-3-phosphate dehydrogenase, G3P: glycerol-3-phosphate, G3P-O-A: glycerol-3-phosphate -O- acyltransferase, PKLR: pyruvate kinase, TKFC: triokinase, VLDL: very-low-density lipoproteins.

Adapted from: Mayes *et al*. *Am J Clin Nutr*; 58: 754S-765S (1993); Rui L. *Compr Physiol*; 4(1): 177-197 (2014). Figure created using Servier Medical Art ®

1.4.3.1 Carbohydrate-dependent Fibroblast Growth Factor-21 Stimulation

Fibroblast Growth Factor 21 (FGF21) is a hormone primarily produced in the liver[87] and to a lesser extent in adipocytes[88] and skeletal muscle[89] and has gained attention in recent years due to its metabolic effects in animal models and humans including body weight reduction and glycemic control[90]. The common pathway that stimulates FGF21 expression is through the lipolytic transcriptional factor PPARα, which is released in response to fasting or ketogenic diets[91]. However, FGF21 upregulation also occurs in response to dietary carbohydrates, particularly fructose. Lundsgaard et al.[92] reported in a randomized cross-over study that healthy males (n=9) fed a hypercaloric carbohydrate-rich diet (80% energy [83% complex carbohydrates and 16% added sugar]) for 3 days had 7-fold higher plasma FGF21 concentrations compared to levels when they were fed a hypercaloric fat-rich diet (80% energy, no added sugar). Similarly, C57BL/6 mice (age 8 weeks) fed a high-sucrose diet (38.5% of carbohydrates) for 15 weeks had 15-fold higher plasma Fgf21 concentrations, gained less weight, and had greater energy expenditure compared to mice fed a standard diet without sucrose[93].

Glucose- or fructose-mediated FGF21 stimulation depends on the transcriptional factor ChREBP. A 5-fold increase in *Fgf21* mRNA was observed in primary hepatocytes from 6-week old male rats overexpressing *Chrebp* [94]. Under high glucose conditions (25mM), a 70% reduction in *Fgf21* mRNA was observed in cells with siRNA targeted *Chrebp* knockdown. No differences in hepatic Fgf21 expression and plasma Fgf21 concentrations were observed in *Chrebp -/-* gavaged with fructose and *Chrebp* +/+ mice gavaged with water[95]. In contrast, hepatic Fgf21 expression and plasma Fgf21 concentrations increased in *Chrebp* +/+ mice gavaged with fructose compared to those gavaged with water. These studies suggest that increases in FGF21 by dietary fructose and glucose is dependent on ChREBP activity.

1.4.3.2 Insulin-like Growth Factor-1 Signaling and FGF21 Blockage of STAT5 in the Liver

Insulin-like Growth Factor-1 (IGF-1), a small peptide consisting of 70 amino acids that shares 40% homology with proinsulin[96], is produced in response to growth hormone (GH)[97]. Liver-derived IGF-1 acts in an endocrine manner and is the main source of circulating IGF-1[98]; whereas locally produced IGF-1 in other tissues acts in an autocrine/paracrine manner[99]. The GH/IGF-1 axis is responsible for 80% of growth in mammals[100]. The pulsatile production of GH in the anterior pituitary gland is tightly regulated by multiple factors including hormones, nutrients, and neuropeptides[101], and the GH actions in the body are mediated by IGF-1[99]. In circulation, IGF-1 is associated with IGF Binding Protein 3 and Acid Labile Subunit (ALS). This forms a complex of 150 kilodaltons[102] that modulates IGF-1 stability and protects it from proteolytic degradation (Figure 1-4).

The binding of GH with GH receptor activates the phosphorylation of Janus Kinase (JAK) 2 in the cytoplasm that initiates a signalling cascade within the cell[103]. The docking site produced by JAK2 recruits different proteins including shc-transforming protein, insulin receptor substrate, protein kinase C phospholipase activators, intracellular calcium and signal transducers and activators of transcription (STATs)[104]. The main transcriptional activator of IGF-1 is STAT5b (Figure1-4)[105, 106]. *Stat5b-/-* mice have a 60% reduction in liver *Igf1* mRNA expression, 30% reduction in serum Igf-1 concentrations, and are 30% smaller than *Stat5b+/+* mice[107, 108]. Congenital defects in *STAT5*B cause severe growth failure and IGF-1 deficiency[109].

Suppressors of cytokine signalling (SOCS) are a family of proteins that downregulate growth factors that utilize the JAK/STAT signalling cascades[110]. Of these, SOCS2 plays a key role in the suppression of STAT5b phosphorylation[111]. Mice that are *Socs2 -/-* have gigantism at

12 weeks of age[112]. Studies in chondrogenic ATDC5 cells demonstrated that overexpression of SOCS2 greatly inhibits STAT5b phosphorylation in response to growth hormone[113], suggesting the negative regulatory function of SOCS2 is considered critical to the regulation of IGF-1. Recent studies have demonstrated that FGF21 is able to stimulate SOCS2 mRNA and protein expression and inhibit longitudinal growth as a consequence[114]. Male and female mice overexpressing FGF21 (*Fgf21-tg*) gained ~40-60% less weight, had smaller tibias, and lower serum IGF-1 concentrations than wild-type mice. This was accompanied by lower Stat5 phosphorylation and greater expression of Socs2 in liver.

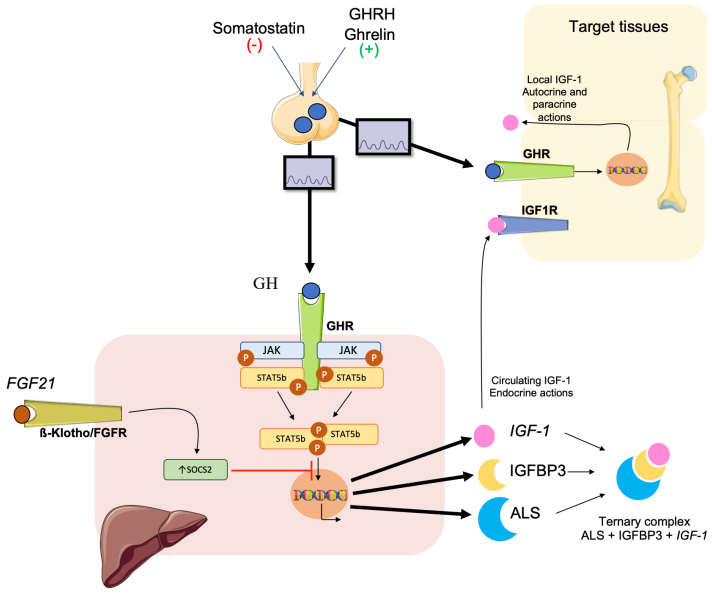

Figure 1-4. Growth hormone signalling in the liver
Growth hormone (GH) is stimulated by the action of growth hormone-releasing hormone (GHRH) and ghrelin and inhibited by somatostatin. Throughout the day, GH is released in a pulsatile manner. In the liver, the binding of GH to the GH receptor (GHR) generates an intracellular cascade that upregulate the transcriptional factor STAT5b initiating the release of insulin-like growth factor-1 (IGF-1).
The binding of fibroblast growth factor 1 (FGF21) to its receptor upregulates the transcription of the suppressor of cytokine signalling 2 (SOCS2) that inhibit the action of STAT5b. In circulation, IGF-1 is found in a ternary complex with the acid labile subunit (ALS) and IGF Binding protein 3 (IGFBP3) that stabilize the molecule. JAK: Janus kinase. P: phosphate molecule. FGFR: Fibroblast growth receptor. IGF1R: IGF-1 receptor. Adapted from: Argente J *et al. EMBO Mol Med*; 9: 1338-1345 (2017). Figure created using Servier Medical Art ®

1.5 Cardiometabolic Disease Risk

1.5.1 Preterm Birth and Cardiometabolic Disease Risk

Preterm infants are at greater risk for cardiometabolic diseases, such as obesity and type 2 diabetes, later in life compared to term infants[115]. This may be due to the adaptive responses of preterm infants for growth and survival away from the uterus despite insufficient energy reserves and immature organs[116].

Several epidemiological studies have reported associations between preterm birth and cardiometabolic disease. For example, in a longitudinal cohort of young adults (mean age 23 years) from Northern Finland, preterm birth <34 weeks of gestation (n=134; 48.5% males) was associated with higher risk of obesity (OR 2.4, 95%CI: 1.2-4.9; *p*=0.01), hypertension (OR 2.4, 95%CI:1.1-5.3; *p*=0.03) and increased waist-hip ratio (mean difference to full term birth 1.7, 95%CI:0.6-2.9; *p*=0.03) compared to infants born at 34-36 weeks of gestation (n=242; 49.6% males) and >37 weeks of gestation (n=344; 48.8% males)[117]. This study excluded adults born small for gestational age. Kaijser et al.[118] conducted a prospective study in Sweden that followed individuals (n=6,425; 59% males) for 20 years (from 1987-2006); 15% were born preterm (<32 weeks of gestation) and using data from hospital registries reported that those born small for gestational age and preterm had a greater risk of type 2 diabetes at a mean age of 60.9 ± 8.2 (HR 1.67, 95%CI: 1.33-2.11) compared to those born at full term. Further, Markopoulou et al.[119] conducted a meta-analysis of 43 studies that included preterm born adults (<37 weeks of gestation, n=18,295; 88% males) and full term born adults (37-42 weeks of gestation, n=294,063; 96% males) and reported that preterm born adults had higher percentage of fat mass (+1.5%; 95%CI: 0.1-2.8; *p*=0.03), systolic blood pressure (+4.22mm Hg; 95%CI: 2.9-5.4; *p*<0.0001), diastolic blood pressure (+2.24mm HG;95%CI: 1.22-3.31; *p*<0.0001), fasting blood

glucose (+0.07 mmol/L;95%CI: 0.02-0.13; p=0.01), and fasting insulin (+16%;95%CI: 6-26; p<0.002) compared to term born adults. When adults born small for gestation age were excluded, the associations were still significant.

1.5.2 Sucrose Consumption and Cardiometabolic Risk

Human colostrum/milk are the first choice of nutrition for preterm infants[120, 121]. The World Health Organization and Health Canada recommends that infants should be exclusively breastfeed for the first 6 months of life[122, 123]. The main disaccharide in human milk is lactose[124]. Sucrose is not present in human milk, and only one study has detected extremely low levels of fructose (7µg/mL) in human milk (n=25) from women with full-term babies at age 1 month[125].

The American Heart Association recommends that children aged <2 years should not consume added sugars[126]. The early introduction of non-milk and sugar-sweetened substances to infants younger than 6 months may increase the risk of obesity and discourage the acceptance of high-quality bitter or sour foods such as green-leaf vegetables later in life[127]. To date, there are no published epidemiological studies on the relationship between early consumption of sucrose by preterm infants and cardiometabolic disease risk; however, there are several studies in older children.

A cross-sectional study of children in upstate New York aged 2-5 years (n=168; 53% males) reported that children that consumed more than 12 oz of fruit juice per day (estimated by 7-day dietary records) were shorter (95.6 cm vs 98.9 cm; p=0.001) and had higher BMI (17.2 kg/m^2 vs 16.3 kg/m^2; p=0.0001) than children that consumed less than 12 oz per day[128]. Secondary cross-sectional analysis of children aged 4.5 years (n=1,549) in the Longitudinal Study of Child Development in Quebec reported that higher sugar-sweetened beverage

consumption between meals (estimated by food frequency questionnaire) was associated with overweight (OR 2.1, 95%CI:1.0-4.7; $p<0.05$) in the children[129]. Greater added sugar in the diet (estimated by two 24-hour recalls) was associated with higher diastolic blood pressure (ß= 0.0206; $p=0.04$) and higher serum triglycerides (ß= 0.1090; $p=0.02$) in a secondary analysis of children aged 7-12 years (n=320; 53% males) that were in the Admixture Mapping of Ethnic and Racial Insulin Complex Outcomes cross-sectional study[130]. Cross-sectional analyses of children aged 8-10 years (n=632; 54% males) with overweight (defined as a BMI ≥85th percentile for age and sex) that are part of the Quebec Adipose and Lifestyle Investigation in Youth Study found that every 100mL of sugar-sweetened beverage consumed (estimated by three 24-hour recalls) was associated with 0.1-unit higher HOMA-IR (ß= 0.097; $p=0.009$) and higher systolic blood pressure (ß= 1.109; $p=0.001$)[131].

1.6 Choline and Growth and Development

Choline is an essential nutrient that is required for membrane structure, cell signalling, lipid metabolism, methylation, and brain development[132]. Choline metabolism can be divided into four main pathways that ultimately generate the following metabolites: phosphatidylcholine, acetylcholine, betaine, and trimethylamine (Figure 1-5)[133]. The metabolism of trimethylamine is beyond the scope of this thesis and will not be covered here as it occurs mainly in the small intestine through anaerobic microbial metabolism[134].

The generation of phosphatidylcholine is the principal metabolic fate of choline and occurs in all nucleated cells in the body[135]. Phosphatidylcholine is the most abundant phospholipid (>50%) in mammalian cellular membranes, and is critical for growth and development[135]. In addition, phosphatidylcholine is the main component required for the export

of hepatic triglycerides throughout the assembly of VLDL in the liver[135]. In fact, choline-deficient diets or reduced phosphatidylcholine synthesis in rats, impair the secretion of VLDL and induce hepatic steatosis and thereby reduced plasma triglycerides concentrations[135, 136]. Moreover, further metabolism of phosphatidylcholine can generate sphingomyelin, a critical sphingolipid during brain development that ensures myelin integrity and axon maturation[137].

The synthesis of phosphatidylcholine occurs predominantly through the CDP-choline pathway and begins with the phosphorylation of free choline to phosphocholine by choline kinase. Phosphocholine is further metabolized to cytidine diphosphate-choline by cytidyltransferase, followed by conversion to phosphatidylcholine in a final reaction catalyzed by choline phosphotransferase[135] (Figure 1-5). In addition, phosphatidylcholine can also be endogenously synthesized in the liver through the sequential methylation of phosphatidylethanolamine using three methyl groups from S-adenosylmethionine catalyzed by phosphatidylethanolamine methyltransferase[135].

Acetylcholine is a major neurotransmitter in the cholinergic system synthesized from the acetylation of choline by choline acetyltransferase. It is essential for memory and learning through the enhancement of feedforward mechanisms in cortical circuits[138], and for pain modulation and cognition. Cholinergic nerve cells obtain choline for the generation of acetylcholine via: 1) uptake of circulating choline that crosses the blood-brain barrier[139], 2) the release of phosphatidylcholine from brain membranes[140, 141]; and 3) the recycling of liberated acetylcholine[142]. Interestingly, in rat brain striatum cells, over stimulation of acetylcholine can deplete structural phospholipids and phosphatidylcholine in membrane reserves when cultured in choline-free medium[140]. Thus, early postnatal exposure to sucrose could potentially compromise

brain structure and neurodevelopment because of choline metabolism and effects on acetylcholine utilization.

Choline can be irreversibly oxidized to betaine by betaine-homocysteine S-methyltransferase (BHMT) in the liver and kidney[143]. Betaine can serve as a methyl donor for the remethylation of homocysteine to methionine (Figure 1-5). The role of choline as a source of methyl groups, through betaine, is metabolically related to the folate and vitamin B12 (Figure 1-5)[133]. Betaine can also serve as an osmolyte[143].

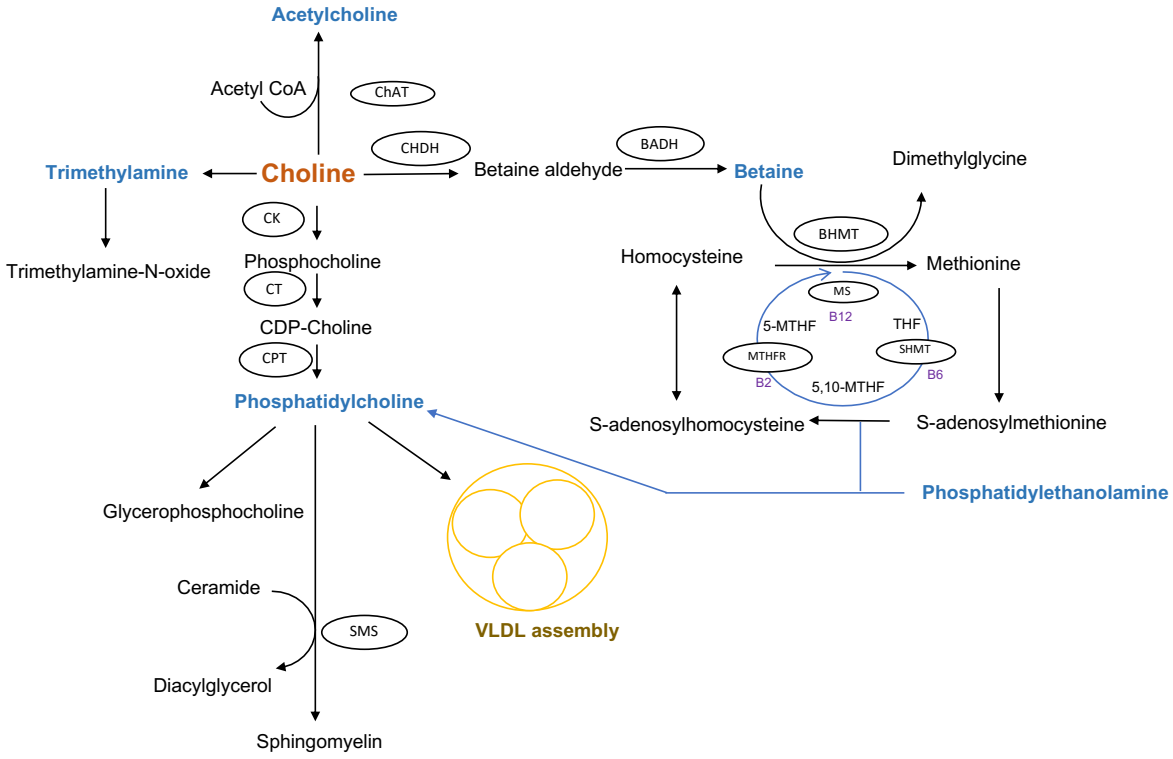

Figure 1-5. Overview of choline metabolism

ChAT: choline acetyltransferase, CHDH: choline dehydrogenase, CK: choline kinase, CT: cytidyltransferase, CPT: choline phosphotransferase, SMS: sphingomyelin synthase, BADH: betaine aldehyde dehydrogenase, BHMT: betaine-homocysteine methyltransferase, MS: methionine synthase, THF: tetrahydrofolate, SHMT: serine hydroxymethyltransferase, 5,10-MTHF: 5,10-methylenetetrahydrofolate, MTHFR: methylenetetrahydrofolate reductase, 5-MTHF: 5-methyltetrahydrofolate.

Adapted from: Wiedeman *et al*. *Nutrients*; 10, 1513 (2018).

Chapter 2: Rationale and Hypothesis

Preterm infants usually require hospitalization in the NICU, where they undergo several life-saving, skin-breaking procedures. Depending on the gestational age at birth, preterm infants can receive up to 400 painful procedures over the course of hospitalization[38]. Untreated pain is associated with adverse effects on neurodevelopment[8]. Thus, it is unethical to not provide any type of analgesia to infants[144].

Oral sucrose is the non-pharmacological standard of care used to manage pain from minor medical procedures[26]. During hospitalization, a preterm infant receives on average between 10-23 painful procedures per day[5] putting them at risk of receiving cumulative amounts of sucrose. For example: if a 2.2 lb (1,000g) preterm infant is given the recommended dose of 0.5-1.0 mL of 24% sucrose for each of 10 procedures/day, this is equivalent to giving 4 tablespoons of sugar (22g) per day to a 22 lb (10kg) toddler[37]. Preterm infants are at risk for cardiometabolic disease, such as obesity, hypertension and type 2 diabetes, later in life[117, 118]. Furthermore, the excessive consumption of sucrose in the form of sugar-sweetened beverages in children, is associated with higher body mass index, dyslipidemia, and decreased pancreatic ß cell function[128, 130, 145]. This suggests that there may be long term adverse metabolic effects of neonatal sucrose treatment on preterm infants. No studies have evaluated the long-term effects of neonatal sucrose treatments on cardiometabolic health.

In mice and preterm infants, the early and repetitive sucrose treatment has detrimental effects on regional brain growth[39] and neurodevelopment[38, 40]. It is unknown if there are also long-term alterations on overall body growth. As previously described, choline and its metabolites have critical roles in growth and brain development; moreover, choline is also involved in lipid metabolism in the liver and the fructose component of sucrose activates several

lipogenic pathways. It is not known if neonatal repeated sucrose treatment has long-lasting consequences on choline metabolism.

I **hypothesize** that there are long-term adverse metabolic effects of early and repeated sucrose treatment during the neonatal period. To test this hypothesis, I conducted studies in a mouse model of neonatal sucrose treatment[39, 40]. Neonatal mice constitute an appropriate animal model to study human prematurity because they are born neurologically and intestinally immature, and the first week of life in a mouse pup corresponds to ~ 24-32 weeks of gestation in humans[146, 147].

I addressed three aims in my thesis research that determined the long-term effects of neonatal sucrose treatment on:

1. Body growth and adiposity.
2. Glucose homeostasis.
3. Hepatic water-soluble choline metabolites.

Chapter 3: Materials and Methods

3.1 Experimental Design

All animal procedures were approved by the Animal Care (certificate # A17-0115) and Biosafety (certificate #B18-0029) Committees of the University of British Columbia and conforms to the Canadian Council on Animal Care guidelines.

Six-week-old female and male C57BL6/J mice were purchased from Centre for Molecular Medicine and Therapeutics mouse colony at BCCHRI. Mice were fed a control diet (D12450K Research Diets, New Brunswick, NJ, USA; diet composition in Table 3-1) and had *ad libitum* access to food and water. They were housed under a standard 12-hour light-dark cycle.

Table 3-1. Maternal and post-weaning diet composition

Nutrient	Caloric Distribution (% of total energy)	Formulation	(g/kg)
Protein	20%	Casein	200
		L-Cystine	3
Carbohydrate	70%	Corn Starch	550
		Lodex 10	150
		Sucrose	4
Fiber		Solka Floc FCC200	50
Fat	10%	Soybean Oil	25
		Lard	20
Mineral Mix	-	S10026B	50
Vitamin Mix	-	V10001C	1
		Choline Bitartrate	2
Energy density	3.82 Kcal/g		

Mineral mix: monohydrate potassium citrate, dibasic calcium phosphate, calcium carbonate, sodium chloride, magnesium sulfate, magnesium oxide, ferric citrate, manganese carbonate hydrate, zinc carbonate, chromium potassium sulfate, copper carbonate, ammonium molybdate tetrahydrate, sodium fluoride, sodium selenite, potassium iodate. *Vitamin mix:* Vitamin E acetate, niacin, biotin, pantothenic acid, vitamin D3, vitamin B12, vitamin A acetate, pyridoxine HCl, riboflavin, thiamine HCl, folic acid, menadione sodium bisulfite.

After one week of acclimation, female mice were bred with age-matched male mice; pregnancy was confirmed via a vaginal plug and males were removed from the cage. To optimize survival and reduce cannibalism of mouse pups, pregnant females were then co-housed with a virgin CD-1 female mouse until the weaning of mouse pups (Figure 3-1). In a previous study, cohousing of C57BL6/J dams with virgin CD-1 females reduced pup stress and improved survival rate[39].

On the day of birth (postnatal day 0, P0), pups were tattooed using a commercial paste (#329A Ketchum Manufacturing INC) for identification purposes. Neonatal female and male pups were randomly assigned to receive one of five treatments: a) sham (only handled); b) sterile water; c) 24% w/v sucrose (#S7903, Sigma); d) 24% w/v fructose (#F2543, Sigma); or e) 24% w/v glucose (#G7528, Sigma). Given that sucrose is a disaccharide of fructose and glucose[42], and each monosaccharide is metabolized differently, I included groups of mice treated with fructose or glucose to investigate if the individual monosaccharide components had biological effects. One pup per sex per litter was assigned to each treatment; treatment groups did not contain littermates. Pups received 10 oral treatments per day between 8:00 AM to 6:00 PM from P1 to P6 (7 days total). Solutions were administrated into the inner cheek of the pup using a single-channel pipette. The dosage was calculated daily so that the pups received 0.2mg sucrose (or glucose or fructose)/g body weight to mimic what it is given to preterm infants at the NICU[26, 39].

Treatments were performed on a heating pad to avoid any heat loss. Each intervention was spaced by 60 minutes to allow recovery and suckling of mouse pups. Treatments were performed in a separate room from the nursing dams to avoid stress due to pup vocalizations. Treated mouse pups were rubbed with nursing female odour before being returned to the dam after each treatment.

At three weeks of age, female and male pups weaned onto the control diet (Table 3-1). Mice were housed by sex in groups of 5 mice/cage and had *ad libitum* access to water and food for the duration of the study. Physiological assessments of glucose homeostasis (described below in section 3.7) were assessed at weaning and 1-3 weeks before euthanasia. At the end of the feeding period, 16-week-old female and male mice were fasted for 5 hours and euthanized by cervical dislocation after isoflurane anesthesia. Blood was collected via cardiac puncture under deep anesthesia and tissues were harvested and snap-frozen in liquid nitrogen for storage at -80°C until further analysis. An overview of the research design is depicted in Figure 3-1.

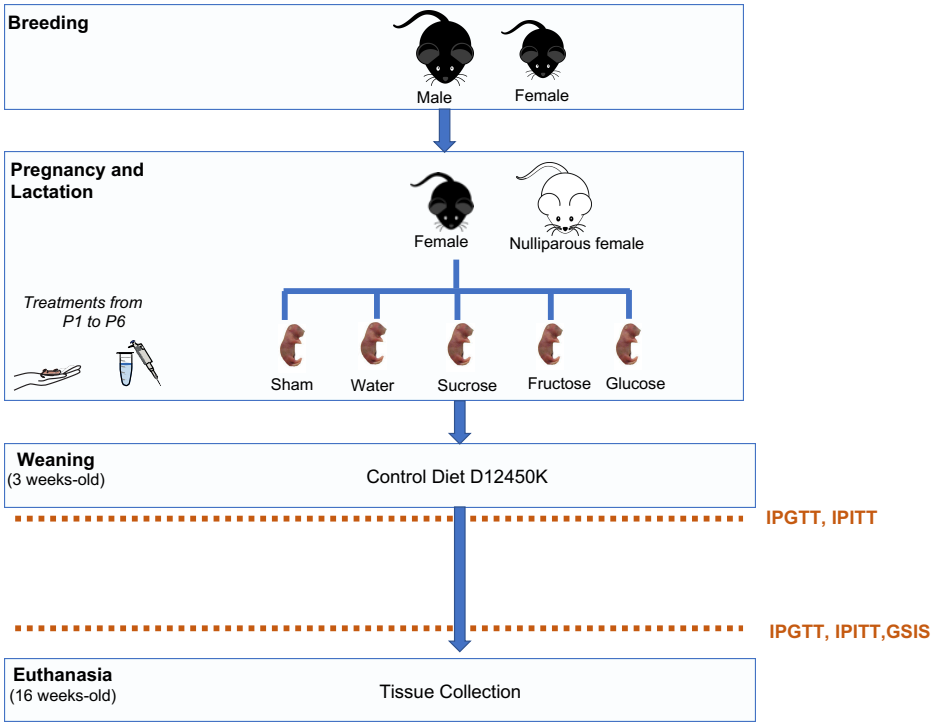

Figure 3-1. Research design overview
IPGTT: Intraperitoneal glucose tolerance test, IPITT: Intraperitoneal insulin tolerance test, GSIS: Glucose-stimulated insulin secretion test.

3.2 Assessment of Growth

Body weight was measured daily from P0-P21; and weekly from weaning until the end of the experiment using a calibrated scale (0.01g).

Body Composition was quantified at age 13 weeks by quantitative magnetic resonance technology (EchoMRI-100 Echo Medical Systems, Houston, TX) which distinguishes fat mass, lean mass and body free fluid based on relaxation times of the hydrogen density[148]. The EchoMRI machine is located in the BCCHRI animal unit and is part of Dr William Gibson's (BCCHRI Scientist; Professor UBC Medical Genetics) laboratory. Each animal was scanned twice and the average of the two scans was used to calculate the percentage of lean and fat mass.

Nose to anus length was measured from the tip of the nose to the tail base on the day of euthanasia under deep anesthesia and before cervical dislocation.

Tibia Length was measured after the removal of non-bone tissue with 2% KOH[149]. Tibias were measured three times using a digital caliper (# 01407A Neiko Stainless Steel, USA). These analyses were conducted in collaboration with Paula Littlejohn (PhD Student, Finlay Lab, UBC Michael Smith Laboratories).

3.3 Serum IGF-1 and FGF21

Blood was collected via cardiac puncture and allowed to clot for 30 minutes on ice followed by centrifugation (4°C for 10 minutes at 8,000 rpm) to separate serum. Serum was divided into 100µL aliquots and stored at -80°C until further analyses. Serum IGF-1 and FGF21 concentrations were quantified by Mouse/Rat IGF-1 ELISA (22-IG1MS-E01 ALPCO Diagnostics) and Mouse/Rat FGF21 ELISA (EZRMFGF21-26K EMD Millipore Corporation) as

per manufacturer's instructions. Assays were performed on serum samples that had not been freeze-thawed.

3.4 Gene Expression

Total RNA was extracted from frozen liver tissue using the AllPrep DNA/RNA Mini kit (#80204 QIAGEN) as per manufacturer's instructions with the addition of on-column-DNase digestion to ensure removal of genomic DNA. RNA concentration was assessed using a Nanodrop spectrophotometer and purity was confirmed by a 260nM/280nM absorbance ratio between 1.9 and 2.1. RNA integrity was assessed by visual observation of 18s and 28s ribosomal RNA bands using agarose gel electrophoresis.

Total RNA (500 ng) was reverse transcribed using the cDNA Reverse Transcription Kit (#4368814, Applied Biosystems). Liver *Igf-1* and *Fgf21* mRNA levels were quantified by TaqMan® real time quantitative PCR (fluorogenic probe) using the ΔΔCt method of relative quantification[150]. Pre-design 5' nuclease probe qPCR assays specific for murine *Igf-1* (Mm00439560_m1; ThermoFisher Scientific), *Fgf21* (Mm.PT.58.29365871.g; Integrated DNA Technologies) and the endogenous control *Hmbs* (Mm.PT.39a.22214827; Integrated DNA Technologies) were used. Each sample was run in duplicate to examine intra-assay variability and repeated in two plates to measure inter-assay variation. Negative controls (no RNA) were always nondetectable.

Table 3-2. Nuclease probe qPCR assays

Gene	Forward (5'-3')	Reverse (5'-3')	Probe (5'-3')	Exon
Igf-1	TCTTCAGTTCGTGTGTGGA	TCCAGCATTCGGAGGGCAC	GGGCTTTTACTTCAACAAGCCCACAGGCT	2-3
Fgf21	CAGCCTTAGTGTCTTCTCAGC	GGGATGGGTCAGGTTCAGA	TCAACACAGGAGAAACAGCCATTCACT	1-1
Hmbs	AAAGATGAGGGTGATTCGAGTG	AAGAATCTTGTCTCCCGTGG	CAGTGTCGGTCTGTATGCGAGCC	2-4

3.5 Immunoblot

Frozen rectus femoris (~ 50 mg) was homogenized in 500µL of cold 1X PBS followed by centrifugation (4°C for 30 seconds at 8,000 rpm). The supernatant was discarded, and the tissue was resuspended in 500µL of lysis buffer, consisting of RIPA buffer (# R0278 Sigma) and protease inhibitor cocktail (#45000 Santa Cruz Biotechnology). Samples were homogenized with stainless steel beads and sonicated on ice for three 15-second pulses at 40% amplitude. Tissue lysates were then centrifuged (4°C for 10 minutes at 8,000 rpm) and the supernatant was stored at -80°C. Protein was quantified by the Bradford Protein Assay[151] using Quick Start™ dye reagent (# 5000205 Bio-Rad).

Samples (20µg protein) were mixed with a loading buffer (consisting of 950 µL 2x Laemmli buffer (#1610737 Bio-Rad) and 50 µL ß-mercaptoethanol) and boiled for 5 minutes at 95°C. The samples were resolved on 10% polyacrylamide gels (consisting of 4% stacking gel and 10% resolving gel). The gel was run at 80V for 15 minutes followed by 110V for 100 minutes. The gels contained the following: dH_2O, Tris Base (Roche), 30% Bis Acrylamide solution (Bio-Rad), 10% SDS (Invitrogen), 10% APS (Sigma), and TEMED (Bio-Rad). The following ladders and standards were run on each gel: 10µL Precision Plus Protein™ (# 1610374 Bio-Rad) and 10µL Unstained Protein Standard *Strep*-tagged (#1610363 Bio-Rad)

The proteins were electrotransferred for 100 minutes at 110V from the gels to polyvinylidene difluoride membranes at 4°C using an ice-cold transfer buffer [dH_20, Tris base (Roche), glycine (Invitrogen), methanol (ThermoFisher)]. The successful transfer was confirmed by visualization of protein bands with Ponceau S staining solution (PON002.1 BioShop). The membrane was then washed twice in a Tris-Buffered Saline (20mM Tris base, 250mM NaCl) +

0.1% Tween-20 solution (TBST) and blocked for 1 hour at room temperature in 5% skim milk in TBST.

After blocking, the membrane was rinsed three times with TBST (15, 5, 5 minutes) and incubated overnight at 4°C on rocker with the primary antibodies: a) polyclonal rabbit anti-IGF1Rβ (#3027 Cell Signaling) at a 1:1000 dilution in 5% BSA TBST; or b) the loading control monoclonal rabbit anti-α-Tubulin (#2125S Cell Signaling) at a 1:1000 dilution in 5% BSA TBST. This was followed by incubation with the secondary antibody, HRP-linked anti-rabbit IgG (#7074 Cell Signaling), at a 1:3000 dilution in 5% skim milk in TBST and HRP-conjugated StrepTactin to detect the protein standard (1:50000 dilution) and kept for 1 hour at room temperature on the rocker. Membranes where washed three times with TBST (15, 5, 5 minutes) and incubated for 5 minutes with a chemiluminescent substrate 1:4 v/v (SuperSignal™ West Pico PLUS. #34579; ThermoFisher Scientific). The membranes were exposed for 10 minutes to X-ray film and the relative protein density was quantified using Image J[152] and normalized to the loading control.

3.6 Histological Analysis of Jejunum

The small intestine was collected on the day of euthanasia and placed in ice cold 1X PBS; length was measured from the pyloric sphincter to the ileocecal valve. Three cross sections of different parts of the jejunum (at 3, 8 and 13 cm from the pyloric sphincter) were fixed in 10% formalin (#HT501128 Sigma-Aldrich) overnight at 4°C and then paraffin-embedded. Tissues were cut into 5µm sections. Tissue sections with >80% of cross section intact on slides were stained with hematoxylin and eosin (by the Histology Core at BCCHRI) and viewed with a brightfield on the optical microscope (Olympus BX61©). At least 20 villi lengths and 20 crypt

depths per mouse were examined and quantified using OlyVIA 2.9© software at 180X magnification.

3.7 Glucose Homeostasis

Glucose tolerance was assessed at ages 3 and 13 weeks by intraperitoneal (IP) glucose tolerance test, and insulin sensitivity was assessed by IP insulin tolerance test at ages 4 and 14 weeks. After a 5 hour fast, an IP injection of D-dextrose (1g/kg body weight in 0.9% NaCl) or insulin (0.75 U/kg body weight in 1X PBS) was given to the mice and blood glucose was quantified at baseline, 15 min, 30 min, 60 min, 90 min and 120 min post-injection using a glucometer (OneTouch Verio ® meter, LifeScan IP Holdings, LLC).

Glucose-stimulated insulin secretion, an *in vivo* indicator of beta-cell function, was measured in adult mice at 15 weeks. After a 5 hour fast, mice were given an IP injection of D-dextrose (1g/kg body weight in 0.9% NaCl) and blood samples were collected by tail snip at baseline and from the saphenous vein at 2, 15 and 30 minutes post injection. Blood samples were allowed to clot and centrifuged (4°C for 10 minutes at 8,000 rpm) to collect serum; the serum was stored at -80°C. Insulin was quantified by a commercial ELISA kit (80-INSMSU-E01 ALPCO Diagnostics).

3.8 Liver Choline Metabolites

Liver water-soluble choline metabolites (free choline, phosphocholine, glycerophosphocholine and betaine) were quantified by high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) using stable isotope-labeled internal standards. Frozen liver tissue (15mg) was homogenized in 1500µL of dH_2O. Ten microliters of

the homogenate (equivalent to 100µg of liver) was transferred to a 1.7mL centrifuge tube, and proteins precipitated with 600µL acetonitrile. Samples were vortexed and centrifuged (4°C for 10 minutes at 20,000 g) and 400µL of the supernatant was transferred to a vial along with 400µL of dH_2O. Samples were run on a Acquity H-class UHPLC and Xevo TQS triple quadrupole mass spectrometer (Waters) with an Agilent Rx-Sil 2.1 X 150mm (# 883700-901 ZORBAX Rx-SIL) column using a hydrophilic interaction chromatographic approach with a binary mobile phase consisting of a) acetronitrile/2% dH_2O with 5mM ammonium formate and 0.1% formic acid; and b) 5mM ammonium formate in dH_2O with 0.1% formic acid. A gradient 70% to 8% acetonitrile over 5 minutes was used to separate the water-soluble choline metabolites before returning to initial conditions. Data were analysed using the Target Lynx software (Waters) and the area of unlabeled analytes to deuterium-labeled internal standards ratios were calculated and compared to known standard curves developed in the lab. Deuterium-labeled internal standards, d9-betaine, d9-choline, d9-phosphocholine were purchased from CDN isotopes and d9-glycerophosphocholine was synthesized from d9 labeled dipalmitoyl glycerophosphocholine (#860352P Avanti) using the Koc-Zeisel method[153]. These analyses were performed by Roger Dyer, Senior Laboratory Technician in the Analytical Core for Metabolomics and Nutrition at BCCHRI.

3.9 Liver Triglyceride Quantification

Frozen liver samples were weighed (50 mg) and homogenized in 500 µL of dH_2O by sonication. Tissue lysates were centrifuged (4°C for 10 minutes at 8,000 rpm) and the supernatant was stored at -80°C. Protein concentrations were determined by the Bradford Protein Assay[151] using Quick Start™ dye reagent (#5000205 Bio-Rad Laboratories, Inc). Lipids were

isolated from liver homogenates by the method of Folch[154] using 6:3:2.25 v/v/v of chloroform/methanol/homogenate. Samples were vortexed thoroughly followed by centrifugation (20°C for 5 minutes at 2,500 rpm); the lower organic layer was then transferred to a new microcentrifuge tube. Lipids were dried for 15 minutes using a nitrogen evaporator, then resuspended in 200µL 1:1 v/v Triton™ X-100 (Sigma-Aldrich)/methanol and sonicated at 40°C for 15 minutes and stored at -80°C. Triglycerides were quantified using a colorimetric kit (Triglyceride Reagent Set, #T7532 Pointe Scientific) and a standard curve (200, 160, 100, 50, 25 mg/dL) generated using a commercial standard (#T7531 Pointe Scientific). Assays were conducted in a 96-well plate, 10 µL each of standards and samples were added into each well in duplicates with 180 µL of warm reagent. The plate was incubated for 5 minutes under constant agitation (700 rpm) and absorbance was read at 500nm. Triglycerides concentrations were normalized to tissue weight and protein concentrations.

3.10 Statistical Analyses

All data points and the mean ± SD are presented. Males and females were analyzed separately. Data normality was assessed by the Shapiro-Wilk test; a p value >0.05 was considered a normal distribution. Non-parametric tests were performed if the data were not normally distributed. Repeated-measures one-way analysis of variance (ANOVA) evaluated changes in weight during the suckling and post weaning periods. My primary objective was to evaluate the differences between the sucrose and water groups, therefore I performed independent sample t-tests to compare both groups. My secondary objective was to analyze the differences between sucrose, fructose and glucose to determine if the effects I found in the sucrose group was attributed to a specific monosaccharide, therefore I performed one-way

ANOVA to compare sucrose to fructose and glucose followed by a Tukey post-hoc test if necessary. Data were analyzed using the software SPSS version 17 (SPSS Inc; IBM, Chicago, US). A p-value <0.05 was considered statistically significant. Results were graphed using GraphPad Prism 8 software.

The sham group (neonatal handled mice; no treatment) was included to validate the water-treated mice as controls. No statistical differences were found in any parameters between these groups (see supplementary material). As such, the sham group was no longer included in the analyses of my thesis and the water treated group was used as my control.

Chapter 4: Growth, Body Composition and Adiposity

4.1 Body Weight

Body weight was assessed daily from birth to weaning (P0-P21) and weekly thereafter until euthanasia at age 16 weeks. There were no differences in birth weight of male or female pups in any of the groups (Figure 4-1A, 4-1B). Female sucrose-treated pups weighed less at the end of the treatment period (P7) compared to the rest of the groups (Figure 4-1C, 4-1E). During the suckling period (P0-P21), sucrose-treated female mice gained less weight compared to the other groups (Figure 4-1G) and were lighter at P21 than glucose- and water-treated mice (Figure 4-2A). No differences in body weight were observed in male pups at any timepoint during the suckling period (Figure 4-1D, 4-1F, 4-1H) or at weaning (Figure 4-2B).

Between ages 3 to 16 weeks, there were no differences in weight gain in females (Figure 4-2C). At age 16 weeks, there were no difference in body weight between any of the treatment groups in female mice (Figure 4-2E). No differences in body weight were observed in male mice (Figures 4-2D, 4-2F).

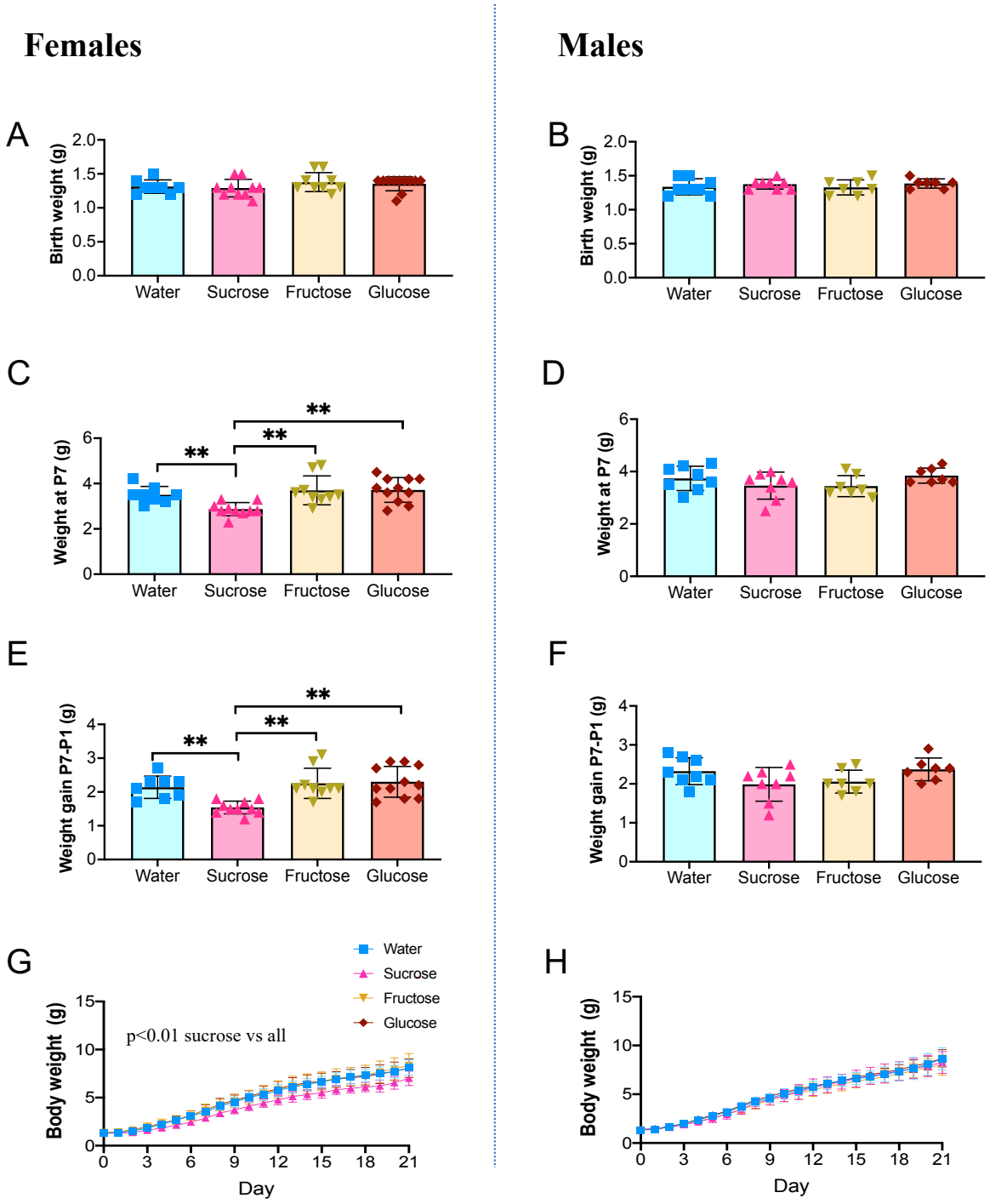

Figure 4-1. Body weight and weight gain during the suckling period
Values presented as mean ± SD. A,B: body weight at birth. C,D: body weight at the end of the treatment (postnatal day 7). E,F: weight gain during the treatment (postnatal day 7 – postnatal day 1). G,H: weight gain during the suckling period. ***p*<0.01; n=7-10 mice per group.

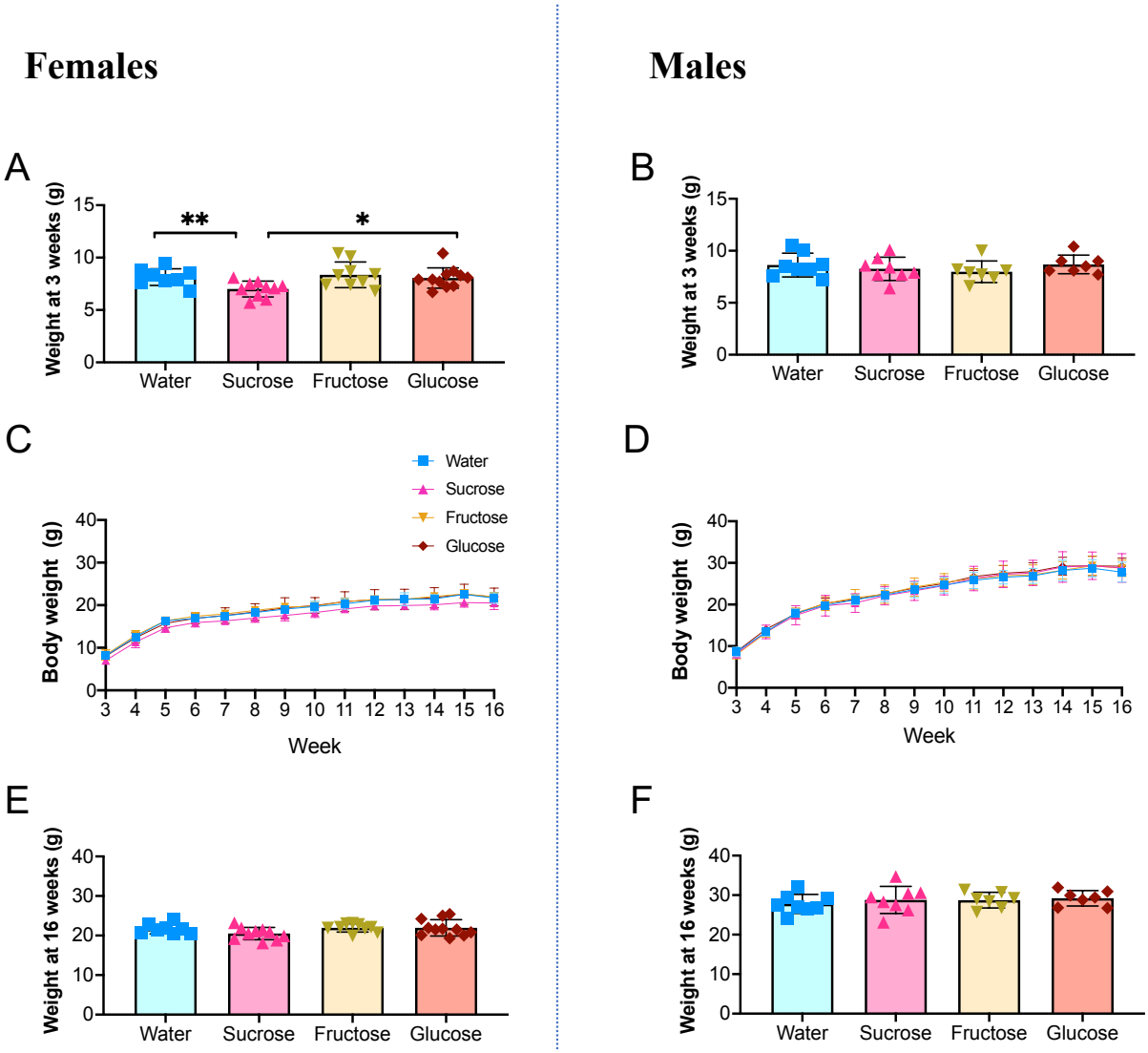

Figure 4-2. Body weight and weight gain

Values presented as mean ± SD. A,B: body weight gain at weaning. C,D: weight gain during the postweaning period. E,F: body weight at adulthood. *p<0.05, **p<0.01; n=7-10 mice per group.

4.2 Length

Nose to anus and tibia length were assessed as indicators of body size at age 16 weeks. Neonatal treatment had no effect on nose to anus length in females (Figure 4-3A). However, sucrose-treated female mice had smaller (p=0.001) tibias compared to the other groups (Figure 4-3C). No differences in body size or tibia length were found in males (Figure 4-3B, 4-3D).

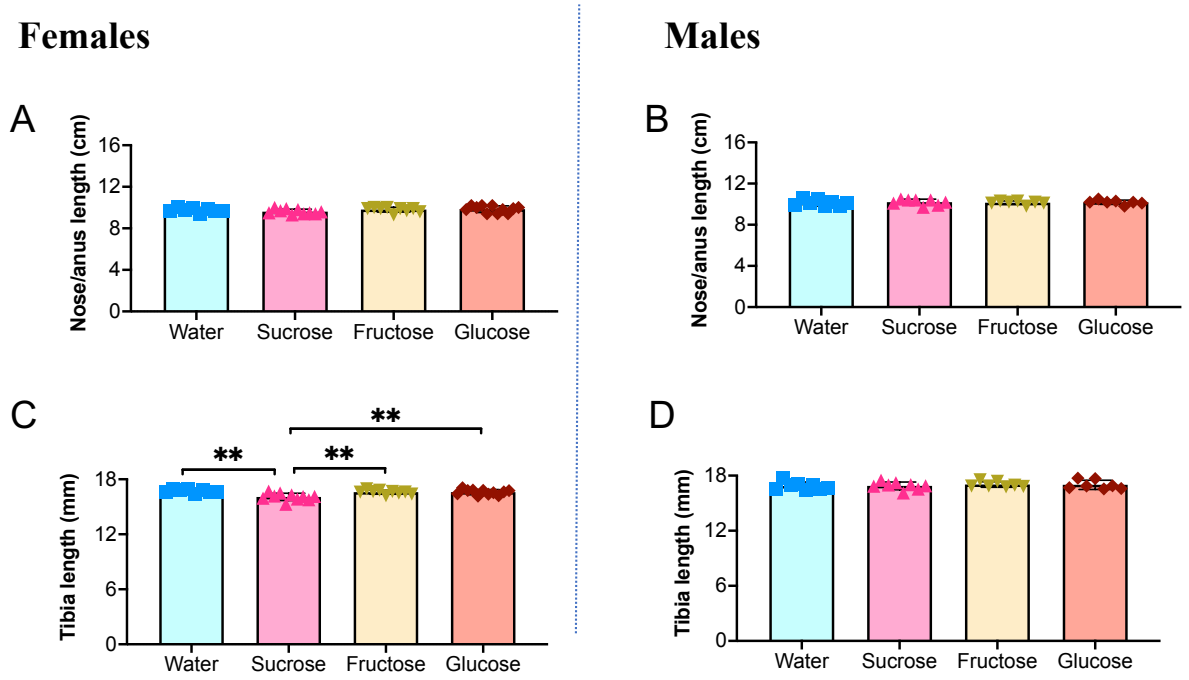

Figure 4-3. Longitudinal growth assessment
Values presented as mean ± SD. A,B: nose to anus length. C,D: tibia length. **p<0.01; n=7-10 mice per group.

4.3 Body Composition and Adiposity

Body composition was assessed at age 13 weeks, prior to physiological assessments of glucose homeostasis. No effect of neonatal treatment on body fat or lean mass percentage in females (Figure 4-4A, 4-4C) or males (Figure 4-4B, 4-4D) was observed.

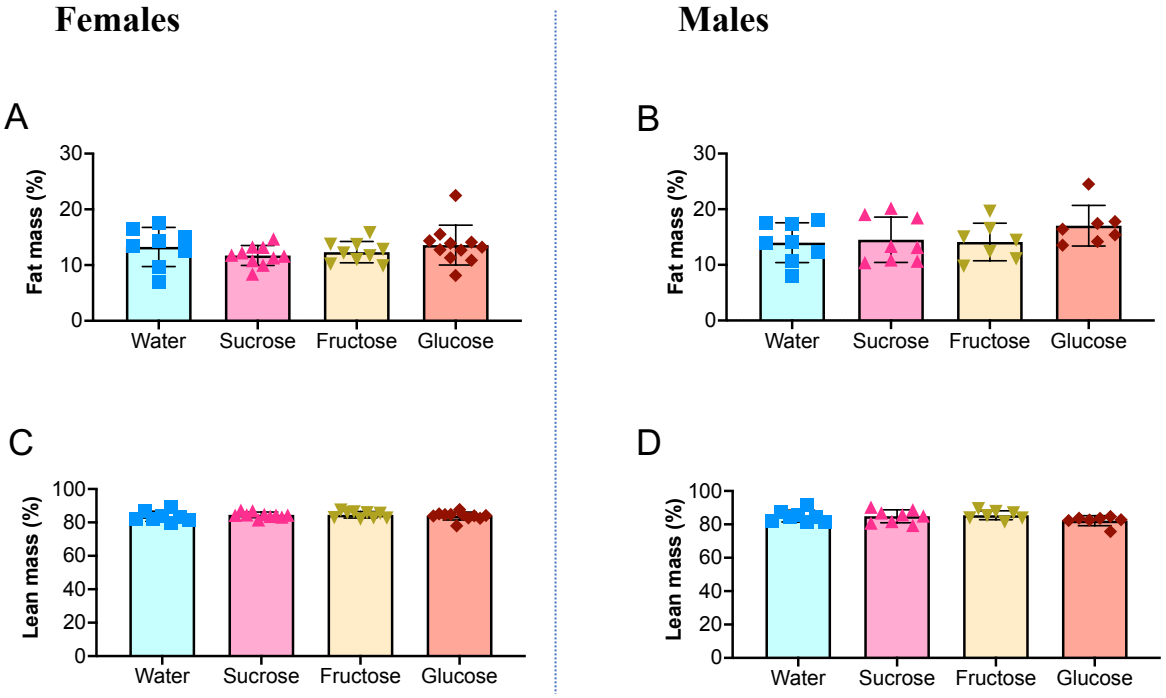

Figure 4-4. Body composition during adulthood
Values were normalized to total body weight and are presented as mean ± SD. A, B: fat mass. C,D: lean mass. n=7-10 mice per group.

Mesenteric, retroperitoneal, gonadal, subcutaneous and brown fat were dissected and weighed. No effect of neonatal treatments on individual fat pad weights or total adiposity were observed in female (Table 4-1) or male (Table 4-2) mice.

Table 4-1. Female total adiposity and individual fat pad weight

	Treatment Group			
Fat Depot (% total body weight)	Water	Sucrose	Fructose	Glucose
Total adiposity	5.15 ± 1.45	4.70 ± 0.87	5.34 ± 1.58	5.34 ± 1.56
Mesenteric	0.77 ± 0.30	0.78 ± 0.23	0.88 ± 0.23	0.81 ± 0.18
Retroperitoneal	0.52 ± 0.21	0.49 ± 0.13	0.54 ± 0.22	0.55 ± 0.18
Gonadal	1.98 ± 0.58	1.65 ± 0.38	2.09 ± 0.62	2.00 ± 0.74
Subcutaneous	1.59 ± 0.42	1.51 ± 0.18	1.64 ± 0.37	1.68 ± 0.46
Brown	0.28 ± 0.04	0.26 ± 0.03	0.27 ± 0.06	0.28 ± 0.05

Values presented as mean ± SD. n=7-10 mice per group.

Table 4-2. Male total adiposity and individual fat pad weight

	Treatment Group			
Fat Depot (% total body weight)	Water	Sucrose	Fructose	Glucose
Total adiposity	6.61 ± 2.05	6.79 ± 2.36	7.27 ± 1.45	7.94 ± 1.75
Mesenteric	0.95 ± 0.34	0.95 ± 0.28	0.99 ± 0.16	1.14 ± 0.20
Retroperitoneal	0.80 ± 0.42	0.92 ± 0.45	1.03 ± 0.22	1.02 ± 0.54
Gonadal	2.85 ± 0.83	2.89 ± 1.08	3.02 ± 0.66	3.37 ± 0.84
Subcutaneous	1.68 ± 0.43	1.68 ± 0.51	1.85 ± 0.46	2.01 ± 0.45
Brown	0.32 ± 0.10	0.33 ± 0.08	0.35 ± 0.06	0.38 ± 0.11

Values presented as means ± SD. n=7-10 mice per group

4.4 Organ Weights

No effect of neonatal treatment on brain, liver, pancreas, adrenal glands, kidney and heart size in female (Table 4-3) or male (Table 4-4) mice.

Table 4-3. Female tissue distribution

	Treatment Group			
Organ (% total body weight)	Water	Sucrose	Fructose	Glucose
Brain	2.07 ± 0.12	2.13 ± 0.14	2.03 ± 0.10	2.07 ± 0.15
Liver	3.95 ± 0.25	3.94 ± 0.25	3.85 ± 0.24	3.91 ± 0.26
Pancreas	1.19 ± 0.17	1.17 ± 0.19	1.07 ± 0.13	1.15 ± 0.12
Adrenal glands	0.05 ±0.01	0.05 ± 0.02	0.04 ± 0.01	0.05 ± 0.01
Kidneys	1.03 ± 0.05	0.97 ± 0.06	0.99 ± 0.06	1.02 ± 0.07
Heart	0.54 ± 0.02	0.57 ± 0.10	0.54 ± 0.05	0.55 ± 0.05

Values presented as mean ± SD. n=7-10 mice per group

Table 4-4. Male tissue distribution

	Treatment Group			
Organ (% total body weight)	Water	Sucrose	Fructose	Glucose
Brain	1.61 ± 0.12	1.57 ± 0.20	1.53 ± 0.09	1.53 ± 0.11
Liver	3.68 ± 0.29	3.43 ± 0.31	3.70 ± 0.38	3.87 ± 0.39
Pancreas	1.02 ± 0.12	1.06 ± 0.32	1.01 ± 0.16	0.92 ± 0.17
Adrenal glands	0.03 ±0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
Kidneys	0.95 ± 0.08	1.02 ± 0.19	0.92 ± 0.05	0.92 ± 0.02
Heart	0.49 ± 0.04	0.53 ± 0.06	0.52 ± 0.04	0.48 ± 0.04

Values presented as mean ± SD. n=7-10 mice per group

4.5 Insulin-like Growth Factor-1 (IGF-1) and Fibroblast Growth Factor-21 (FGF21)

I investigated whether the differences in body weight and bone length of the adult female sucrose-treated mice compared to water-treated mice was accompanied by differences in serum IGF-1 concentrations[99]. Adult sucrose-treated female mice had lower serum IGF-1 concentrations compared to water-treated female mice (577.32 ± 126.14 ng/mL vs 742.99 ± 143.57 ng/mL respectively; p=0.028, Figure 4-5A). I further investigated if the differences in serum IGF-1 concentrations were accompanied by differences in liver *Igf-1* mRNA expression but found no effect of neonatal treatments (Figure 4-5C). I also assessed IGF-1 receptor protein expression in skeletal muscle and found no effect of neonatal treatment (Figure 4-5I).

Dietary sucrose, fructose and glucose stimulate expression (mRNA and protein) of liver FGF21[155, 156],which inhibits IGF-1[114]. I postulated that the lower serum IGF-1 concentrations in adult sucrose-treated female mice would be accompanied by a higher FGF21 levels. However, I observed no effect of neonatal sucrose treatment on liver *Fgf21* mRNA or serum FGF21 concentrations in female mice (Figure 4-5E, 4-5G). No differences in serum IGF-1 and FGF21 concentrations or liver *Igf*-1and *Fgf21* mRNA were observed in male mice (Figure 4-5B, 4-5D, 4-5F, 4-5H).

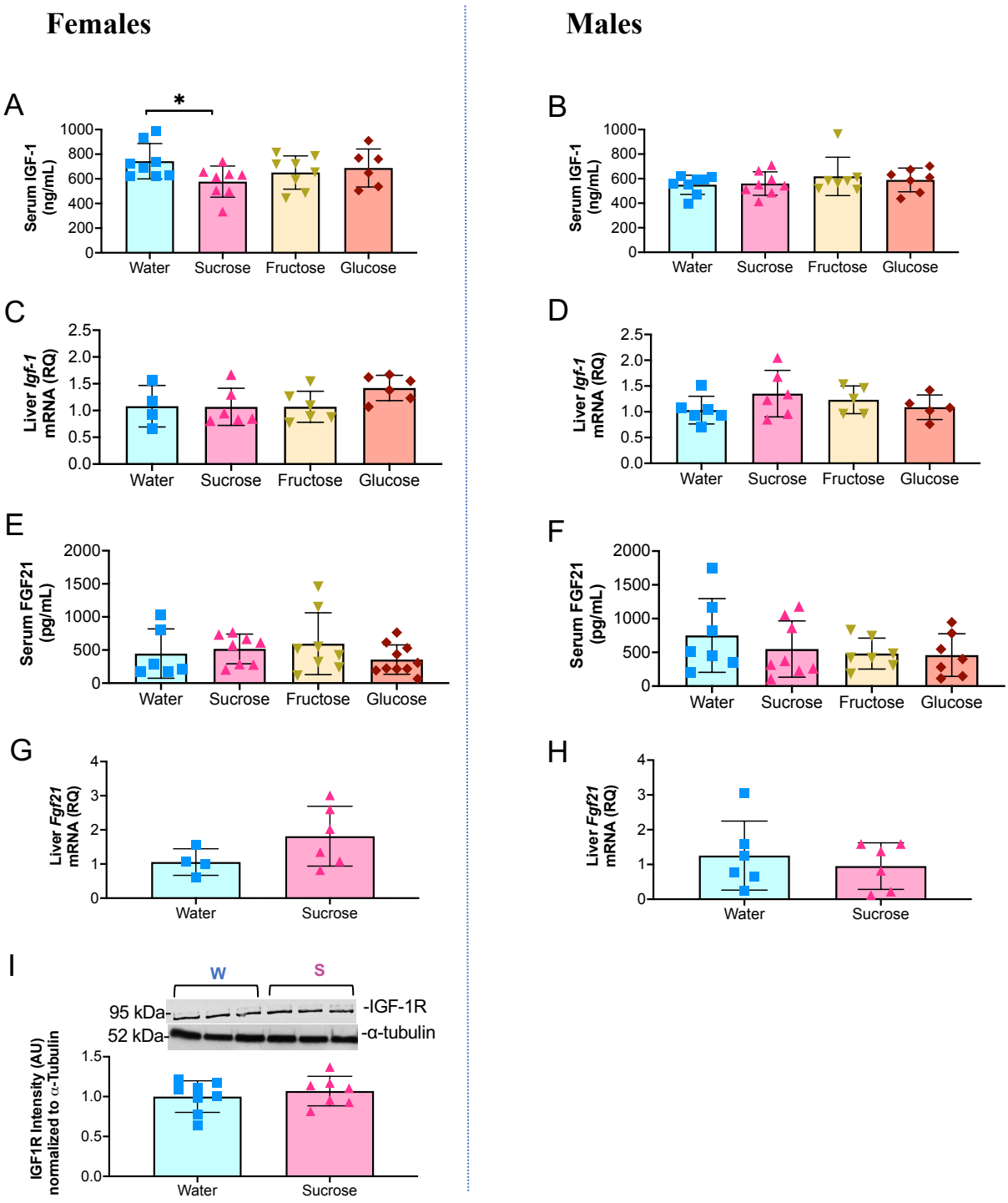

Figure 4-5. Serum IGF-1 and FGF21 concentrations and liver *Igf-1* and *Fgf21* mRNA
Values presented as mean ± SD. A,B: serum IGF-1 concentrations. C,D: liver *Igf-1* mRNA expression. E,F: serum FGF21 concentrations. G,H: liver *Fgf21* mRNA expression I: expression of the IGF-1 receptor in skeletal muscle (rectus femoris). *$p<0.05$; n=4-8 mice per group.

4.6 Small Intestine Morphology

Neonatal mice have an immature intestine, which may be sensitive to exposure to dietary components other than milk[147]. I investigated if the repeated sucrose treatment during the neonatal period affected small intestinal morphology. I postulated that the negative effect of sucrose on body size and growth could be related to alterations in intestinal morphology, thereby disturbing nutrient absorption. In addition, IGF-1 has well-known trophic effects in the small intestine and promotes villi and crypt proliferation and regeneration[157]. Given that sucrose digestion occurs predominantly in the jejunum, I performed histological analysis of jejunum from the female mice. I observed no differences in villi length, crypt depth or villi/crypt ratio in the sucrose-treated female mice compared to the water-treated female mice (Figures 4-6B, 4-6C, 4-6D).

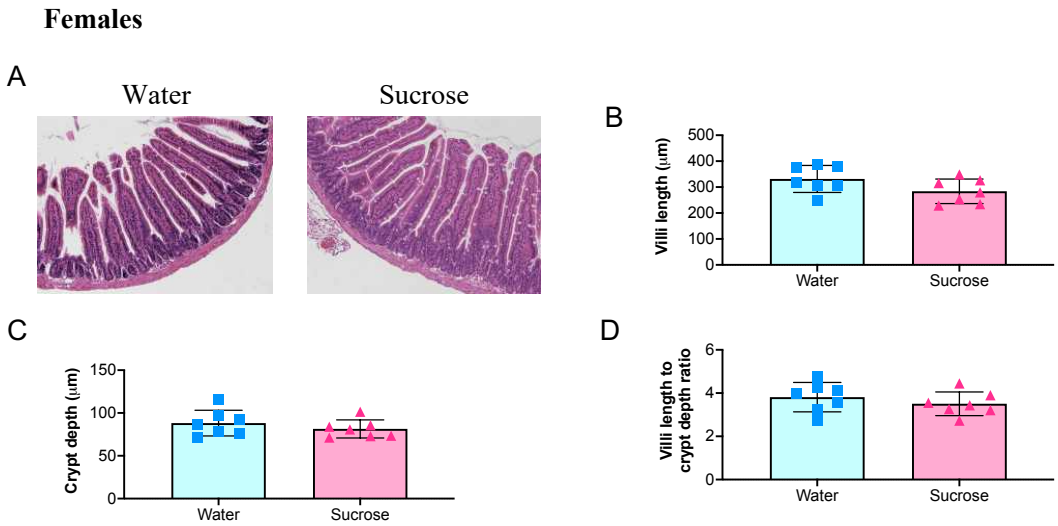

Figure 4-6. Morphology of female adult jejunum
Values presented as mean ± SD. A: Overview of adult female jejunum villi and crypts. B: villi length. C: crypt depth. D: villi/crypt ratio. n=7 mice per group.

Summary of Findings

My results suggest that in female mice, repeated neonatal sucrose treatment reduced weight gain during the suckling period and reduced tibia length in adulthood. This was accompanied by lower serum IGF-1 concentrations in adult females, suggesting a role for an IGF-1-dependent pathway. Furthermore, I found no effect of fructose or glucose, suggesting that the effects I observed are specific to sucrose and not to its monosaccharide components. No effects of neonatal sucrose treatments on growth or IGF-1 were observed in male mice. Neonatal sucrose treatment had no effect on adiposity or body composition in female or male mice.

Chapter 5: Assessment of Glucose Homeostasis

5.1 Glucose Metabolism at Weaning

I conducted IP glucose and insulin tolerance tests to assess glucose tolerance and insulin sensitivity in the pups at weaning. No differences were observed in fasting blood glucose (Figure 5-1A, 5-1B), glucose tolerance (Figure 5-1C, 5-1D), or insulin tolerance (Figure 5-1E, 5-1F) in male or female mice.

5.2 Glucose Metabolism During Adulthood

I observed no differences in fasting blood glucose (Figure 5-2A, 5-2B), glucose tolerance (Figure 5-2C, 5-2D), insulin tolerance (Figure 5-2E, 5-2F), fasting serum insulin concentrations (Figure 5-3A, 5-3B), or GSIS (Figure 5-3C, 5-3D) in male or female adult mice from the different treatment groups.

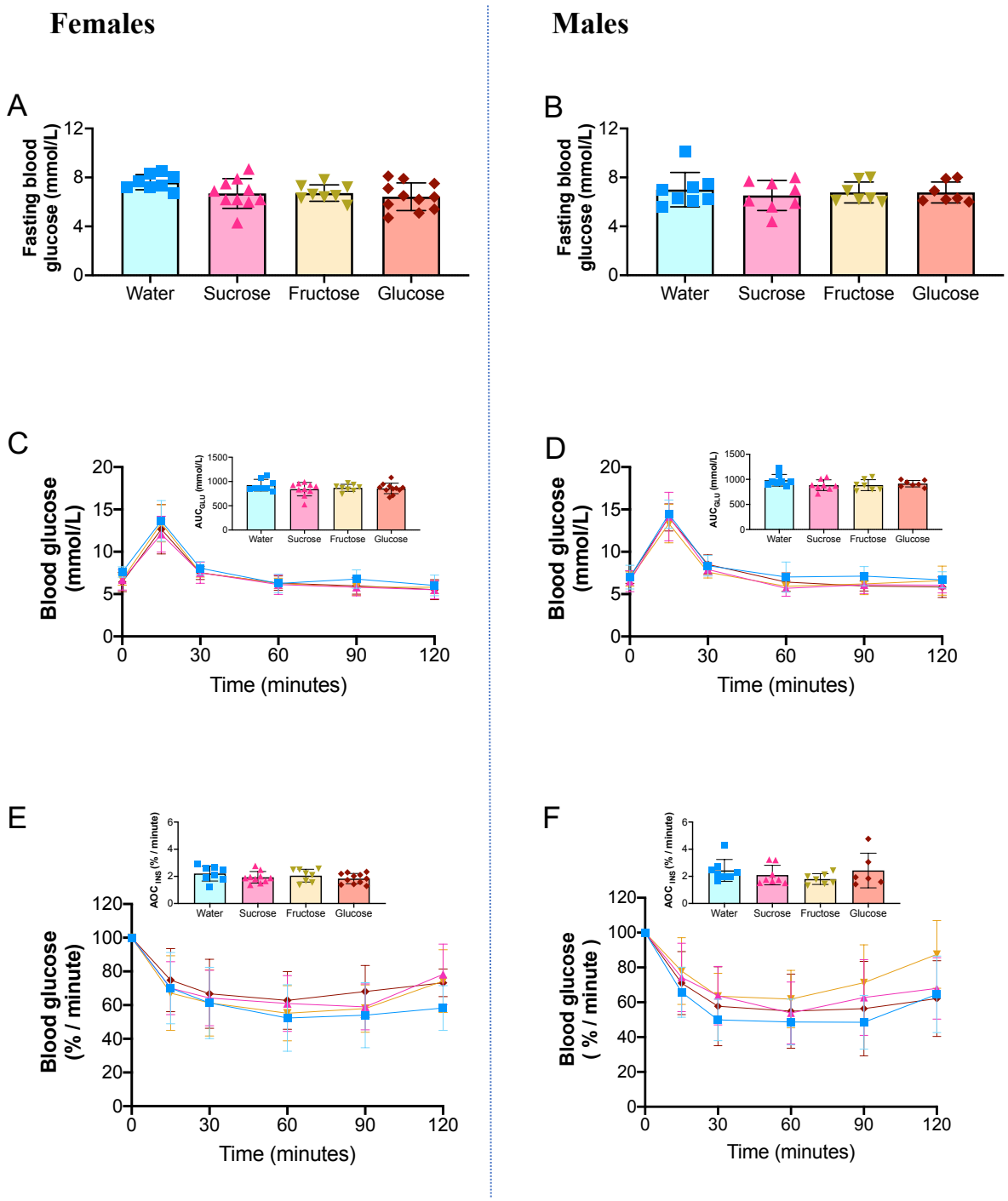

Figure 5-1. Glucose and insulin tolerance in mice at weaning.

Values presented as mean ± SD. A,B: fasting blood glucose in 3-week old mice. C, D: intraperitoneal glucose tolerance test and area under the curve (AUC) in 3 week-old mice. E,F: intraperitoneal insulin tolerance test and area over the curve (AOC) in 4 week-old mice. n=7-10 mice per group.

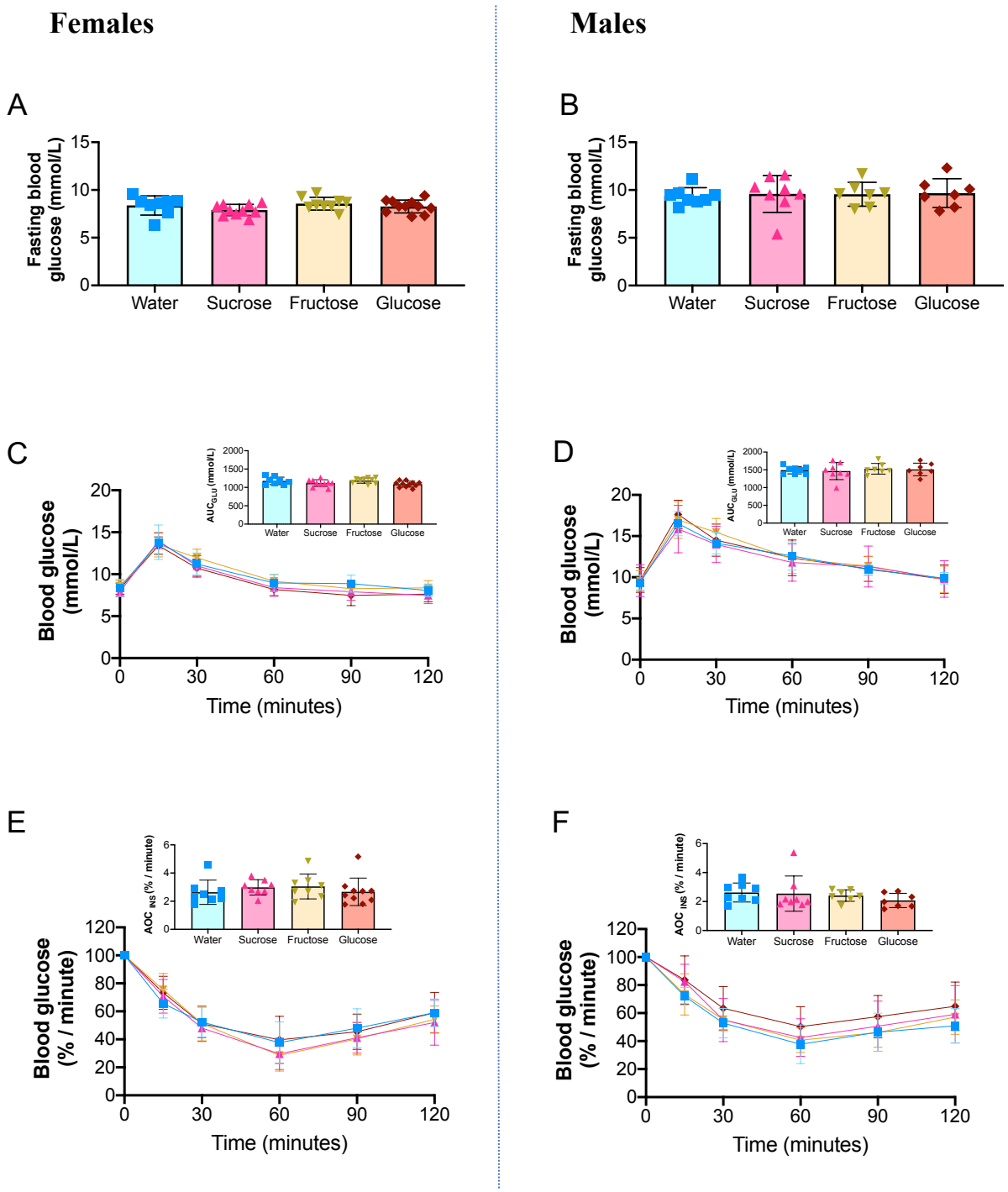

Figure 5-2. Glucose and insulin tolerance in adult mice.

Values presented as mean ± SD. A,B: fasting blood glucose in 13-week old mice. C, D: intraperitoneal glucose tolerance test and area under the curve (AUC) in 13 week-old mice. E,F: intraperitoneal insulin tolerance test and area over the curve (AOC) in 14 week-old mice. n=7-10 mice per group.

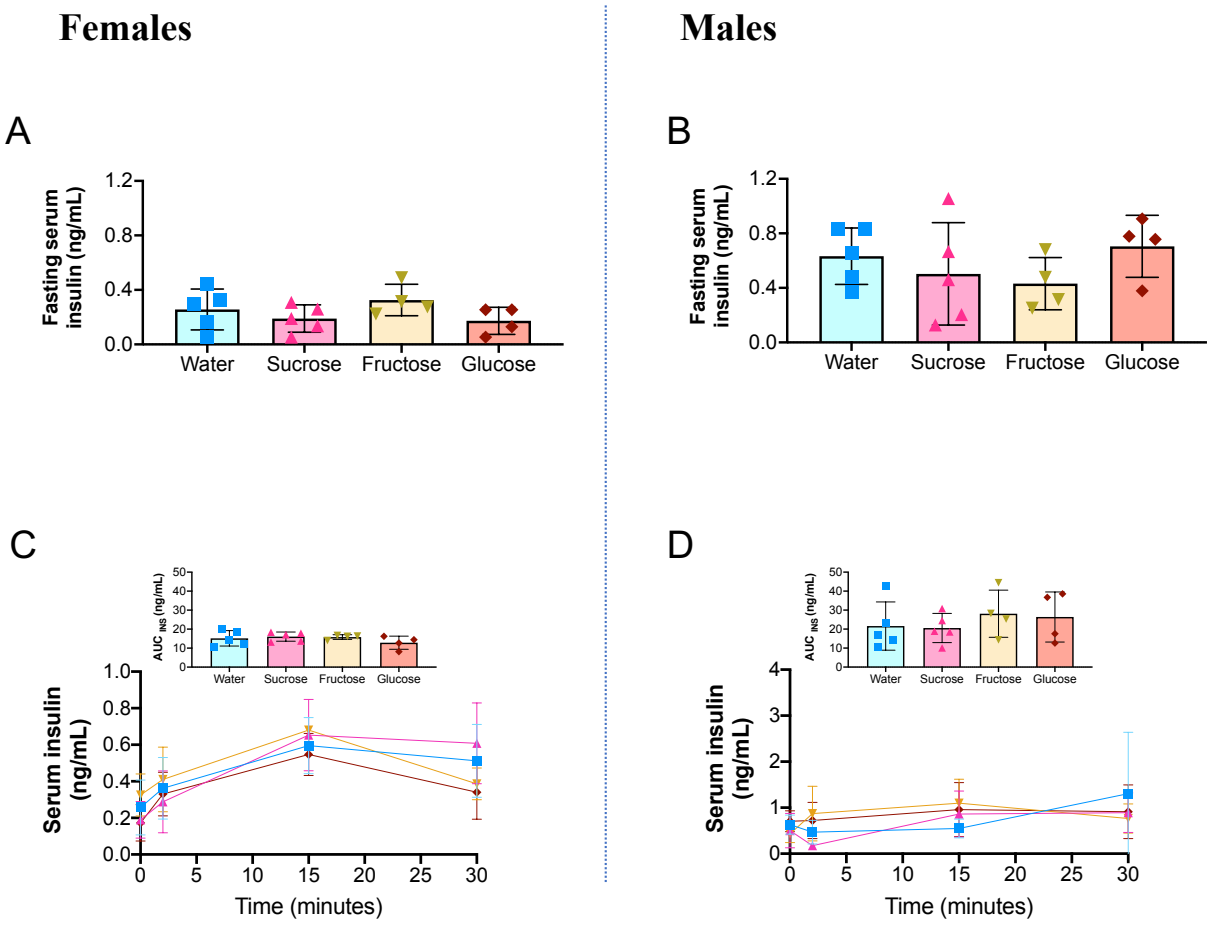

Figure 5-3. Glucose-stimulated insulin secretion in adult mice.
Values presented as mean ± SD. A,B: fasting serum insulin in 15 week-old mice. C, D: glucose-stimulated insulin secretion test and area under the curve (AUC) in 15 week-old mice. n=4-5 mice per group.

Chapter 6: Liver Choline Metabolites

Choline is essential for growth, brain development, lipid metabolism, and cellular methylation reactions[132]. Given that fructose, a component of sucrose, activates lipogenic pathways and disturbs choline metabolism in the liver[74] I postulated that neonatal sucrose treatment may affect choline metabolism in the liver.

Interestingly, I observed that adult female mice that received neonatal sucrose treatment had lower free choline (Figure 6-1A), phosphocholine (Figure 6-1C) and glycerophosphocholine (Figure 6-1E) concentrations in the liver, and higher betaine concentrations in the liver (Figure 6-1G) compared to water-treated female mice. These findings suggest that in female mice, neonatal sucrose treatment has long-term impact on liver choline metabolism. I found no differences in liver water-soluble choline metabolites in the adult male mice (Figure 6-1B, 6-1D, 6-1F, 6-1H).

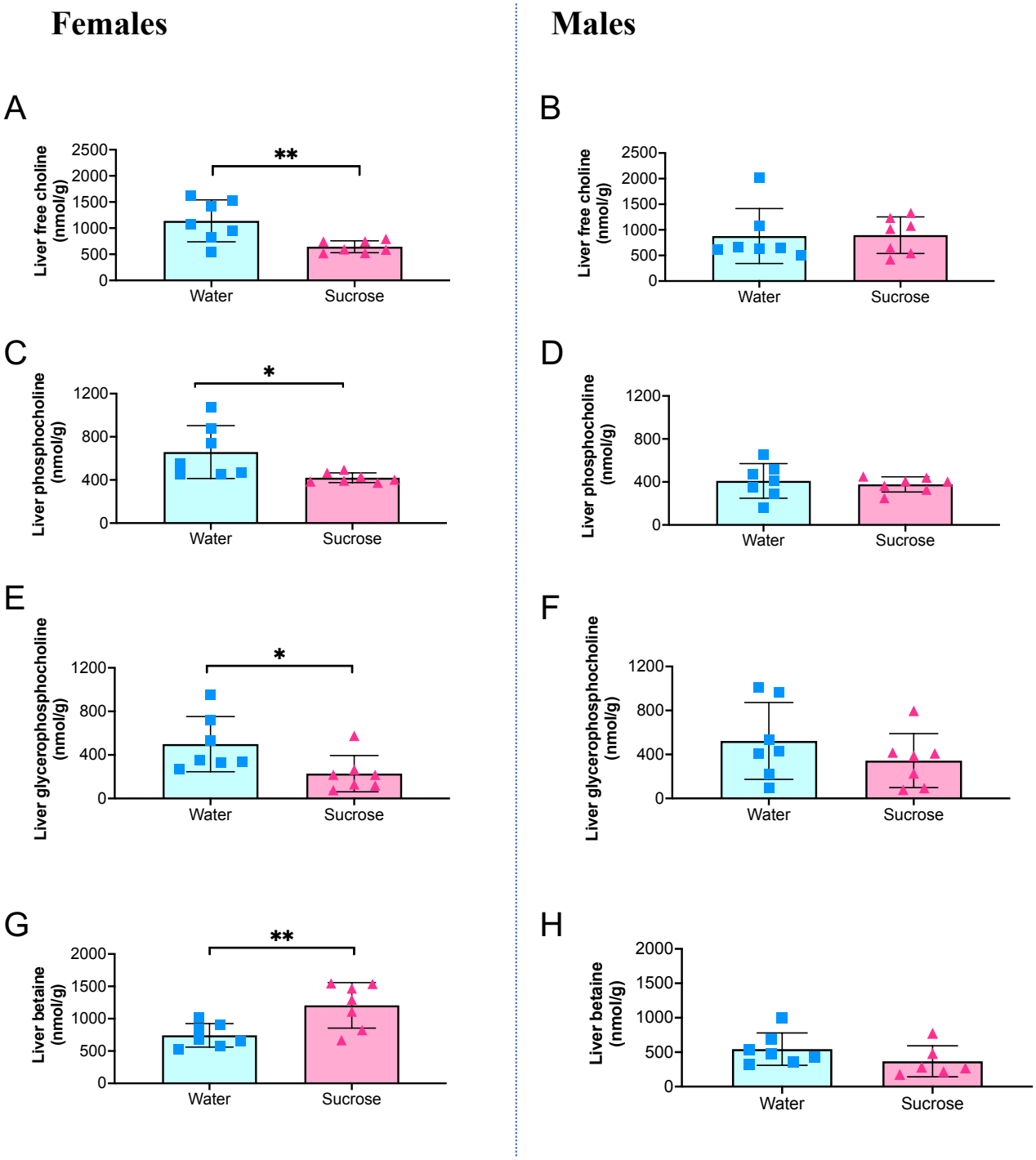

Figure 6-1. Water-soluble choline metabolites in the liver

Values presented as mean ± SD. A,B: free choline. C, D: phosphocholine, E,F: glycerophosphocholine, G,H: betaine. *p<0.05, **p<0.01; n=6-7 mice per group.

Phosphocholine is a major substrate for synthesis of phosphatidylcholine, a necessary component of very-low-density lipoproteins[135] that transport triglycerides from the liver. Therefore, I postulated that the lower liver phosphocholine concentrations in neonatal sucrose-treated adult female mice may be accompanied by triglyceride accumulation in the liver. However, I found no differences in liver triglyceride concentrations between neonatal sucrose-treated and water-treated adult female mice (Figure 6-2A, 6-2C); a similar finding was also observed in the male mice (Figure 6-2B, 6-2D).

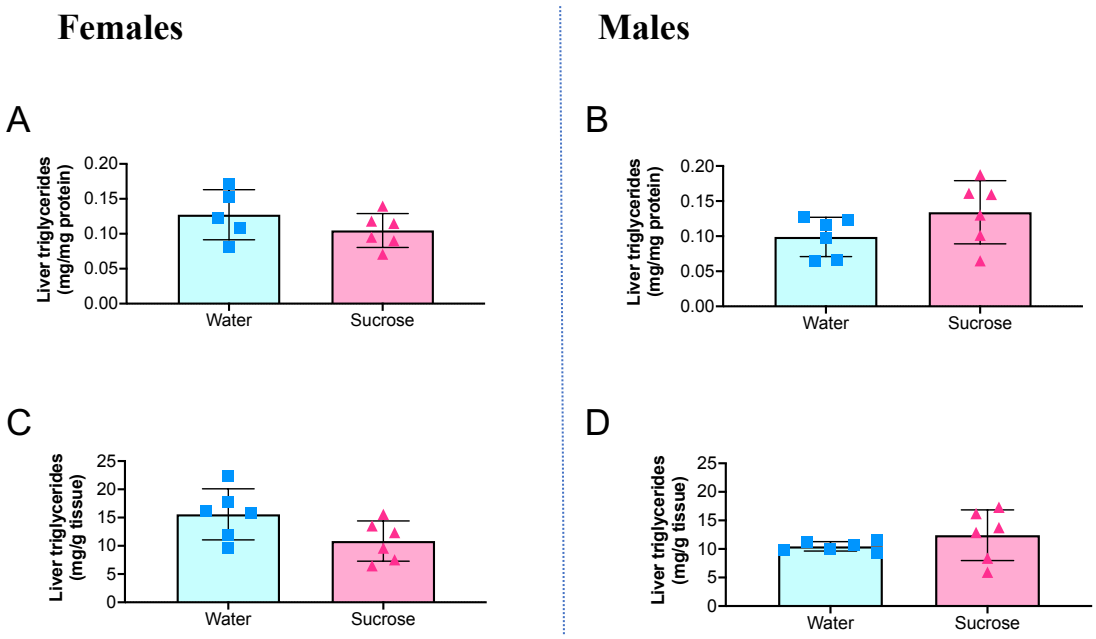

Figure 6-2. Liver triglycerides
Values presented as mean ± SD. A,B: data presented in mg/mg of protein. C, D: data presented in mg /g tissue. n=6 mice per group.

Chapter 7: Discussion

I evaluated the long-term effects of neonatal sucrose treatment on growth, adiposity, glucose homeostasis and liver choline metabolism using a mouse model[39]. To my knowledge, there are no published studies exploring the metabolic effects of neonatal sucrose treatment. My research directly addresses the knowledge gaps highlighted recently by the American Academy of Pediatrics[29] that more research is needed on the safety and long-term effects of neonatal sucrose treatment. My thesis research identified three main findings. First, I found sex-specific differences in growth. Female mice that received sucrose during the neonatal period gained less weight during the suckling period and were smaller at weaning. By adulthood, the weight of sucrose-treated females was similar to the other groups, suggesting catch-up growth. However, sucrose-treated female mice had reduced tibia length in adulthood, suggesting enduring effects on growth. These effects were accompanied by reduced serum IGF-1 concentrations in adulthood, suggesting a role for IGF-1 mediated pathways in the effects of sucrose treatment on growth. Second, I observed alterations in liver water-soluble choline metabolites in neonatal sucrose-treated adult female mice. Given the important role of choline in brain development, alterations in liver choline metabolism could contribute to the adverse effects of neonatal sucrose treatment on brain size and neurodevelopment[39, 40]. Third, contrary to my hypothesis, I observed no effects of neonatal sucrose treatment on adiposity or physiological assessments of glucose homeostasis.

7.1 Effects on Growth

I found that sucrose-treated female mice gained less weight at the end of the treatment (P7) and during the suckling period compared to the rest of the female groups. The first published study[39] with the same mouse model that I used also reported differences in weight gain. Mice receiving oral sucrose during the neonatal period gained less weight at the end of the treatment (mean difference of weight P7-P1) compared to water controls. However, this difference did not reach statistical significance (p=0.078) possibly because male and female mice were analyzed together.

During the post-weaning period, the weight differences I observed during the suckling period were no longer present, and the female sucrose-treated mice had similar body weights at age 16 weeks compared to the rest of the groups. My findings are in agreement with the studies by Tremblay et al.[39, 40] that reported no effect of neonatal sucrose treatment on final body weight of adult mice (age 13 weeks). In preterm infants, only a small randomized controlled trial (n=43) has evaluated weight gain in sucrose-treated preterm infants (<32 weeks of gestation) at hospital discharge and report no difference between sucrose-treated infants and infants treated with water[158].

It is not clear why sucrose treatment in the neonatal period affects growth and body weight. However, experiments in older mice have reported similar results. Eight week-old C57BL/6J mice fed a high-sucrose diet (38.5% of the energy) for 15 weeks were found to gain less weight, and this was accompanied by increased energy expenditure and FGF21 expression in the liver (mRNA) and plasma (protein)[93]. It is well known that dietary fructose and glucose activate ChREBP, a transcriptional factor in the liver that stimulates FGF21 synthesis[159]. Activation of FGF21 downregulates expression of STAT5b, a main transcriptional regulator of

IGF-1 expression[114]. I speculated that lower serum IGF-1 concentrations would be accompanied by higher serum FGF21 concentrations. However, I found no effect of neonatal sucrose treatment on serum FGF21 or liver Fgf21 mRNA expression in adult mice suggesting the growth and body size differences I observed involves pathways unrelated to FGF21. Nevertheless, there is a possibility that sucrose treatment during the neonatal period produced an acute and transient increase in FGF21 expression and thereby downregulated systemic IGF-1 concentrations during critical timepoints in development leading to the differences in body weight gain and bone size. There is also the possibility that oral sucrose treatment reduces milk intake during treatment. Along this line, young female Sprague-Dawley rats (age 28 days; n=44) given a 13% sucrose solution *ad libitum* for 8 weeks consumed 30% less food than those receiving tap water[160].

Another interesting finding I observed, is that tibia length was smaller in adult female mice treated with neonatal sucrose. This finding suggests that the early disturbances in growth also impacted tibia chondrocyte activity in the growth plate, as the highest tibia elongation rates in mice occurs in the first weeks after birth (~2.6 mm increments per week)[161]. Sucrose could also directly interfere with bone growth during this important developmental time point. In this context, numerous studies in adult rats have reported negative effects of sucrose-containing diets on bone density, strength and mineral concentration and that these effects are more prominent in female mice than male mice[162-164]. For example, Tjäderhane et al.[162] reported that young Wistar female rats (age 21 days; n=36) fed a high sucrose diet (43g / 100g of diet) for 5 weeks had lighter and weaker bones (tibia and femur), lower bone density and decreased calcium/phosphorous bone concentrations than females fed a high starch diet (43g / 100g of diet). Interestingly in humans, a cross-sectional study reported that children aged 2-5 years with high consumption of sugar (>12 oz of industrial fruit juice per day; estimated by 7-day dietary

records) were shorter than children with lower consumption of sugar (<12 oz of industrial fruit juice per day)[128]

The mechanisms underlying the effects of sucrose on bone are not clear but several different mechanisms have been proposed. For example, dietary sucrose-stimulated hyperinsulinemia may induce hypercalciuria and reduce reabsorption of calcium in the kidneys; thereby, reducing bone mineral concentration[165, 166]. Further, *in vitro* studies demonstrated that under high glucose conditions (49.5 mmol/L), MG-63 (human osteosarcoma) cells have impaired response to IGF-1 stimulation and decreased cell growth suggesting inhibition of osteoblast proliferation[167]. From my findings, it is difficult to speculate on the underlying mechanism for my observations because I only assessed one indicator of tibia growth and did not measure growth plate width, bone mineral density, periosteal bone formation or chondrocyte proliferation. Future studies should be aimed at investigating the effects of neonatal sucrose treatment on these parameters.

Given the differences in growth and bone size that I observed, I speculated that neonatal sucrose treatment may affect growth factors. Despite finding lower serum IGF-1 concentrations in female mice that received neonatal sucrose treatment, I found no effect on liver *Igf-1* mRNA expression. Many posttranscriptional and posttranslational modifications regulate IGF-1 protein expression such as miRNAs silencing[168] and proteolytic processing of proIGF-1 to mature IGF-1[169]; therefore, mRNA transcripts do not necessarily correlate with protein expression[168, 169]. Further studies are required to evaluate the regulatory mechanisms of IGF-1 that occurred in neonatal sucrose-treated female mice.

I further speculated that the lower serum IGF-1 concentrations would be associated with changes in IGF-1R expression, however, I found no differences in IGF-1R protein expression in

skeletal muscle. Further research is required to evaluate IGF-1R functionality by evaluating IGF-1R phosphorylation or downstream IGF-1 signalling pathways such as phosphatidylinositol 3-kinase (PI3K)/Akt or the Shc/mitogen activated protein kinase (MAPK)[170].

The effects of neonatal sucrose treatment on growth and bone size were sexually dimorphic with no effects observed in male mice. The underlying mechanism that accounts for this sex-specific phenotype are unknown but may involve a combination of hormonal and genetic influences[171]. Although the expression of sex steroids occurs during puberty, in males there is a transitory release of testosterone during the late embryonic period up to the first week of life that is critical for brain development [172]. It is also well-known that growth hormone can be stimulated by testosterone [173]; therefore, it is possible that the disruption of growth elicited by sucrose during the neonatal period did not affect males due to the transitory protection conferred by testosterone. Furthermore, the releasing pattern of growth hormone in female mice is characterized by frequent peaks and shorter intervals whereas in male mice the peaks are higher and less frequent[174]. Therefore, neonatal sucrose treatment could impact growth hormone patterns by interfering with the frequent peaks of growth hormone in female mice.

7.2 Effects of Neonatal Sucrose on Choline Metabolites

My study is the first to report on effects of neonatal sucrose treatment on hepatic choline metabolism in adulthood. Importantly, all of my treatment groups were weaned onto a control diet, which provided adequate choline as choline bitartrate (Table 3-1). Free choline and phosphocholine are substrates required for the synthesis of phosphatidylcholine by the CDP-choline pathway [135]. In my study I found that neonatal sucrose-treated female mice had lower hepatic concentrations of free choline and phosphocholine as well as lower

glycerophosphocholine, a product generated by the deacylation of phosphatidylcholine (Figure 1-5). I was unable to quantify phosphatidylcholine in my samples, but I postulate that liver phosphatidylcholine would also be lower in neonatal sucrose treated female mice. Given that phosphatidylcholine is required for the assembly of VLDL, an important carrier of choline and long chain fatty acids[135, 175], my findings suggest that there could be disturbances in the delivery of these important nutrients to extra-hepatic tissues, including the brain. This could potentially explain the reduced regional brain size in adult mice[39] and impairments in neurodevelopment in both mice and preterm infants[38, 40] that received neonatal sucrose treatment.

In my study, hepatic betaine concentrations were higher in neonatal sucrose-treated adult female mice than in neonatal water-treated adult female mice. Betaine can be obtained from the diet or it is generated by the irreversible oxidation of choline, which occurs predominantly in liver, kidney and retina[143]. The control diet that the mice were fed did not contain betaine. The higher liver betaine could be from enhanced synthesis or a reduction in the use of betaine to re-methylate homocysteine to methionine. It is also unclear why choline metabolites are altered in neonatal sucrose-treated female mice but not male mice. Further research is required to investigate the underlying mechanisms accounting for these sex-specific differences.

7.3 Effects on Adiposity

Fructose and glucose metabolism in the liver stimulate several lipogenic pathways[72]. The adverse effects of sucrose on adiposity is attributed to fructose, which has been shown to increase adiposity and visceral fat in both mice and humans[176]. Although I speculated that neonatal fructose- and sucrose-treated mice would have greater adiposity I found no effects of the treatments on fat mass or visceral adiposity. My results contrast the few studies that have

investigated the effects of dietary fructose on body composition during the suckling period. Neonatal mice suckling from dams receiving a 10% fructose solution[172] or suckling rats that were fed with rat-milk substitutes (50% of rat milk lactose substituted by fructose)[173] had greater fat pad weight, and adipocyte area and diameter compared to control mice[177, 178]. It could be that the duration and the amounts of fructose and sucrose provided by the neonatal treatments are much less than those found to affect adiposity.

7.4 Effects on Glucose Homeostasis

Blood glucose and insulin concentrations acutely rises after consumption of solutions containing either sucrose or glucose[177, 179]. I found no effect of neonatal sucrose, glucose or fructose treatment on glucose homeostasis in mice at weaning or adulthood. My intervention may not have been long enough to cause disturbances in glucose homeostasis as detrimental effects of dietary sucrose/fructose/glucose occurs after several weeks of feeding. Further, it is well-stablished that switching from consumption of sugar-sweetened solutions to water improves glucose and insulin tolerance[180]. As such, it is possible that disturbances in glucose homeostasis occurs only during the neonatal treatment period and disappears later during development.

7.5 Differential Effects of Sucrose, Fructose and Glucose

It is unclear why there is an effect of neonatal sucrose treatment on growth and body size in female mice but no effects of fructose- or glucose-treatments. One possibility is that the hedonic properties of sucrose, could discourage the consumption of breastmilk during the critical developmental window with long-lasting effects on growth. In adult rats and mice there is a greater preference for sucrose solutions than glucose or fructose[181, 182]. In suckling rats, the

mRNA expression of the intestinal fructose transporter, GLUT 5, is very low (less than 70%) compared to expression in post-weaning rats[63]. However, fructose absorption is stimulated if it is co-ingested with glucose[65]. It may be that fructose given as a single monosaccharide in the neonatal oral treatment was not absorbed, but the fructose component in the sucrose-treated female mice was fully absorbed and metabolized in the small intestine because of co-ingestion with glucose, and thereby, elicited the biological effects.

7.6 Conclusions and Limitations

The overarching summary of my findings is depicted in Figure 7-1. Female mice that received neonatal sucrose treatment gained less weight during the suckling period and were smaller at weaning. In adulthood, these female mice had similar body weight to the other females but had smaller tibias and lower serum IGF-1 concentrations, suggestion some long-term effects on growth. This was accompanied by alterations in liver choline metabolites suggesting long term effects of neonatal sucrose treatment on liver choline metabolism; the biological relevance of these findings remain to be determined. There were no effects of neonatal sucrose treatment adiposity or glucose homeostasis in male or female mice.

It is important to consider the limitations in my study. The effects of neonatal sucrose treatment on bone are limited to tibia length as I did not thoroughly evaluate tibia strength, width, mineral concentrations, or chondrocytes activity. I did not measure tissue-specific production of IGF-1, which could account for the lower serum IGF-1 concentrations. I quantified total IGF-1R protein in skeletal muscle and did not assess activity, which may have been affected by neonatal sucrose treatment. I quantified hepatic water-soluble liver choline metabolites but

did not assess liver and brain phosphatidylcholine, sphingomyelin and acetylcholine, which are also important for growth and brain development.

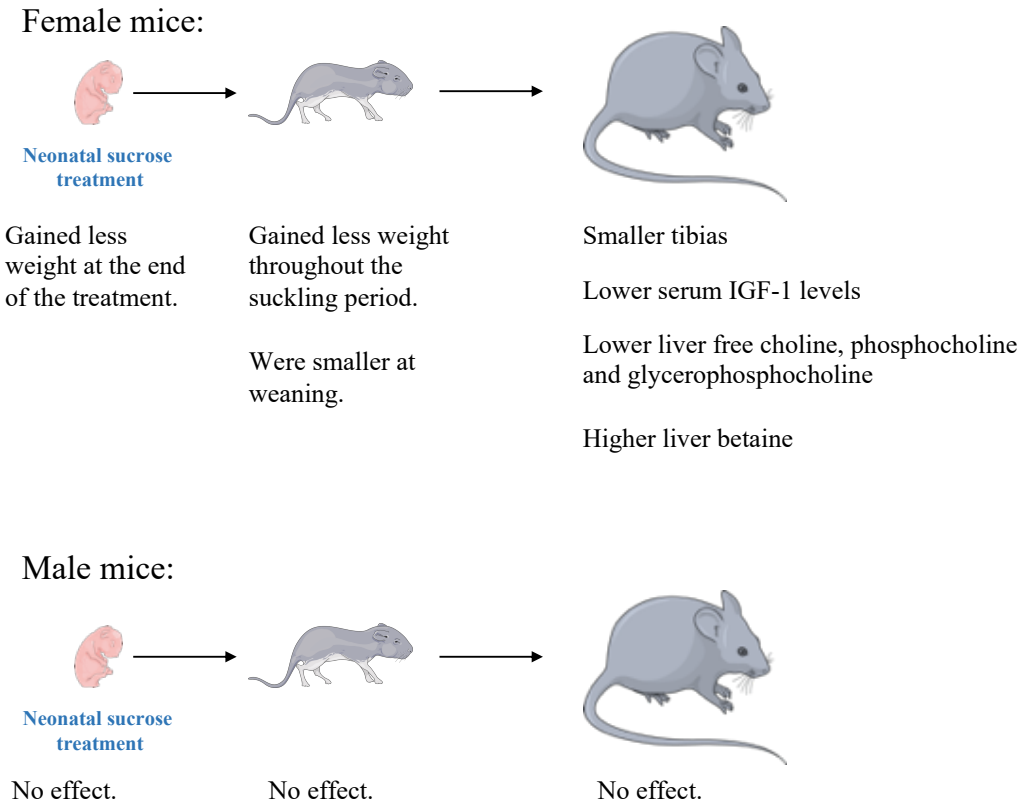

Figure 7-1. Overarching summary of thesis

7.7 Future Directions

There are several directions for further research. Studies assessing isotopic tracing of the sucrose molecules in neonatal mice to assess its metabolic fate immediately after the first treatment (P1), and at the end of the cumulative treatments (P7) would improve our understanding of sucrose metabolism during the early neonatal period. More elaborate assessments of growth factors such as growth hormone, circulating IGF-1 binding proteins, and the functionality of IGF receptors during the treatment and posttreatment are required.

Assessment of bone growth, such as chondrocyte activity and tibia-specific IGF-1 production would improve the understanding of how neonatal sucrose affects bone. Determining if the hedonic properties of sucrose affects suckling from the dams and milk intake by the pups will provide insight into whether reduce milk intake underlies reduced growth in the sucrose-treated pups. Further research should also evaluate other hormonal factors such as glucocorticoids or thyroid hormone[183], that have key roles in growth and chondrocyte activity of the growth plate.

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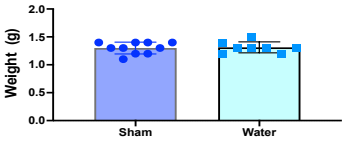
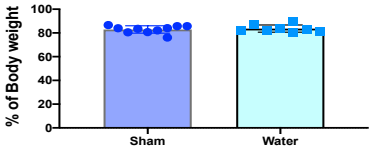
Appendix

Females

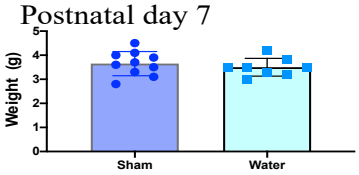
Birth weight



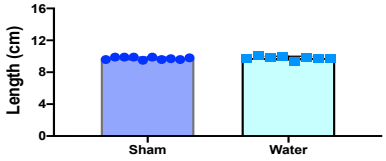
Lean mass



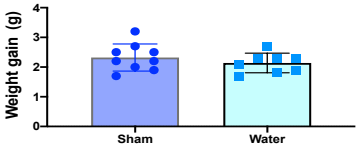
Postnatal day 7



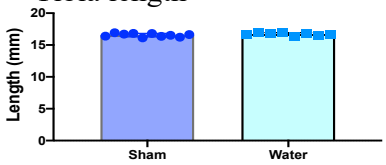
Nose to anus length



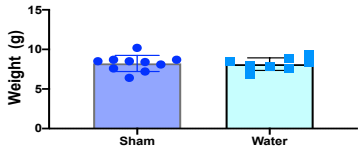
Weight gain (P7-P1)



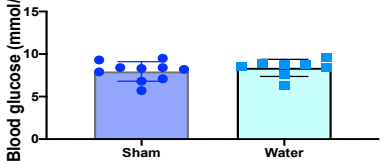
Tibia length



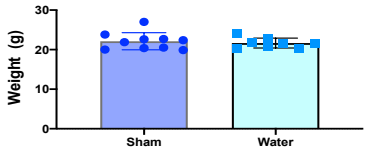
Weight at weaning



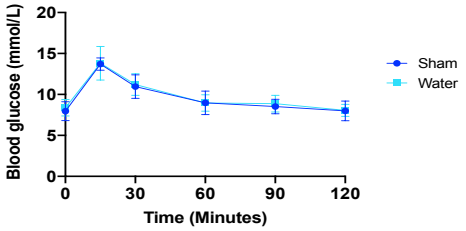
Fasting blood glucose



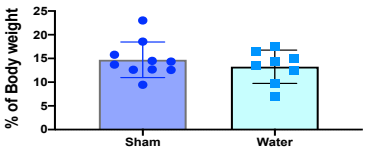
Weight at adulthood



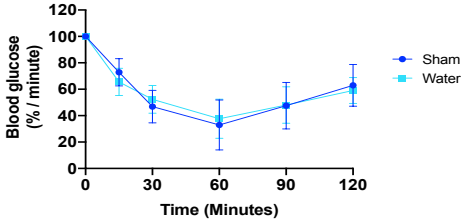
IPGTT adulthood



Fat mass

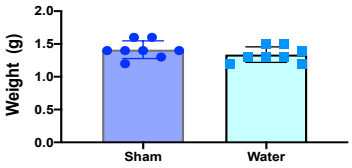


IPITT adulthood

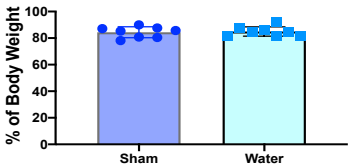


Males

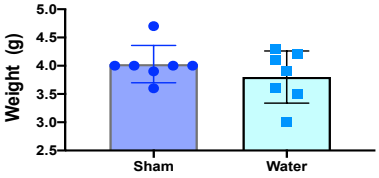
Birth weight



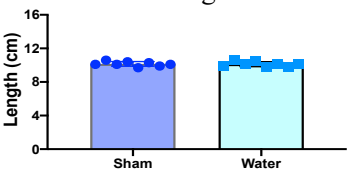
Lean mass



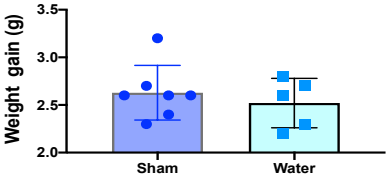
Postnatal day 7



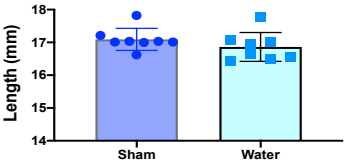
Nose to anus length



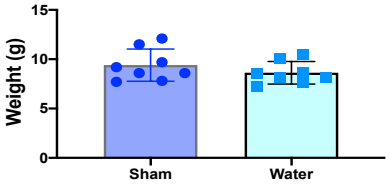
Weight gain (P7-P1)



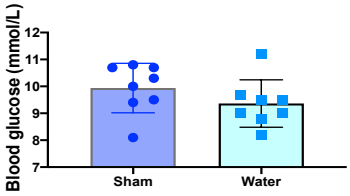
Tibia length



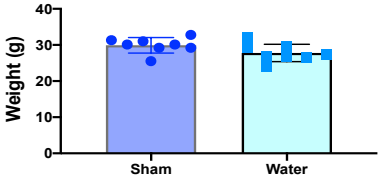
Weight at weaning



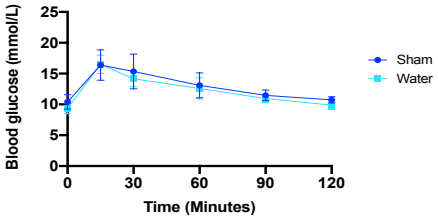
Fasting blood glucose



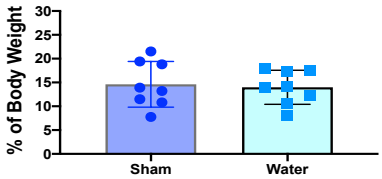
Weight at adulthood



IPGTT adulthood



Fat mass



IPITT adulthood