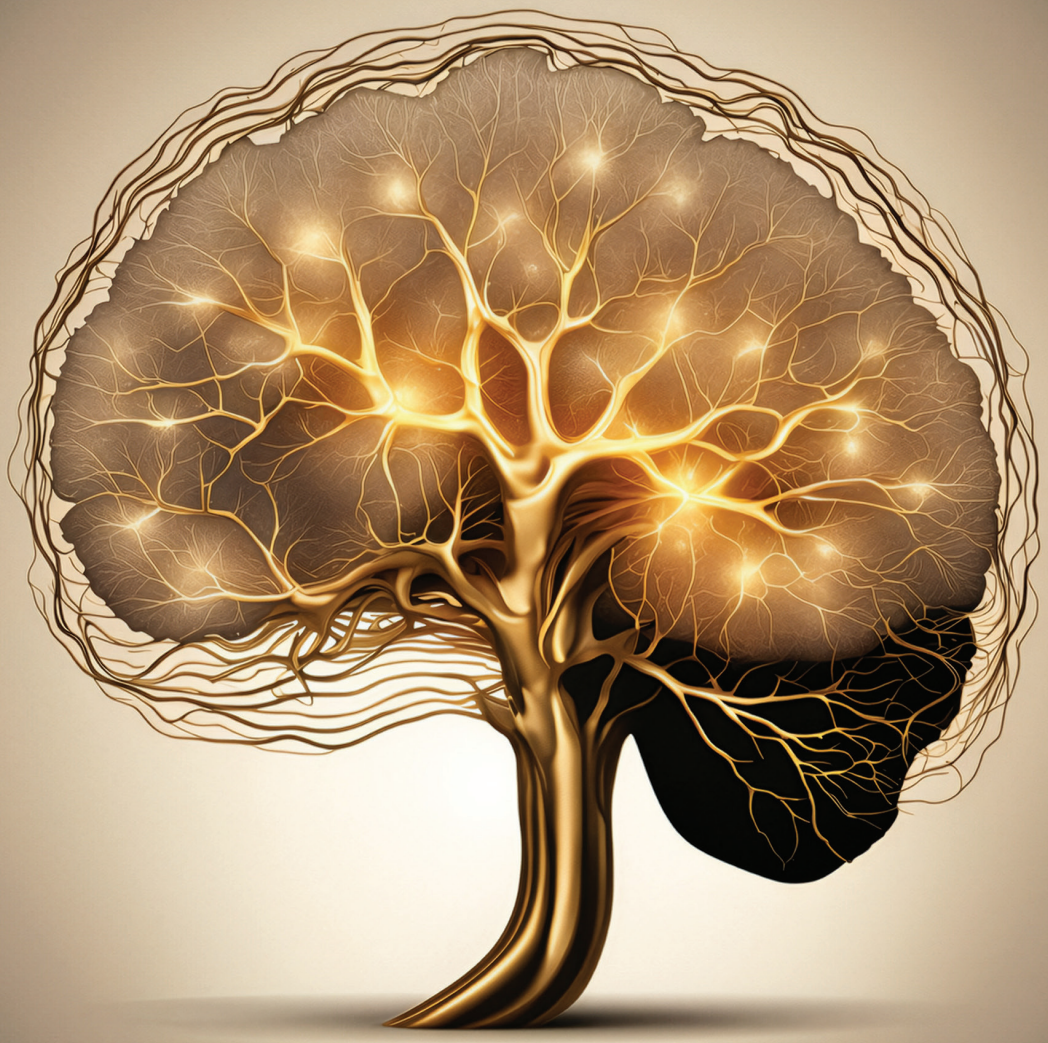
Food for Thought:

How Human Milk Oligosaccharides contribute to neural plasticity and cognitive development throughout the lifespan



Sylvia Docq

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by

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Table of contents

1

Chapter 1

General introduction

The benefits of breastmilk

Breastmilk as a primary nutritional source is recommended by the WHO (WHO, 2024) for at least the first six months of life, and preferably continues to be part of an infant's diet for the first few years. Human breastmilk is globally recognized as the golden standard for infant nutrition, because it is specifically tailored to the individual infant's nutritional and developmental needs (Ballard & Morrow, 2013, de Weerth et al., 2023). Unlike infant formula, it contains all the necessary nutrients and bioactive molecules, such as immunoglobulins, cytokines, chemokines, hormones, mucins, oligosaccharides and glycans. These bioactive molecules promote a prosperous development and immediate beneficial health outcomes, ranging from protection against infection to boosting the infant immune system and intestinal development. The beneficial effects of these molecules also extend beyond infancy, by mediating childhood health outcomes in metabolic disease (de Weerth et al., 2023). Considering all these protective effects on children's health related outcomes, maternal milk is shown to provide a plethora of health benefits which enable infants to thrive in the early years of life and beyond.

Human Milk Oligosaccharides are a key component of infant nutrition

One of the important components present within breastmilk are glycans called Human Milk Oligosaccharides (HMOs). HMOs are synthesized in the maternal mammary alveolar cells and are composed out of only 5 different monosaccharides. Nonetheless, they attain a high structural complexity, which has yielded to over 150 HMOs currently identified (Le Doare et al., 2018; Townsend, 2019). The monosaccharides which are used as building blocks for HMOs are glucose (Glc), galactose (Gal), which together make up lactose, N-Acetyl-Glucosamine (GlcNAc), fucose (Fuc), and sialic acid (Neu5Ac). Depending on the terminal sugar appendage of sialic acid, fucose or neither, three main classes of HMOs have been identified; the sialylated HMO's which contain sialic acid, the fucosylated HMOs which contain fucose, and the neutral HMOs which have neither sialic acid nor fucose attached to the core lactose group.

Tri-saccharides can be synthesized by appending fucose or sialic acids to the lactose residue, leading to formation of, amongst others, 2'-FL (Fuc(α1-2)Gal(β1-4) Glc), 3'-SL (Neu5Ac (α 2-3)Gal(β1-4)Glc) and 6'-SL (Neu5Ac(α 2-6)Gal(β1-4)Glc) (Ayechu-Muruzabal et al., 2018). 2'-FL, 3'-SL and 6'-SL are found to be the most prominent HMOs residing in human breastmilk (Vandenplas et al., 2018). Unlike other mammals, which mainly receive 3'-SL and 6'-SL via maternal milk, human milk exhibits a very high concentration of 2'-FL (Urashima et al., 2001). Further

properties of HMOs include their complex diversity within milk, which varies over time as lactation and infant development progresses (Kunz et al., 2000). The colostrum, i.e. milk solely secreted very close around the parturition date, yields the highest absolute HMO content which then tapers off as time passes (Elwakiel et al., 2018). Next to HMO variation as a function of time, interpersonal variations in HMO content also persist in terms of quantity and availability of certain HMOs within the milk. Research has shown that HMO milk composition is linked to the maternal blood group characteristics, identified as the Secretor (Se) and Lewis (Le) status of the mother (Bode, 2012; Schneider, Al-Shareffi & Haltiwanger, 2017; Tonon et al., 2019). The Se and Le genes determine the availability of ABO blood group antigens and their expression in the mammary glands is linked to the presence of FUT2 and FUT3 enzymes, respectively, which in turn regulate the synthesis of certain fucosylated HMOs such as 2'-FL, LNFP1 and LNDFH II (Tonon et al., 2019). Mutations in the Se gene inactivate FUT2, resulting in milk from non-secretor (Se-) women lacking or having only traces of α1-2 fucosylated HMOs. Similarly, mutations in the Le gene inactivate FUT3, causing milk from Lewis-negative (Le-) women to lack or have only traces of α1-4 fucosylated HMOs (Totten et al., 2012).

Overall, HMOs serve as metabolic substrates for the microbiome residing within the gut, while also exercising a protective anti-pathogenic role around epithelial cell surfaces (Bode, 2012; Wise et al., 2018; Falsaperla et al., 2024; Xu et al., 2025; Urashima et al., 2025). They occur in great abundance and complexity within breastmilk based on several characteristics such as time, diet and blood group status.

The basics of brain development and plasticity

To examine how HMOs contribute to brain development and neural plasticity, a brief overview on how these processes take place is needed. It should be noted that while the greater majority of brain development occurs in the very early years in life, several stages of brain development have no clear end point in terms of age. The brain is susceptible to plasticity changes as a result of environmental influences such as diet and learning, but also in response to tissue damage due to genetic or acquired disease and physical trauma. Therefore, processes such as synaptogenesis, myelination, and within the hippocampus and olfactory bulb also neurogenesis, still occur within the adult brain.

Brain development unfolds in several stages, primarily during early life, though it continues throughout life to support neural plasticity (Sanes, Reh & Harris, 2012). The process begins with neurogenesis, where neural stem cells proliferate to produce

new neurons. From the moment of their birth, these neurons start to differentiate, gradually acquiring their specific identities and functions. Initial differentiation occurs at the site of origin, and as neurons migrate to their final destinations in the brain, they continue to differentiate. Neural migration, guided by mechanisms such as radial and tangential migration and chemical guidance, ensures that neurons reach their correct locations. Upon arrival, neurons undergo a final differentiation step and acquire their very distinct functions as they integrate into their assigned neural network. During neuronal migration, axons form to facilitate inter-neuronal communication, followed by synaptogenesis, where synaptic connections are established to create neural circuits. Synaptic pruning within these circuits offers further refinement through the elimination of unnecessary synaptic connections. Finally, these circuits are further optimized through myelination, where axons are insulated with myelin sheets to enhance signal transmission strength and speed. The brain's development is influenced by critical periods during which it is particularly sensitive to environmental inputs, and by experience-dependent plasticity, which allows neural circuits to be shaped by life experiences.

It is important to note that brain development occurs on different time scales. Regions responsible for higher-order processing, such as the prefrontal cortex, take significantly longer to mature compared to primary sensory and motor cortices and subcortical structures. While basic sensory and motor functions are established early, the prefrontal cortex, which is crucial for complex cognitive processes, continues to develop well into early adulthood. Secondly, while mammalian species differ in the time scale during which brain development events occur, the underlying processes are shared by all species.

The role of fucosylated and sialylated HMOs in brain development and plasticity

During infancy, brain development is impacted by several factors such as nutrition, which has cascading downstream influences on cognitive development throughout the years (Nyaradi, Hickling, Foster, Oddy, 2013). Human milk is proven to be beneficial for neurodevelopment, and its beneficial effects have been attributed to both sialylated and fucosylated Human Milk Oligosaccharides (HMOs) (Chiurazzi et al., 2021), a relationship mediated by the gut microbiome (Cheng & Yeung, 2021; Carabotti et al., 2015; Falsaperla et al., 2024; Li et al., 2025).

1) Sialic acid in brain development and plasticity

Sialic acid, obtained from dietary sources, is utilized in the assembly of gangliosides and polysialic acid (PSA) chains (Cheng & Yeung, 2021). Gangliosides are sialylated

brain glycosphingolipids, mostly found in myelin and neurons, and are involved in axon myelination interactions, axon stability, axon regeneration, and serve as modulators of neuronal excitability (Wang, 2009; Sipione et al., 2020). During early-life neurodevelopment, ganglioside density and structural complexity increase as the infant brain matures (Xu et al., 2025). When measured during adulthood, the majority of the complex gangliosides fall into one of four key classes (GM1, GD1a, GD1b, GT1b), which together account for 97% of the gangliosides within the brain (Schnaar, Gerardy-Schan & Hildebrandt, 2014; Sipione et al., 2020).

Polysialic acid (PSA) is involved in neural plasticity through its post translational modification of Neural Cell Adhesion Molecules (NCAM) via a process called 'polysialylation' (Bonfanti, 2006; Wang, 2012). Polysialylation occurs at different stages of neurodevelopment and neurogenesis (Fan, McMath & Donovan, 2023). The majority of neurons contain PSA at some point during the differentiation process, and PSA has also been associated with long-distance cell migration, neurite outgrowth, axonal pathfinding, and synaptogenesis (Bonfanti, 2006). While growing fiber tracts express PSA, it should be noted that during the myelination phase, PSA is downregulated as it is hypothesised to function as an inhibitor for myelination (Fewou et al., 2007). NCAM is a transmembrane glycoprotein that is regulated by the *NCAM1* gene, occurs in three major isoforms generated by alternative splicing, and mediates neural development, learning and memory, synaptic plasticity and cell signalling (Gascon, Vutskits & Kiss, 2007; Vukojevic et al., 2020). NCAM in its base form ensures synaptic stability, while attaching the polysialic acid tails to it induces synaptic plasticity (Muller et al., 1996; Bonfanti, 2006; Weledji & Assob, 2014; Wang, 2012). Therefore, the regulation of the polysialylation of NCAM in turn, regulates the plasticity of neuronal networks, which have downstream consequences for cognitive functions. Newly formed granule cells in the dentate gyrus of the hippocampus exhibit heightened plasticity during their maturation and display a high expression of PSA-NCAM, highlighting its importance in neural plasticity and learning (Sahay, Wilson & Hen, 2011; Oomen et al., 2014; Bonfanti, 2006). The fact that brain regions involved in learning and memory are high in PSA-NCAM content further supports the relevance of sialic acid and its application to these cognitive processes. Intervention trials in piglets have established that the supplementation of sialylated HMOs increases the sialic acid brain content during infant brain development (Wang et al., 2007; Wang, 2009; McVeagh & Miller, 2008).

One of the first notable effects reported of sialylated HMO supplementation on neurodevelopment is their influence on the myelination processes, as is evident by an increase in myelination-related genes, myelin-associated glycoproteins, and

myelin basic protein (encoded by the *Mbp* gene) (Obelitz-Ryom et al., 2019). While myelination is traditionally related to long-lasting neurodevelopmental events, myelin has also been fundamental for memory formation, a process which has been coined as "myelin plasticity" (Xin & Chan, 2020; Khelfaoui, Ibaceta-Gonzalez, & Angulo, 2024). Studies have shown that myelin plasticity is involved in cognitive processes such spatial memory consolidation, fear learning and working memory (Steadman et al., 2020; Shimizy et al., 2023, Pan et al., 2020). While there are no studies linking HMOs to MBP, the myelin basic protein and ganglioside GM1 have been found to interact through the binding of up to four GM1 carbohydrate groups to their binding sites on the myelin basic proteins (Ong & Yu, 1984) Furthermore, highly myelinated regions such as the corpus callosum and cerebellum display an increase in ganglioside-bound sialic acid, hence offering further support for the involvement of sialic acid in axonal myelination (Jacobi et al., 2016) and as a potential supportive role in myelin plasticity. The study by Mudd et al. (2017) has indicated that the supplementation effects of sialylated HMOs are dose dependent and have been found to be specifically constrained to distinct regions such as the corpus callosum, hippocampus and prefrontal cortex, which are essential key regions in cognition, memory and learning.

Finally, another neurodevelopmental process under the influence of sialylated HMOs is the synthesis of important brain metabolites and neurotransmitters such as an increase in glutamate, which has been shown to support neurite sprouting and synaptogenesis (Wang et al., 2021; Falsaperla et al., 2024). In rodents, we also find additional support for the neuroprotective effects of dietary 3'-SL and 6'-SL during a social disruptor stress paradigm on the generation of new neurons (Tarr et al., 2015), and improved Long Term Potentiation (LTP) at one year of age (Oliveros et al., 2016).

2) Fucose in brain development and plasticity

When examining the role of fucose in the developing and mature brain, several studies indicate that fucosylated glycoconjugates play a crucial role in neuronal development, learning, and memory (Mountford et al., 2015; Tosh et al., 2019). Since 2'-FL cannot cross the blood-brain barrier (BBB), its effects are likely mediated through the incorporation of cleaved fucose at synapses (Murrey et al., 2006) or via metabolites from the gut-brain axis (GBA). Interestingly, fucose is rapidly incorporated into hippocampal glycoproteins following learning experiences, suggesting that fucosylation is tightly linked to local protein synthesis in response to synaptic stimulation (Schneider, Al-Shareffi & Haltiwanger, 2017; Vazquez et al., 2015; Munni et al., 2025). Fucosylation via FUT1 and FUT2 has also been shown

to play a direct role in synapse formation, neurite outgrowth, sensory neuron pathfinding, and neurite migration. Moreover, the fucosylation of Synapsin Ia and Ib, key proteins involved in neurotransmitter release and synapse formation, regulates synaptic turnover and stability within primary hippocampal neurons (Murrey et al., 2006; Schneider, Al-Shareffi & Haltiwanger, 2017).

In addition, 2′-FL supplementation has been found to potentiate LTP (Vazquez et al., 2016; Oliveros et al., 2016), underscoring its significant role in memory formation and synaptic plasticity. Two key genes involved in mediating 2′-FL's influence on LTP and plasticity, as highlighted by these studies, are the Brain-Derived Neurotrophic Factor (*Bdnf*) gene and the Postsynaptic Density Protein (*Psd95*) gene, also known as *Dlg4*. BDNF supports the survival of existing neurons and promotes the growth of new neurons and synapses, while PSD95 is essential for the synaptic scaffolding necessary to stabilize postsynaptic structures during plasticity events (Meyer, Bonhoeffer, & Scgeuss, 2014). Vazquez et al. (2015) observed that rats supplemented with 2'-FL exhibited increased BDNF protein levels in the striatum and hippocampus, along with elevated PSD95 expression in the frontal cortex and hippocampus. Among the various isoforms of the *Bdnf* gene, isoform III is particularly crucial for activity-dependent plasticity, which is integral to LTP and memory formation (Altieri et al., 2004). This isoform is regulated by CREB, a transcription factor activated during synaptic activity, which promotes synaptic strengthening by driving receptor incorporation at the synapse and supporting dendritic growth. While isoform III plays a central role, other *Bdnf* gene variants, such as isoforms I and IV, also significantly influence plasticity and memory, with their expression patterns varying depending on the cognitive process involved (Bach et al., 2023, bioRxiv preprint).

Finally, although the NCAM protein is closely associated with sialic acid in mediating plasticity, the expression of NCAM1 within the hippocampus has also been found to be influenced by a combination of BMOs and HMOs, including 2′-LF and LNnT, in piglets (Fleming et al., 2020). These findings suggest that multiple HMOs coordinate their molecular effects across different stages of protein synthesis and their subsequent downstream impacts. Piglet studies also show modest increases in the relative and absolute volumes of the cortex and corpus callosum following fucose supplementation (Fleming et al., 2020). In rodent studies, FL supplementation at one year of age enhanced LTP (Oliveros et al., 2016), aligning with the sialylated HMO findings reported by Oliveros et al. (2018). An intriguing finding from these studies is the potential for sex-dependent effects, with a combination of 3'-SL and 2'-FL influencing dopamine transporter expression in the ventral tegmental area

and leptin expression within the nucleus accumbens in females specifically (Tuplin et al., 2021).

To summarise, both fucosylated and sialylated HMOs appear to be necessary contributors to several processes involved with neural development, ranging from differentiation, axon and circuit formation, and myelination to neurogenesis, with downstream effects on learning, memory, LTP, and cognition, which may be mediated by a few key development and neuroplasticity genes such as *Ncam1*, *Bdnf*, *Mbp* and *Dlg4*. Animal studies suggest that these downstream effects of HMO on cognition can be long-lasting, but it remains uncertain whether these effects are also present within the infant and adult human brain. While it is prudent to exercise caution when extrapolating these findings to humans, the empirical evidence in mammalian studies and the observational studies in infants so far support the significant role of these oligosaccharides in brain development (Lis-Kuberka & Orczyk-Pawilowicz, 2019).

The downstream effects of HMO on behaviour and cognition

1) Preclinical studies

Despite differences in natural HMO content between species, supplementing animals with additional HMOs produces beneficial cognitive outcomes across different animal species (Docq et al., 2020; Fan, McMath & Donovan, 2023). Various animal studies on the effects of individually administrated 2′-FL, 3'-SL and 6'-SL have so far revealed that HMOs enhance learning and memory (Vazquez et al., 2016; Oliveros et al., 2018), spatial memory (Obelitz-Ryom et al., 2019) and reduce stress-induced anxiety (Tarr et al., 2015), irrespective of the age of administration (e.g. before or after weaning). Vazquez et al. (2014 & 2016) found that oral the administration of 2′-FL in adult rats enhanced the learning speed in the operant conditioning tasks). In addition, the 2′-FL treated groups performed significantly better than controls (who did not receive HMOs) when tested for spatial learning and working memory. Oliveros et al. (2016) further corroborate these findings. In their study, they showed that oral 2′-FL administration during the lactation period improved the memory and learning capabilities of rats, as shown by the novel object recognition task (NORT), Y maze test. These marked improvements were apparent both after weaning and during adulthood. Similar beneficial findings have also been reported for the sialylated HMOs. Oliveros et al. (2018) examined the effects of 6′-SL and Neu5Ac (administered separately to rat pups during the lactation period) versus a control group. Here they found that the two groups who received either sialylated HMO (6'-SL or Neu5Ac) performed better in the NORT,

Y maze and operant learning. In addition, in adulthood, these groups also displayed enhanced LTP. Of the sialylated HMOs in this study, 6'-SL proved to have the most potent beneficial effects on the memory tasks.

2) Clinical studies

When examining the effects of HMOs on future academic outcomes, longitudinal observational studies are commonly employed where the breastmilk content of the mother is analysed and then linked with behavioural outcomes, at several critical time points (e.g. 1 month, 6 months, 12 months, 18 months, 24 months). Depending on the study, these behavioural outcomes are assessed through different scales probing for various markers of cognitive development, such as the Bayley Scales of Infant Development (Bayley-III) (Berger et al., 2020 and Oliveros et al., 2021), Mullen Scales of Early Learning and Ages (Cho et al., 2021), Ratings of Executive Functioning (Willemsen et al., 2023) and Stages and Ages Questionnaires (ASQ) (Ferreira et al., 2021, Rozé et al., 2022). Depending on the questionnaires used, distinctions were made between language and communication, motor skills, problem solving, and social skills. Furthermore, these studies span different countries and thus different maternal and infant cohort characteristics are at play, such as the United States (Berger 2020), Malawi (Jorgensen et al., 2021), Korea (Cho et al., 2021), Brazil (Ferreira et al., 2021) or the Netherlands (Willemsen et al., 2023). Due to the large heterogeneity in study design and both ethnic and secretor status of the cohorts included, detailed comparisons between these studies are difficult to achieve with great certainty. Nonetheless, general trends do emerge from these different studies and will therefore be discussed.

Recent studies have explored the complex relationship between HMOs and infant cognitive development, revealing both promising and varied outcomes. Oliveros et al. (2021) investigated the relationship between HMOs and infant development, specifically focusing on the impact of 2-fucosyllactose (2'-FL) and 6-sialyllactose (6'-SL) on composite cognitive scores and motor development at 18 months. Their study found that higher levels of 2'-FL and 6'-SL in breast milk were positively associated with improved cognitive scores (6'-SL) and motor outcomes (2'-FL and 6'-SL). Berger et al. (2020) used the same assessment method and found that higher concentrations of 2'-FL in breastmilk at one month postpartum were positively associated with cognitive development at 24 months, while higher levels of DSLNT were linked to lower cognitive scores. However, when measuring HMO content at six months, these associations shifted, with other HMOs, such as LNH and FLNH, showing positive correlations with cognition scores, while 2'-FL levels at 6 months of age no longer were related to the 24-month-old cognition scores. Willemsen et

al. (2023) confirmed that higher early concentrations of 2'-FL and other fucosylated HMOs are related to better executive functioning at three years when infants were exclusively breastfed. However, when infants received a diet of part breastmilk, part infant formula, higher levels of sialylated HMOS were associated with worse outcomes, which raises questions on the influence of sialylated HMOs during development. Lastly, Cho et al. (2021) found that high levels of sialylated HMOs such as 6'-SL and 3'-SL were associated with improved cognitive and language outcomes in Korean infants at 24 months of age.

The effects of different classes of HMOs become more complicated once researchers include the secretor status of the mother in their analysis. Jorgensen et al. (2021) highlighted the importance of maternal secretor status, finding that infants of secretor mothers with higher levels of total fucosylated and total sialylated HMOS, displayed improved language development at 18 months. In the case of non-secretor mothers, positive correlation between the sialylated HMO LSTb and working memory and executive function were observed, while no effects of fucosylated HMOs are reported. The positive effects of high HMO content have been further highlighted by Ferreira et al. (2021), who found that lower breastmilk HMO concentrations were linked to higher risks of developmental problems, while research involving preterm infants by Rozé et al. (2022) indicated that HMOs like LNFP III may positively influence cognitive outcomes, particularly in secretor mothers.

Collectively, these studies emphasize the nuanced and multifactorial nature of the impact of HMOs on infant development, with maternal secretor status, feeding practices, and specific milk HMO composition and timing, playing critical roles in their developmental effects. The overall trend indicates that, depending on the maternal secretor status, ethnicity and the time point of measuring both HMO content and cognitive outcomes, certain HMOs either are beneficial or detrimental to such developmental outcomes. Especially the inclusion of fucosylated HMOs within the diet yielded positive results in consecutive developmental outcomes, while surprisingly an increase in sialylactose seemed to produce mixed results depending on the study. These findings further highlight that there is no such thing as a perfect and uniform milk composition that will yield the best outcomes for all children, but that a personalised diet that takes all individual characteristics into account would likely be the most suitable source of nutrition, as it reflects the individual infant's dietary needs at a given point in time. It should be noted that these relationships are correlational at best and therefore should be used as

a guideline for further studies elucidating the different effects of fucosylated and sialylated HMOs.

Outstanding questions regarding HMOs role in cognitive development

An increased recognition of HMOs as important contributors to infant development has kick started the search to a better mechanistic understanding of its functioning. Nonetheless, there are still several outstanding questions on the relationship between HMO and neonatal brain development, which warrant further investigation. One of the main issues in the research on HMOs conducted so far is that the majority have focused on singular HMOs or very limited combinations of HMOs, which does not reflect a naturalistic situation as maternal milk provides a combination of different HMOs. While it remains unknown how a complex combination of fucosylated and sialylated HMOs may produce downstream developmental and cognitive outcomes, it should be noted that specific gut bacteria utilise different classes of HMOs. This would mean that a greater variety of HMOs yields a greater variety in gut bacteria, and consequently a different mixture of metabolites which have distinct downstream effects on development and cognition. In addition, due to the large variability between these studies' experimental design and methods used, comparing the effects of different HMOs between studies is difficult. These limitations call for a larger, unified study in which the effects of different HMOs on complex cognitive functioning are systematically compared, when administered both independently as in conjunction (Wichmann et al., 2024).

In sum, it is to date unclear whether and how HMOs influence neurodevelopment, and whether there is a synergistic effect of the different types of HMOs in their modulation of these processes. Answering these questions allow for a deeper understanding of the precise function and role of HMOs on development and cognition. Therefore, this thesis aims to elucidate the influence of dietary HMOs alone and in combination on cognition and development through infancy until adulthood.

In **Chapter 2**, we reviewed the cognitive developmental outcomes from fucosylated and sialylated Human Milk Oligosaccharides (HMOs) administration in rodents and piglets. We first introduce the background on the importance of HMOs in infant nutrition for a healthy gut, immune system regulation and how HMOs could contribute to improved cognitive outcomes in children. As there are few direct studies investigating the link between HMOs and brain development in humans, the focus of the review is on murine and piglet models instead. The cognitive measures included in the review article span behavioural tests with the emphasis

on memory and learning behaviour. The main behavioural findings summarised in Chapter 2 highlight a few key aspects of the influence of HMOs on development and cognition. Overall, administering HMOs during infancy leads to improved memory and learning when assessed during infancy and adulthood, an effect that remains apparent once the cognitive load of the task is increased as the animals age. Finally, the review further highlights that other factors such as sex could also yield a distinct behavioural profile (Chapter 3). Finally, we proposed potential underlying mechanisms through which HMOs exert their beneficial actions on animal cognition, such as the regulation of PSA-NCAM and neural plasticity (Chapter 4), the interplay of the immune system and cognition and the microbiome.

In the first phase of our research project, we wanted to investigate the effects of HMO administration on cognitive and emotional development during infancy and adolescence. In **Chapter 3** we therefore investigate the immediate early life (neuro)developmental outcomes of fucosylated and siayllated HMOs (in singles and in various combinations) in both male and female rats from PND 8 – PND 49. All animals were assessed on the rate of eye opening (X^2 test), weight gain during infancy, and exploratory + anxiety behaviour in the elevated plus maze and open field test (MANOVA).

During the final phase of the research project, we wanted to assess whether there were long term effects of infant HMO administration (in singles and in combinations) on memory and cognition, and whether performance during memory could be linked to variations in neural plasticity or neurogenesis. In **Chapter 4**, all males bred and used in Chapter 3 were subjected to the novel object recognition test (NORT), followed up by the novel object location test (NOLT) when they were between 8 and 12 months old. On the final day of the NOLT 50% of the animals were injected with BrdU to label the rate of neurogenesis during the novel location task as a cytoarchitectural substrate of neural plasticity and learning. In addition, the gene expression levels of *Mbp, Dlg4, NCam1* and *Bdnf* within the mPFC and hippocampus were measured to assess if the HMO administration would affect neural plasticity.

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Chapter 2

The Protective and Long-lasting Effects of Human Milk Oligosaccharides on Cognition in Mammals

Sylvia Docq, Marcia Spoelder, Wendan Wang, Judith R. Homberg

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Abstract

Over the last few years, research indicated that Human Milk Oligosaccharides (HMOs) may serve to enhance cognition during development. HMOs hereby provide an exciting avenue in the understanding of the molecular mechanisms that contribute to cognitive development. Therefore, this review aims to summarize the reported observations regarding the effects of HMOs on memory and cognition in rats, mice and piglets. Our main findings illustrate that the administration of fucosylated (single or combined with Lacto-N-neoTetraose (LNnT) and other oligosaccharides) and sialylated HMOs results in marked improvements in spatial memory and an accelerated learning rate in operant tasks. Such beneficial effects of HMOs on cognition already become apparent during infancy, especially when the behavioural tasks are cognitively more demanding. When animals age, its effects become increasingly more apparent in simpler tasks as well. Furthermore, the combination of HMOs with other oligosaccharides yields different effects on memory performance as opposed to single HMO administration. In addition, an enhanced hippocampal long-term potentiation (LTP) response both at a young and at a mature age are reported as well. These results point towards the possibility that HMOs administered either in singular or combination forms, has long-lasting, beneficial effects on memory and cognition in mammals.

Keywords: Human Milk Oligosaccharides; cognition; brain development; animal behaviour; fucosyllactose; sialyllactose; Long Term Potentiation

Introduction

The natural composition of breast milk is well recognized as the golden standard of infant nutrition (WHO, 2020) and is associated with long-term health benefits (Feldman & Eidelman, 2003; Boquien, 2018; Vandenplas et al., 2018; Bar et al., 2016; Nolan et al., 2019; Carucci et al., 2020; Munlit et al., 2017; Rajani et al., 2018; den Dekker et al., 2016). Studies have shown that exclusive breastfeeding is accompanied by a reduced risk for developing medical conditions during childhood such as gastrointestinal infections (e.g. necrotizing enterocolitis)(Bar et al., 2016; Nolan et al., 2019). Indications that breastfeeding confers protective effects in the onset and course of allergic diseases such as atopic dermatitis, food allergy and asthma have also emerged over the recent years (Carucci et al., 2020; Munlit et al., 2017; Rajani et al., 2018). Such protective effects of breastfeeding have been attributed to multiple factors related to the gut, as it is found that breastfeeding can improve immune functioning, promote a healthy gut microflora (Doare et al., 2018). Apart from the gut, bioactive components within breast milk such as the adipokines (e.g. leptin, ghrelin), help regulate appetite control and energy intake. Breast milk also contains growth factors, such as neuronal growth factors (NGF) and epidermal growth factors (EGF), which exert trophic effects on the neonatal nervous system and enhance gastrointestinal mucosal maturation respectively (Doare et al., 2018; Çatli et al., 2014; Gila-Diaz et al., 2019). In recent years, the mental health benefits that breastfeeding provides have garnered much more attention in neuroscientific research. Notably, breastfeeding is associated with improved cognitive development, as demonstrated by improved IQ scores (Kramer et al., 2008) and a reduced risk of childhood behavioural disorders (Vandenplas et al., 2018), (Poton et al., 2018; Horta et al., 2015). These findings also coincide with studies showing enhanced brain development parameters, such as white matter development in frontal and temporal regions (Denoi et al., 2013) and maturation of the basal ganglia and thalamus (Herba et al., 2013). Overall, these studies indicate that there are clear developmental and cognitive benefits related to breastfeeding and breast milk, which raises the question: which breast milk factors facilitate cognitive development?

Breast milk is a complex liquid which contains many different lipids (such as the Milk Fat Globule rich in phospholipids and long chain fatty acids), an assortment of vitamins (Vitamin A, B, C, D K), sialic acid (both in free form and bound to oligosaccharides, glycoproteins and glycolipids) and other biologically active components, some of which affect neurodevelopment (Allen-Blevins et al., 2017; Andreas et al., 2015; Mudd & Dilger, 2017, Wang et al., 2017). Of particular interest to infant nutrition and development are the Human Milk Oligosaccharides (HMOs).

These non-digestible carbohydrates are the third most abundant class of breast milk components, and over 200 HMOs, comprised out of 5 monosaccharides (glucose, galactose, N-Acetyl-Glucosamine, fucose and sialic acid) have thus far been identified (Ayechu-Muruzabal et al., 2018). HMOs have recently moved into the spotlight of cognitive research due to its widespread effects on infant development and cognition (Vandenplas et al., 2018; Le Doare et al., 2018; Andreas et al., 2015). There are three main families of HMOs; the non-fucosylated neutral HMOs, (e.g. Lacto-N-neoTetraose (LNnT)), the fucosylated HMOs (e.g. 2'Fucosyllactose (2'-FL)) and the sialylated (SL) HMOs (e.g. 3'-Sialyllactose (3'-SL) and 6'-Sialyllactose (6'-SL)) (Ayechu-Muruzabal et al., 2018; Hegar et al., 2019). Oligosaccharides are present in all mammalian milk (Urashima et al., 2001). However, what makes human milk unique compared to other mammalian milk is that it contains the largest diversity of complex oligosaccharides [(Urashima et al., 2001; Ten Bruggencate et al., 2014) and high concentrations of 2'-FL. It should be noted that the presence of 2'-FL is subject to large inter individual variation depending on the Lewis antigen blood group system of the mother, which encompasses two genes; the Lewis gene (Le gene or FUT-3 gene) and the Secretor gene (Se gene or FUT-2 gene) (Kunz et al., 2017). Depending on genetic expression, women are either defined as 'secretors' (Se+), or 'non secretors' (Se-), and Lewis positive (Le+) or Lewis negative (Le-) (Ayechu-Muruzabal et al., 2018; Kunz et al., 2017). Both Secretor and Lewis genes are responsible for yielding fucosyltransferase-2 (FUT-2) and fucosyltransferase-3 (FUT-2) respectively, which append fucose to the core oligosaccharides. Depending on which of these FUT enzymes are active, different oligosaccharides will be created; as FUT2 expression results in the synthesis of 2'-FL, while FUT3 expression has been associated with the formation of LNFP-II instead (Austin et al., 2016; Thurl et al., 2010; Austin et al., 2019). These polymorphisms essentially give rise to four major milk groups within the human population; as both genes can be active, inactive, or either one of the two is active, hereby resulting in a variable HMO content in breast milk (Thurl et al., 2010). Around 60 - 72% of the maternal population are secretors, and the milk of these 'secretor mothers' contains an overall higher concentration of HMOs in breastmilk as compared to non-secretors (Ayechu-Muruzabal et al., 2018, Kunz et al., 2017; Azad et al., 2018, Paganini et al., 2019). All in all, a large variability exists within the human population concerning the exact proportions of different HMOs (Austin et al., 2016). Moreover, HMOs are also subject to dynamic changes within the same breastfeeding female, depending on factors such as circadian rhythm, lactation stage, maternal diet, and maternal genetic background (Vandenplas et al., 2018; Le Doare et al., 2018; Andreas et al., 2015; Austin et al., 2016; Thurl et al., 2010; Austin et al., 2019; Azad et al., 2018; Paganini et al., 2019; Lus-Kuberka et al., 2019; Sanchez et al., 2013).

Supplementation of infant formula with HMOs renders the composition and downstream effects of infant formula to become closer to those of breastmilk. One of the well-documented advantages of HMOs is its prebiotic role and the capacity to regulate the immune system in the periphery. HMOs can exert antimicrobial and antiviral effects by binding to pathogens which reach the mucosal surfaces in the gut, or by directly binding to the gut epithelial receptors, effectively blocking the access of pathogens (Le Doare et al., 2018; Andreas et al., 2015). Experimental studies in infants showed enhancing effects on the immune response of additional 2'-FL supplementation. Goehring and colleagues (2016) observed that infants who were fed breastmilk or a 2'-FL enriched formula had lower concentrations of plasma inflammatory cytokines (IL-1α, IL-1ß, IL-6, TNF-α) when compared to children fed the ordinary (non-enriched) infant formula. Furthermore, ex vivo stimulation of peripheral blood mononuclear cells (PBMCs) yielded lower levels of TNF-α and IL-6 when infants were breastfed or were on a 2-'FL enriched diet. Enriching infant formula with 2'-FL and LNnT also renders the gut microbiome composition and its metabolites (propionate, butyrate and lactate) of formula infants closer to that of breastfed infants (Steenhout et al., 2016). It stands to reason that, if the supplementation of HMOs to infant formula produces immunological and health responses similar to those of breastfed infants, this may also partly account for cognitive outcomes (Kramer et al., 2008). Indeed, apart from HMOs involvement in immune functioning, a recent study by Berger and colleagues (2020) reported that the amount of 2'-FL, measured in mother's breast milk one month after birth, predicted improved cognitive outcomes in two-year-old children. Since it is known that alterations in the immune system impacts brain development and later life cognitive functioning (Bilbo & Schwarz, 2012), it is possible that the HMO mediated immune response provides a route via which HMOs could contribute to cognition. Thus, investigating how HMOs impact underlying neural mechanisms of their associated cognitive outcomes will provide valuable insight in HMOs' role in brain development and functioning.

While there have been correlational studies exploring the role of HMOs on development in humans, no direct human study has thus far investigated both immune and cognitive outcomes with HMO analysis in breast milk or upon HMO supplementation in infant formula. However, direct studies on the effects of HMOs and cognition have been undertaken in murine models and piglets. While there are obvious differences between species, several animal models have been used extensively in behavioural research due to their translational value in brain development and behaviour. The behavioural tasks used in animal models in probing various cognitive functions are well validated (Wallace et al., 2015).

Moreover, since the life span of rodents in particular, is relatively short, animal models allow the investigation of the most sensitive developmental period to HMO supplementation. In addition, behavioural studies in animals can be corroborated by more invasive measures in vivo, granting a live view on the underlying neurobiological processes. One method commonly used in rodent memory studies is electrophysiology. Long Term Potentiation (LTP) involves the strengthening of synapses in response to prior stimulation during memory formation and retrieval. This produces a long-lasting shift in synaptic strength and is therefore an important underlying mechanism of synaptic plasticity and memory (Wiera et al., 2017). Findings derived from preclinical work could prove to be informative and may serve as input to future longitudinal studies on the contribution of HMOs to the cognitive development of humans.

This review's aim is twofold. Firstly, it aims to summarize the effects of HMOs in animal research and their subsequent cognitive and electrophysiological outcomes. Special consideration is given to the type of HMO used (e.g. fucosylated (2'-FL), neutral (LNnT) and sialylated (3'-SL, 6'-SL), the age of the animals upon HMO administration, the used cognitive task complexity, and the age of the animals during testing. Its second purpose is to provide additional interesting avenues for future research to explore. The search for relevant articles was conducted in Pubmed in the period of 1979 until August, 2020, using a specialized search string comprised of both Mesh terms and key words in the title and abstract (Appendix A). This resulted in the inclusion of nine articles that contained 1) an animal model, 2) HMOs and 3) cognitive behavioural tests.

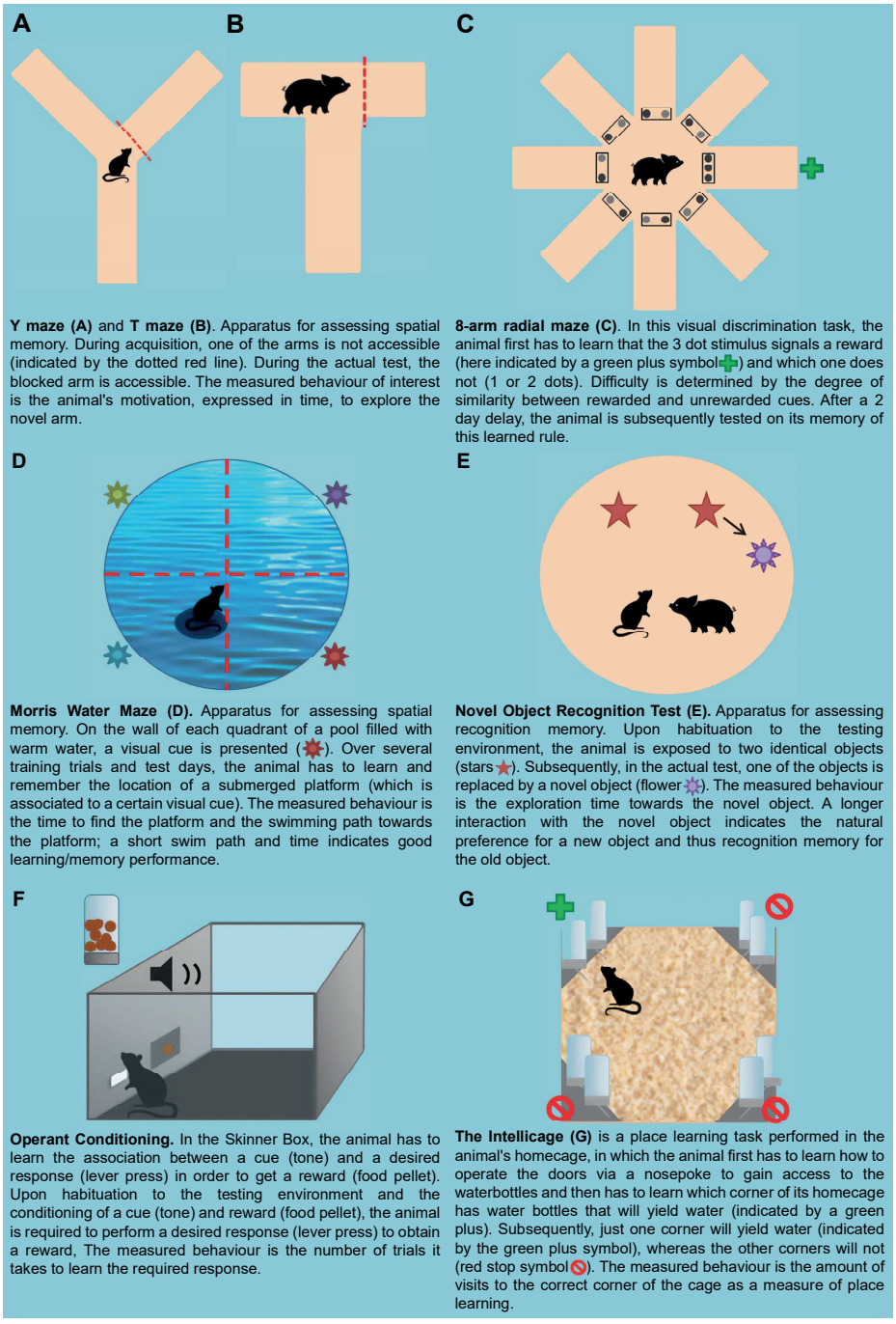
Assessing the effects of HMOs on cognitive measures in animal models

Rodents and piglets are naturally curious and intelligent animals, which results in their frequent use as an animal model for the assessment of cognition in a wide variety of behavioural tasks (Gieling et al., 2011; Antunes & Biala, 2012; Vorhees & Williams, 2014; Song et al., 2019; Kiryk et al., 2020). Behavioural tests are a valid, minimally invasive way to expose underlying cognitive processes, under the condition that the animal is capable of, and facilitated in, expressing such processes externally. In the context of HMO research, the focus has mainly been on memory and learning behaviour as cognitive capabilities. In the following sections, we will first graphically present an overview of the animal tests which investigated the consequences of HMOs on cognition. Subsequently, we present the main findings

of the selected nine articles, grouped by the type of HMO (fucosylated or sialylated), in Table 1. Thereafter, the main results will be described, which is then followed by a discussion about the implications of the findings reported in the investigations.

The type of behavioural tests used to study the effects of HMOs on cognition make use of either the intrinsic rewarding value of an animal's natural curiosity in new exposures (Fig. 1A, B and E) (Gieling et al., 2011; Antunes & Biala, 2012), the aversion to uncontrolled swimming without a platform to rest on (Fig. 1D) (Vorhees & Williams, 2014) or the willingness to obtain an extrinsic reward like food or water (Fig. 1C, F and G) (Song et al., 2020; Kiryk et al., 2020). Since animals prefer to be exposed to new items or environments to explore, the time spent to explore this new item or environment can be used as a measure for spatial or recognition memory. The willingness to obtain a food or water reward is commonly measured in operant conditioning tasks in either a skinner box or an Intellicage (Song et al., 2020; Kiryk et al., 2020). Operant conditioning tasks encompass associative learning paradigms, in which certain behaviour is reinforced via a reward or a punishment. In operant conditioning, different reinforcement schedules exist, such as the Fixed Ratio (FR) schedule, in which animals have to reach a certain criterion before they receive a reward. For example, an FR(4) schedule requires 4 correct responses from the animal in order for it to obtain a reward.

Overall, these cognitive tasks can be grouped by the level of complexity; as tasks that require a few trials are easier to perform than a task that requires weeks of training. Considering this, we have grouped the Y maze, T maze, Morris Water Maze (MWM) and the Novel Object Recognition Test (NORT) as simple cognitive tests, and the 8-arm radial maze and the operant tasks (Skinner box and Intellicage) as the complex cognitive tasks.



Y maze (A) and **T maze (B)**. Apparatus for assessing spatial memory. During acquisition, one of the arms is not accessible (indicated by the dotted red line). During the actual test, the blocked arm is accessible. The measured behaviour of interest is the animal's motivation, expressed in time, to explore the novel arm.

8-arm radial maze (C). In this visual discrimination task, the animal first has to learn that the 3 dot stimulus signals a reward (here indicated by a green plus symbol) and which one does not (1 or 2 dots). Difficulty is determined by the degree of similarity between rewarded and unrewarded cues. After a 2 day delay, the animal is subsequently tested on its memory of this learned rule.

Morris Water Maze (D). Apparatus for assessing spatial memory. On the wall of each quadrant of a pool filled with warm water, a visual cue is presented. Over several training trials and test days, the animal has to learn and remember the location of a submerged platform (which is associated to a certain visual cue). The measured behaviour is the time to find the platform and the swimming path towards the platform; a short swim path and time indicates good learning/memory performance.

Novel Object Recognition Test (E). Apparatus for assessing recognition memory. Upon habituation to the testing environment, the animal is exposed to two identical objects (stars). Subsequently, in the actual test, one of the objects is replaced by a novel object (flower). The measured behaviour is the exploration time towards the novel object. A longer interaction with the novel object indicates the natural preference for a new object and thus recognition memory for the old object.

Operant Conditioning. In the Skinner Box, the animal has to learn the association between a cue (tone) and a desired response (lever press) in order to get a reward (food pellet). Upon habituation to the testing environment and the conditioning of a cue (tone) and reward (food pellet), the animal is required to perform a desired response (lever press) to obtain a reward, The measured behaviour is the number of trials it takes to learn the required response.

The Intellicage (G) is a place learning task performed in the animal's homecage, in which the animal first has to learn how to operate the doors via a nosepoke to gain access to the waterbottles and then has to learn which corner of its homecage has water bottles that will yield water (indicated by a green plus). Subsequently, just one corner will yield water (indicated by the green plus symbol), whereas the other corners will not (red stop symbol). The measured behaviour is the amount of visits to the correct corner of the cage as a measure of place learning.

Figure 1. Summary of the behavioural tests used in the HMO studies. The type of animal placed inside the test (rodent or piglet) corresponds to the animal model used in the behavioural paradigms included in this review.

Table 1. Summary behavioural studies.

Study	Species	HMO component & dose	Age & duration administration	Age test	Tests	Key results
Fucosylated HMOs						
Oliveros et al., 2016	*Lister Hooded Rats*	**2'-FL** *(1g/ KG/BW)*	From PND 3 – weaning	Long Term study: 1) 4-6 weeks 2) 1 year old	NORT Y maze MWM LTP (only at 1 year)	At 6 weeks of age no differences in behaviour (*n= 12)* were found. At 1 years of age, 2'-FL rats showed improved performance in the NORT and Y- maze paradigms. No effect was observed in the MWM. LTP was more intense and long lasting in the 2'-FL supplemented groups (*n= 10*)
	Sprague Dawley Rats	**2'-FL** *(1g/ KG/BW)*	From PND3 until week 6	Short Term study: 6 weeks	LTP	LTP was more intense and long lasting in the 2'-FL supplemented groups (*n= 10*).
Vazquez et al., 2016	*Sprague Dawley Rats*	**2'FL** *(350 mg/kg/BW via AIN-93M diet)* **L-Fucose (Fuc)** *(equimolar amounts of fuc and 2'-FL via AIN-93M diet)*	3-4.5 months old for 5 weeks	Started at 2.5-4 months old	Operant conditioning (FR1) LTP	2'-FL but not fuc displayed enhanced LTP. Vagotomy inhibited the effects of oral 2'-FL on LTP (*n= 10*) and operant learning paradigms (*n= 10*).
Fleming et al., 2020a	*Pigs (1050 Cambro genetics)*	Three groups: 1. **Oligofructose (OF)** *5g/L* 2. **OF + 2'-FL** *5g/L OF + 1g/L 2'-FL* 3. **Control** *Nothing*	PND 2 - 33	PND 22	NORT	Pigs (*n= 12*) who received Oligofructose (OF) displayed enhanced object recognition when tested 1 hour after being habituated to the two objects. When pigs consumed both 2'-FL and OF, they showed improved recognition memory after a 48h delay.

Table 1. Continued

Study	Species	HMO component & dose	Age & duration administration	Age test	Tests	Key results
Fleming et al., 2020b	*Pigs (1050 Cambro genetics)*	Four groups: 1. **HMOs (2'FL + LNnT)** *1g/L 2'-FL + 0.5g/L LNnT* 2. **BMOs** *12,4g/L* 3. **HMOs + BMOs** *1g/L 2'-FL + 0.5g/L LNnT + 12.4g/L BMOs* 4. **Control.** *Nothing*	PND 2 - 33	PND 22	NORT	Pigs (*n= 12)* who received only HMOs displayed enhanced object recognition when tested 1 hour after being habituated to the two objects. When pigs consumed both HMOs and BMOs, they showed improved recognition memory after a 48h delay.
Vazquez et al., 2015	*Sprague Dawley Rats*	**2'FL** *(1g/kg/BW) via oral gavage during acute administration* *and* **2'-FL** *(350mg/Kg/BW) via AIN-93G diet, during short time feeding*	Acute administration: when rats were 3 months old Short time feeding: from 2.5-4 months, for 5 weeks	Operant tests started when administration started. LTP was performed after administration period.	Operant conditioning (FR1) LTP	2'-FL groups performed better in operant learning paradigms (rats *n= 10*, mice *n= 28*) and showed an enhanced LTP response (rats and mice *n= 8*). The long time supplementation of 2'FL also increased the expression of molecules involved in storage newly acquired memories (BDNF, PSD-95 phosphorylated CamKII,…).
	C57BL/6 mice	**2'FL** *(350mg/Kg/BW via AIN-93G diet)*	Long time feeding: from 2- 3.5 months old, for 12 weeks		Intellicage (FR1, FR4, FR8) LTP	

Table 1. Continued

Study	Species	HMO component & dose	Age & duration administration	Age test	Tests	Key results
Sialylated HMOs						
Oliveros et al., 2018	*Sprague Dawley Rats*	**Neu5Ac** **6'-SL** *(Dose ranged from 400mg/Kg/BW to 2600mg/Kg/BW based on theoretical model)*	From PND 3 until weaning	After weaning 1 year old	NORT Y maze NORT Y maze Intellicage LTP	No effects detected after weaning (*n= 10*). At 1 year old, sia (Neu5Ac and 6'-SL) exposed rats (*n= 8*♀) showed improved performance on all the behavioural tests (NORT, Y-maze, Intellicage) and showed enhanced LTP (*n= 10*) when compared to the control group. Of the SL supplemented animals, the 6'-SL group performed better than the Neu5AC group
Wang et al., 2007	Piglets *Landrace / Large White cross*	**Sialic Acid (ingredient of Casein glycomacropeptide cGMP))** *(4 groups of animals with their own dose each; 0mg/L (control), 140mg/L; 300mg/L; 635mg/L and 830mg/L)*	From PND 3 until end of experiment	PND 21 – PND 35	8-arm Radial maze	Supplemented groups *(n=12 – 14 per group)* required less trials to learn the required response, with a dose - response correlation for the difficult task.

Table 1. Continued

Study	Species	HMO component & dose	Age & duration administration	Age test	Tests	Key results
Obelitz-Ryom et al., 2019	Pre term delivered (experimental groups) Piglets *Landrace x Yorkshire x Duroc*	**Sialyllactose** *(6'-SL + 3'-SL)(380mg/L)* **Lactose (control)** *(6000mg/L)*	PND1 – PND19	PND13 – PND18	Spatial T-maze	Four experimental groups were included in the study; PRE-SAL(*n= 10*♀, *10*♂), PRE-CON (*n=9*♀, *11*♂),TERM-CON (*n= 9*♀, *5*♂) and TERM-SAL (*n= 6*♀, *6*♂). TERM CON piglets reached learning criteria of 80% correct choices on day 3, PRE-SAL on day 4 and PRE-CON on day 5. More PRE-SAL piglets reached the T maze learning criteria compared to PRE-CON piglets. Upregulation of genes for sialic acid metabolism, myelination and ganglioside biosynthesis were present in the hippocampus of SL supplemented preterm piglets.
	Term delivered piglets (reference groups) *Landrace x Yorkshire x Duroc*	**Lactose (control)** *(6000mg/L)* **Pig's milk** (under natural rearing conditions)				
Fleming et al., 2018	Piglets (no breed specified)	**Sialyllactose** *(380mg/L)*	PND2 – PND22	PND15 – PND22	NORT	No effects (*n= 17*) were observed.

NORT: Novel Object Recognition Test, MWM: Morris Water Maze, LTP: Long Term Potentiation. BMO: Bovine Milk Oligosaccharide. When provided, strains of species have been included in the table. In all studies presented here, the HMOs were administered orally. All animals used in the studies were male, unless otherwise specified. When the experimental groups have not been detailed in the key results column, the reported n indicates the number of animals per experimental group of that study.

Effects of HMOs on cognition in mammals

Main behavioural findings

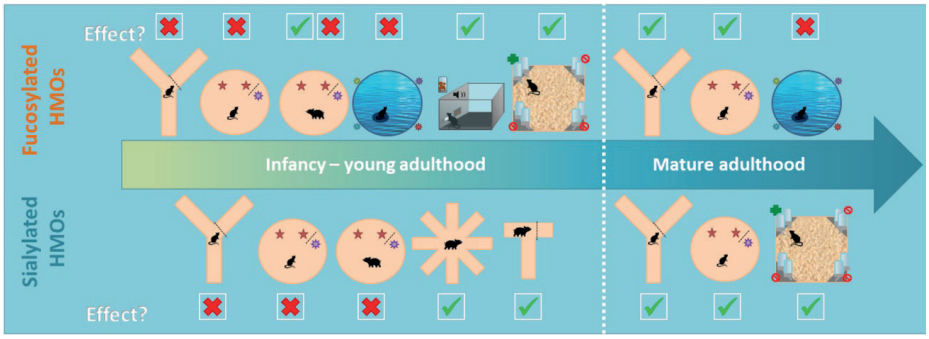


Figure 2. Graphical summary of behavioural tests results. The results have been grouped based on the type of HMO (Fucosylated versus Sialylated), animal model (rodents versus piglets) and the age of when the behavioural test has been performed. Infancy – young adulthood has been defined as the period ranging from PND1 – 6 months of age, while mature adulthood encompasses animals of 1 year old. Red crosses indicate that no significant differences were observed between the HMO and the control group, while green check marks indicate that positive effects due to HMO supplementation were reported. Details on the nature of such effects are summarized in Table 1.

Supplementing mammals with additional HMOs leads to beneficial cognitive outcomes under certain specific circumstances (Table 1, Figure 2). In general, both fucosylated and sialylated HMOs contribute to an improved memory performance and faster learning speed (tests described in Figure 1A – 1G) when tested in mature adulthood, irrespective of the age of administration of these HMOs (e.g. during infancy or adulthood) (Oliveros et al., 2015; Oliveros et al., 2018; Vazquez et ak., 2016; Vazquez et al., 2015; Wang et al., 2007; Obelitz-Ryum et al., 2019; Fleming et al., 2020a; Fleming et al., 2020b; Fleming et al., 2018).

Simple cognitive tasks

When rodents performed spatial and recognition memory tests during adolescence and early adulthood, no effects of either fucosylated or sialylated HMOs, as assessed by the NORT (when tested 24 hours after the acquisition phase), MWM and the Y maze, were reported. Contrary to the rodent studies, three piglet studies showed that supplementing HMOs during the lactation period resulted in improved spatial memory (T maze) in infancy (Obelitz-Ryom et al., 2019) and object recognition (NORT) (Fleming et al., 2020a; Fleming et al., 2020b). Supplementing only oligofructose or the combination of 2'_FL and LNnT increased object recognition when piglets were tested one hour after the acquisition phase. When tested

48 hours later, only the piglets who received a combination of either Bovine Milk Oligosaccharides (mostly neutral non fucosylated oligosaccharides) and 2'FL and LNnT (Fleming et al., 2020a), or Oligofructose and 2'-FL (Fleming et al., 2020b) displayed long-term recognition memory. In mature adulthood (older than 1 year), rodent studies also found significant differences in both the Y maze and the NORT for both sialylated and fucosylated HMOs. However, the sialyllactose piglet study performed by Fleming and colleagues (2018) yielded no results. In this study, they found no differences between the sialyllactose group and control group on the NORT performed during infancy.

Complex cognitive tasks

When considering the tasks that probe conditioning and learning capabilities and in which the cognitive difficulty could be varied, such as the 8-arm radial maze (Wang et al., 2007) and operant tasks (Oliveros et al., 2018; Vazquez et al., 2016; Vazquez et al., 2015), beneficial effects of HMO already surface at a young age in rats, mice and piglets alike. These effects also persist throughout adulthood. Perhaps the beneficial effects of HMOs become especially apparent upon increments on the cognitive load to meet the task demands.

Effects of HMOs on Long Term Potentiation (LTP)

The method of *in vivo* LTP induction in the studies listed here involved the implanting of stimulating electrodes on the Schaffer's collateral of the dorsal hippocampus and 2 to 4 recording electrodes in the stratum radiatum underneath CA1 (Oliveros et al., 2015; Oliveros et al., 2018; Vazquez et al., 2016; Vazquez et al., 2015). A high frequency stimulation (200-Hz trains of pulses, 100ms each and presented repeatedly with 1-minute intervals) was delivered to the Schaffer's collateral and 30 minutes later the field excitatory post-synaptic potentials (fEPSPs) were recorded. Enhanced LTP responses are reported in all these studies (Oliveros et al., 2015; Oliveros et al., 2018; Vazquez et al., 2016; Vazquez et al., 2015), both after weaning and during adulthood, when animals were supplemented with fucosylated or sialylated HMOs.

Discussion

To the best of our knowledge, this review is the first to summarise the effects of fucosylated and sialylated HMOs on cognition and electrophysiological brain recordings in rodents and piglets. The effects of both types of HMOs uncovered in the reported investigations unequivocally point towards long lasting beneficial

effects on cognition and memory, which is further supported by changes in the underlying physiological mechanisms as measured by LTP (Oliveros et al., 2015; Oliveros et al., 2018; Vazquez et al., 2016; Vazquez et al., 2015).

Most of the reported animal studies, included in Table 1, revealed that HMOs enhance learning and memory. For the simple cognitive tasks, the effects of HMOs are not unequivocal, as differences are observed between the animal model used, task parameters, the dosage used and age of administration and testing. It should be noted that in most of the studies, the HMO dosage was comparable to concentrations found in human milk Austin et al., 2016; Thurl et al., 2010; Austin et al., 2019; Azad et al., 2018; Lis-Kuberka & Orczyk-Pawilowicz, 2019), and effects of HMO supplementation were already visible at these physiological relevant dosages.

In rodents, no significant effects on spatial memory or long-term recognition memory are reported when the animals' age ranges from juvenile to young adulthood. In piglets, HMOs are found to affect spatial memory and intermediate recognition memory but not long-term recognition memory when they were fed only HMOs during infancy. Inter species differences between rodents and piglets may help to explain why effects of HMO administration are visible in piglets but not in rodents when tested at a very young age. The third trimester in human gestation coincides with the first ten postnatal days of rat pups, while the neurodevelopmental trajectory and morphological properties of piglet brains are much more comparable to humans (Gieling et al., 2011; Pressler & Auvin, 2013; Semple et al., 2013; Radlowski et al., 2014). This complicates the comparison of the effects of oral delivery of HMOs between piglets and rodents. Differences reside in the immediate environment upon birth and the extent to which the brain and body have developed at that point, as neonatal rat pups would be more comparable to prenatal piglets in the final days before parturition and there are no studies performed in the cognitive effects of HMOs on piglets in young adulthood. This interspecies difference in developmental stage upon birth and subsequent postnatal period might contribute to the heterogeneity in the findings between species on simple behavioural tests such as the NORT and the T maze.

Nevertheless, one cannot exclude the possibility that other factors than mere species differences may be at play, for example, the test parameters used in the studies. In the NORT of the rodent studies, the retention interval (time between acquisition phase and test phase) was 24 hours, which is considered to be fairly long and is considered to be a measure of long-term recognition memory (Antunes & Biala, 2012). In the piglet studies, different retention intervals, ranging from 1 hour (intermediate) to

48 hours (long-term), were used. It is possible that similar enhancing effects of HMO administration on recognition memory (NORT) reported by Fleming and colleagues (2020a and 2020b) would have been found in juvenile rodents if the retention interval was 1 hour instead of 24 hours, and if the rodents had been fed a similar combination of oligosaccharides as the piglets received. However, when probing such a long-term recognition memory of one year old rodents, an improved recognition memory is observed in the HMO supplemented animals, together with improved spatial memory as measured by the Y maze. As long-term recognition memory was not observed in juvenile piglets and rodents when supplied with only one HMO, but was observed in piglets when they were given a combination of oligosaccharides, this may not be a simple matter of species differences. Another explanation could be that within the developing brain, there are different processes at play when retrieving a newly consolidated memory (one hour later) versus an older memory (24 – 48 hours later), which may require more resources, such as the combination of various types of oligosaccharides. Interestingly, when piglets were supplemented with a complex mixture of oligosaccharides (HMOs and BMOs or Oligofructose), they displayed an improved long-term recognition memory. Perhaps the effects when HMOs form combinations, or are provided with other oligosaccharides are more potent, and thus easier to discern, than the effects of singular HMOs on memory.

Other factors such as gender and sample size could also contribute to the heterogeneity of the simple behavioural test findings, but it is uncertain to what extent these factors may have influenced the results. Only two sialyllactose studies (and no fucosyllactose study), used both males and females; one rodent study by Oliveros and colleagues (2018) and one piglet study by Obelitz-Ryom and colleagues (2019). However, no separation based on gender was performed in the analysis. As studies on postnatal administration of compounds, such as the study by Shumake and colleagues (2014) have demonstrated gender specific effects in rats, it stands to reason that early life HMO supplementation could produce gender specific outcomes. Nonetheless, when comparing the findings generated by Oliveros et al. (2018) and Obelitz-Ryom et al. (2019) with the exclusively male studies of the same species and HMO administered, the behavioural results remained very similar. Furthermore, most of the studies employed comparable sample sizes (n=10 – 12 on average) and effects of HMOs on cognition were already reported in studies with the lower sample sizes. While potential effects of variation in sample size cannot be completely excluded, HMO supplementation already produces beneficial results in experiments with lower sample sizes. Therefore, the heterogeneity in findings between studies are more likely due to a combination of factors such as species and task parameters as previously discussed.

When both piglets and rodents were tested on complex cognitive tasks from a young age onwards, HMOs exerted a beneficial effect on learning and memory. Therefore, it is possible that the HMOs effects become more apparent when cognitive load is increased, either due to task difficulty or due to aging. This may explain why the beneficial effects of HMOs are especially visible when the tasks are cognitively more strenuous, such is the case with the 8-arm radial maze or the operant tests, as increases in cognitive load make brain limitations more discernible.

While behavioural tests on learning and memory at a young age in general yielded mixed results, HMO supplementation did significantly improve LTP from a young age onwards. Interestingly, while in both young adult (2.5 months old) and mature adult (1 year old), just one HFS application was sufficient to induce LTP, very young rodents (6 weeks old) required a second high frequency stimulation (HFS) to induce LTP. Nonetheless, HMO administration resulted in an enhanced LTP response in both younger and older rodents alike. It is possible that LTP might be a more sensitive measure to investigate the beneficial effects of HMOs on cognitive outcomes at a young age. Furthermore, under normal circumstances, the LTP response is reduced in older rats as a natural result of aging (Oliveros et al., 2015). This natural reduction in LTP response was not encountered when the animals were supplemented with HMOs. On the contrary, supplementation with HMOs facilitated an enhanced LTP response. Because LTP is a measure of synaptic plasticity, it stands to reason that synaptic plasticity benefits from HMOs both in the short-term as in the long-term. Therefore, supplementation of HMOs, both sialylated and fucosylated, in infancy could have long-lasting protective effects on the molecular underpinnings of learning and memory.

Potential underlying mechanisms

There are a few possible factors which could account for the cognition enhancing effects of HMOs in mammals.

In the case of sialylated HMOs, Polysialylated Neural Cell Adhesion Molecules (PSA-NCAM) could be upregulated. The PSA-NCAM complex is upregulated in newborn, immature neurons and growing fiber tracts during embryogenesis and has been linked to increased synaptic plasticity (Bonfanti, 2006; Weledji & Assob, 2014; Murrey & Hsieh-Wilson, 2008; Sahay et al., 2011). Furthermore, PSA-NCAM is highly expressed in adult brain regions with high degrees of plasticity and neurogenesis, such as the olfactory bulb and the hippocampus (Murrey & Hsieh-Wilson, 2008). Improved neural plasticity and the survival of newborn neurons contributes to cognition and memory (Sahay et al., 2011). Therefore, it is possible that sialylated HMOs are capable of influencing neurogenesis, which in turn contributes to the

reported improvement in cognition. This suggestion is further supported by Oliveros and colleagues (2018). These authors found an increase in PSA-NCAM in 6'-SL supplemented animals. However, the role of fucosylated HMOs in plasticity and neurogenesis is currently not well understood and requires further investigation.

A second possible factor is the improved immune functioning due to the supplementation of HMOs and their well-established role in the immune system. As mentioned in the introduction, immune factors also contribute to cognitive functioning (Bilbo & Schwarz, 2012), though there are multiple hypotheses on how this may occur. One hypothesis states that perinatal immune activation directly affects neurodevelopmental pathways necessary for learning and memory, which leads to reduced neurotransmitter function, a reduction in hippocampal presynaptic proteins and impaired LTP (Bilbo & Schwarz, 2012). A second hypothesis postulates that early life immune activation indirectly determines the adult response to an infection with a pathogen, either via exaggerated pro inflammatory cytokines or via a decrease in anti-inflammatory cytokines. This in turn could lead to downstream changes in cognition and neural function (Bilbo & Schwarz, 2012). As HMOs are capable of regulating the neonatal cytokine response in the periphery (Le Doare et al., 2018; Lis-Kuberka et al., 2019; Goehring et al., 2016; Yu et al., 2016), it is possible that they also exert their enhancing effects on cognition via the immune system.

A last possible factor through which HMOs may improve cognition involves the microbiome. HMOs contribute to the microbiome composition within the gut and therefore could interact with the brain via the resulting bacterial metabolites such as the Short Chain Fatty Acids (Dalile et al. 2019). As certain gut bacteria are specific for the utilization of sialylated HMOs and other bacteria for the fucosylated HMOs, a larger variety of HMOs may go hand in hand with a larger yield of specific gut bacteria capable of metabolizing these HMOs, and thus determining their subsequent metabolites (Bode, 2015). Interactions between single HMOs and the microbiome have been previously reported by Tarr and colleagues (2015). They demonstrated that the administration of sialylated HMOs changed the microbial composition in the gut of mice, which in turn led to a reduction in anxiety-related behaviour and a maintenance of neurogenesis. The influence of the gut-brain-axis has also been touched upon by Vazquez and colleagues (2016), as they found that ablating the vagal nerve, which is part of the gut-brain-axis, diminished the beneficial effects of orally supplied 2'-FL on LTP. Similar to these results, Kuntz and colleagues examined the metabolic fate of 2'-FL and found that 2'-FL was not directly incorporated in the brain but required an intact gut microbiome for the generation of fucose metabolites which are subsequently taken up into the systemic circulation and

organs (Kuntz et al., 2019). In addition, it is possible that combinational HMOs may generate better effects than alone. This idea has already been demonstrated at the level of the growth and function of gut bacteria (Thongaram et al., 2017; Lawson et al., 2020). Different HMOs are processed by different bacteria which contain either sialidases or fucosidases to cleave sia and fuc of the carbohydrates (Bode, 2015). In turn, another group of bacteria can feed on the HMOs once the fuc and sia moieties are removed. These bacterial interactions, which depend on the HMOs present in the gut, may exert downstream effects on memory and cognition via the gut-brain axis. In light of potential downstream effects of the microbiome on behaviour, environmental housing conditions which affect the microbiome should also be considered (Lees et al., 2014) in this context, though it is uncertain to what extent the microbiotic variations due to husbandry may have influenced the effects of HMO supplementation on subsequent behaviour. Finally, another important factor to consider in the context of the microbiome are gender specific effects. While infant sex is reported to be largely unrelated to the HMO composition within human breastmilk (Azad et al., 2018), another study by Moossavi and colleagues (2019) found that the milk microbiota vary dependent on infant sex. This could potentially be attributed to cross interactions with the gut microbiome of the infant, as gender differences have been reported there (Moossavi et al., 2019). As HMOs interact with both the milk and the gut microbiome (Ramani et al., 2018; Borewicz et al., 2020), it is therefore possible that sex dependent variations could lead to differential cognitive outcomes of HMO supplementation.

Conclusions

The observation that HMOs are capable of enhancing cognition has initiated the search for a better mechanistic understanding of its functioning. Nonetheless, there are still several outstanding questions on the relationship between HMO and neonatal brain development, which warrant further investigation. An important aspect that needs to be addressed is the apparent age-related differences when assessing various cognitive tests. This point illustrates one of the current issues on HMO research in animals, as the tools currently used may not be sensitive enough to fully explore the range to which HMOs may affect brain development and cognition. Thus, one of the more complex tools could be the use of challenging operant tasks, such as the Trial Unique Delayed Non-Matching to Location (TUNL) measuring spatial memory and pattern separation (Oomen et al. 2013), the 5-Choice Serial Reaction Time Task measuring attention and motor impulsivity (Higgins et al., 2017), or delayed reinforcement tasks measuring choice impulsivity (Dunnett et

al., 2012), ideally performed in the animal's home cage. The difficulty of such tasks can be varied and may thus be more suited to test cognitive functioning in young animals, as at a young age only effects of HMOs were found in difficult tasks.

Another important issue is that due to the large variability between the experimental design and methods used across studies, comparing the effects of different HMOs between studies is difficult. Such variability includes the age of testing, the tests and experimental parameters, the HMO components used, the gender of the animals, variation in sample sizes, the environmental conditions and the variation in (neuro)developmental stage during which the animals were supplemented the HMOs. These limitations call for a larger, unified study in which the effects of different HMOs on complex cognitive functioning are systematically compared, when administered both independently and as in conjunction. In such a unified study, all these factors can be accounted for, enabling a systematic comparison.

A last important issue is that most HMO studies so far have focused on singular HMOs, with the exception of the two most recent studies performed by Fleming in 2020. The focus on singular HMOs is a limitation because it does not reflect a naturalistic situation where maternal milk provides a combination of different HMOs (Bode, 2012). Therefore, considering the interactions of HMOs when supplemented in combination would provide valuable insights on the influence of the gut microbiome and its downstream effects on cognition and development.

While research on the cognitive implications of HMOs is still in its infancy, the early findings reporting its long-lasting beneficial effects on memory and cognition are promising. Further studies on the exact molecular mechanisms, ranging from immune functioning to neuroplasticity and the microbiome will prove to be useful in deepening our understanding of how HMOs and their interactions contribute to cognition and development.

3

Chapter 3

Short-term benefits of fucosylated and sialylated Human Milk Oligosaccharide supplementation on development and cognition during infancy and adolescence in rats

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Abstract

Introduction: 2'-fucosyllactose (2'-FL), 3'-sialyllactose (3'-SL) and 6'-sialyllactose (6'-SL) occur in varying concentrations in breastmilk and exert beneficial effects on development. Variations in Human Milk Oligosaccharide (HMO) content in infant nutrition may have differential downstream effects on concurrent developmental outcomes. We aimed to elucidate the short-term outcomes of 2'-FL, 3'-SL and 6'-SL in physical and behavioural development.

Methods: Two-hundred-and-twelve male and female Long Evans rat pups were administered 2'-FL, 3'-SL and 6'-SL, either alone or in various combinations, from PND8 until PND49. Their weight gain over time, and rate of eye opening were assessed during infancy, and anxiety and exploratory behaviours during adolescence.

Results: All HMO treated groups, with the exception of 3'-SL, were significantly lighter, and both the 3'-SL and one of the triple mixes were the first to have both eyes opened. In the open field, when male and female data were combined, 3'-SL-treated animals entered the center more frequently and spent more time there, while also spending less time in the border region. In contrast, the 3'-SL+6'-SL, 6'-SL, control, and (6:1:2) triple-mix groups spent more time in the border. When analysed separately by sex, females in the (6:1:2) triple-mix group showed reduced locomotor activity, while no locomotor effects were observed in males. Finally, triple mix animals travelled the most on the Elevated Plus Maze when compared to the control group.

Conclusions: Our findings demonstrate that different HMOs influence various developmental outcomes. The 3'-SL and triple HMO mix groups showed the earliest eye-opening times, indicating a potentially accelerated rate in neural development. In behavioural tests, 3'-SL enhanced exploratory behaviour and reduced anxiety, while locomotor effects of the triple-mixed groups differed by task: females showed reduced activity in the open field, whereas triple-mix groups travelled more on the Elevated Plus Maze. The data suggest that HMOs promote beneficial developmental outcomes which may be sex dependent.

Keywords: 2'-fucosyllactose, 3'-sialyllactose, 6'-sialyllactose, development, emotion

Introduction

Early-life nutrition plays a pivotal role in long-term health outcomes. Infants rely on maternal milk during the first six months of their lives and benefit from specific nutrients present in maternal milk in terms of physical and cognitive maturation. Human milk bolsters the infant's immune system by contributing the gut's defense to pathogens, aids gastrointestinal maturation, and shapes gut microbiome (Le Doare et al., 2018). Of particular interest are Human Milk Oligosaccharides (HMOs), which are the third most abundant class of components in breastmilk after lactose and lipids and have widespread effects on infant development and cognition (Andreas, Kampmann & Le Doare, 2015; Vandenplas et al., 2018). HMOs are necessary nutrients to ensure proper infant development over the period of supplementation (Andreas, Kampmann & Le Doare, 2015; Vandenplas et al., 2018). They are synthesized in the maternal mammary alveolar cells and are composed out of only 5 different monosaccharides. Nonetheless, they attain a high structural complexity, which has yielded to over 200 HMOs currently identified (Le Doare et al., 2018; Townsend, 2019). The monosaccharides are used as building blocks for HMOs are glucose (Glc), galactose (Gal), N-Acetyl-Glucosamine (GlcNAc), fucose (Fuc), and sialic acid (Neu5Ac). Tri-saccharides can be synthesized by appending fucose or sialic acids to the lactose residue, leading to formation of, amongst others, 2'-FL (Fuc(α1-2)Gal(β1-4)Glc), 3'-SL (Neu5Ac (α 2-3)Gal(β1-4)Glc) and 6'-SL (Neu5Ac(α 2-6)Gal(β1-4)Glc) (Ayechu-Muruzabal et al., 2018). Importantly, 2'-FL, 3'-SL and 6'-SL are found to be the most prominent HMOs in human breastmilk (Vandenplas et al., 2018).

Unlike other mammals, human milk has a very high concentration of 2'-FL. Nonetheless, most other species including pigs and rats, mainly receive 3'-SL and 6'-SL via maternal milk, indicating a large variation in concentration and ratio of HMOs in mammalian milk (Urashima et al., 2001). In addition, HMO content in maternal milk varies as a function of time, environmental factors and maternal biological characteristics such as blood group, maternal diet, and ethnical background. The flexible adaptation of HMO content over time ensures that, for every individual, their HMO intake is adjusted accordingly to meet the infants' developmental demands. This is also evident when considering the fact that the ratio and concentration of different HMOs in breastmilk change dynamically over time during infant development to meet the developmental demands in immune functioning, gut microbiota composition and brain development (Wang et al., 2007; ten Bruggencate et al., 2014). As HMOs persist in matrixes in the maternal milk, it is very likely that these HMOs interact with one another, hereby potentially producing

synergistic effects. Lawson et al. (2019) found that HMOs support gut bacteria through cross-feeding, where certain HMOs promote specific bacteria, and the by-products of these bacteria help others to grow. This synergistic effect, supported by other studies (Thongaram et al., 2017; Hoeflinger et al., 2014; Bunesova et al., 2016), highlights the importance of HMOs in shaping the gut microbiome, which is key to understanding how components of human milk influence brain development and later behaviour.

Research on the impact of HMOs on infant developmental outcomes has been underway in the last few decades. One of the most marked areas in development from childhood to adulthood, next to cognitive and physical development, is the increased aptitude at emotional regulation. This ability to modify emotional reactions in stressful or demanding situations is a skill that develops gradually from toddlerhood through early adulthood (Tottenham, 2017). Emotional regulation is tied to the development of brain systems, especially the bidirectional connection between the medial prefrontal cortex (mPFC) and the amygdala (Ghasghaei & Barbas, 2002; Quirk & Beer, 2006). This interaction, seen across mammals such as rhesus monkeys (Ghasghaei & Barbas, 2002) and rats (Quirk & Beer, 2006), evolves with age. The amygdala develops earlier during childhood and adolescence, while the mPFC matures in the early twenties. In adults, greater mPFC activity dampens amygdala responses, whereas children show different connectivity patterns, indicating a sensitive developmental period (Silvers et al., 2017; Casey et al., 2019). Research suggests amygdala-to-mPFC connectivity emerges earlier than the reverse, reflecting a gradual maturation of emotional regulation (Gabard-Durnham et al., 2014).

Furthermore, in the context of HMOs, Tarr et al. (2015) found that that the supplementation of sialylated HMOs to infant rats reduced social disruptor induced anxiety, hereby pointing to a role of HMOs not only in cognitive development or physical development, but also in emotional development. Further support for how HMOs may influence neurodevelopmental events on a systems connectivity level has been procured by Pisa et al. (2021 & 2023) and Hauser et al. (2021). They examined the influence of sialylated HMOs on neurodevelopmental outcomes through the use of knockout models in which the dam produced either 3'-SL deficient milk (Pisa et al., 2021), 6'-SL deficient milk (Hauser et al., 2021) or 3'-SL and 6'-SL deficient milk (Pisa et al., 2023). These knockout studies have shown how HMOs could potentially contribute to early neurodevelopmental processes by influencing gene expression and steering circuit formation in the medial prefrontal cortex. Finally, Rajhans et al. (2023) have found that 6’-SL, mediated by myelination, was associated with the development of social skills, while 2’-FL was associated

with improved language outcomes in children. This study provides an additional argument for the involvement of both fucosylated and sialylated HMOs in cognitive and emotional development in early life.

Because different gut bacteria process HMOs, they produce metabolites and glycans that influence neurodevelopment. Since sialylated and fucosylated HMOs require specific bacteria, their combination in the diet may shape a distinct microbiota and metabolic profile with unique effects. Therefore, we hypothesise that the combination of different HMOs function together in synergy, and that a specific ratio of HMOs provided during developmental critical windows produces a unique profile of long-lasting beneficial effects on neurodevelopment and emotional outcomes in mammals.

To test this hypothesis, a longitudinal behavioural study has been conducted using rats which were administered either a single, a double or triple HMO combination from PND8 until PND49. These rats were subjected to behavioural tests from infancy until adulthood. 2'-FL, 3'-SL, 6'-SL, in single form, 3'-SL + 6'-SL in combined form, or 2'-FL + 3- SL + 6'-SL in varying ratios were the elected HMO groups in this study due to their widespread effects on cognition and development in prior studies. As outcome parameters we included eye opening and weight gain, representing common physical parameters to track neonatal development in rodents. While weight gain is seen as an overall indicator of health and development, eye opening is also considered to be an indicator of brain maturation (Gandhi, Chang & Stryker, 2005). In addition, the animals were subjected to the Elevated Plus Maze (EPM) test and Open Field Test (OFT), which are commonly used in animal models and provide insights into exploratory behaviour and anxiety (Campos et al., 2013).

Method

Animals

Long Evans rats breeding pairs were purchased from Envigo (US) and used for the in-house breeding of the rats used in this study. Litters exceeding 10 pups were culled down to 10 pups on PND 0-1 to ensure the dam could provide adequate care, and pups were weaned at PND21. We attempted to maintain an equal distribution of males and females within the litters. Animals were housed in a reversed day/night cycle (12:12; lights off at 9AM, on at 9PM). The housing room's ambient temperature was set at a steady 20°C, with 40% humidity. Food and water were supplemented *ad libitum*. All animals were housed socially in type IV tecniplast

cages (either in pairs or in triples). Animals of the same sex and same HMO groups were housed together to prevent pregnancies and coprophagy of feces from other HMO treatment groups. A total of 116 males and 96 females were used in this study (Table 1). The experimental procedures were performed under a project license from the Central Committee on Animal Experiments (Centrale Commissie Dierproeven, The Hague, The Netherlands), in full compliance with the legal requirements of Dutch legislation on the use and protection of laboratory animals (Animal Testing Act). All efforts were made to reduce the number of animals used and their suffering.

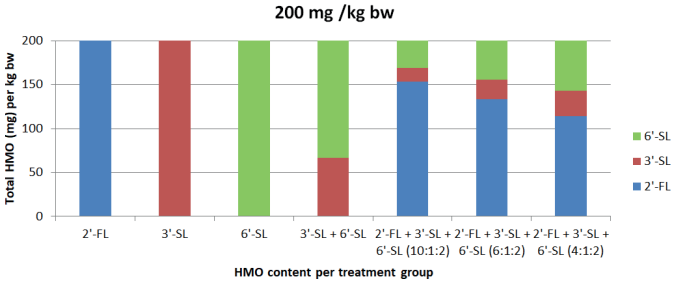
Table 1. HMO groups sizes per gender. 10:1:2, 6:1:2 and 4:1:2 refer to the HMO ratios used in the three mix groups 2'-FL+3'-SL, +6'-SL.

	3'-SL	2'-FL	3'-SL+6'-SL	Control	6'-SL	10:1:2	6:1:2	4:1:2
Male	13	15	16	11	13	16	16	16
Female	14	12	11	16	14	8	10	11

HMO administration and treatment groups

Three different HMOs (manufactured at Jennewein Biotechnologie, Germany) were included in this study; 3'-Sialyllactose (3'-SL), 6'-Sialyllactose (6'-SL) and 2'-Fucosyllactose (2'-FL). These HMOs were supplemented to young animals (from PND 8 until PND 49) as either single HMOs or in different combinations (Figure 1). This yielded the following eight treatment groups; the control group which only received drinking water, then the single HMO groups; 3'-SL, 6'-SL and 2'-FL separately, 3'-SL and 6'-SL combined (double mix group, ratio 1:2) and the triple mix groups of 2'-FL, 3'-SL and 6'-SL (ratios 10:1:2, 6:1:2 and 4:1:2). These ratios were based on the ratios of fucosylated and sialylated HMOs naturally occurring in breastmilk, where 2'-FL is the most abundant HMO, followed by 6'-SL and then 3'-SL (Liu et al., 2023; Soyyılmaz et al., 2021). It should be noted that the groups 2'-FL + 6'-SL and 2'-FL + 3'-SL are not included in this study, due to the use of a limited number of animals. We have chosen 3'-SL+6'-SL as the double mix group as they are the HMOs that naturally occur in rat milk, of which we know that they have neurodevelopmental effects (Tarr et al. 2015). Thus, including this group allows us to compare our findings with other researchers.

The administration age and range (PND8 – PND49), together with the dose fluctuations were elected because they encompass different developmental periods. In terms of development, rat pups on (PND7-8) corresponds to a perinatal infant, and rat pups on PND 21 correspond with a 2-3 year old child (Semple et



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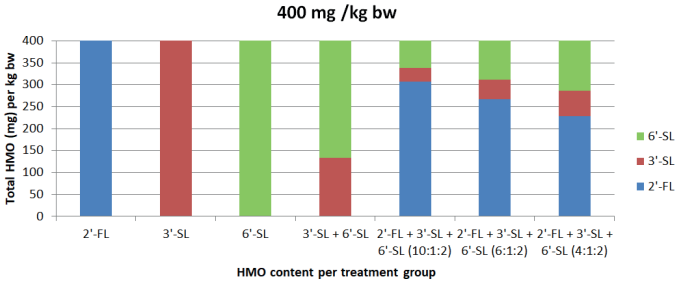

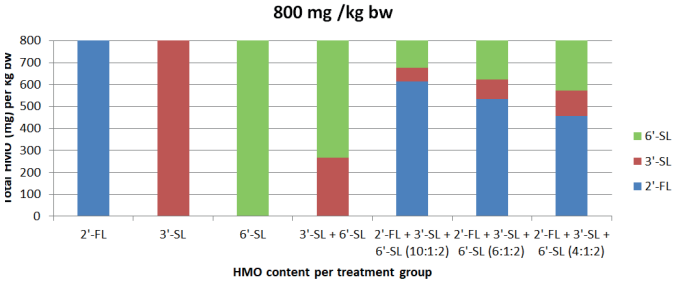

D

	200mg/kg bw			400 mg/kg bw			800 mg/kg bw		
	2'-FL	3'-SL	6'-SL	2'-FL	3'-SL	6'-SL	2'-FL	3'-SL	6'-SL
2'-FL	200,00			400,00			800,00		
3'-SL		200,00			400,00			800,00	
6'-SL			200,00			400,00			800,00
3'-SL + 6'-SL		66,67	133,33		133,33	266,67		266,67	533,33
2'-FL + 3'-SL + 6'-SL (10:1:2)	153,85	15,38	30,77	307,69	30,77	61,54	615,38	61,54	123,08
2'-FL + 3'-SL + 6'-SL (6:1:2)	133,33	22,22	44,44	266,67	44,44	88,89	533,33	88,89	177,78
2'-FL + 3'-SL + 6'-SL (4:1:2)	114,29	28,57	57,14	228,57	57,14	114,29	457,14	114,29	228,57

Figure 1. HMO concentrations. Graphs A, B and C depict the exact dosage of HMOs per treatment group, where A is the maintenance dose post weaning, and B and C are the initial dosages administered during lactation from PND 8 PND 14 (B) and PND 15-PND 21 (C). D contains the detailed information of HMO quantity per treatment group.

al., 2013). The termination of HMO administration on rodent PND49 was elected as it corresponds to an 18 year old human. The HMOs were supplemented per litter, meaning that one litter would receive 3'-SL, the next litter would receive 6'-SL, and so forth, to keep the microbiome of the litters intact.

All the HMO mixtures were prepared one day in advance at the start of an experimental cohort using the drinking water of the animal facility, aliquoted and frozen at -20°C until they were required for administration. On the day of administration, the required number of aliquots were prepared. The person administering the HMO mixtures and performing the behavioural experiments was kept blind to the identity of the components throughout the study.

Finally, the HMO dosage fluctuated over time depending on the developmental age of the pups; from PND 8 – PND 14 animals were given a dose of 400mg/Kg/Bw a day via oral gavage, from postnatal day (PND) 15 – 21 they received a dose of 800mg/kg/Bw a day via oral gavage, and from PND 22 – 49 they received a dose of 200mg/Kg/Bw a day via their drinking water. The variations in dosage were implemented to reflect the natural fluctuations in HMO milk content during the lactation period to end up with a constant level of HMOs by approximation during the whole lactation period (Duncan et al., 2009). When animals were weaned, a lower, chronic maintenance dose was provided to mimic the decrease in HMO intake that normally takes place after weaning, until the last early life behavioural tests were concluded at PND 49 (Figure 2), hereby covering the entire developmental range (Semple, 2013). The lower dosage was chosen because children do not tend to ingest as many dietary HMOs once breastfeeding ceases (which could be around the age of 2-3 years old).

Physical and behavioural testing

All animals were transported in their homecage to the testing room and kept in the testing room under low light (10 lux) conditions. Behaviour was measured using one overhead camera and analysed offline using Noldus Ethovision. Animals were tested in the elevated plus maze test on PND 34-35, and the open field test on PND 38-39 (Figure 3).

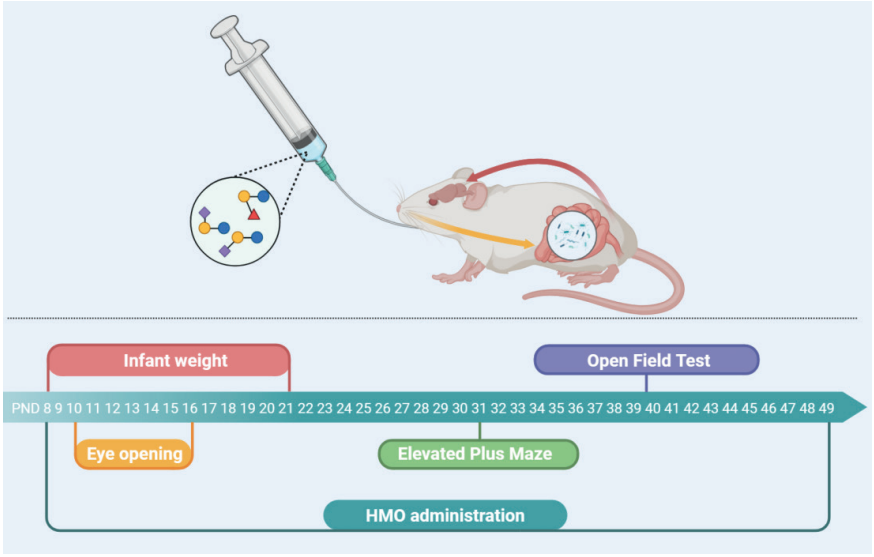

Figure 2. Experimental timeline. The green bar indicates the HMO administration period going from PND8 - PND49. The registration of weight and eye opening occurred over time, while the EPM and OFT were performed only once each. EPM = elevated plus maze; OFT = open field test; PND = postnatal day. Created with BioRender.com.

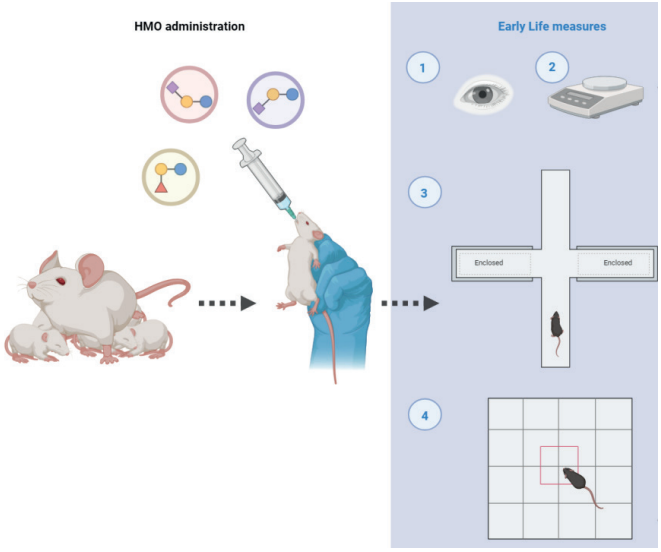

Figure 3. Experimental design early life HMO study in both male and female rats. Pups were administered HMO from PND8 until PND49 and (1) eye opening from PND10-PND16, (2) weight from PND8-PND42, (3) Elevated Plus Maze at PND34-35 and (4) Open Field at PND 38-39 were recorded. Upon conclusion of the last behavioural experiment, all female animals were sacrificed, while the male animals were kept for late life measures. Created with BioRender.com.

Eye Opening and weight

Pups from PND 8 until PND 16 were assessed once a day, at 9 AM, on the number of eyes they had opened on a given day. To this end, the dam was separated from her pups temporarily until all pups in the litter were visually inspected for eye opening, had been given their HMO treatment and had been weighed. Upon conclusion of the inspection and assessment of the litter, the dam was reunited with the pups. The home cage in which the litter remained was placed on a heated mat (temp: 30 °C) to ensure the pups' body temperature would not drop in the absence of the mother. Gloves were changed every time a new cage was being checked. The whole HMO administration, eye opening check and weighing procedure per cage took about 15 minutes per cage every day.

Elevated Plus Maze (EPM)

The EPM is a plus shaped apparatus, with on one axis two closed arms, and on the other axis two open arms. Each arm was 10 cm wide and 50 cm long, and the centre of the plus maze was a square of 10x10 cm. The EPM was kept in the same room through the entire course of the project, with the same environmental settings; ambient temperature was 20°C, humidity was at 40% and the light intensity in the open arms was kept at 10 lux. Each animal was put in the centre of the plus maze, facing an open arm, and were then recorded for 5 minutes on the plus maze. Between each animal, the apparatus was thoroughly cleaned with 40% Ethanol and left to dry. In the event an animal would jump off the plus maze, that animal was retested 2-3 days later one last time on the plus maze.

Open Field Test (OFT)

The open field setup consisted of a white square, 50cm x 50cm x 50cm (LxWxH). The open field was kept in a room at 20°C. Humidity was at 40% and the light intensity in the field was maintained at 10 lux. A camera was mounted above the open field arenas to record the behaviour for offline behavioural scoring using Noldus Ethovision (version 15.00). The border region was defined as the area spanning 15 cm in width from each wall, surrounding the center region which was a square in the middle of the arena measuring 20 cm x 20 cm (LxW). The amount of time animals spent in either the border or the center of the arena and the frequency with which they move between zones are commonly used as measures of anxiety, locomotor activity or exploratory drive. In general, rats who are anxious avoid open spaces and stay closer to the walls, while rats who were curious were more willing to explore the entire arena (Lynn & Brown, 2010). Each animal was placed in the center of the open field and recorded for 15 minutes. After 15 minutes, animals were returned to their homecage and the arenas were thoroughly cleaned with

40% Ethanol and left to dry. The open field arenas were white for maximum contrast for camera detection.

Statistical analysis

For statistical data analysis, IBM SPSS version 20.0 was used. Differences were statistically significant when the p value was < 0.05. In the event of significant differences between one HMO treatment from the control group, red diamonds are used in the graphs above their corresponding bars. The main focus of the data analysis was the comparison of the individual treatment groups with the control group. Furthermore, as animals were bred in 4 large batches over time, the data was examined for potential batch effects prior to each analysis and corrected when batch effects were significant by including which batch the animals originated from as a covariate. Only relevant data with significant values are included in the main body of this article.

Body weight: The pre-weaning body weight of all animals was recorded daily from PND8 until PND 21. A Repeated Measures ANOVA, with within-subject factor "time" and between-subject factors "HMO treatment", "batch" and "sex", was performed on the weight data collected from PND 8 up until PND 21. As no interaction effects between HMO treatment and sex were found, the analysis was repeated but with the sexes were pooled. Because the assumption of the Mauchly's Test of Sphericity was violated, a Greenhouse-Geisser correction was used in the analysis. In the event the repeated measures ANOVA yielded a significant (interaction) effect, a consecutive MANOVA was performed to identify on which day(s) the groups differed significantly from each other. The MANOVA was used because it controls for the testing for multiple dependent variables simultaneously while retaining the same between-subjects factors 'sex' and 'HMO treatment' factors (Pallant, 2020), with Wilks' Lambda (Λ) as outcome value. Finally, a Least Significant Difference (LSD) post-hoc test was used to further determine which groups differed significantly from one another. This post-hoc test performs pairwise comparisons between group means but is less conservative than the Bonferroni post-hoc test, henceforth reducing type II errors.

Eye opening: Eye opening was included as an early life measure as it is considered as marker of early postnatal brain development in rodents (Gandhi, Cang & Stryker, 2005). Eye opening was recorded and categorized as either '0 eyes open', '1 eye open' or '2 eyes open', from PND12 until PND16. We were interested in two particular outcomes; which proportion of animals, per HMO treatment, had 1 eye open, and which proportion of animals, per HMO treatment group, had both eyes open.

To analyse these data, a Chi Square test (X^2) was performed from PND12 up until PND16, as the X^2 statistic determines the presence of a significant difference between two proportions (McHugh, 2013). The Chi-square test examines the proportion of pups in a given HMO treatment group that had two or one eyes open versus pups that did not have both eyes open within the same HMO treatment. This test was subsequently followed up with Cramer's V (ϕc) to express the strength of the reported correlation between HMO treatment and eye-opening status on the days on which the X^2 test statistic yielded significant results. Correlation coefficients of >0.05< 0.10 are considered weak, correlation coefficients of >0.10 < 0.15 are considered moderate, and correlation correlations of >0.15 are considered strong or when > 0.25 very strong (Akoglu, 2018). Due to the nature of ϕc, no direction in relation (i.e. positive or negative correlation) can be inferred, thus we therefore limit ourselves in expressing this as a measure of relationship strength. Effects are deemed significant at a p value of ≤ .05.

Open Field Test: The behaviour of animals in this test fluctuated over time. Therefore, we conducted the data analysis for the open field in multiple steps.

Analysis 1: examination of effects of sex and HMO treatment on locomotor and exploratory behaviour over 15 minutes. First, to examine the general effects of HMO treatment on locomotor behaviour and exploration behaviour, a two-factor MANOVA was performed, with sex and HMO treatment as between-subject factors. The independent variables included in this analysis were distance travelled, velocity (both indices of locomotor behaviour), frequency of entering the center and the border zone and the time spent in the center and border zone of the arena (which we and others consider to be indices of not only exploratory behaviour, but also anxiety, Lynn & Brown, 2010).

Analysis 2: examination of effects of HMO treatment on exploratory behaviour per time bin. Based on the results of the previous analysis, we used a two-factor repeated measures ANOVA over three timepoints (timebin 0-5 min, 5-10 min and 10-15 min). Because HMO treatment did not affect locomotor behaviour directly, our focus was on the exploratory related measures 'frequency of entering the border zone' versus the 'center zone', and 'time spent in the border zone' versus the 'center zone'. This analysis was followed up with an LSD post-hoc test where appropriate.

Elevated Plus Maze: To analyse the EPM data, a two-factor MANOVA was performed, with sex and HMO treatment as between-subject factors. The behavioural parameters of interest were 'velocity', 'distance travelled', 'time spent in open

arm' versus 'closed arm' versus 'center', and frequency of entering the 'open arm', 'closed arm' or 'center'. When significant effects of HMO treatment on any of these parameters were found, an LSD post-hoc test was performed.

Results

Infant weight

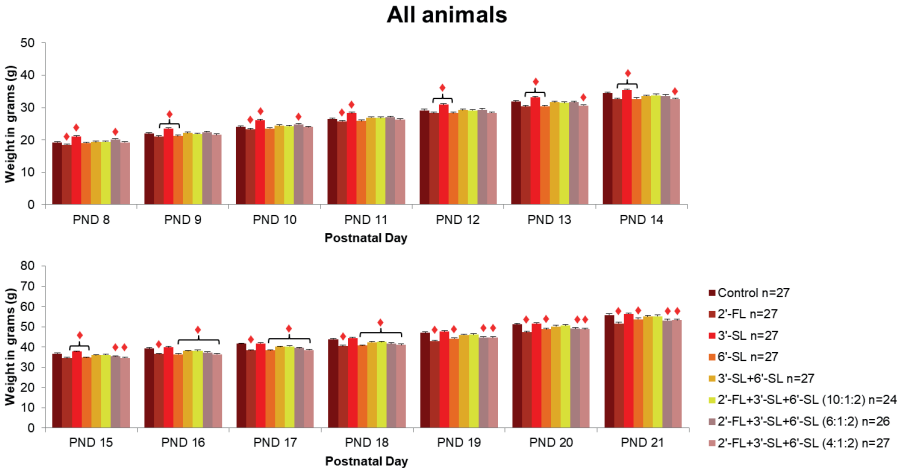

Figure 4. The effect of HMO treatment on body weight gain over time before weaning in male and female rats combined. This figure displays the weight gain for each treatment group recorded over time. Because of a lack of sex differences, males and females were combined in the analysis and the graphs. The bars represent mean +/- S.E.M. of body weight in grams. The red diamond above the bars indicate that these groups differed significantly from the control group, when it is placed above an accolade, the accolade covers all the treatment groups that differ significantly from the control group.

As shown in Figure 4, there was a significant effect of HMO treatment on body weight gain over time ($F_{(15.881, 440.133)} = 4.416$, $p < .001$, $\eta^2 = .137$). In addition, there was an effect of sex on weight gain over time ($F_{(2.269, 440.133)} = 9.826$, $p = .001$, $\eta^2 = .034$). As the weight gain over time due to HMO supplementation was not dependent on sex (Time*Sex*HMO interaction effect was $F_{(15.881, 440.133)} = .943$, $p = .520$, $\eta^2 = .032$), we did not make any distinction based on sex in the subsequent analyses, but focused on the effect of HMO on weight gain per day instead, corrected for batch effects.

A consecutive MANOVA, followed up with a LSD post-hoc test revealed that, over multiple days, HMO treatment groups differed significantly from one another (Wilks' $\Lambda_{(182, 1703.641)} = 8.177$, $p < .001$, $\eta^2 = .371$). Significant differences between HMO

treatments and the control group are depicted, per postnatal day, in Table S 1. From PND 8 up until PND 15, the 3'-SL group weighed significantly more than the control group, and from PND 16 onwards this difference between the control group and 3'-SL disappeared. All other treatment groups tended to be lighter than the control group, with significant differences for the single HMO treated groups 2'-FL from PND8 – PND 21, and 6'-SL on PND 9 and PND 12 onwards. The mix groups (double and triple mix) were also significantly lighter than the control group; 3'-SL + 6'-SL on PND 16 up until PND 18, 2'-FL+3'-SL+6'-SL(10:1:2) on PND 16 and PND 17, 2'-FL+3'-SL+6'-SL(6:1:2) on PND 8, PND 10 and PND 15 until PND 21, and finally 2'-FL+3'-SL+6'-SL(4:1:2) from PND 13 onwards.

In summary, HMO administration produced varying effects on pre-weaning body weight gain. The 3'-SL administered group was the heaviest, and animals fed fucosyllactose (FL) in their diet were the lightest in comparison.

Eye opening

Figure 5 shows the eye opening data for one eye open (Figure 7A) and both eyes opened (Figure 7B), split by sex.

Figure 5A. One eye open, split by male and female: Significant effects of HMO treatment on single eye opening were found on PND13 ($X^2_{(7)} = 25.783$, $p = .001$) for females, but not for males ($X^2_{(7)} = 9.412$, $p = .224$), and on PND15 ($X^2_{(7)} = 14.789$, $p = .039$) for males, but not for females ($X^2_{(7)} = 10.213$, $p = .177$). On these two days, there was a strong correlation between the pups having one eye opened and their HMO treatment for females on PND13 ($\phi c = .518$, $p = .001$) and males on PND15 ($\phi c = .357$, $p = .039$). Further examination of these proportions via MANOVA followed by an LSD post hoc test, revealed that on PND 13 the female 3'-SL supplemented group had the greatest proportion of pups with one eye opened when compared to all other treatment groups and the control group ($MD_{Control} = -.36$, $p < .001$, $SE = .079$). On PND 15, all the male treatment groups had the least number of pups with one eye open when compared to the control group.

Figure 5B. Two eyes open split by male and female: A significant effect of HMO treatment on double eye opening were found for both males and females on PND13 (males: $X^2_{(7)} = 19.248$, $p = .007$, females: $X^2_{(7)} = 29.377$, $p < .001$), only males on PND14 ($X^2_{(7)} = 17.319$, $p = .015$), and both males and females on PND15 (males: $X^2_{(7)} = 49.258$, $p < .001$, females: $X^2_{(7)} = 25.909$, $p = .001$). On these three days, there was a strong correlation between the pups having both eyes opened and their HMO treatment on PND13 (males: $\phi c = .407$, $p = .007$, females: $\phi c = .553$, $p < .001$), PND 14

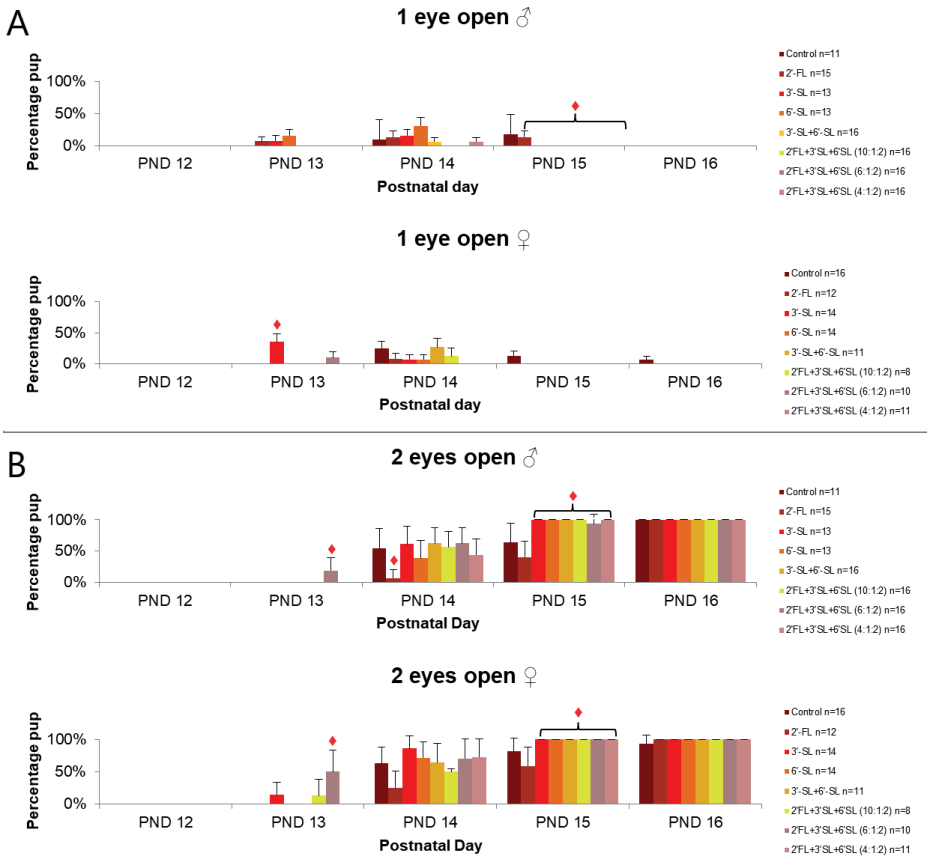

Figure 5. The effect of HMO treatment on eye opening in male and female HMO supplemented animals during PND12-PND16. The bars represent the cumulative percentage of animals that had either one eye open (A) or both eyes open (B) on a given day, with no distinction between males and females. The red diamond above the bars on PND 13, 14 and 15 indicates significant differences in X^2 values between HMO treatment groups and the control, the accolade above the bars indicates that the significant differences from the control group span all treatment groups that fall under the accolade.

(males: ϕc =.361, p =.034), and PND15 (males: ϕc = .652, p <.001, females: ϕc = .520, p =.001). Further examination of these proportions via MANOVA followed by a LSD post hoc test, revealed that on PND 13 both the male and the female 6:1:2 mix supplemented group had the greatest proportion of pups with both eyes opened when compared to all other treatment groups and the control group (male: $MD_{Control}$= .38, p <.001, SE= .118, female: $MD_{Control}$= 1, p < .001, SE= .194). On PND 14, the male 2'-FL group had the smallest proportion of pups with both eyes opened

when compared to the other treatment groups except for 6'-SL, and the control group ($MD_{Control}$=-.96, $p < .014$, SE= .383). Finally, on PND 15, both 2'-FL treated animals (males: $MD_{Control}$= -.47, p =.022, SE= .203, female: $MD_{Control}$= -.46, p = .017, SE= .188), and all other HMO treated animals had the most pups with both eyes opened when compared to the control group.

To summarise, female 3'-SL treated animals were among the first to have started eye opening on PND 13, while the 6:1:2 mix treated animals were the first to have both eyes open on PND 13 (both males and females). As animals age, we find that on PND 15, both male and female HMO treated animals, except for 2'-FL, has the most pups with opened eyes.

Open Field Test (OFT)

Analysis 1: examination of effects of sex and HMO treatment on locomotor (velocity and distance moved) and exploratory / anxiety-like (frequency entry center vs border, and time spent in center vs border) behaviour recorded over 15 minutes. HMO treated animals (Figure 6A-D) differed significantly from one another in the time the animals spent in the center ($F_{(7, 192)}$= 2.203, p =.036, η^2= .074) (Figure 5C, and induced trends for the time spent in the border ($F_{(7,192)}$= 1.858, p=.079, η^2= .063) and the frequency of border region entries ($F_{(7,192)}$= 1.865, p=.077, η^2= .064). Furthermore, there were significant sex differences between males and females in both the distance they moved ($F(_{1, 7587717.336})$= 8.965, p=.003, η^2 = .045, and their velocity $F(_{1, 7587717.336})$= 8.826, p=.003, η^2= .045. Finally, in terms of velocity for the females the (6:1:2) group was significantly slower and moved less.

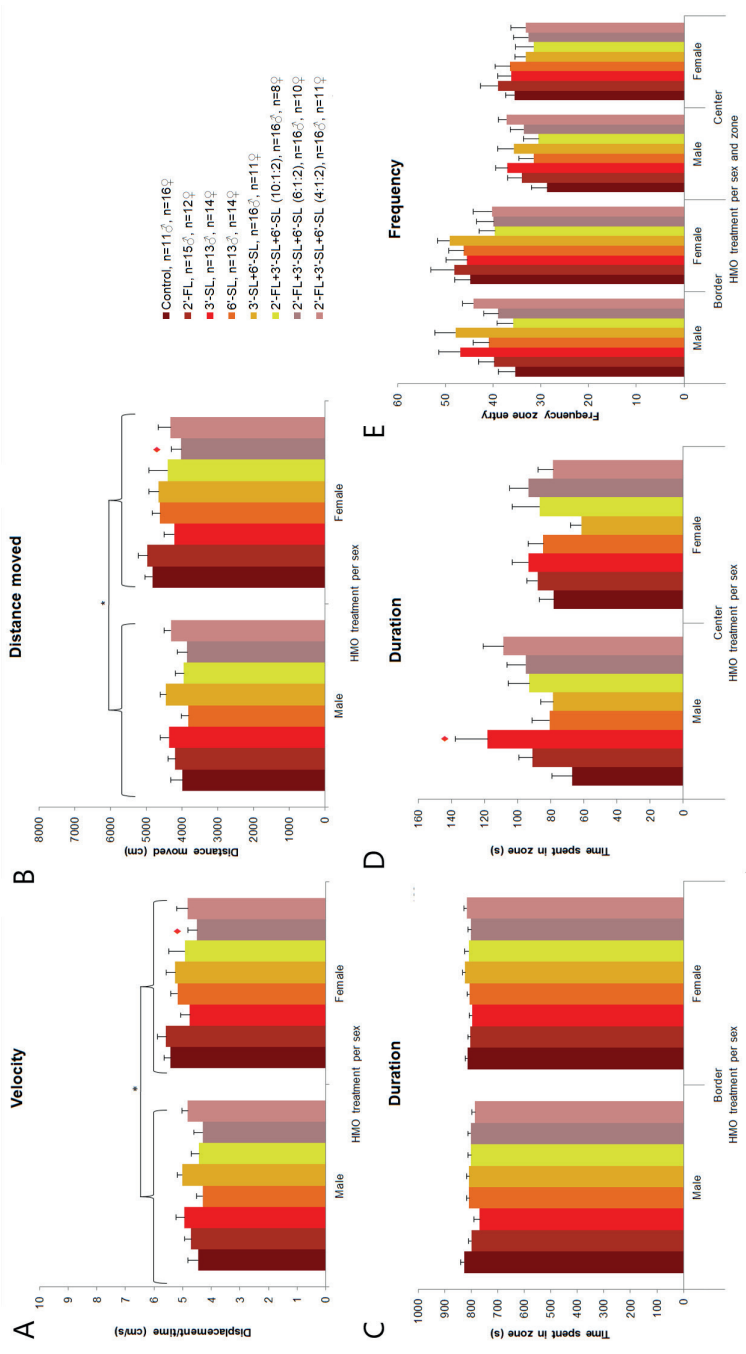

Figure 6. Locomotor and explorative behaviour in the Open Field Test over the entire task duration (0-15 min), split per sex. **A) Velocity**: Speed (measured in cm /s) per sex. **B) Distance moved:** the amount of cm animals travelled in the open field per sex. **C) Time spent in zone:** The amount of time (s) animals spent in the border region of the arena (left side of the graph), or the center of the arena (right side of the graph) per sex. **D) Frequency:** Frequency of crossovers from one zone to the other per sex. **Symbols:** Horizontal bars with a diamond on each end and an asterisk above them represent significant differences between an HMO treatment group and the control group. Horizontal accolades spanning all 8 treatment groups for females and males, connected with a horizontal bar with an asterisk without diamonds on each end, represent significant differences between males and females. Data are presented as mean +/- S.E.M., significance is set at $p < .05$.

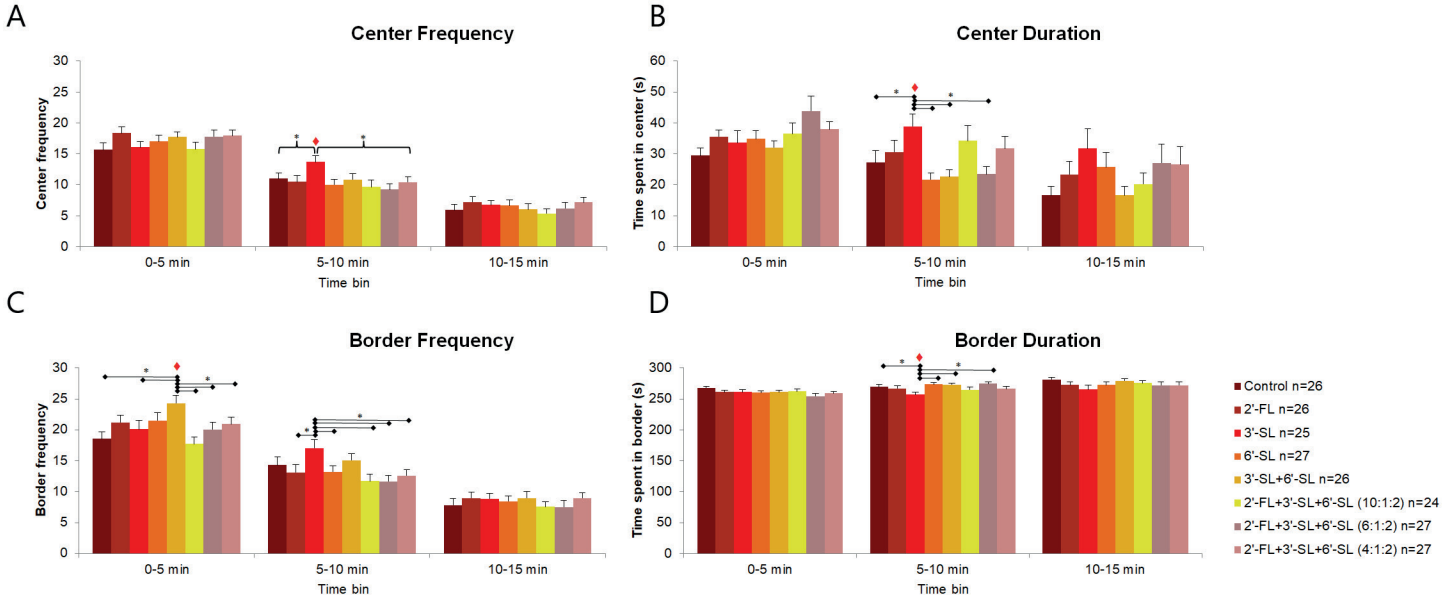

Figure 7. The effect of HMO treatment on open field test parameters (A) Frequency of center visits, divided per time bin. (B) Time spent in the center area of the open field arena per time bin. C) Frequency of border regions, divided per time bin. (D) Time spent in the border area of the open field arena per time bin. Data are presented as mean +/- S.E.M. Horizontal bars with a small, black diamond on each end indicate significant differences between the bars it connects. The asterisks indicate that the p value is < .05. The horizontal accolade on section A indicates that all these groups which are covered by the accolade, differ significantly from the 3'-SL group which falls outside of it. Bars with a red diamond above them indicate that the HMO treated group differs significantly from the control group.

Analysis 2: examination of effects of HMO treatment on exploratory behaviour per time bin. For the analysis of center and border behaviour per time bin, the male and female animals were pooled due to the lack of significant sex differences in the exploratory behaviours as show in Figure 7A-D. The exploratory behavioural variables included in this analysis were time spent in the border and center and frequency of entering border and center, at three different time bins (0-5 min, 5-10 min, 10-15 min). The time spent and frequency with which they preferred to navigate the different zones, significantly varied over time, depending on HMO treatment ($F_{(42, 880.560)}$= 1.956, p <.001, Wilks' Λ= .658, η^2 = .067). Furthermore, we also found that the exploratory behaviours of the animals fluctuated significantly over time ($F_{(6,187)}$ = 94.356, p <.001, Wilks' Λ = .248, η^2= .752), and that exploratory behaviours varied significantly per HMO treatment ($F_{(21, 546.128)}$) = 1.982, p =.006, Wilks' Λ= .971, η^2= .068).

Frequency of center visits. The MANOVA revealed a significant effect of HMO treatment on the frequency of center visits during the 5-10 min timebin ($F_{(7, 200)}$= 2.076, p= .048, η^2=.068 (Figure 7A). A follow up LSD analysis revealed that the animals who received 3'-SL entered the center significantly more than all the other treatment groups; 2'-FL (*MD* = 3.22, *SE* = 1.329, p=.016), 3'-SL+6'-SL (*MD*= 2.87, *SE*= 1.329, p = .032), Control (*MD*= 2.72, *SE*= 1.329, p = .042), 2'-FL+3'-SL+6'-SL (10:1:2) (*MD* = 4.05, *SE*= 1.356, p = .003), 2'-FL+3'-SL+6'-SL (6:1:2) (*MD*= 4.46, *SE*= 1.304, p =.342) and 2'-FL+3'-SL+6'-SL (4:1:2) (*MD*= 3.28, *SE*= 1.317, p =.014).

Time spent in center. Further analysis revealed that the effect of HMO treatment on time spent in the center was significant during the 5-10 min time bin ($F_{(7, 200)}$) = 3.024, p=.005, η^2= .096) (Figure 7B). A follow up LSD corrected post-hoc test revealed that animals who received 3'-SL spent the most time in the center of the open field when compared to the control group (*MD*= 11.6418, *SE*= 5.00081, p =.021), the 3'-SL + 6'-SL (*MD*= 16.3034, *SE*=5.00081, p= .001) , 6'-SL (*MD*= 17.1695, *SE*= 4.95520, p= .001) and 2'-FL+3'-SL+6'-SL (6:1:2) (*MD*= 15.3769, *SE*= 4.95520, p =.002).

Frequency entry border area. For the frequency of border area entries, we found significant effects of HMO treatment during time bin 0-5 min ($F_{(7, 200)}$) = 2.721, p = .010 and η^2= .087) and time bin 5-10 minutes ($F_{(7,200)}$) = 2.579, p=.014 and η^2= .083) (Figure 7C). A follow up with a LSD post-hoc test revealed that, for time bin 0-5 min, the 3'-SL + 6'-SL group entered the border region significantly more frequently when compared to the control group (*MD*= 5.73, *SE*= 1.690, p =.001), 3'-SL (*MD*= 4.19, *SE*= 1.707, p= .015), 2'-FL+3'-SL+6'-SL (10:1:2) (*MD*= 6.56, *SE*=1.725, p <.001), 2'-FL+3'-SL+6'-SL (6:1:2) (*MD*= 4.23, *SE*= 1.675, p=.012), 2'-FL+3'-SL+6'-SL (4:1:2) (*MD*= 3.34, *SE*= 1.675, p=.047). During timebin 5-10 min, the 3'-SL supplemented

group entered the border region significantly more frequently than the 2'-FL (*MD*= 3.93, *SE*= 1.601, *p*=.015), 6'-SL (*MD*= 3.86, *SE*= 1.586, *p* =.016), 2'-FL+3'-SL+6'-SL (10:1:2) (*MD*= 5.33, *SE*= 1.633, *p* =.001), 2'-FL+3'-SL+6'-SL (6:1:2) (*MD*= 5.38, *SE*= 1.586, *p*=.001), and 2'-FL+3'-SL+6'-SL (4:1:2) (*MD*= 4.49, *SE*= 1.586, *p*=.005) supplemented groups. Also, animals who received 3'-SL + 6'-SL spent significantly more time in the border when compared to 2'-FL+3'-SL+6'-SL (10:1:2) (*MD*= 3.33, *SE*=1.618, *p* <.041) and 2'-FL+3'-SL+6'-SL (6:1:2) (*MD*= 3.37, *SE*= 1.570, *p*=.033).

Time spent in border. Finally, for time spent in the border region we found an HMO effect in time bin 5-10 min ($F_{(7:200)}$= 2.634, *p* = .013, η^2= .084) (Figure 7D). A follow up LSD post-hoc test revealed that, for time bin 5-10 min, the animals who received 3'-SL spent significantly less time in the border region when compared to rats who received control (*MD*= -12.5055, *SE*= 5.17273, *p*= .017), 3'-SL+6'-SL (*MD*= -16.3748, *SE*= 5.17273, *p*=.002), , 6'-SL (*MD*= -16.9070, *SE*= 5.1255, *p*=.001) and 2'-FL + 3'-SL + 6'-SL (6:1:2) (*MD*= -17.7070, *SE*= 5.1255, *p*=.001).

In summary, we found, apart from a small reduction in locomotor behaviour in (6:1:2)-mix-treated animals, no effects of HMO treatment on locomotor behaviour in the open field test. We did find significant effects of treatment with sialylated HMOs on exploratory behaviour, especially in the second time bin (5 – 10 min). The 3'-SL group crossed the border between the center area and border area of the open field arena the most and spent most time in the center of the field, while the 3'-SL+6'-SL, control, 6'-SL and (6:1:2) triple mix groups spent most time in the border of the field.

Elevated plus maze

In the EPM (Figure 7A – C), significant differences between HMO treated animals were found on multiple exploratory variables ($F_{(77.000,\ 11110.048)}$= 1.560, *p*= .002, Wilks' Λ = .540, η^2= .084). Further examination (Figure 6A-C) revealed that the HMO groups were significantly different from one another for the factors 'distance moved' ($F_{(7,194)}$= 2.313, *p*=.027, η^2= .077), 'frequency entry closed arms' ($F_{(7,194)}$= 2.277, *p* =.030, η^2= .076), and time spent in center ($F_{(7,194)}$= 3.139, *p*=.004, η^2= .102). No significant differences between sexes were found ($F_{(11.000,\ 184.000)}$= 1.075, *p*= .384, η^2= .060). Therefore, the results and subsequent interpretation will generalize over both sexes. A follow-up with an LSD post hoc test revealed the next results.

Time zones. No effect of HMO treatment on the time spent in the open and closed arms was found. However, time spent in the center was reduced by almost all treatments when compared to the control group; 3'-SL (*MD*= 13.0704, *SE*= 4.72562,

p=.006), 3'-SL+6'-SL (*MD*= 20.000, *SE*= 4.68082, *p* < .001), 6'-SL (*MD*= 11.0781, *SE*= 4.72562, *p*=.020), 2'-FL+3'-SL+6'-SL (6:1:2) (*MD*= 13.0519, *SE*= 4.68082, *p*=.005) and 2'-FL+3'-SL+6'-SL (4:1:2) (*MD*= 11.0667, *SE*= 4.68082, *p*=.019) supplemented rats (Figure 8A).

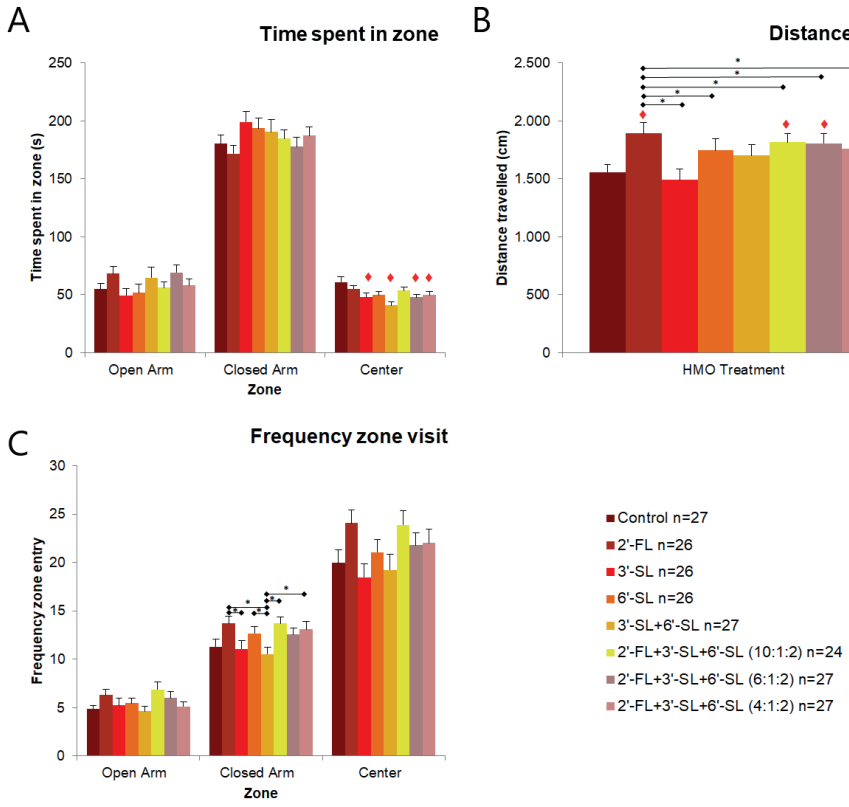

Figure 8. The effect of HMO treatment on elevated plus maze test parameters. (A) Time spent in the open arm, closed arm and center zone, (B) Frequencies of entering the open arm, closed arm and center zones. (B) (C) Distance moved. Data are presented as mean +/- S.E.M. Horizontal bars with a black diamond on each end link a pair of HMO groups which differ significantly from one another. The asterisks indicate significant differences between HMO groups (*with a significance level of* $p < .05$). Bars with a red diamond indicate a significant difference between an HMO treated group and the control group.

Frequency zone visits. No effect of HMO treatment on the frequency on the open arms and center was found. However, 2'-FL supplemented animals entered the closed arms more often than 3'-SL (*MD*= 2.6154, *SE*= 1.08731, *p*=.017), and 3'-SL+6'-SL (*MD*= 3.1738, *SE*= 1.0772, *p*=.004) animals. The 3'-SL+6'-SL group also entered the

closed arm significantly less when compared to 6'-SL (*MD*= -2.1353, *SE*= 1.07720, *p*=0.049), 2'-FL+3'-SL+6'-SL (10:1:2) (*MD*= -3.1898, *SE*=1.09982, *p*=.004) and 2'-FL+3'-SL+6'-SL (4:1:2) (*MD*= -2.556, *SE*= 1.06699, *p*=.018) groups. Finally, 2'-FL+3'-SL+6'-SL (10:1:2) supplemented rats entered the closed arms more frequently compared to the control group (*MD*= 2.4120, *SE*=1.09982, *p*=.029) (Figure 8B).

Distance moved. 2'-FL supplemented animals travelled a significantly larger distance compared to the control group (*MD*= 333.8787, *SE*= 122.53386, *p*=.007) and 3'-SL supplemented animals (*MD*= 400.0779, *SE*= 123.68443, *p*= .001). 3'-SL supplemented animals also moved significantly less than 6'-SL (*MD*= -252.2052, *SE*= 123.68443, *p*= .043), 2'-FL+3'-SL+6'-SL (10:1:2) (*MD*=-323.2352, *SE*= 126.23490, *p*=.011), 2'-FL+3'-SL+6'-SL (6:1:2) (*MD*= -312.0435, *SE*= 122.53386, *p*=.012) and 2'-FL+3'-SL+6'-SL (4:1:2), (*MD*= -266.3366, *SE*= 122.53386, *p*=.031) supplemented animals. Finally, distance was increased by the triple mix treatments 2'-FL+3'-SL+6'-SL (10:1:2) (*MD*= -257.0360, *SE*= 125.10778, *p*=.041) and 2'-FL+3'-SL+6'-SL (6:1:2) (*MD*= -245.8443, *SE*= 121.37237, *p*=.044) supplemented animals (Figure 8C) when compared to the control group.

In summary, distance moved on the elevated plus maze was significantly increased in all HMO treated animals except for the 3'-SL supplemented animals, in particular the triple – mixed groups. Furthermore, the HMO treated groups spent significantly less time in the center of the plus maze when compared to the except for the groups high in fucosyllactose, i.e. 2'-FL and (10:1:2) triple mix animals.

Discussion

We found that different HMOs exerted different developmental outcomes throughout infancy and adolescence. More specifically, HMO treated animals, with the exception of the 3'-SL treated group, were on average significantly lighter, with fucosyllactose treated animals being the lightest. Furthermore, depending on sex, the female 3'-SL and both female and male (6:1:2) triple mix treated animals were the first HMO groups started eye opening on PND13. On PND 15, all HMO groups, except for 2'-FL, had the most pups with eyes opened. In the open field test, the 3'-SL group crossed the border between the center area and border area of the open field arena the most and spent most time in the center of the open field, while 3'-SL+6'-SL, 6'-SL and (6:1:2) triple mix spent the most time in the border of the field. Finally, in the elevated plus maze test, distance moved was lowest in the 3'-SL supplemented animals but increased in the triple-mix groups compared to controls. Most HMO-treated groups

spent significantly less time in the center of the plus maze compared to the control group, except for the 2'-FL and 2'-FL+3'-SL+6'-SL (10:1:2) groups. Overall, the data suggest that the 6:1:2 triple mix promotes development, that the 6:1:2 and 10:1:2 triple mix groups increase distance moved in the elevated plus maze, and that the 6:1:2 mix group spent more time in the border of the open field relative to the 3'-SL group. Finally, we found that the 3'-SL group developed most quickly, was the least anxious (more time and visits in the center of the open field), and moved the least in the elevated plus maze test.

One of the earliest markers which could be indicative of HMOs' influence on neurodevelopment we recorded was eye opening. The effects of HMOs on eye-opening were dependent on the type of HMOs that were administered, sex specific and fluctuated over time. During eye opening, the 6:1:2 mix group was the first to have both eyes opened, while only the females of the 3'-SL group had started eye opening on PND13. It is possible that different HMOs, or their potential downstream metabolites, interact with different neurodevelopmental pathways within the brain. As we have not collected brain samples at the time of eye opening in our study, it is difficult to ascertain which neurobiological processes are at play in the background. However, as it is commonly found that HMOs are a critical component for neural development, it is likely that they exert their neurodevelopmental actions via a variety of molecular mechanisms, such as alterations in plasticity, myelination and circuit formation (Wang, 2009; Berger et al., 2023, Hauser et al., 202, Rajhans et al., 2023), which can lead to different behavioural outcomes down the line. In fact, other studies have examined the molecular effects of a lack of dietary HMOs on gene expression in the medial prefrontal cortex at the time of eye opening. Hauser et al. (2021) found that the normal wildtype (WT) pups, who were raised on 6'-SL deficient milk, displayed a downregulation of genes involved in neural circuit formation (e.g. gangliosides and PSA -NCAM) in the prefrontal cortex at the time of eye opening. Furthermore, these 6'-SL deprived WT pups displayed altered tryptophan metabolism when compared to control mice. As the authors demonstrated that the lack of a specific HMO during infancy leads to a disruption in gene expression for neural circuit formation, the presence of certain HMOs in the infant's diet seems a prerequisite for neurodevelopmental processes to occur normally. Such alterations in circuit formation and tryptophan metabolism under 6'-SL deficient conditions thus highlight the importance and role of sialic acid in neural development during critical time periods such as eye opening. In our study we found that a diet high in sialic acid, or a complex mixture of fucosyllactose and sialyllactose, yielded a different rate of eye opening. Guan et al. (2017) describe eye opening as an indicator of visual cortex maturation. In this case, different HMO

treatments may contribute to an increased cortical maturation, which seems to occur in a sex dependent manner as well. While this does not answer the question whether an abundance of certain HMOs like in our present study would then lead to an accelerated circuit formation, the fact remains that the presence or absence of HMOs during infancy influence developmental processes occurring at critical developmental timepoints, such as eye opening. Future studies are required to further investigate the neurobiological effects of HMOs, in single forms and various combinations, to better understand their (combined) effects on brain development.

During adolescence, HMOs were found to affect anxiety-like behaviour. More specifically, 3'-SL supplemented animals displayed the lowest levels of anxiety in the EPM and OFT. An effect on emotional behaviour has also been reported by Tarr et al (2015), who fed mice 3'-SL or 6'-SL in a social disruptor stress paradigm. They found that the supplementation of sialylated HMOs for two weeks prior to stressor exposure was effective in ameliorating the negative effects of stress on anxiety behaviour and rescued the social disruption stressor induced reduction in immature neurons in the hippocampal dentate gyrus. Regarding locomotor activity on the EPM, we found that 2'-FL supplementation, in single or mixed form, led to an increase in the distance moved in the EPM test. To the best of our knowledge, no other murine study has examined activity behaviour in adolescence because of HMO administration. However, a recent study by Hung et al. (2021) showed that the acute administration of 2'-FL immediately post intracerebral haemorrhage stroke improved locomotor activity. Taken these and the EPM findings together it is possible that dietary 2'-FL produces a more active phenotype. An additional interesting finding is the sex difference in locomotor activity in the OFT when the data was examined over the full duration of 15 minutes. Within the (6:1:2) mix group, females of this group showed a significant reduction in both distance moved and the velocity with which they moved, when compared to the control group. This finding was not found in the males of the (6:1:2) mix treatment group. Hence, there may be sex differences in the effects of a specific combination of fucosylated and sialylated HMOs which warrant further investigation. Furthermore, due to the differences in locomotor activity between the OFT and the EPM for the triple mix (6:1:2) group, it is possible that the OFT locomotor activity is more anxiety driven while the EPM locomotor activity is more exploration driven.

In the present study, HMO administration pre- and post-weaning (PND8-42) affected developmental outcomes during infancy and behaviour already during adolescence (PND22-42). Of interest, not all studies which started HMO supplementation in the pre-weaning period yielded short term results in adolescence. For instance, Oliveros

et al. (2016) supplemented rats from PND3 until weaning with 1g KG/BW of 2'-FL and only reported improved cognitive performance when they were 1 year old but not at 6 weeks of age. This delayed effect of administration is also reported in their sialylated HMO study (Oliveros et al., 2018) where the pre-weaning supplementation of Neu5Ac and 6'-SL only produced cognitive effects when animals were 1 year old. The discrepancy between our findings and those of Oliveros may relate to procedural differences. One of the key differences between our study and Oliveros' study was that the latter used a cross-fostering paradigm when rearing their pups on a sialylated specific diet (Oliveros et al. 2018). In their design, the pups designated for the HMO diet were fostered to already lactating dams whose milk at that point in lactation, was almost devoid of sialylated HMOs, and thus the dietary sialic acid intake could be more accurately controlled. In addition, in their 2'-FL study in 2016; they tested animals both in early life and in late life, but those were not the same experimental animals.

We hypothesized that combining HMOs may have synergistic effects and exert beneficial effects on development. In line with this hypothesis, we observed that the 6:1:2 triple mix promoted eye opening. This group also displayed increased distance moved in the elevated plus maze compared to amongst others the control group and spent more time in the border of the open field relative to the 3'-SL group. As to whether a change in locomotor activity and preference to stay close to borders is a beneficial development is hard to determine. Our findings do suggest that the combination of HMOs differently affects brain development.

A strength of this study is that we tested HMO in singular form and in combination and adjusted the dose according to the developmental stage of the animals. Moreover, we tested both male and female rats, providing a more complete picture of the effects of HMO administration by accounting for both sexes. Administrating HMOs from infancy (PND8) until sexual maturity (PND49) ensured that several (neuro)developmental milestones such as visual development (eye opening, the last sense to develop), emotional and locomotor development (OFT and EPM) were under the influence of HMOs. By including this entire developmental period from infancy to late teens, we gained more information on how HMOs may affect the cognitive and emotional development at different stages. A limitation of the present study is that HMO administration was still ongoing during behavioural testing in our study, albeit at a lower dose (200mg/kg BW) from PND21 onwards. Therefore, we cannot rule out the possibility that HMOs acutely influenced various developmental, behavioural and emotional parameters. Additional studies in which the effects of acute versus chronic supplementation at different time points

throughout life would shed more light on this matter. Secondly, the control group still received HMOs naturally via the dam's maternal milk, which contains 3'-SL and trace amounts of 6'-SL (Urashima et al., 2001). Therefore, the inclusion of a negative control group via a cross-fostering paradigm where the maternal milk has been depleted could be a viable solution in future studies. Furthermore, it is important to note that the translation of the effects of HMOs on the development of rats to human infants requires the utmost caution as there are marked species differences, such as the naturally occurring HMO content in rat milk versus human milk, and therefore also differences in the composition of the microbiome that can process these HMOs to generate the downstream metabolites which may further impact brain development. Another key difference is the fact that rats are an altricial species as opposed to humans who are in part altricial and in part precocial species in terms of neonatal development. However, researchers such as Semple et al. (2013) have summarised how the rodent ages were to translate to human age based on neurodevelopmental markers and developmental stages such as proliferation, migration, myelination and so on. The setup of this study encompassed every developmental stage so the effects of HMOs throughout development are charted. In addition, prior studies have found marked effects of dietary sialylated and fucosylated HMOs in rats and infants alike (Vazquez et al., 2015; Oliveros et al., 2016, Oliveros et al., 2018, hereby at least providing common ground for potential underlying metabolic and developmental mechanisms in both species.

Conclusion

In conclusion, our study shows that HMO supplementation from the pre-weaning phase up to adolescence modifies eye opening, anxiety-related behaviour and activity. These results support the notion that HMO administration during critical time periods has marked effects on brain development and behaviour, with different outcomes between sexes. Furthermore, we provide the first indications of potential synergistic effects of HMOs, though additional studies are required where such synergy can be examined closer in a dose-controlled manner. Nonetheless, this further highlights the importance of having the right balance and variety of dietary HMOs for the developing infant and beyond.

Supplementary material

Table S 1. Comparisons between the control group and HMO groups for weight gain over time. Reported in this table are the mean differences (MD) and standard errors (SE) of the significant comparisons ($p < .05$*, $p < .01$**, $p < .001$ †) between the control group and the other treatment groups, based on the multivariate ANOVA LSD post hoc results. The comparison pairs in this table are formulated as control vs one of the treatment groups, with no distinction between sexes. 10:1:2, 6:1:2 and 4:1:2 refer to the mix group ratios of the 2'-FL +3'-SL+6'-SL treatment groups.

Control, vs:	**PND 8**	**PND 9**	**PND 10**	**PND 11**	**PND 12**	**PND 13**	**PND 14**
2'-FL n=27	0.704 (.332)*	0.952 (.351)**	0.728 (.365)*	0.798 (.379)*	0.908 (.406)*	1.456 (.410)†	1.761 (.431)†
3'-SL n=27	-1.21 (.333)†	-1.524 (.351)†	-2.058 (.365)†	-1.805 (.380)†	-1.699 (.407)†	-1.364 (.411)**	-1.056 (.431)*
6'-SL n=27		0.759 (.351)*			0.851 (.406)*	1.228 (.410)**	1.687 (.431)†
3'-SL+6'-SL n=27							
(10:1:2) n=24							
(6:1:2) n=26	-0.932 (.336)**		-0.810 (.368)*				
(4:1:2) n=27						1.157 (.410)**	1.764 (.431)†
Control, vs:	**PND 15**	**PND 16**	**PND 17**	**PND 18**	**PND 19**	**PND 20**	**PND 21**
2'-FL n=27	2.127 (.442)†	2.730 (.481)†	3.221 (.516)†	3.455 (.544)†	3.999 (.617)†	4.012 (.705)†	4.151 (.781)†
3'-SL n=27	-1.089 (.443)*						
6'-SL n=27	1.927 (.442)†	2.904 (.481)†	3.209 (.516)†	3.166 (.544)†	1.759 (.609)†	2.501 (.705)†	2.032 (.781)*
3'-SL+6'-SL n=27		1.271 (.481)**	1.461 (.516)**	1.496 (.544)**			
(10:1:2) n=24		1.176 (.500)*	1.302 (.537)*	1.427 (.565)*			
(6:1:2) n=26	1.271 (.446)**	2.006 (.486)†	2.071 (.521)†	2.243 (.549)†	2.349 (.623)†	2.193 (.712)**	2.633 (.789)**
(4:1:2) n=27	2.087 (.442)†	2.841 (.481)†	3.091 (.516)†	2.803 (.544)†	2.384 (.617)†	2.442 (.705)**	2.458 (.781)**

Table S 2. Results X^2 test for eye opening. The 'observed' value in the table indicates the actual observed number of animals that had both eyes open, while the 'expected' value is what is predicted value based on the model generated by the X^2 test. The percentage of pups with both eyes opened is included after the observed values as well. 10:1:2, 6:1:2 and 4:1:2 refer to the HMO ratios used in the three mix groups 2'-FL+3'-SL, +6'-SL.

HMO	PND 13	PND 14	PND 15
3'-SL	Observed: 2 out of 27 – 7.4% Expected: 1.4	Observed: 20 out of 27 – 74% Expected: 14.9	Observed: 27 out of 27 – 100% Expected: 24.2
2'-FL	Observed: 0 out of 27 – 0.0% Expected: 1.4	Observed: 4 out of 27 – 14.8% Expected: 14.9	Observed: 13 out of 27 – 48.1% Expected: 24.2
3'-SL+6'-SL	Observed: 0 out of 27 – 0.0% Expected: 1.4	Observed: 17 out of 27 – 62.9% Expected: 14.9	Observed: 27 out of 27 – 100% Expected: 24.2
Control	Observed: 0 out of27 – 0.0% Expected: 1.4	Observed: 16 out of 27 – 59.2% Expected: 14.9	Observed: 20 out of 27 – 74% Expected: 24.2
6'-SL	Observed: 0 out of 27 – 0.0% Expected: 1.4	Observed: 15 out of 27 – 55.5% Expected: 14.9	Observed: 27 out of 27 – 100% Expected: 24.2
10:1:2	Observed: 1 out of 24 – 4.1% Expected: 1.2	Observed: 13 out of 24 – 54.1% Expected:13.2	Observed: 24 out of 24 – 100% Expected: 21.5
6:1:2	Observed: 8 out of 26 - 30.7% Expected: 1.3	Observed: 17 out of 26 – 65.3% Expected: 14.3	Observed: 25 out of 26 – 96.1% Expected: 23.3
4:1:2	Observed: 0 out of 27 – 0.0% Expected: 1.4	Observed: 15 out of 27 – 55.5% Expected: 14.9	Observed: 27 out of 27 – 100% Expected: 24.2

4

Chapter 4

Long-term synergistic effects of fucosylated and sialylated human milk oligosaccharides on memory and plasticity

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Abstract

Introduction: Human Milk Oligosaccharides (HMOs) promote cognitive development and improve memory functioning. While HMOs are found in different combinations and ratios across lactation, it is unclear if a combination of HMOs in different ratio's has a larger effect on cognitive development relative to single HMOs. We hypothesized that the combination of fucosylated and sialylated HMOs work in synergy and exert a greater improvement of cognitive functioning and promote sustained neural plasticity in later life.

Methods: Hundred-and-ten male Long Evans rats were administered the HMOs 2'-FL, 3'-SL and 6'-SL, either in singles or in various combinations, from PND8 until PND49. During adulthood, they were tested on object recognition and location memory. Finally, we assessed mRNA expression levels of *Bdnf, DGL4, MBP* and *NCAM1* within the medial prefrontal cortex and dorsal hippocampus, and neurogenesis levels based on BrdU+ neurons in the dentate gyrus.

Results: 2'-FL+3'-SL+6'-SL (10:1:2) and (6:1:2) mix treatments, but not the single HMO treatments, significantly improved recognition memory. No significant effects of HMO treatment on location memory were found, though the 2'-FL+3'-SL+6'-SL (10:1:2) mix's performance differs significantly from baseline. The 2'-FL+3'-SL+6'-SL (10:1:2) and (4:1:2) supplemented groups displayed an increased number of BrdU+ neurons within the hippocampus. Finally, the triple mix groups displayed a significant downregulation in *Bdnf* gene expression in the medial prefrontal cortex.

Conclusions: In conclusion, early life fucosylated and sialylated HMOs operate in concert to improve recognition memory, increase hippocampal neurogenesis and reduce *Bdnf* gene expression in the medial prefrontal cortex in late adulthood. Further research is essential to elucidate the mechanisms through which HMOs affect neurodevelopment and cognition throughout life.

Keywords: 2'-fucosyllactose, 3'-sialyllactose, 6'-sialyllactose, recognition memory, pattern separation, neurogenesis

Introduction

Breastmilk is recommended by the WHO (2024) as the primary nutritional source for at least the first six months of life and ideally remains part of an infant's diet for several years. Globally recognized as the gold standard for infant nutrition (Ballard & Morrow, 2014; de Weerth et al., 2023), it is uniquely tailored to an infant's needs. Unlike infant formula, breastmilk contains essential nutrients and bioactive molecules like immunoglobulins, cytokines, and oligosaccharides, which support immune function, protect against infections, and aid intestinal development (Townsend, 2019). These benefits extend beyond infancy, influencing long-term health outcomes such as metabolic disease (de Weerth et al., 2023). Maternal milk thus provides numerous health advantages that help infants thrive. Considering all these protective effects on children's health related outcomes, maternal milk is shown to provide a plethora of health benefits which enable infants to thrive in the early years of life and beyond.

Of particular interest among these bioactive molecules are the Human Milk Oligosaccharides (HMOs). HMOs are complex carbohydrates synthesized in the mammary alveolar glands, and are the third most abundant class of nutrients present in maternal milk (Ballard & Morrow, 2013, Andreas, Kampmann & Le Doare, 2015; Vandenplas et al., 2018). In general, HMOs consist of three main categories; fucosylated (FL), sialylated (SL) and neutral HMOs, and their quantity and ratio within breastmilk dynamically vary over time as a function of a wide range of maternal and environmental factors, such as maternal genetic background, maternal bloodtype and maternal diet (Jorgensen et al., 2021; Ferreira et al., 2021; Bode, 2012). It is generally believed that the high individual variability of HMO content over time and populations reflects the infant's specific nutritional demands at any given point in time (Wang et al., 2007; ten Bruggencate et al., 2014).

HMO's function and importance for the developing infant is multifold; HMOs are critical nutrients in promoting healthy immune system functioning and contribute to a balanced gut microbial composition with downstream effects on brain development and cognitive functioning, mediated by the Gut Brain Axis (GBA). Upon ingestion, HMOs are metabolized in the gut by bacteria, producing metabolites like short-chain fatty acids (SCFAs) and sialic acid, which further influence brain functioning (Dalile et al., 2019). It is therefore assumed that the right HMO balance in infant nutrition is a requirement for healthy brain development and downstream cognitive outcomes throughout life, a phenomenon also coined as Lactocrine Programming (Bartol & Bagnell, 2018, Willemsen et al., 2023). This

phenomenon can be best explained as the concept that bioactive components in maternal milk foster long lasting effects on cognitive and behavioural development. As HMOs are among those bioactive components, it stands to reason that they are capable of exerting long lasting effects by contributing to various neuronal circuits and neurodevelopmental processes during early life which may ultimately result into downstream effects on cognition and behaviour.

Research on the impact of HMOs on cognitive development has progressed significantly over the last two decades. While conducting clinical trials with human infants presents challenges, animal studies consistently show that HMOs benefit neural development, neurogenesis, circuit formation, and memory function. Specifically, studies on individually administered 2'-Fucosyllactose (2'-FL), 3'-Sialyllactose (3'-SL), and 6'-Sialyllactose (6'-SL) reveal that these HMOs enhance learning and memory (Vazquez et al., 2016; Oliveros et al., 2018), improve spatial memory (Obelitz-Ryom et al., 2019), and reduce stress-induced anxiety while maintaining normal neurogenesis rates during stressful conditions (Tarr et al., 2015), regardless of the timing of administration (e.g., before or after weaning).

Key mechanisms underlying memory formation include adult hippocampal neurogenesis, which is partly mediated by brain sialic acid levels (Tarr et al., 2015). Newly formed granule cells in the dentate gyrus exhibit hyper-excitable membrane properties, facilitating long-term potentiation (LTP) that supports synaptic plasticity and memory formation (Yirmiya & Hoshen, 2011). These granule cells, along with CA1/CA3 neurons, express high levels of the development marker PSA-NCAM, suggesting that sialic acid from HMOs enhances hippocampal plasticity through these neurons (Bonfanti, 2006). Tarr et al. (2015) demonstrated that administering sialylated HMOs maintained neurogenesis even under social stress, which typically reduces new neuron generation (as indicated by BrdU/DCX markers). Such increased neurogenesis was found to correlate with improved memory performance and pattern separation, i.e. the ability to distinguish similar contexts, which are crucial for learning and cognitive flexibility (Sahay et al., 2011; Oomen et al., 2014a; Anacker & Hen, 2017).

Additionally, both fucosylated and sialylated HMOs uniquely contribute to neural development and plasticity by influencing key neurodevelopmental processes, such as neural migration, myelination, synaptic plasticity regulation, and neurogenesis (Semple et al., 2013). The incorporation of fucose from fucosylated HMOs into hippocampal glycoproteins during learning suggests that fucosylation may collaborate with local protein synthesis in response to synaptic stimulation

and LTP potentiation, further supporting memory formation (Murrey & Hsieh-Wilson, 2008; Vazquez et al., 2015, 2016; Oliveros et al., 2016). Concurrently, dietary sialic acid derived from sialylated HMOs plays a critical role in pre- and postnatal brain development and contributes to synaptic plasticity, neurogenesis, myelination, and memory formation (Wang et al., 2007; Tarr et al., 2015). One of the primary molecular mechanisms by which sialic acid influences neural plasticity and memory is through its attachment to Neural Cell Adhesion Molecules (NCAM) in a polysialylated form, resulting in increased PSA-NCAM expression in key brain regions involved in cognition and memory (Muller et al., 1996; Bonfanti, 2006; Fewou et al., 2007; Weledji & Assob, 2014; Wang, 2012).

Given the high variability in HMO content in breastmilk, it is likely that different HMOs work synergistically when supplied together. This has already been demonstrated in gut bacteria growth and function (Lawson, 2019; Thongaram et al., 2017; Hoeflinger et al., 2014; Bunesova et al., 2016). Since gut-related mechanisms are closely linked to brain development, behaviour, and cognition, we propose that the synergistic effects of HMOs may extend to neural processes, potentially enhancing memory and cognitive function across the lifespan. To investigate this, we designed a study which examines cognitive performance and also directly measures neurogenesis. Specifically, we hypothesize that combined supplementation of fucosylated and sialylated HMOs will synergistically boost memory performance and hippocampal plasticity, in part by promoting neurogenesis and increase in neuroplasticity markers such as *Bdnf, Mbp, Dlg4* and *Ncam1*. To this end, we supplemented adult male rats with 2'-FL, 3'-SL, and 6'-SL (individually and in combination) from PND 8 to PND 49. Memory performance was assessed using both the Novel Object Recognition Task (NORT) and the Novel Object Location Task (NOLT), which probe recognition memory and spatial memory, including pattern separation (Sahay et al., 2011; Oomen et al., 2014a). Furthermore, we assessed neurogenesis in the hippocampus and the expression of neuroplasticity factors in the doral hippocampus (dHPC) and medial prefrontal cortex (mPFC), regions that play a key role in memory processes. Specifically, we focused on *Dlg4, Mbp, Ncam1* and *Bdnf*, as they are genes that are mediated by the fucosylated and sialyllated HMOs (Altieri et al., 2004; Meyer, Bonhoeffer & Scgeuss, 2014; Vukojevic et al., 2020; Weledji & Assob, 2014; Obelitz-Ryom et al., 2019). DLG4 is a scaffolding protein essential for synaptic signaling and the organization of synaptic complexes, playing a vital role in maintaining synaptic structure and plasticity in neurons (Meyer, Bonhoeffer & Scgeuss, 2014). MBP is critical for the formation and maintenance of the myelin sheath, which insulates nerve fibers to ensure rapid signal transmission and proper neural function (Obelitz-Ryom et al., 2019; Xin & Chan, 2020). NCAM1 is involved in cell-cell adhesion,

influencing neural development, synaptic plasticity, and remodeling, which are essential for learning, memory, and neural repair processes (Gascon, Vutskits & Kiss, 2007; Vukojevic et al., 2020; Bonfanti, 2006; Weledji & Assob, 2014). Finally, BDNF is responsible for neuroplasticity, neural survival and cogntion and memory (Altieri et al., 2004; Bach et al., 2023). This combined approach allows us to assess both behavioural outcomes and potential underlying molecular mechanisms of HMOs on memory and plasticity.

Method

Animals

Long Evans breeding rats were purchased from Envigo (US) and used for the in-house breeding of the rats used in this study. Litters exceeding 10 pups were culled down to 10 pups on PND 0-1 to ensure the dam could provide adequate care, and pups were weaned at PND21. We attempted to maintain a 50-50 male to female ratio. Animals were housed in a reversed day/night cycle (12:12; lights off at 9AM, on at 9PM) so that handling, HMO administration and behavioural testing was carried out during the dark hours, which was their active period of the day. The housing room's ambient temperature was set at a steady 20°C, with 40% humidity. Food and water were supplemented ad libitum during the course of this study. All animals were housed socially in type IV tecniplast cages (either in pairs or in triples). Animals of the same sex and same HMO groups were housed together to prevent pregnancies and coprophagy of feces from other HMO treatment groups. A total of 110 males were used in this study, these were the same males as used in Chapter 3. The experimental procedures were performed under a project license from the Central Committee on Animal Experiments (Centrale Commissie Dierproeven, The Hague, The Netherlands), in full compliance with the legal requirements of Dutch legislation on the use and protection of laboratory animals (Animal Testing Act). All efforts were made to reduce the number of animals used and their suffering.

Experimental groups

The animals used in this study have been supplemented with HMO's from PND 8 until PND 49. 8 experimental groups were included in this study; 3'-SL, 6'-SL and 2'-FL for the single HMOs, 3'-SL + 6'-SL (1:2 ratio) for the double mix, and for the triple mixes, the treatments were 2'-FL + 3'-SL + 6'-SL at ratios (10:1:2), (6:1:2) and (4:1:2), described in detail in Chapter 3. The final group was the control group, who did not receive any HMOs but were administered normal drinking water in the exact same manner as the other HMO animals did. In gross alignment with development-

dependent variation in HMO concentration in mother milk, the HMO dosage they received and the administration method, changed over time; from PND 8 – PND 14 animals were given a dose of 400mg/Kg/Bw a day via oral gavage, from PND 15 – 21 they received a dose of 800mg/kg/Bw a day via oral gavage, and from PND 22 – 49 they received a dose of 200 mg/ Kg/Bw a day via their drinking water. Memory tests were conducted when rats were 10-12 months old (Figure 1).

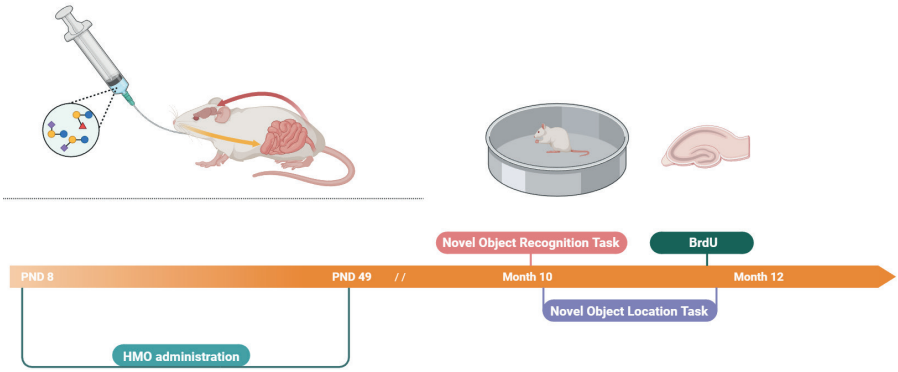

Figure 1. Timeline. Male rats who were administered HMOs 3'-SL, 6'-SL or 2'-FL, in single or in combined form during infancy and adolescence, conducted two memory tests (Novel Object Recognition Test and Novel Object Location Task) during late adulthood. Half of these animals were injected with BrdU for hippocampal neurogenesis staining on the final NOLT trial and were sacrificed three days later, while the remaining half of the animals not receiving BrdU were sacrificed 48 hours after the final trial. Created with BioRender.com.

Memory tests

Testing conditions: On each testing day, animals were transported during their active phase to the testing room at least one hour before the experiment began. This room maintained the same day/night cycle as the original housing room. The transportation period was included as part of the habituation process. Prior to the memory tests, animals had been assessed during infancy and adolescence on eye opening, weight, the elevated plus maze, and the open field test.

Testing apparatus, objects and setup: Two identical grey circular arenas (diameter: 100 cm; wall height: 50 cm; elevated 30 cm from the ground) were used. Each arena contained embedded magnets in the baseplate to ensure consistent object placement via magnetic coasters. A camera was mounted above each arena for offline scoring. Objects included black and white tealights (acquisition phase) and a blue glass object (testing phase) for the NORT; and red cups and red rubber hoops for

the NOLT (see Supplementary Material S1). Arenas and objects were cleaned with 70% ethanol after each trial. No external cues were present in the arena or testing room. Environmental conditions were consistent: temperature at 20°C, humidity at 40%, and arena light intensity at 20 lux. All trials lasted 3 minutes, with a one-hour interval between acquisition and testing.

Shared Experimental Procedure: Animals were habituated to handling and the testing environment for two weeks prior to the experiments. Habituation to objects occurred on two separate days, with one rest day between each. The same acquisition protocol was used for both the NORT and NOLT: animals were placed in the arena with two identical objects for 3 minutes, then removed. After a one-hour inter-trial interval, they were returned for the testing phase. One rest day was maintained between each testing day.

Novel Object Recognition Test

The Novel Object Recognition Test (NORT) assesses recognition memory by measuring exploration of a novel object. A trial on a given day consists of two phases; the acquisition phase in which animals can explore the identical object pairs for 3 minutes, followed by the testing phase one hour later. During the testing trial, one familiar object remained while the other was replaced by a novel object. (Figure 2A and B). Increased exploration of the novel object is taken as evidence of recognition memory (Lueptow, 2017). order and object positioning were counterbalanced across animals.

Novel Object Location Task

The Novel Object Location Task (NOLT) assesses spatial memory and pattern separation (Oomen et al., 2014; van Goethem, van Hagen, & Prickaerts, 2018). In this task, both acquisition and testing involve two identical objects, but one object is displaced in the testing phase (Figure 2C). Exploration of the displaced object indicates recognition of the spatial change.

Object positions were labeled as P1 (center), P3 (intermediate displacement), and P5 (furthest displacement). Two testing conditions were defined: Easy condition (P5): Large displacement from the original location, assumed easier to detect (low pattern separation demand). Difficult condition (P3): Small displacement, considered more difficult to detect (high pattern separation demand).

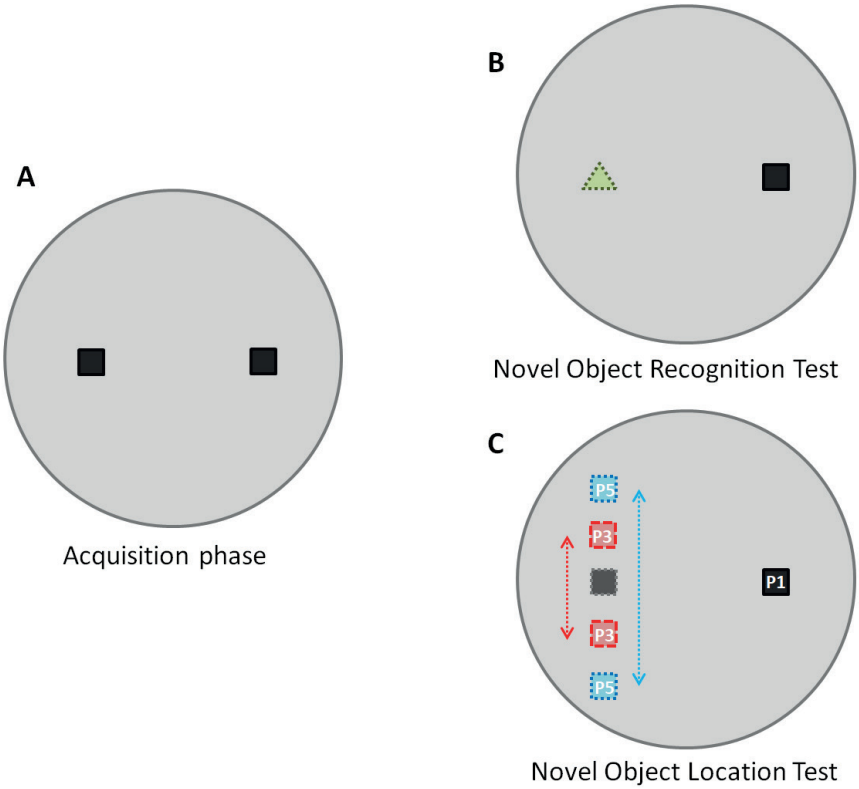

Figure 2. Graphical overview of the Novel Object Recognition Test and the Novel Object location test. **A** This represents the baseline location of the objects which is the same for both the NORT and the NOLT. **B** This is the testing phase of the NORT, where one of the familiar objects (black square) is replaced by a a novel object (green triangle) on either the left or the right side of the arena. **C** This is the testing phase of the NOLT, with Position 1 (P1) being the baseline. Here one of the two objects is displaced to either position 3 (P3, red boxes) or position 5 (P5, blue boxes). Position 3 is a small displacement which represents the difficult condition of the task, while Position 5 is a large displacement which represents the easy condition of the task. Objects used in this task are included in the supplementary material section.

During testing, the displaced object was moved along an axis with five embedded magnet positions (P5-P3 north, P1 center, P3-P5 south), placed on either the left or right side (Figure 2C and Figure S1). The testing sequence and object pairs were counterbalanced across treatment groups to avoid bias. Object pairs were alternated between testing days to preserve novelty. One day of rest was maintained between each testing day.

Recording and scoring: All sessions were video recorded and scored manually with Behavioral Observation Research Interactive Software (BORIS) (Friard &

Gamba, 2016), to determine the amount of time animals spent exploring left and right objects in both acquisition and testing phases. Exploration was defined as direct nose contact with the object; time spent sitting on the object was excluded (Lueptow, 2017). The following values were determined: a1: time spent on the left object in acquisition; a2: time spent on the right object in acquisition; a3: time spent on unchanged object in testing; b: time spent on novel/displaced object in testing; e1: total exploration time in acquisition; and e2: total exploration time in testing.

The preference for a novel/displaced object was expressed as discrimination index (DI), with 0<DI<1 indicating the preference for the novel/displaced object and -1<DI<0 indicating the preference for the unchanged object (van Goethem, van Hagen & Prickaerts, 2018). For the NORT, discrimination indices were calculated across all groups along this formula; DI: $\frac{b-a3}{e2}$. For the NOLT, indices were calculated separately for P3 (difficult) and P5 (easy) conditions. Finally, a formula correction for the discrimination index was applied due to an object preference in some of the tested animals during the NORT; DI corrected = $(\frac{b - average(a1 + a2 + a3 + a4)}{e1 + e2})$.

NOLT retest with BrdU injection: 2 days after having been tested on each displacement position in the NOLT, half of each HMO treatment group, selected at random, underwent one additional NOLT trial (with 50% of the animals being tested on the P1 position and the other 50% of the animals on the P3 position during the displacement phase). The rationale behind these two positions was that the P1 location is the 'no displacement' control condition for the P3 displacement. During this additional trial, all the animals were administered BrdU via i.p. injection to facilitate neurogenesis labelling during this memory task. Animals were randomly assigned to either the P1 or the P3 task condition during this additional trial.

BrdU administration: The animals were injected once with 5-bromo-2'-deoxyuridine (Sigma-Aldrich, B5002-1G), at a dose of 100mg/Kg Bw, on the final testing day of the Novel Object Location Task, one hour prior to the acquisition phase. This enabled the thymidine analogue to be taken up by the body by the time they were being tested on the novel location (2 hours after injection).

Sacrifice

Rats were sacrificed either three, (BrdU treated animals, 10 – 12 months of age) or four (the non-BrdU injected rats) days after concluding the NOLT. The rats who received BrdU were transcardially perfused after pentobarbital anaesthesia, while the rats who did not receive BrdU were decapitated after a light isoflurane anaesthesia. Perfusion was performed after a lethal IP pentobarbital injection, first

by rinsing using (PBS) x1, followed up by tissue fixation with 4% paraformaldehyde (PFA). Upon sufficient fixation, brains were extracted and subsequently stored in 4% PFA solution overnight. The brains obtained after decapitation were immediately extracted and snap frozen on dry-ice, after they were wrapped in aluminium foil and stored at -70°C.

qPCR

Sample preparation: Brains obtained from the non BrdU treated animals were sectioned at 100 µm thickness and captured on RNA free slides, upon which ±10 punches per brain region (dHPC and mPFC) were collected in 2 mL Eppendorf tubes using a 1.5mm punching needle, for later use in RT-qPCR analysis. All slicing and punching were performed at -12°C to -14°C using the Leica CM3050 S Research Cryostat. The Eppendorf tubes containing the brain punches, were immediately put on dry ice after collection, and stored at -70°C.

RNA isolation: Five animals per HMO treatment group were randomly selected for gene expression analysis. As both the dHPC and the mPFC were selected as brain regions of interest, gene expression of all target genes was analysed for the same 40 animals in both regions. The samples were spread over multiple extraction runs, contemplating equal distribution of the HMO treatment groups over the different runs. RNA was isolated from the punched brain material, using the QIAGEN RNeasy mini kit (GIAGEN, Hilden, Germany), according to manufacturer's standard protocol including DNase digestion (attachment 5), performed by the QIAGEN QIAcube automated nucleic acid extractor (QIAGEN), elution volume set at 50 µL. 1% (v/v) ß-mercaptoethanol was added to the RLT buffer as a reducing agent. Lysing of the samples was performed using 5 mm stainless steel beads by the QIAGEN TissueLyser (QIAGEN) for two times 2 minutes at 20 Hz. DNase treatment was performed by using RDD buffer containing 12.5% (v/v) DNase I incubation mix. Once isolated, the RNA concentration of the samples was measured using the Tecan Infinite F nano+ plate reader (Tecan, Männedorf, Switzerland). Subsequently, the RNA isolates were stored at -70°C.

cDNA synthesis: Reverse transcription was performed for the synthesis of single stranded complementary DNA (cDNA) from 450 ng (dHPC)/500 ng (mPFC) total RNA, according to the Bioline SensiFAST cDNA synthesis Kit protocol (Bioline, London, England). Four no-RT controls were added (one per RNA isolation run). cDNA synthesis was performed using the Thermo Fisher Scientific MiniAmp Thermal Cycler (Thermo Fisher Scientific, Waltham, Massachusetts, US) (annealing: 10 minutes at 25°C, reverse transcription: 15 minutes at 42°C, inactivation:

5 minutes at 85°C, whereafter the samples were cooled down and kept at 4°C). After synthesis, the cDNA samples were diluted 1:4, using RNase-free water as diluent, divided over two 96-wells plates, sealed and stored at -20°C.

qPCR: mRNA expression levels of *Bdnf*, *Ncam1*, *Dlg4* and *Mbp* were measured in the dHPC en mPFC. DLG4 (also known as PSD-95), MBP (Myelin Basic Protein), and NCAM1 (Neural Cell Adhesion Molecule 1) are crucial for the proper functioning and development of the nervous system. Together, these genes contribute to the structural integrity and functional efficiency of neural circuits. *Bdnf (isoform III)*, *Ncam1*, *Dlg4* and *Mbp* expression levels were compared to reference genes. *Pgk1*, *Gapdh* and *Ywhaz* were used as reference genes for the dHPC, and *Pgk1*, *Hprt1*, and *Actb* were used as reference genes for the mPFC. Gene expression was normalized to reference genes selected for their stable expression within each brain region under the experimental conditions. For the dHPC, *Pgk1*, *Gapdh*, and *Ywhaz* were used; for the mPFC, *Pgk1*, *Hprt1*, and *Actb* were selected. These genes were identified through pilot studies conducted on the same brain regions and literature as stable housekeeping genes for these brain regions (Bustin et al., 2009). The stability of the housekeeping genes in the samples were set according to the geNorm normalization output calculated in qbase+ software.qPCR sample preparation was performed using a 10 µM gene-specific primer mix, 1X SensiFAST SYBR No-Rox mix, mixed by the QIAGEN QIAgility pipetting robot (QIAGEN, Hilden, Germany) (QIAGEN GIAgility software version 4.17.1) using the QIAGEN Rotor-Disc 100 (QIAGEN). No-template controls were added for each gene of interest/reference gene. Amplification of the samples was performed using the QIAGEN Rotor-Gene Q (QIAGEN), according to the following programme: 95°C for 2 min, 40 amplification cycli: 95°C for 5 sec, 60°C for 10 sec and 72°C for 20 sec, followed by a melt curve 75-95°C. For all samples duplicates were tested. Basic analysis was performed using qbase+ (Biogazelle, Zwijnaarde, Belgium) (software version 3.4), using $\Delta Ct \leq 0.5$ as cut-off for reliable replicates, and $M \leq 0.5$ and $\Delta CV \leq 0.2$ as cut-off values for expression stability of reference genes.

Immunohistological staining

Samples: The experimental groups included were the control group (n=5), the 2'-FL+3'-SL+6'-SL (10:1:2) mix group (n=5), the 2'-FL+3'-SL+6'-SL (6:1:2) mix group (n=5) and the 2'-FL+3'-SL+6'-SL (4:1:2) mix group (n=5). These treatment groups were selected as they were the only ones who have proven to be effective in influencing behaviour and gene expression. These animals were randomly selected from the BrdU administered animals from each HMO treatment group. 5 hippocampal slices per animal were stained and BrdU and Doublecortin positive cells were manually counted using the CellCounter option in ImageJ.

Sample preparation and reagents: Transcardially perfused brains obtained from the BrdU injected animals were retrieved from the -70°C storage freezer, divided in half by splitting the two hemispheres and one hemisphere was consequently sliced on the microtome at 40 µm thickness. The other hemisphere was returned to the freezer storage for future histological experiments. The newly acquired brain slices were captured and stored in 12 well plates in a buffer solution of 1x PBS with 0.02% Sodium Azide until they were ready to be stained. We stained for neurogenesis markers and included BrdU anti-mouse (1:200,Leuner; Glasper & Gould, 2009) (ThermoFischer Scientific, B35128) to label the novel neurons, Doublecortin anti-rabbit (DCX) (1:200, Kuipers, Schroeder & Trentani, 2015) (Synaptic Systems, 326003) to label the immature neurons and NeuN anti-chicken (1:500, Coquand et al., 2024) (Millipore, ABN91) to label the mature neurons. For fluorescent labelling, we used the following secondary polyclonic antibodies (1:200): anti-chicken 488 (ThermoFischer Scientific A32931), anti-mouse 546 (ThermoFisher Scientific, A-11030) and anti-rabbit 648 (1:200) (ThermoFisher A-21245).

Staining protocol: On the first day of the staining, the free-floating sections were mounted on superfrost coated slides and left to air dry for 40 minutes. The slides were then washed in PBS x1 for 3 x 10 minutes and then incubated in 2N HCL (Honeywell Fluka, 07102-1l-GL) at 37°C in a warm waterbath for 30 minutes. After the HCL treatment, the slides were quenched in 1x Borate Buffer (ThermoFisher Scientific, 28341) for 10 minutes and rinsed again for 3 x 10 minutes. The tissues were then subsequently blocked using a BSA based blocking buffer. The slides were then incubated overnight with the primary antibodies at 4°C inside a humid chamber. On the second day of the staining, the slides were washed for 3x 10 minutes in PBS1x and then incubated for 3 hours with the secondary antibodies in the dark at room temperature. After the secondary incubation was completed, the slides were washed for a final time for 3x 10 minutes and were then allowed to air dry in the dark before the fluorsave and coverslip were added.

Imaging: Slides were imaged using the AxioObserver 7 with Sample Finder AI at 40x magnification. The Zeiss Axio Observer with Sample Finder AI is a widefield system that allows researchers to perform high-resolution multi-colour automated imaging (Zeiss, 2020). As elected markers of interest, we included BrdU as a marker of newborn cells, Doublecortin (DCX) as a marker for immature neurons, and NeuN as a marker of mature neurons. Tilescan images were stitched and processed using Image J (version 1.54f), and BrdU+ and DCX+ cells were manually counted using the Cell Counter plugin and an overlay mask (illustrated in Figure 3) was drawn to ensure the cells were detected consistently in the same zone across all animals. Cell

counting focused on the CA1, CA2, CA3, Hilus, Molecular Layer in Dentate Gyrus, and the regions along the projections of the performant pathway (above the MCL layer in the DG projecting to CA3) and the Schaffer's collateral (projecting from CA3 along the ventral side of the CA1 region).

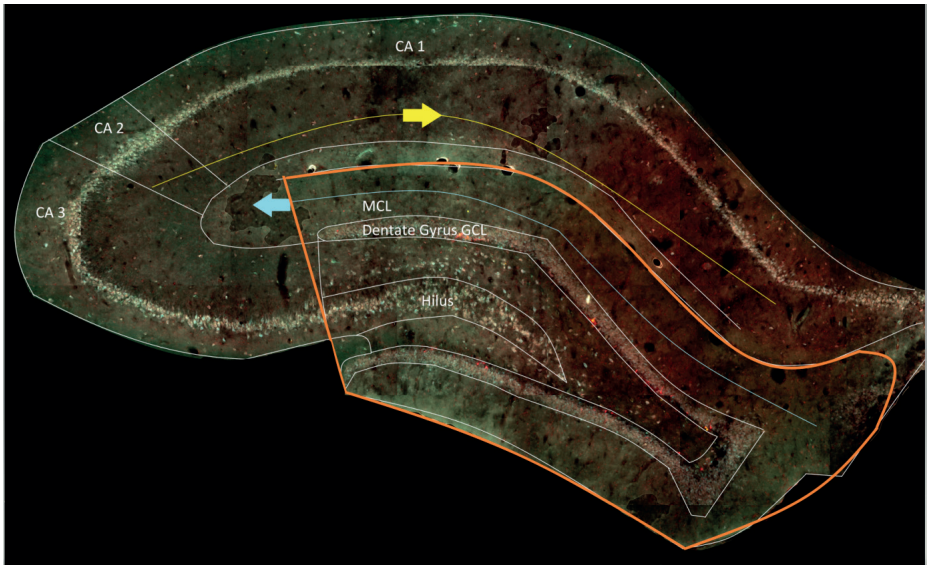

Figure 3. Mask overlay for counting BrdU and DCX+ cells. The dorsal hippocampus was divided in several subregions; encircled in orange is what considered to be the entirety of the dentate gyrus, consisting of the molecular cell layer (MCL), granule cell layer (GCL) and the hilus (which extends from CA3). CA1 comprises of both CA1 and the subiculum, CA3 and CA2 have been separated during counting though a combined CA2+CA3 variable has been included as the transition from CA3 to CA2 is not clear with the current staining. Finally, the pale blue line delineates the trajectory of the medial and lateral entorhinal cortex input via the performant pathways projecting through the dentate gyrus into CA3 (as indicated by the blue arrow), and from the CA3 region projecting through the ventral side of CA1 (as pointed out by the yellow arrow) lie the Schaffer's collateral fibers.

Statistical analysis

For statistical analysis, IBM SPSS version 20.0 was used. Prior to the final data analysis, preliminary checks for object and side preference were performed. As an object preference was found in one of the object pairs used for the NORT, Animals who spent less than 10 seconds in total on exploring both objects during the acquisition phase were excluded all together from the analysis (Asiminas et al., 2022). The analysis for both the NORT and the NOLT was performed in two steps. First, we determined if the animals were capable of detecting the novel object or the displacement of the familiar object. To this end, we assessed if the discrimination index for each group was significantly larger than 0 using a one sample t-test.

Then we assessed the discrimination index differences between each of the HMO treatment groups and the control group using a paired sample t-test. Values higher than 0 and < +1 indicate a preference for the novel or displaced object, and values lower than 0 and > -1 indicate a preference for the familiar or stationary object. Ex vivo data were also analysed, first examined for a main treatment effect using the MANOVA, followed up with an exploratory student t-test for treatment to control group comparisons. Multiple comparisons were controlled using the Benjamini-Hochberg procedure to limit the false discovery rate (FDR) to 5%. P-values were ranked in ascending order, and an adjusted significance threshold was calculated for each based on their rank and the total number of tests. Tests with p-values below their corresponding threshold were considered statistically significant. Finally, to assess the correlation between ex vivo data and behavioural data, Pearson correlation analyses were conducted. Correlation coefficients of >0.05 < 0.10 were considered weak, correlation coefficients of >0.10 < 0.15 were considered to be moderate, and correlations of >0.15 were considered strong (Akoglu, 2018; Prematunga, 2012). Differences were considered to be statistically significant when the p value was < 0.05. In the event of significant differences between one HMO treatment from the control group, red diamonds are used in the graphs above their corresponding bars, and significant differences between one HMO treatment from 0 is indicated using a blue triangle. Only relevant data with significant values are included in the main body of this article.

Results

Novel Object Recognition Task

Novel object detection. The one sample t-test revealed that all treatment groups had a significantly higher discrimination index than 0 (Figure 4). 3'-SL (M=.1904, SD= 1.3616), $t(8)$= 4.195, p=.003, 2'-FL (M= .2125, SD=.17462), $t(14)$= 4.713, p <.001, 3'-SL + 6'-SL (M= .2141, SD= .12359), $t(12)$= 6.245, p <.001, Control (M=.1403, SD= .13891), $t(9)$= 3.195, p=.011, (10:1:2) mix (M= .2608, SD= .10936), $t(10)$= 7.909, p <.001, (6:1:2) mix (M= .1347, SD= .53668), $t(11)$= 5.847, p <.001, and (4:1:2) mix (M= .2282, SD= .11250), $t(10)$= 6.727, p < .001.

HMO effect on object recognition. Interestingly, the (10:1:2) mix group (M=.2608, SD= .10936, $t(19)$= -2.219, p = .039), and the (6:1:2) mix group (M= 2.774, SD= .16438, $t(20)$, -2.087, p = .05) were found to display a significantly higher discrimination index compared to the control group.

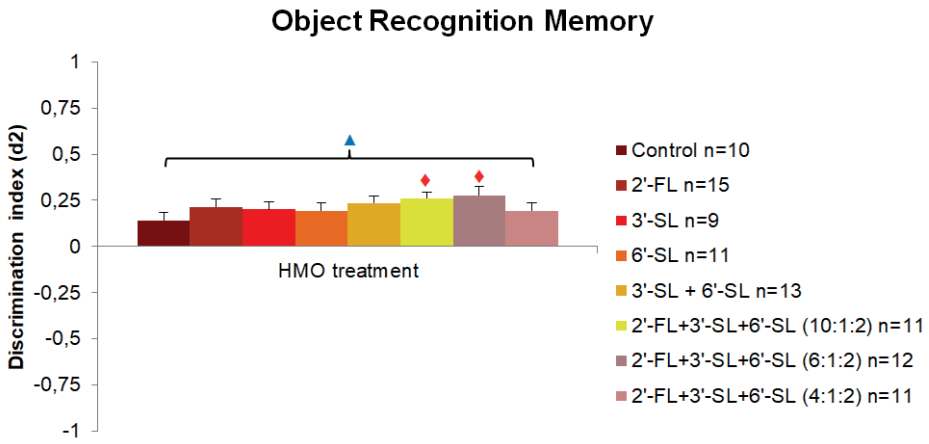

Figure 4. Novel Object Recognition memory (NORT) in male rats supplemented with HMOs during infancy and adolescence. NORT performance was tested at 10-12 months of age. Data are presented as mean +/- S.E.M. of the discrimination index. The red diamond indicates the groups whose discrimination index was significantly different from the control group. The blue triangle indicates which groups differ significantly from 0. All groups' novel object recognition were significantly above 0 and the mix groups (10:1:2) and (6:1:2) performed significantly better than the control group.

Novel Object Location Task

Novel location detection. 3'-SL + 6'-SL had a significantly higher discrimination index than 0 in the P3 location (Figure 5A), (M= .1360, SD= .23070, t(13)= 2.205, p=.046). In the same 3'-SL+6'-SL group a trend was also present for the P5 condition (M= .1606, SD= .34431), t(14)= 1.807, p= .092). In addition, a trend was detected in the 6'-SL group for the P3 location (M= .1921, SD= .31545, t(11)= 2.109, p= .059). The (10:1:2) mix group was able to distinguish the displacement in the P5 condition (Figure 5B) (M= .2374, SD= .27702, t(14)= 3.320, p= .005), but no other groups detected the object displacement towards either the P3 or P5 location.

Effect of HMO treatment on NOLT performance. There was a significant effect of HMO treatment in recognising the object displacement in the difficult (P3) condition for the (4:1:2) mix group when compared to the control group, (M= -1.1448, SD= .14018, t(22)= 2.229, p= .036)

In summary, the 2'-FL+3'-SL+6'-SL (10:1:2 and 6:1:2) mix groups displayed better object recognition memory compared to the control group. In the NOLT only the 10:1:2 mix group could recognise the displacement in the easy task condition (P5 displacement) and 3'-SL+6'-SL could recognise the displacement in the difficult task condition (P3 displacement).

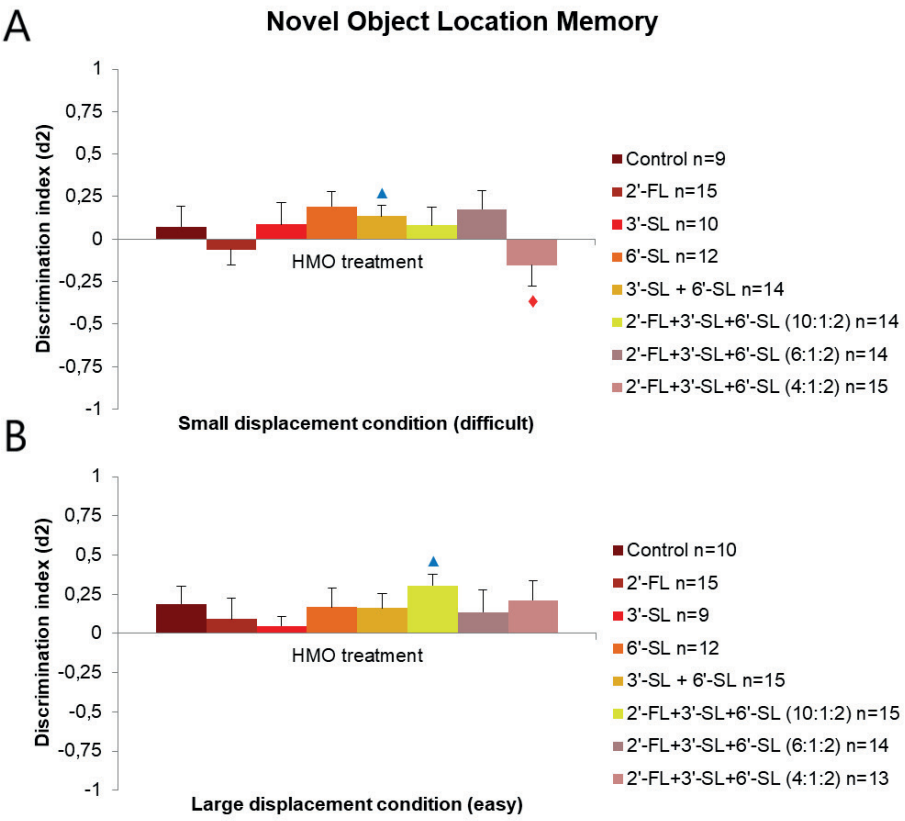

Figure 5. Novel Object Location memory (NOLT) performance in rats supplemented with HMOs during infancy and adolescence. A) Performance on the difficult discrimination (P3) task in which the object was displaced 32 cm from its starting position. B) Performance on the easy discrimination (P5) trial in which the object was displaced 16 cm from its starting position. Data are presented as mean +/- S.E.M. The blue triangle marks the groups whose discrimination index differed significantly from 0.

qPCR

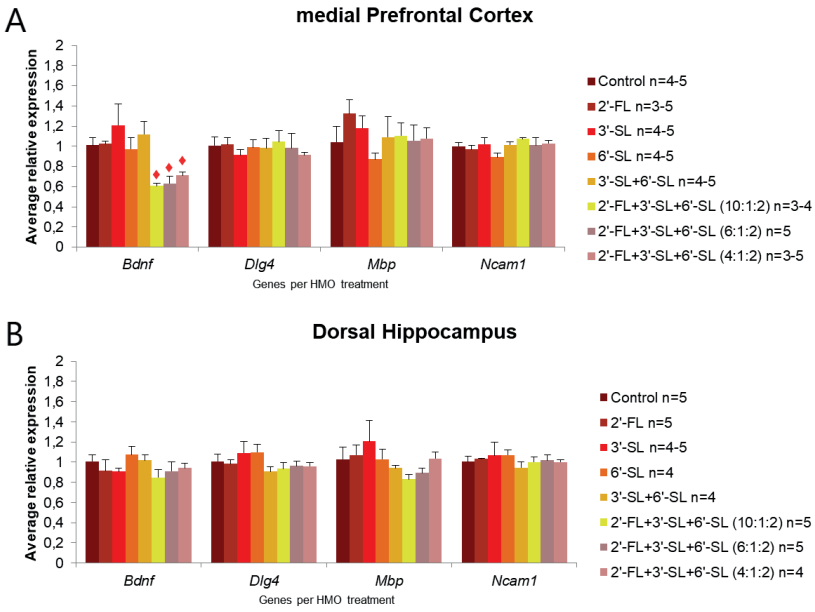

Figure 6. Gene expression in the medial prefrontal cortex (mPFC) (A) and dorsal hippocampus (dHPC) (B) of early life HMO exposed rats. Bars are clustered together per gene (*Bdnf, Dlg4, Mbp, Ncam1*) and per region (mPFC vs dHPC) and represent the relative mRNA gene expression levels for each experimental group. Data are presented as mean +/- S.E.M. The numbers within the bars indicate the group size of each corresponding HMO treatment. Red diamonds indicate a significant difference between the control group and the experimental group. Significant differences between the control group and 2'-FL+3'-SL+6'-SL (10:1:2), (6:1:2) (, and (4:1:2)) mixes were found in the levels of mPFC *Bdnf* expression.

Global analysis (MANOVA) to examine treatment effects

A MANOVA was performed to examine the effects of HMO treatment on the mRNA expression levels of *Bdnf, Dlg4, Mbp* and *Ncam1* within the dorsal hippocampus and medial prefrontal cortex. A main effect of treatment was found; $F(_{56,\ 53.777})$= 1.696, p =.027, Wilks' Λ=.004, partial η^2= .543. Closer examination shows that HMO treatment significantly influences *BDNF* expression within the mPFC $F(_{7,\ 16})$= 4,848, p =.004, partial η^2= .680. A follow up with LSD corrected post hoc test revealed that the 10:1:2 (*MD*= -.4147, *SE*= .15147, p = .015) and 6:1:2 (*MD*= -.3764, *SE*= .11733, p =.005) mix group had a significantly downregulated expression of BDNF when compared to the control group (Figure 6).

Comparison HMO treatment with control

***Bdnf*:** Significant average relative changes in *Bdnf* mRNA expression levels were found in the mPFC, when comparing the control group to the 2'-FL+3'-SL+6'-SL (10:1:2) ($t(6)= 1.060$, $p=.007$), 6:1:2 triple mix ($t(8)= 3.722$, $p= .006$), and 4:1:2 triple mix ($t(6)= 2.972$, $p= .025$) groups. No significant differences between the control and 3'-SL (mPFC $t(7)= -.979$, $p= .36$, dHPC $t(7)= 1.349$, $p= .219$), 2'-FL (mPFC $t(6)=-.147$, $p=.88$, dHPC $t(8)=.744$, $p=.478$), 3'-SL+6'-SL (mPFC $t(7)=-.777$, $p=.462$, dHPC $t(7)= -.144$, $p= .889$), 6'-SL (mPFC $t(7)= .292$, $p=.779$, dHPC $t(7)= -.652$, $p=.535$); 2'-FL+3'-SL+6'-SL (10:1:2) (dHPC $t(8)= 1.573$, $p=.154$), 2'-FL+3'-SL+6'-SL (6:1:2) (dHPC $t(8)= .897$, $p=.396$) and 2'-FL+3'-SL+6'-SL (4:1:2) (dHPC $t(7)= .806$, $p=.447$) groups were found.

***Dlg4*:** No significant differences in *Dlg4* mRNA expression levels were found between the control group and any of the HMO treated groups; 3'-SL (mPFC $t(7)= 1.004$, $p= .349$, dHPC $t(7)=-.659$, $p=.531$), 2'-FL (mPFC $t(7)= -.074$, $p=.943$, dHPC $t(8)= .316$, $p=.760$), 3'-SL+6'-SL (mPFC $t(7)= .197$, $p=.849$, dHPC $t(7)= 1.134$, $p=.294$), 6'-SL (mPFC $t(7)= .173$, $p=.867$, dHPC $t(7)= -.846$, $p=.426$), 2'-FL+3'-SL+6'-SL (10:1:2) (mPFC $t(5)=-.267$, $p=.80$, dHPC $t(8)= .822$, $p=.435$), 2'-FL+3'-SL+6'-SL (6:1:2) (mPFC $t(7)= .149$, $p=.886$, dHPC $t(8)= .555$, $p=.594$), or 2'-FL+3'-SL+6'-SL (4:1:2) (mPFC $t(6)=1.087$, $p=.319$, dHPC $t(7)=.573$, $p=.584$).

***Mbp*:** No significant differences in *Mbp* mRNA expression levels were found between the control group and any of the HMO treated groups; 3'-SL (mPFC $t(8)= -.698$, $p= .505$, dHPC $t(7)=-.776$, $p=.463$), 2'-FL (mPFC $t(8)= -1.330$, $p=.220$, dHPC $t(8)= -.276$, $p=.790$), 3'-SL+6'-SL (mPFC $t(8)= -.185$, $p=.858$, dHPC $t(7)= .633$, $p=.547$), 6'-SL (mPFC $t(7)= .146$, $p=.388$, dHPC $t(7)= -.005$, $p=.996$), 2'-FL+3'-SL+6'-SL (10:1:2) (mPFC $t(5)=-.267$, $p=.80$, dHPC $t(8)= .822$, $p=.435$), 2'-FL+3'-SL+6'-SL (6:1:2) (mPFC $t(8)= -.051$, $p=.961$, dHPC $t(8)=1.075$, $p=.314$), and 2'-FL+3'-SL+6'-SL (4:1:2) (mPFC $t(8)=-.187$, $p=.856$, dHPC $t(7)=-.040$, $p=.970$) groups.

***Ncam1*:** No significant differences in *Ncam1* mRNA expression levels were found between the control group and any of the HMO treated groups; 3'-SL (mPFC $t(8)=-.317$, $p= .759$, dHPC $t(7)= -.453$, $p=.664$), 2'-FL (mPFC $t(8)= .589$, $p= .572$, dHPC $t(8)=-.512$, $p=.623$), 3'-SL+6'-SL (mPFC $t(8)= -.283$, $p= .784$, dHPC $t(7)=.749$, $p=.479$), 6'-SL (mPFC $t(8)= 2.034$, $p= .076$, dHPC $t(7)= -.804$, $p=.448$), 2'-FL+3'-SL+6'-SL (10:1:2) (mPFC $t(7)= -.1,848$, $p= .107$, dHPC $t(8)= .080$, $p=. 938$), 2'-FL+3'-SL+6'-SL (6:1:2) (mPFC $t(8)= -.139$, $p= .893$, dHPC $t(8)=-.180$, $p=.862$), and 2'-FL+3'-SL+6'-SL (4:1:2) (mPFC $t(8)= -.682$, $p= .514$, dHPC $t(7)=.053$, $p=.959$) groups.

To summarise, there is a marked reduction in *Bdnf* expression in the mPFC in the 2'-FL + 3'-SL + 6'-SL mix groups (10:1:2, 6:1:2 and 4:1:2) when compared to the control group, but no other variations in gene expression were found in either the mPFC or dHPC.

Exploratory correlation analysis between gene expression levels and memory performance within the mPFC and dHPC for all HMO treatment groups

Bdnf: No significant correlations between *Bdnf* mRNA expression levels within the dHPC and NORT and NOLT discrimination indexes were found. However, within the mPFC, *Bdnf* expression was positively correlated with recognition memory in the 6'-SL group (r= .98, p =.016, n=4) but negatively to recognition memory in the 4:1:2 mix group (r=-.9, p = .023, n=3). Finally, a negative correlation between mPFC *Bdnf* expression and object location memory (hard condition) was found in the 3'-SL+6'-SL group, (r=-.992, p = .008, n=4).

Dlg4: No significant correlations between mPFC *Dlg4* mRNA expression levels and memory indices were found. Within the dHPC, positive correlations with the object location hard condition were found for the 2'-FL (r=.89, p= .045, n=5) and (4:1:2) mix (r=.998, p= .036, n=3) groups. Lastly, a positive correlation between the easy condition of the object location memory and dHPC *Dlg4* gene expression was present in the 3'-SL + 6'-SL mix group (r=.98, p= .017, n=4).

***Mbp*:** Within the mPFC, the (6:1:2) mix group object location memory for the hard condition was negatively correlated with gene expression. When examining the dHPC, a reduced *Mbp* mRNA expression level correlated with improved memory recognition in the 3'-SL group (r= -.96, p=.043, n=4) and object location memory (easy condition) for the 10:1:2 mix group (r= -.953, p= .047, n=4).

***Ncam1*:** Within the mPFC, a significant negative correlation between *Ncam1* mRNA expression levels and object location (hard condition) for the 2'-FL+3'-SL+6'-SL (4:1:2) mix group (r= -.959, p= .036, n=3) was found. Furthermore, there was a negative correlation between Ncam1 gene expression in the dHPC and object recognition memory in the 3'-SL group (r= -.99, p= .015, n=4) and the 2'-FL+3'-SL+6'-SL (10:1:2) (r= -.901, p=.037, n=5) group, and between *Ncam1* mRNA expression levels and object location memory (easy condition) in the 2'-FL+3'-SL+6'-SL (4:1:2) mix group (r= -.968, p= .041, n=4).

In summary, memory performance of HMO treated animals correlated with multiple genes in a region-specific manner, while the control group displayed no such pattern. Overall, a reduced expression of dHPC *Mbp* and *Ncam1* was associated with better object location memory in the 2'-FL, 3'-SL+6'-SL and 2'-FL+3'-SL+6'-SL (4:1:2) group, while an increase in dHPC *Bdnf* was associated with better recognition memory in the 6'-SL group. Finally, the 6'-SL group showed better recognition memory with increasing mPFC *Bdnf* levels (Figure 7).

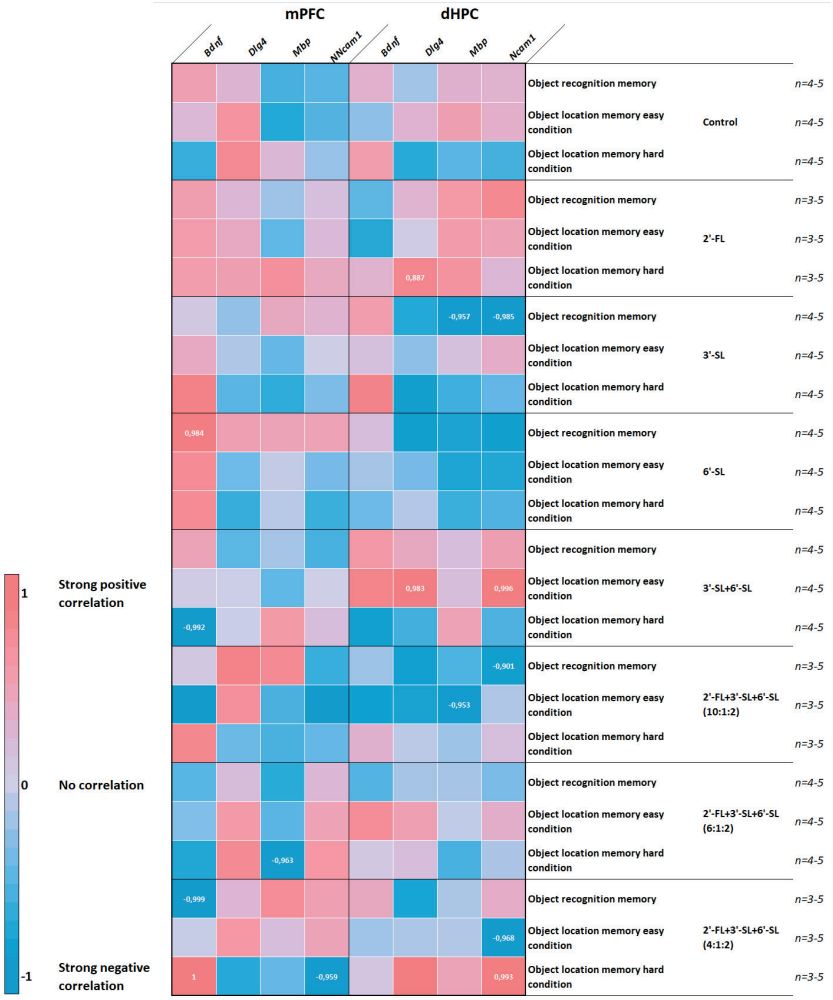

Figure 7. Correlation heatmap of qPCR genes, NORT and NOLT discrimination indices. Displayed in the figure above is a colourgradient heatmap, in which negative correlation coefficients are indicated by a blue colour, and positive correlation coefficients are marked with a red colour. The level of colour saturation represents the correlation coefficient's strength. Only the significant correlations ($p < .05$) are filled in the cells.

Immunohistological staining

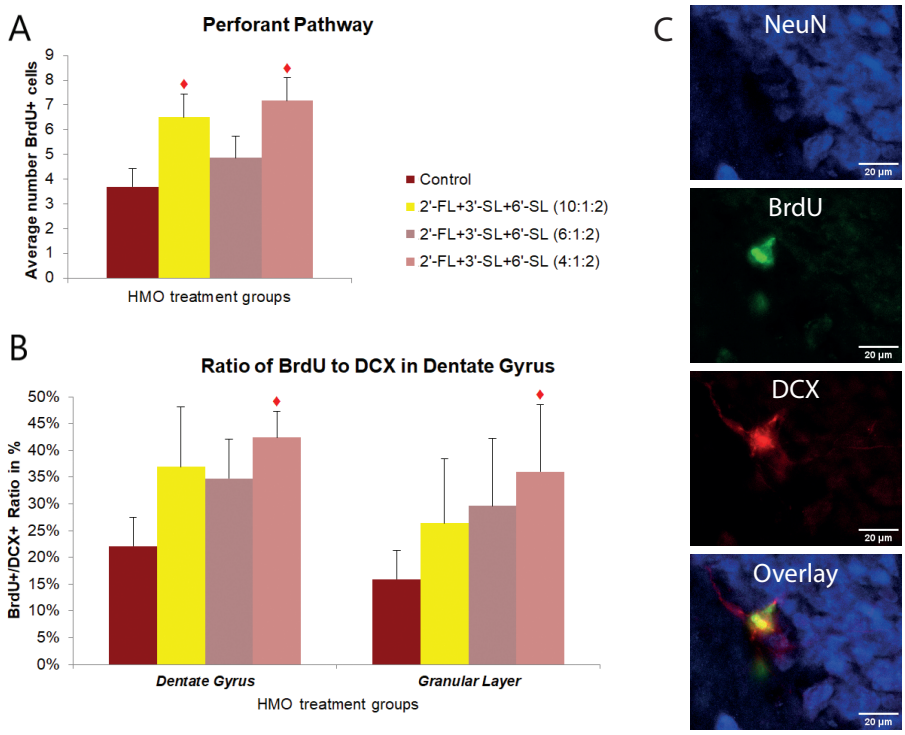

Figure 8. BrdU+ cell count graphs along the perforant pathway (A) and BrdU to DCX ratio (B) for the control and 2'-FL+3'-SL+6'-SL triple mix groups. Data are presented as either the average amount of counted BrdU+ cells (A) or the BrdU to DCX ratio expressed in percentages % (B). Red diamonds above the error bars indicate significant differences between the control group and the experimental group. Data are presented as mean +/- S.E.M. (C) Overview of dentate gyrus BrdU + and DCX +, obtained from the 2'-FL+3'-SL+6'-SL (4:1:2) mix group. For the subsequent counting and analysis, we did not include a separate category for neurons expressing BrdU+ DCX+ simultaneously, but we counted the DCX + and BrdU + cells separately.

Global analysis (MANOVA) to examine treatment effects

To further analyse the data, a MANOVA was conducted to examine whether there was a main treatment effect of HMOs on BrdU+ and DCX + proliferation (Figure 8A-C and Figure S2). No main effect of HMO treatment on neurogenesis markers was found, $F(_{39.000,\ 12.593})= 1.050$, $p = .489$, partial $\eta^2= .760$, Wilks' $\Lambda = .014$.

Due to the large number of comparisons (14 dependent variables), we will be including the Univariate results next. Based on these results, we do see a main effect of HMO treatment on BrdU+ cells within the region of the perforant pathway, $F(_{3,16})= 3.306$, $p= .047$, partial $\eta^2= .383$ (Figure 8A), for both the 10:1:2 mix ($MD_{control}=$

2.8160, *SE*= 1.2279, *p* = .036) and the 4:1:2 mix ($MD_{control}$= 3.4800, *SE*= 1.2279, *p* =.012). However, when applying the FDR correction on the univariate results there were no significant effects on HMO treatment.

Exploratory comparison HMO treatment with control

A paired samples t-test was conducted, in which each HMO group was compared to the control group (Figure 8A - 8C and S2). The number of BrdU+ cells located in the perforant pathway region (for location, see Figure 3) was significantly increased for the 2'-FL+3'-SL+6'-SL (10:1:2) mix group and the control group in (*t*(8)= -2.392, *p*=.044), and also for the 2'-FL+3'-SL+6'-SL (4:1:2) mix group and the control group (t(8)= -2.932, *p*=.019). The BrdU to DCX ratio was significantly different between the 2'-FL+3'-SL+6'-SL (4:1:2) mix group and the control group in the dentate gyrus (*t*(8)= -.2810, *p*=.023) and granule cell layer (*t*(8)= -3.164, *p*=.013). No other significant effects of HMO treatment on the remaining hippocampal regions have been detected.

Discussion

In line with our hypothesis, we found that the 2'-FL+3'-SL+6'-SL 10:1:2 and 6:1:2 HMO groups, but not the single and dual HMO supplemented groups, displayed a significant improvement in recognition memory. However, object location memory was not affected by early life HMO treatment, although the 10:1:2 mix (P5) and the 3'-SL + 6'-SL group (P3) were capable of detecting the displacement whereas the control group could not. All triple mix HMO groups displayed a significant downregulation in *Bdnf* gene expression in the mPFC. Furthermore, memory performance of HMO treated animals correlated with multiple genes in a brain region-specific manner; such as the mRNA expression levels of *Bdnf, Mbp* and *Ncam1* in the mPFC, and the mRNA expression levels of *Dlg4*, *Mbp* and *Ncam1* in the dorsal hippocampus. Finally, the 2'-FL+3'-SL+6'-SL (4:1:2 and 10:1:2) supplemented groups displayed an increased number of BrdU+ neurons within the perforant pathway region.

We found that early life HMO treatment differentially affected NORT and NOLT performance. Since the NORT and NOLT probe different neural circuits for recognition memory (entorhinal cortex based) and spatial pattern separation (dorsal hippocampus based), respectively (Wilson et al., 2013; Oomen et al., 2014), it is possible that HMOs target very specific neurocircuitries. This idea could serve as a potential explanation for the HMO group differences between easy and difficult

task conditions within the NOLT as well. The 2'-FL rich mix (10:1:2) was beneficial to recognise a large displacement, while the 3'-SL + 6'-SL group performed better under the small displacement conditions. It should be noted that the control group was not able to detect the displacement during either condition of the NOLT, which could be attributed to task difficulty. The findings however suggest that cognition is suboptimal at baseline in laboratory rats and that HMOs have a positive effect on memory performance. It is possible that for more complex task demands, very specific ratios of sialylated and fucosylated HMOs were required, and that the margin in which these ratios may operate is very small (i.e requiring a very specific blend of different types of HMOs for a given to ask to maximize the outcomes). It is also possible that the NOLT task was too difficult for the animals. No spatial cues were used in the NOLT task, and while the NOLT protocol we employed (van Goethem & Prickaerts, 2018) was successfully used for animals between 3-5 months of age, it could be that further adjustments were required to accommodate the capabilities of the older animals of 10-12 months of age used in this study. The navigational strategy the animals used when determining the novel object location could have been under the influence of an age-dependent loss of function within the hippocampus (Berdugo-Vega et al., 2020). As ageing coincides with changes in hippocampal circuits with downstream effects on spatial memory consolidation and formation, the NOLT performance could be an indication that the HMO treatment is not sufficient in preventing the detrimental effects of natural ageing on spatial navigation. As recognition memory, tested at the same time as spatial memory in our study, was enhanced by HMO treatment, it further lends credence to the idea that HMO may affect brain development and circuit formation and functioning in a highly specialised manner.

The results of the gene expression study, in particular the reduced *Bdnf* expression in HMO treated groups despite improved object recognition memory, were surprising but may offer new perspectives. While an increase in *Bdnf* expression is often linked to improved memory functioning and plasticity (Meyer, Bonhoeffer, & Scheuss, 2014; Bach et al., 2024), the specific effects of the different Bdnf isoforms are still unclear. Our study may suggest that improved memory performance could be associated with a decrease, rather than an increase, in some Bdnf transcripts, such as transcript variant III. The *Bdnf* gene expresses 4 different isoforms and comprises nine distinct promoter regions, with the various transcripts possibly serving unique functions within the brain (Altieri et al., 2004). It would therefore be valuable to repeat the qPCR analysis targeting other Bdnf transcripts (for example transcript I or IV) to see whether *Bdnf* expression changes as a function of HMO treatment. Moreover, since *Bdnf* is initially synthesized as a precursor (proBdnf),

which is subsequently cleaved into its mature form, and because proBdnf and mature Bdnf have opposite effects, the proteolytic processing of proBdnf is crucial for the cellular outcomes of *Bdnf* expression (Miranda et al., 2019). Finally, it remains possible that the animals experienced some anxiety or stress during the final experiment, despite efforts to minimize stressful factors during housing and testing, which might also influence *Bdnf* expression.

Regarding neurogenesis, we found that HMO treatment increased the number of BrdU+ neurons in the perforant pathway region. Interestingly, as typically an increased rate of neurogenesis is related to improved memory and pattern separation, we found no such effect in this study. In fact, the 2'-FL+3'-SL+6'-SL (4:1:2) group had the largest number of newborn neurons but did not perform better than any of the other HMO or control groups on either memory task. At first glance this seems to contradict our findings in the 2'-FL+3'-SL+6'-SL (10:1:2) group, as that group performed significantly better in the memory tasks, and also had significantly more neurogenesis. However, this could be explained by the hippocampal region in which the neurogenesis took place, as only the number of newborn neurons was significantly increased in the dentate gyrus in only the 4:1:2 group. Furthermore, neurogenesis does not only incur a better memory. Scott et al. (2021) recently illustrated that an increase in neurogenesis also mediates forgetting. This could explain why the (4:1:2) group had the poorest performance in location memory despite the significant increase in the number of newborn neurons.

A primary limitation of this study is the exclusive use of males. Therefore, it is not known if the present findings generalize to females, or whether there are sex specific effects of a prior HMO treatment over time. Secondly, while the control group was not supplemented with additional HMOs, they still received 3'-SL and trace amounts of 6'-SL through their maternal milk as rat milk naturally contains certain sialylated HMOs (Urashima et al., 2001). Perhaps a negative control group in which the rat milk is completely devoid of HMOs could be included in a future study, as is the case in the studies performed by Hauser and Pisai. These studies have indicated that HMOs are a necessary nutrient in the formation and maintenance of cortical circuits involved in various cognitive outcomes including memory. Including such a negative control group would serve to disentangle the influence of particular HMOs and their combinations in the molecular machinery underlying neurodevelopment. Thirdly, the ex vivo analyses for both gene expression and histology were underpowered, and we cannot exclude the possibility that different effects, or lack thereof, may surface as the sample size increases. Therefore, the ex vivo analyses should be interpreted with caution as they currently serve an exploratory purpose

to point future research in a potential interesting direction. Nonetheless, other studies have demonstrated that dietary HMOs influence gene expression within multiple brain regions (Fleming et al., 2020; Hauser et al., 2021; Pisa et al., 2021; Pisa et al., 2023). Furthermore, while the correlation study was underpowered, the correlation coefficient was very high, which provides cause for further investigation with a larger sample size. Employing a whole transcriptome analysis in larger groups should be considered to obtain a more detailed picture on the molecular downstream effects of HMO supplementation on neural development, plasticity and cognitive outcomes. Fourth, although NeuN staining was included in the immunohistochemical protocol to provide morphological context and help identify mature neurons, NeuN-positive cells were not quantified separately in this study. Consequently, while DCX-positive cells are interpreted as immature neurons in the process of maturation, and BrdU-positive cells as proliferating precursor cells, the exact proportion of newly generated neurons reaching full maturation remains undetermined. Future studies including quantitative NeuN analysis and co-localization with BrdU and DCX markers would be valuable to assess the degree of neuronal maturation and integration more precisely. Furthermore, due to the neuronal labeling protocol included in the study, we have no information which of the newly generated neurons were included in the task performance. Therefore, we are presently unable to ascertain how HMO treatment may influence memory performance beyond the scope of neuronal proliferation and gene expression. Fifth and last, animals were sacrificed 48 hours after the final trial and the genes included in this study may operate at different timelines (Mathisen, Johnson & Kawczak, 1997; Heo et al., 2018; Xu et al., 2019). A future study design accounting for these individual gene timelines may yield valuable insights on the exact effects of early life HMO supplementation throughout the lifespan.

Conclusion

The findings within this study could be indicative of synergistic functioning of different HMOs when administered during early life, such as improving object recognition memory, decreasing the *Bdnf* isoform III gene expression within the mPFC and increasing the rate of hippocampal neurogenesis. Further research is required to unravel the mechanisms through which HMOs may influence neurodevelopment and cognition throughout life.

Supplementary material

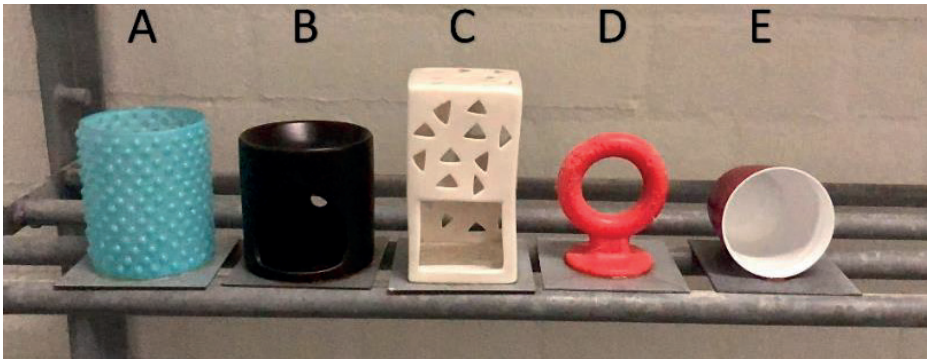

Figure S1. Objects used in the NORT and the NOLT. NORT: A) The blue glass was the novel object, B and C were the familiar objects, NOLT: D) Red hoops and E) Red cups.

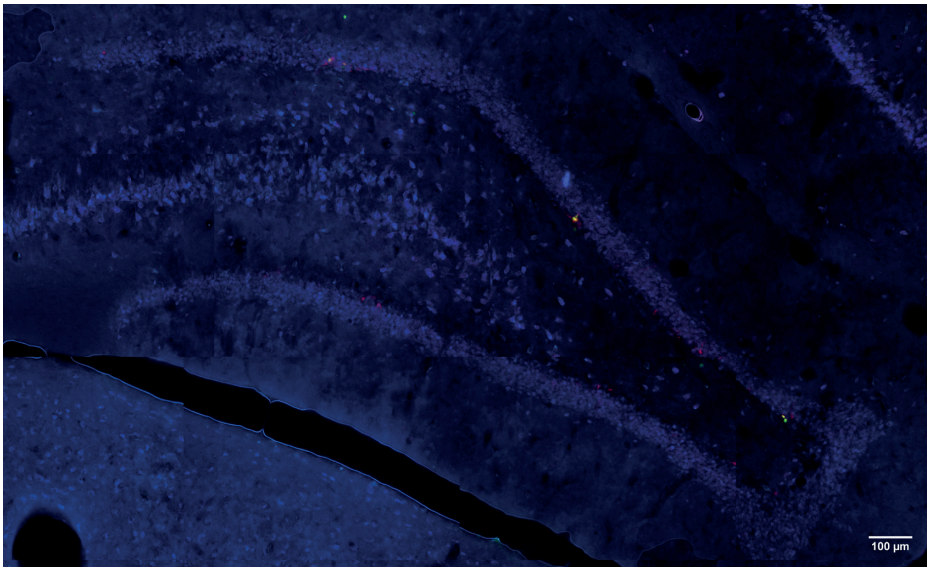

Figure S2. Composite image of whole dentate gyrus staining of 2′-FL+3′-SL+6′-SL (4:1:2) HMO treated group. Blue cells are Neun, red cells are DCX+ and green cells are BrdU+. In the event BrdU+ overlaps DCX+ cells they appear as yellow.

Table S2. Neurogenesis cell counting results. Displayed in this table are the group averages of the counted BrdU + and DCX + cells per HMO group in their respective region where they were detected. In the third column, the ratio of BrdU + cells / DCX + cells have been calculated by taking the total number of BrdU + cells, divided by the total number of DCX + cells, per subject. Based on this method, the group average percentages are calculated and included in the last column. 10:1:2, 6:1:2 and 4:1:2 refer to the mix group ratios of the 2'-FL +3'-SL+6'-SL treatment groups.

HMO	Avg BrdU+						Avg DCX+		BrdU+/DCX+ ratio		BrdU+ paths	
	DG GCL	Hilus	Total DG	CA3	CA2	CA1	DG GCL	Total DG	DG GCL	Total DG	Perforant pathway	Schaffer's Collateral
10:1:2	17	5.6	22.6	11.4	8.2	26.6	73	73	26%	36%	9.6	9
6:1:2	20.2	4.4	24.6	9.4	3.4	27.4	81	83	30%	33%	6.8	12.4
4:1:2	26.2	5.6	31.8	12.4	6	27.6	73	75	36%	42%	11.2	13.4
Control	13	3.8	16.8	8.8	4.8	25.2	80	80	16%	21%	5.2	7.6

5

Chapter 5

General discussion

Thesis objective and main findings

A healthy start of life is crucial for our future health and mental well-being later on. Infants rely in their first six months of life on maternal milk, which provides specific and non-specific factors that have long-term consequences for early metabolism. Human breastmilk enhances the immature immunologic system, promotes gastrointestinal mucosal maturation and alters gut microflora of the neonate and strengthens host defense mechanisms against infective and other foreign agents. Following the termination of breastfeeding, there is evidence of ongoing protection against illness due to protective influences on the immune system mediated via human milk. While almost all women can breastfeed, there are a small number of mothers who cannot nurse their children, be it due to underlying health conditions (HIV, drug abuse, breast cancer, lactation failure, etc.) or treatment for these conditions (anti-viral therapy, mastectomy, chemotherapy, etc.). In these cases, infant formula is an essential alternative to ensure the proper growth and development of the infant. To improve infant formula, it is essential to understand how breastmilk's various human milk components may beneficially foster infant development.

In this project we focused on one of those key components in breastmilk which are the Human Milk Oligosaccharides (HMOs). In the past, nutritional and paediatric research has highlighted the importance of these complex oligosaccharides in terms of the development of the immune system and facilitation of its proper functioning, and how these HMOs contribute to gut microbiome health with downstream effects on both physical and mental development. As prior longitudinal studies have indicated, there also exist marked differences between infants who were fed either breastmilk or infant formula devoid of HMOs. The most prevalent finding these studies is that the breastfed infants cognitively outperformed the infants who were solely raised on formula. For example, meta-analysis studies where breastfed and formula fed children were compared on academic outcomes during childhood and adolescence report that children who were breastfed had an average gain in IQ score of 3.44 points (Horta, de Mola & Victora, 2015), even when factors such as gestation duration, maternal education and birth weight were accounted for (Anderson, Johnstone & Remley, 1999). This has prompted the present study to further investigate how these HMOs may contribute. Preclinical studies conducted in the past two decades have highlighted the importance of fucosylated and sialylated HMOs, especially in the context of cognitive and memory functioning.

The aim of this thesis was to generate additional insight in how these different types of HMOs may contribute to early life behavioural and developmental outcomes in infancy and adolescence, and how they may also still guide our cognitive capabilities in later life, thus essentially sketching a picture of HMOs' influence over our mental capacity throughout our entire lifespan. Furthermore, we aimed to investigate whether providing a complex mixture of different HMOs provides additional benefits over only supplementing one particular type.

Discussion of Chapters

In our **second chapter**, we reviewed the state of the art of HMO supplementation research in preclinical mammalian studies in terms of cognitive functioning. Here, we discussed how sialylated and fucosylated HMOs appear to induce marked improvements in both working and spatial memory in mammals, accelerate the learning rate during operant tasks and also potentiate the LTP response both during infancy and adulthood. A key observation in our review is that the beneficial effects of HMOs become more apparent during infancy when behavioural tasks have an increased cognitive load. As animals age, also the performance on the simpler behavioural tasks is positively affected by prior fucosylated and sialylated HMO administration. Based on these findings, we concluded in our review that HMOs can exert long-lasting beneficial effects on cognition and memory. However, the variability across these studies, ranging from animal species, age, duration and dose of administration, called for the need of one unified study which employed the same study design over an entire lifespan, where different classes of HMOs and their combinations are supplemented during key neurodevelopmental windows ranging from early infancy until adolescence. To this end, we designed one major longitudinal study in which eight experimental HMO treatment groups (2'-FL, 3'-SL, 6'-SL and combinations thereof) were established. These HMO treated rat pups were subjected to a comprehensive test battery to document physical development, (emotional) behavioural development from infancy until adolescence (**Chapter 3**) and memory functioning in adulthood (**Chapter 4**).

In **Chapter 3**, we delved into the immediate, short-term effects of HMO supplementation on physical and behavioural development during infancy and adolescence in both males and females. Here we found that different HMOs generated different behavioural phenotypes depending on the HMO treatment. Firstly, when 3'-SL and the 6:1:2 triple mix were provided, animals were the fastest to have both eyes opened while the control animals and 2'-FL fed animals were the slowest Furthermore, the

supplementation of single 3'-SL resulted in animals who were the least anxious in the Open Field Test, while animals fed a 2'-FL enriched diet were the lightest and displayed an increase in exploratory drive during the behavioural tests. A final finding is that there were also sex differences apparent during adolescence within the 6:1:2 mix group, as females displayed decreased locomotor activity compared to the control group, while in the male population no such effect was observed. These early life findings seem to point to the possibility that different HMOs and their combinations contribute to distinctly different developmental profiles, which may also have a sex dependent component. Further research is required to elucidate the pathways through which HMOs may shape behavioural and potential cognitive differences between both sexes in adolescence and later life.

In **Chapter 4**, we continued our search for the long-term effects of HMO supplementation in adulthood with the males also used in **Chapter 3**. These males were tested on both recognition memory and object location memory when they were around 10 months old. Here we found that the HMO triple mixes (2'-FL + 3'-SL + 6'-SL), but not the single HMO treatments, had a significantly improved object recognition memory, but no effects of HMO treatment on object location memory were found. Interestingly, we found a significant downregulation of *Bdnf* gene expression levels in the medial prefrontal cortex of all triple mix groups when compared to the control group. While a downregulation of BDNF could be associated with stress, we would expect to see this with all animals. Furthermore, these groups also had an increased ratio of BrdU+ to Doublecortin+ cells in the dentate gyrus, and the number of BrdU+ cells in the performant pathway region were doubled in (10:1:2) and (4:1:2) treatment groups.

Based on the results generated in both our short term and long-term outcome studies, we conclude that HMOs have several significant beneficial effects on concurrent physical and cognitive development and that synergistic action between these HMOs are an important factor in this. Specifically, HMO administration during infancy resulted in variations in weight gain depending on the treatment delivered, improved memory functioning and increased neurogenesis. These beneficial effects of HMOs on recognition memory functioning seem to be more pronounced when HMOs were supplied in a complex mixture of both fucosylated and sialylated oligosaccharides. Finally, our results also indicate that HMOs may possess the capacity to influence brain development and functioning in both a sex and region-specific manner, opening up exciting avenues for future research which could even yield precious insights into neurodevelopment as a whole.

Potential mechanisms underlying HMO's synergistic influence on neural plasticity and cognition

Our findings from Chapters 3 and 4 reveal synergistic effects of fucosylated and sialylated HMOs on cognition and neural plasticity. While our studies addressed both short-term and long-term effects of HMOs on cognitive and neural development, these findings prompt several new questions and potential mechanisms that require further exploration. A central pathway by which HMOs may exert these widespread effects on cognition and neural plasticity is the gut-brain axis (GBA). The GBA is integral to neural development and plasticity through its complex influence on neurochemical signaling. In the section below, we explore several interconnected mechanisms of interest that may inspire future research into HMOs' influence on neural outcomes.

1. Human Milk Oligosaccharides interact with the Gut Brain Axis

A significant body of research supports the beneficial effects of HMOs in both infants and animals. Upon ingestion, HMOs are not directly digested in the stomach; instead, they act as a growth substrate for beneficial gut microbiota, which, in turn, produce metabolites that influence multiple systems, including the brain (Bode, 2012; 2015; Dalile et al., 2019). Depending on the infant's diet—and thus the ratio and concentration of different HMOs consumed—a specific gut microbiotic profile develops. Microbiota capable of digesting these specific HMOs will thrive, sometimes at the expense of those unable to efficiently utilize these compounds. This enzymatic digestion process triggers a cascade of effects on metabolite production, ultimately impacting brain development. This further underscores the level of specificity and adaptive flexibility required in an infant's diet over time (along with the infant's age) to maximize beneficial developmental outcomes.

The Gut-Brain Axis (GBA) is of great importance to the healthy development of an individual. The rapid changes and maturation of the intestinal microbiota during the first years after birth coincide with the hallmark rapid structural and functional changes in the central nervous system (de Weerth, 2017). Brain regions important for cognitive functioning, such as the hippocampus, can be shaped by the gut microbiota. Their metabolites (SCFAs) are capable of steering synaptogenesis via BDNF (Brain Derived Neurotrophic Factor) modulation, myelination, and microglia development, which participate in the shaping of neural circuits in the developing brain (de Weerth, 2017; Dalile et al., 2019). In addition, they are also capable of altering the glutamate and GABA levels within the hypothalamus (Bridgman et al.,

2017). Dysfunction of the GBA could have pathophysiological consequences and negatively impact both physical and mental health.

Interactions between single HMOs and the microbiome have previously been reported. Tarr et al. (2015) demonstrated that administration of 3'-SL and 6'-SL changed the microbial composition which led to behavioural and neuronal changes. 6–8-week-old mice were fed a diet, enriched with either 3'-SL, 6'-SL or a normal diet prior and during social stress exposure. Control mice were shown to be more anxious, with a reduction in immature neurons in the dentate gyrus when compared to mice fed either a 3'-SL or 6'-SL enriched diet. These results show that ingested sialylated HMOs improve anxious behaviour and that one of the potential supporting mechanisms of this is the GBA. The influence of the GBA has also been touched upon by Vazquez et al. (2016), as they found that ablating the vagal nerve, which is part of the Gut-Brain Axis, diminished the beneficial effects of orally supplied 2'-FL on LTP.

To summarise, HMOs contribute to the gut microbiome composition and therefore could interact with the brain via the GBA, bacterial metabolites such as the SCFAs (Tarr et al., 2015; Dalile et al., 2019) and the supply of sialic acid and fucose in neural glycosylation processes (Varki & Kornfeld, 2022). As certain gut bacteria are specific for the utilization of sialylated HMOs and other bacteria for the fucosylated HMOs, it stands to reason that a larger variety of HMOs goes hand in hand with a larger yield of specific gut bacteria capable of metabolizing these HMOs, and thus determining their subsequent metabolites (Bode, 2015). This points towards synergistic effects of HMOs because of the growth of certain bacteria (most notably bifidobacteria) which results in a specific yield of SCFAs, with possible downstream effects on cognition. Therefore, there is a great need to study the effects of specific combinations of HMO to find out what downstream effects it has on the gut microbiome and subsequent modulation of the central nervous system.

2. The role of glycobiology in HMOs' interactions with neurodevelopmental processes

To understand how HMOs may contribute to neural developmental processes, we need to discuss the basic principles of glycobiology first. Every cell in our body is covered in a thick layer of glycans, providing cells with a complex surface texture. Glycans are branching structures that attain even a higher level of complexity than proteins or DNA and are excreted to the cell surface via the endoplasmatic reticulum-Golgi pathway. They are involved in nearly every bodily process and play a central role in cell-to-external environment communication, such as during the onset of viral infections or

via alterations in the glycan coat in cancer cells rendering them invisible to the immune system (Varki & Kornfeld, 2022). The overarching glycan class can subsequently be divided into several subclasses, such as HMOs, polysaccharides, glycoproteins (NCAM and glycosylated neurotransmitter receptors), glycolipids (sphingolipids, gangliosides and cerebrosides) and proteoglycans (aggrecan). It should be noted that glycan structures are not the direct result of genetic expression but are recognised as secondary gene products.

Of particular relevance are HMOs' indirect effects on neurodevelopment through gut-brain interactions and their direct roles in neural glycosylation processes. Sialylated HMOs, for instance, supply sialic acid, which is essential for the formation of sialylated glycoproteins like PSA-NCAM and glycolipids like gangliosides. These compounds play essential roles in synaptic plasticity, axonal growth, and cell adhesion, all fundamental for healthy neural circuitry formation.

3. Prenatal oligosaccharide enrichment of the developing infant brain

One of the items we did not include in our study revolves around the fact that infants are already exposed to HMOs and their terminal sialic acid and fucose sugars in utero (Wise et al., 2018). Our main focus was on the effects of HMOs supplied postnatally, but we did not examine or accounted for sialic acid or fucose supply prenatally. Nonetheless, as HMOs are involved in neurodevelopmental processes and are already present within the embryonic brain, it stands to reason that next to breast feeding alternative sources exist. Firstly, HMOs 2'-FL, 3-FL and 6'-SL have previously been found in the amniotic fluid, where they are hypothesised to function as prebiotics and define the developing infant's microbiome at this stage (Wise et al., 2018).

4. Epigenetic modification due to HMO supplementation

We found that specific combinations of HMOs produce long lasting effects. A possible mechanism of action could be that the HMOs guide epigenetic changes in critical brain regions. The reason we propose this is that HMO supplementation was suspended at PND 49, and all the animals were kept on the exact same diet since. While it is plausible that their microbiotic profile was altered during the supplementation, once the HMOs were no longer included in the diet, it is more likely that the microbiome of the different groups has become more similar again over time. This means that the different effects in adulthood are caused by changes during development when they were given HMOs, and one way to have such long lasting changes is through variation in epigenetic regulation. Further research in which the microbiome profile and an epigenetic profile is generated will shed more light on these potential underlying mechanisms.

Concluding Remarks and Future Directions

In conclusion, this chapter proposes several mechanisms through which HMOs may influence cognitive and neural plasticity outcomes, providing a basis for future research. HMOs may support neurodevelopment through a combination of gut-brain axis interactions, glycan-mediated cellular processes, prenatal exposure, and long-lasting epigenetic modifications. Future studies should explore specific HMO combinations and their downstream effects on the microbiome, neurotransmitter systems, and neural plasticity, particularly focusing on the complex interplay of microbiota-driven metabolites and glycobiology within the developing brain. Additionally, investigating the epigenetic landscape shaped by early HMO exposure could reveal novel insights into the mechanisms by which early nutrition influences lifelong cognitive outcomes.

Beyond infancy, the potential therapeutic applications of HMOs warrant further exploration. The long-lasting effects observed in experimental models suggest that HMOs may play a role in sustaining gut and brain health across the lifespan. While current research predominantly focuses on early-life development, an emerging question is whether HMO supplementation could offer neuroprotective benefits in aging populations. To date, no clinical studies have been conducted to evaluate the effects of HMOs on cognitive maintenance or neurodegeneration in later life, yet their prebiotic properties and influence on neuroactive molecule production suggest promising avenues for investigation. For instance, HMOs promote gut microbial diversity and stimulate the synthesis of neurotransmitters such as gamma-aminobutyric acid (GABA) and serotonin, which are crucial for cognitive function and mental health. Animal studies indicate that HMOs modulate behaviour, learning, memory, and other cognitive processes, highlighting their potential impact on human brain health. Consequently, future research should examine whether HMO supplementation at strategic life stages could mitigate cognitive decline or even contribute to the prevention of neurodegenerative diseases such as Alzheimer's disease (AD). While no direct evidence currently supports the role of HMOs in preventing Alzheimer's, their known benefits for gut and brain function make them a compelling target for future clinical trials. By bridging the gap between preclinical findings and human applications, research on HMOs could ultimately inform dietary interventions, from infant nutrition to potential supplements for aging individuals, with the goal of optimizing cognitive health across the lifespan.

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Chapter 6

Appendix

English summary

Breast milk is considered the gold standard for infant nutrition during the first years of life. What makes breast milk so unique is its distinctive composition, ranging from nutrients such as proteins, fats, and vitamins to bioactive components that promote the child's growth, gut microbiota, and immune function. Even after breastfeeding ends, there is still evidence of long-lasting protection against diseases due to the boost breastfeeding gives to the immune system. However, breastfeeding is not always an option, for example, due to practical considerations or medical reasons. In these cases, infant formula serves as a complete alternative to ensure that infants still receive all the essential nutrients for growth and development.

While infant formula provides a complete nutritional alternative to breast milk, there are still differences in its composition, which can contribute to differences in immune function and cognitive development between children raised on breast milk and those raised on formula. One of these critical differences lies in the presence of Human Milk Oligosaccharides (HMOs). HMOs are complex milk sugars that play a crucial role in the development of the immune system, the composition of the gut microbiome, and both physical and mental development. Although HMOs are made up of up to five components (glucose, galactose, glucosamine, sialic acid, and fucose), this results in a broad spectrum of over 150 different HMOs in breast milk, and the quantity and ratio of these HMOs also vary according to the stage of lactation and other factors such as the mother's diet and blood type. This indicates that there is no universal composition of breast milk that is equally effective for all infants, but that breast milk is dynamically adjusted to meet the specific needs of the individual child. This important nuance is currently lacking in the synthesis of infant formula, as it contains only a limited number of HMOs. Furthermore, it is still unclear whether HMOs interact with each other to produce synergistic effects, although studies on the gut microbiome do provide some indications.

In recent decades, HMOs have also been studied in the context of brain development and cognition, exploring areas such as emotional and social development, memory, and learning ability. Several animal studies show that HMOs rich in fucose and sialic acid impact multiple critical brain areas, such as the medial prefrontal cortex and hippocampus, and processes related to these cognitive domains. These processes include the regulation of genes related to neural plasticity, myelination, memory, and the formation of neural circuits within the aforementioned areas. Since HMOs are processed by various bacteria in the gut, they trigger a cascade of metabolites that influence various neurodevelopmental processes. Different bacteria are required

to metabolize sialylated and fucosylated HMOs, which may result in a very specific microbiotal composition with unique effects, depending on the exact combination of HMOs in the diet.

Our hypothesis is that the combination of different HMOs works synergistically, and that a specific ratio of HMOs, provided during critical developmental periods, produces a unique profile of long-lasting beneficial effects on neurodevelopment and emotional outcomes in mammals.

To test this hypothesis, we conducted a longitudinal behavioural study with rats that were administered a single, double, or triple combination of fucosylated and sialylated HMOs from PND8 to PND49. Behavioural tests were conducted from infancy to adulthood. The HMO groups in the study were 2'-FL, 3'-SL, 6'-SL, and their various combinations, as previous studies indicated that these HMOs have broad effects on cognition and development. Physical parameters such as eye opening and weight gain were measured, as well as emotional regulation and anxiety behaviour using the Elevated Plus Maze (EPM) and Open Field Test (OFT).

Chapter 2 reviews the impact of HMO supplementation on cognitive functions in preclinical mammalian studies. It emphasizes that sialylated and fucosylated HMOs improve memory, learning speed, and long-term potentiation (LTP), both in infancy during development and in adulthood. These effects are most noticeable during infancy, especially when tasks are cognitively demanding. As the animals age, HMOs also have positive effects on the performance of simpler tasks, suggesting that HMOs have long-lasting positive effects on cognitive functions. The variability in study results indicated the need for a unified, long-term study where task difficulty could be managed, which led to the design of our present longitudinal study with eight HMO treatment groups.

Chapter 3 focuses on the short-term effects of fucosylated and sialylated HMOs during early development from pup to adolescence. Different HMOs caused different behavioural outcomes, with some treatments accelerating eye opening and reducing anxiety, while others increased exploratory behaviour. Sex differences were also observed in locomotor activity, particularly with the 2'-FL + 3'-SL + 6'-SL HMO mix (ratio 6:1:2), which affected males and females differently. This suggests that HMOs may influence developmental profiles in a sex-dependent manner, requiring further investigation.

Finally, **Chapter 4** describes the long-term effects of HMO supplementation during childhood on working memory in adulthood. Male rats treated with a triple HMO mix (2'-FL + 3'-SL + 6'-SL) showed significant improvements in object recognition memory, but there were no clear effects of HMOs on distinguishing different locations. Additionally, HMO treatment altered *Bdnf* gene expression in the medial prefrontal cortex, while the hippocampal gene expression was also correlated with better overall memory performance. Furthermore, differences in the rate of hippocampal neurogenesis were also observed in the triple mix groups.

Overall, the study demonstrated that HMOs offer significant benefits for both physical and cognitive development, both in the short and long term, with the combination of fucosylated and sialylated HMOs being particularly effective. These findings suggest that HMOs influence brain development in a sex- and region-specific manner, opening up new research possibilities in neurodevelopment.

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Dutch summary | Nederlandse samenvatting

Moedermelk wordt beschouwd als de gouden standaard voor babyvoeding gedurende de eerste levensjaren. Wat borstvoeding zo uniek maakt is zijn unieke compositie, variërende van nutriënten zoals eiwitten, vetten en vitamines, tot ook bioactive componenten welke de groei, darmflora en het immuun functioneren van het kind bevorderen. Zelfs na het beëindigen van de borstvoeding zijn er nog steeds aanwijzingen voor de langdurige bescherming tegen ziekten dankzij de boost welke borstvoeding geeft aan het immuunsysteem. Borstvoeding is echter niet altijd een optie, bijvoorbeeld omwille van praktische overwegingen of medische redenen. In deze gevallen bestaat kunstvoeding als volwaardig alternatief om er voor te zorgen dat zuigelingen nog steeds alle broodnodige voedingsmiddelen binnen krijgen om te groeien en zichzelf te kunnen ontwikkelen.

Hoewel flessenvoeding qua nutritie een volwaardig alternatief is op borstvoeding, zijn er nog steeds verschillen in de compositie van flessenvoeding, wat ook kan bijdragen aan verschillen in het immuun functioneren en de cognitieve ontwikkeling tussen kinderen grootgebracht op moedermelk en kinderen grootgebracht op flessenvoeding. Een van deze kritieke verschillen bevindt zich in de aanwezigheid van Humane Melk Oligosacchariden (HMOs). HMOs zijn complexe melksuikers welke een cruciale rol spelen in de ontwikkeling van het immuunsysteem, de samenstelling van het microbioom in de darmen en ook de fysieke en mentale ontwikkeling. Hoewel HMOs opgebouwd zijn uit maximaal vijf componenten (glucose, galactose, glucosamine, sialic acid en fucose) genereert dit een breed spectrum van meer dan 150 verschillende HMOs in moedermelk, en de hoeveelheid en onderlinge verhouding van deze HMOs verschilt ook naargelang het stadium van lactatie en andere factoren zoals dieet en bloedgroep van de moeder. Dit geeft ook aan dat er geen één universele samenstelling is van moedermelk welke voor alle zuigelingen even effectief is, maar dat moedermelk dynamisch aangepast is op de specifieke behoeften van het individuele kind. Dit is een belangrijke nuance welke momenteel nog ontbreekt in de synthese van kunstvoeding aangezien deze tot op heden slechts een beperkt aantal HMOs bevat, en tot op heden is het ook onduidelijk of HMOs ook onderlinge interacties hebben welke leiden tot synergetische effecten, hoewel studies naar het darm microbioom hier wel aanwijzingen voor bevatten.

De afgelopen decennia zijn HMOs ook onderzocht in de context van hersenontwikkeling en cognitie, waarbij domeinen zoals emotionele en sociale ontwikkeling, geheugen en leervermogen werden geëxploreerd. Verscheidene (dieren)studies tonen aan

dat HMOs rijk in fucose en sialic acid op meerdere kritieke hersengebieden, zoals de mediale prefrontale cortex en de hippocampus, en processen ingrijpen gerelateerd aan deze cognitieve domeinen. Deze processen omvatten onder meer de regulatie van genen gerelateerd aan neurale plasticiteit, myelinatie en geheugen, en ook de aanleg van neurale circuits binnen deze voorgenoemde gebieden. Aangezien HMOs door verschillende bacteriën in de darmen worden verwerkt, geven ze aanleiding tot een cascade van metabolieten die verschillende neuro-ontwikkelingsprocessen beïnvloeden. Doordat verschillende bacteriën nodig zijn om de sialylerende en fucosylerende HMOs te metaboliseren, kan dit een zeer specifieke microbiotische samenstelling opleveren, met unieke effecten, afhankelijk van de exacte combinatie van HMOs in het dieet.

Onze hypothese is dat de combinatie van verschillende HMOs synergetisch samenwerkt, en dat een specifieke verhouding van HMOs, verstrekt tijdens kritieke ontwikkelingsperioden, een uniek profiel van langdurige gunstige effecten op neuro-ontwikkeling en emotionele uitkomsten bij zoogdieren produceert.

Om deze hypothese te testen, hebben we een longitudinale gedragsstudie uitgevoerd met ratten die van PND8 tot PND49 een enkele, dubbele of driedubbele combinatie van gefucosyleerde en gesialyleerde HMOs kregen. Gedragsproeven werden uitgevoerd van de kindertijd tot volwassenheid. De groepen HMOs in de studie waren 2'-FL, 3'-SL, 6'-SL, en hun verschillende combinaties, omdat eerdere studies aangaven dat deze HMOs breed effect hebben op cognitie en ontwikkeling. Er werden fysieke parameters zoals oogopening en gewichtstoename gemeten, alsmede emotionele regulatie en angstgedrag met behulp van de Elevated Plus Maze (EPM) en Open Field Test (OFT).

In **hoofdstuk 2** wordt de impact van HMO-suppletie op cognitieve functies in preklinische zoogdierstudies beoordeeld als onderdeel van een review. Er wordt benadrukt dat sialylated en fucosylated HMOs het geheugen, de leersnelheid en de langetermijnpotentiatie (LTP) verbeteren, zowel in de kindertijd tijdens de ontwikkeling als op volwassen leeftijd. Deze effecten zijn het meest merkbaar tijdens de zuigelingenfase, en vooral wanneer de taken cognitief veeleisender zijn. Naarmate de dieren ouder worden, hebben HMOs ook al positieve effecten bij de uitvoer van eenvoudigere taken, wat suggereert dat HMOs langdurige positieve effecten hebben op cognitieve functies. De variabiliteit in de resultaten van de studies duidde wel op de behoefte aan één uniforme, longitudinale studie waarbij de taak in moeilijkheidsgraad kan worden aangepast, wat leidde tot onze huidige longitudinale studie met acht HMO-behandelingsgroepen.

Hoofdstuk 3 richtte zich op de kortetermijneffecten van gefucosyleerde en gesialyleerde HMOs tijdens de vroege ontwikkelingsfase van jonge pup tot adolescentie. Verschillende HMOs veroorzaakten verschillende gedragsresultaten, waarbij sommige behandelingen de oogopening versnellen en angst verminderden, terwijl andere HMO (combinaties) exploratief gedrag bevorderden. Eveneens werden er ook sekseverschillen waargenomen in locomotor gedrag, met name bij de 2'-FL+3'-SL +6'-SL HMO-mix (ratio 6:1:2), die mannen en vrouwen verschillend beïnvloedde in gedrag. Dit wijst erop dat HMOs mogelijk de ontwikkelingsprofielen op een sekse-afhankelijke manier beïnvloeden, wat verder onderzoek vereist.

Tot slot beschrijft **hoofdstuk 4** de langetermijneffecten van HMO-suppletie tijdens de kindertijd op het werkgeheugen in de volwassenheid. De mannelijke ratten die behandeld werden met een triple HMO-mix (2'-FL + 3'-SL + 6'-SL) vertoonden significante verbeteringen in met het herkennen van bekende objecten, maar er waren geen duidelijke effecten van HMOs op het onderscheid van verschillende locaties. Daarnaast veranderde HMO-behandeling de *Bdnf* genexpressie in de mediale prefrontale cortex bij de 2'-FL + 3'-SL + 6'-SL mix groepen, terwijl de hippcampale genexpressie gecorreleerd was aan een beter geheugenprestatie in het algemeen. Bovendien werden verschillen in de mate van hippocampale neurogenese ook geobserveerd tussen de drie triple mix groepen.

Over het geheel genomen laat de studie zien dat HMOs significante voordelen bieden voor fysieke en cognitieve ontwikkeling op zowel korte als op lange termijn, waarbij de combinatie van fucosylated en sialylated HMOs bijzonder effectief is. Deze bevindingen suggereren dat HMOs de hersenontwikkeling op een sekse- en regio-specifieke manier beïnvloeden, wat nieuwe onderzoeksmogelijkheden in hersenontwikkeling opent.

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References

Akoglu, H. (2018). User's guide to correlation coefficients. *Turkish Journal of Emergency Medicine, 18*(3), 91–93. https://doi.org/10.1016/j.tjem.2018.08.001

Allen-Blevins, C. R., You, X., Hinde, K., & Sela, D. A. (2017). Handling stress may confound murine gut microbiota studies. *PeerJ, 5*, e2876. https://doi.org/10.7717/peerj.2876

Altieri, M., Marini, F., Arban, R., Vitulli, G., & Jansson, B. O. (2004). Expression analysis of brain-derived neurotrophic factor (BDNF) mRNA isoforms after chronic and acute antidepressant treatment. *Brain Research, 1000*(1–2), 148–155. https://doi.org/10.1016/j.brainres.2003.12.028

Anacker, C., & Hen, R. (2017). Adult hippocampal neurogenesis and cognitive flexibility: Linking memory and mood. *Nature Reviews Neuroscience, 18*(6), 335–346. https://doi.org/10.1038/nrn.2017.45

Anderson, J. W., Johnstone, B. M., & Remley, D. T. (1999). Breast-feeding and cognitive development: A meta-analysis. *The American Journal of Clinical Nutrition, 70*, 525–535. https://doi.org/10.1093/ajcn/70.4.525

Andreas, N., Kampmann, B., & Mehring Le-Doare, K. (2015). Human breast milk: A review on its composition and bioactivity. *Early Human Development, 91*(11), 629–635. https://doi.org/10.1016/j.earlhumdev.2015.08.013

Antunes, M., & Biala, G. (2012). The novel object recognition memory: Neurobiology, test procedure, and its modifications. *Cognitive Processing, 13*, 93–110. https://doi.org/10.1007/s10339-011-0430-z

Asiminas, A., Lyon, S.A., Langston, R.F., Wood, E.R. (2022). Developmental trajectory of episodic-like memory in rats. Frontiers in behavioral neuroscience, 16, 969871. Doi: https://doi.org/10.3389/fnbeh.2022.969871

Austin, S., de Castro, C. A., Bénet, T., Hou, Y., Sun, H., Thakkar, S. K., Vinyes-Pares, G., Zhang, Y., & Wang, P. (2016). Temporal change of the content of 10 oligosaccharides in the milk of Chinese urban mothers. *Nutrients, 8*, 346. https://doi.org/10.3390/nu8060346

Austin, S., De Castro, C. A., Sprenger, N., Binia, A., Affolter, M., Garcia-Rodenas, C. L., Beauport, L., Tolsa, J. F., & Fischer Fumeaux, C. J. (2019). Human milk oligosaccharides in the milk of mothers delivering term versus preterm infants. *Nutrients, 11*, 1282. https://doi.org/10.3390/nu11061282

Ayechu-Muruzabal, V., van Stigt, A., Mank, M., Willemsen, L., Stahl, B., Garssen, J., & van't Land, B. (2018). Diversity of human milk oligosaccharides and effects on early life immune development. *Frontiers in Pediatrics, 6*, 239. https://doi.org/10.3389/fped.2018.00239

Azad, M. B., Robertson, B., Atakora, F., Becker, A. B., Subbarao, P., Moraes, T. J., Mandhane, P. J., Turvey, S. E., Lefebvre, D. L., Sears, M. R., & Bode, L. (2018). Human milk oligosaccharide concentrations are associated with multiple fixed and modifiable maternal characteristics, environmental factors, and feeding practices. *The Journal of Nutrition, 148*, 1733–1742. https://doi.org/10.1093/jn/nxy175Bach, S. V., Bauman, A. J., Hosein, D., Tuscher, J. J., Ianov, L., Greathouse, K. M., Henderson, B. W., Herskowitz, J. H., Martinowich, K., & Day, J. J. (2023). Distinct roles of *Bdnf I* and *Bdnf IV* transcript variant expression in hippocampal neurons. *bioRxiv*. https://doi.org/10.1101/2023.04.05.535694

Ballard, O., & Morrow, A. L. (2013). Human milk composition: Nutrients and bioactive factors. *Pediatric Clinics of North America, 60*(1), 47–74. https://doi.org/10.1016/j.pcl.2012.10.002

Bar, S., Milanaik, R., & Adesman, A. (2016). Long-term neurodevelopmental benefits of breastfeeding. *Current Opinion in Pediatrics, 28*, 559–566. https://doi.org/10.1097/MOP.0000000000000389

Bartol, F. F., & Bagnell, C. A. (2018). Lactocrine programming. *Encyclopedia of Reproduction, 2*, 814–820. https://doi.org/10.1016/B978-0-12-801238-3.64699-0

Berdugo-Vega, G., Arias-Gil, G., Lopez-Fernandez, A., Artegiani, B., Wasielewska, J. M., Lee, C., Lippert, M. T., Kempermann, G., Takagaki, K., & Calegari, F. (2020). Increasing neurogenesis refines hippocampal activity rejuvenating navigational learning strategies and contextual memory throughout life. *Nature Communications, 11*, 135. https://doi.org/10.1038/s41467-019-14026-z

Berger, P. K., Plows, J. F., Jones, R. B., Alderete, T. L., Yonemitsu, C., Poulsen, M., Ryoo, J. H., Peterson, B. S., Bode, L., & Goran, M. I. (2020). Human milk oligosaccharide 2'-fucosyllactose links feedings at 1 month to cognitive development at 24 months in infants of normal and overweight mothers. *PLoS ONE, 15*(9), e0228323. https://doi.org/10.1371/journal.pone.0228323

Berger, P. K., Ong, M. L., Bode, L., & Belfort, M. B. (2023). Human milk oligosaccharides and infant neurodevelopment: A narrative review. *Nutrients, 15*(3), 719. https://doi.org/10.3390/nu15030719

Bilbo, S. D., & Schwarz, J. M. (2012). The immune system and developmental programming of brain and behavior. *Frontiers in Neuroendocrinology, 33*, 267-286. https://doi.org/10.1016/j.yfrne.2012.08.006

Bode, L. (2012). Human milk oligosaccharides: Every baby needs a sugar mama. *Glycobiology, 22*(9), 1147-1162. https://doi.org/10.1093/glycob/cws074

Bode, L. (2015). The functional biology of human milk oligosaccharides. *Early Human Development, 91*(11), 619-622. https://doi.org/10.1016/j.earlhumdev.2015.09.001

Bonfanti, L. (2006). PSA-NCAM in mammalian structural plasticity and neurogenesis. *Progress in Neurobiology, 80*(3), 129-164. https://doi.org/10.1016/j.pneurobio.2006.09.006

Borewicz, K., Gu, F., Saccenti, E., Hechler, C., Beijers, R., de Weerth, C., van Leeuwen, S. S., Schols, H. A., & Smidt, H. (2020). The association between breastmilk oligosaccharides and faecal microbiota in healthy breastfed infants at two, six, and twelve weeks of age. Scientific Reports, 10, 4270. https://doi.org/10.1038/s41598-020-61024-z

Boquien, C-Y. (2018). Human milk: An ideal food for nutrition of preterm newborns. *Frontiers in Pediatrics, 6*, 1-9. https://doi.org/10.3389/fped.2018.00295

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., & Wittwer, C. T. (2009). The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clinical Chemistry, 55(4), 611–622. https://doi.org/10.1373/clinchem.2008.112797

Bunesova, V., Lacroix, C., & Schwab, C. (2016). Fucosyllactose and L-fucose utilization of infant *Bifidobacterium longum* and *Bifidobacterium kashiwanohense*. *BMC Microbiology, 16*(1), 248. https://doi.org/10.1186/s12866-016-0851-4

Çatlı, G., Dündar, N. O., & Dündar, B. N. (2014). Adipokines in breast milk: An update. *Journal of Clinical Research in Pediatric Endocrinology, 6*, 192-201. https://doi.org/10.4274/jcrpe.1531

Campos, A. C., Fogaça, M. V., Aguiar, D. C., & Guimarães, F. S. (2013). Animal models of anxiety disorders and stress. *Revista Brasileira de Psiquiatria, 35*(Suppl 2), S101–S111. https://doi.org/10.1590/1516-4446-2013-1139

Carabotti, M., Scirocco, A., Maselli, M. A., & Severi, C. (2015). The gut-brain axis: Interactions between enteric microbiota, central and enteric nervous systems. *Annals of Gastroenterology, 28*(2), 203-209.

Carucci, L., Nocerino, R., Paparo, L., Di Scala, C., & Canani, R. B. (2020). Dietary prevention of atopic march in pediatric subjects with cow's milk allergy. *Frontiers in Pediatrics, 8*, 1-9. https://doi.org/10.3389/fped.2020.00440

Casey, B. J., Heller, A. S., Gee, D. G., & Cohen, A. O. (2019). Development of the emotional brain. *Neuroscience Letters, 693*, 29-34. https://doi.org/10.1016/j.neulet.2017.11.055

Cheng, Y-J., & Yeung, C-Y. (2021). Recent advances in infant nutrition: Human milk oligosaccharides. *Pediatrics and Neonatology, 62*, 347-353. https://doi.org/10.1016/j.pedneo.2020.12.013

Chirazzi, M., Cozzolino, M., Reinelt, T., Nguyen, T. D., Chie, S. E., Natalucci, G., & Miletta, M. C. (2021). Human milk and brain development in infants. *Reproductive Medicine, 2*(2), 107-117. https://doi.org/10.3390/reprodmed2020011

Cho, S., Zhu, Z., Li, T., Baluyot, K., Howell, B. R., Hazlett, H. C., Elison, J. T., Hauser, J., Sprenger, N., & Wu, D. (2021). Human milk 3'-sialyllactose is positively associated with language development during infancy. *American Journal of Clinical Nutrition, 114*(2), 588–597. https://doi.org/10.1093/ajcn/nqaa295

Dalile, B., Van Oudenhove, L., Vervliet, B., & Verbeke, K. (2019). The role of short-chain fatty acids in microbiota–gut–brain communication. *Nature Reviews Gastroenterology & Hepatology, 16*(8), 461-478. https://doi.org/10.1038/s41575-019-0157-3

de Weerth, C. (2017). Do bacteria shape our development? Crosstalk between intestinal microbiota and the HPA axis. *Neuroscience and Biobehavioral Reviews, 83*, 458–471. https://doi.org/10.1016/j.neubiorev.2017.09.016

Deoni, S. C. L., Dean, D. C., Piryatinsky, I., O'Muircheartaigh, J., Waskiewicz, N., Lehman, K., Han, M., & Dirks, H. (2013). Breastfeeding and early white matter development: A cross-sectional study. *Neuroimage, 82*, 77-86. https://doi.org/10.1016/j.neuroimage.2013.05.090

den Dekker, H. T., Sonnenschein-van der Voort, A. M. M., Jaddoe, V. W. V., Reiss, I. K., de Jongste, J. C., & Duijts, L. (2016). Breastfeeding and asthma outcomes at the age of 6 years: The Generation R Study. *Pediatric Allergy and Immunology, 27*, 486-492. https://doi.org/10.1111/pai.12576

Duncan, P.I., Raymond, F., Fuerholz, A., Sprenger, N. (2009). Sialic Acid Utilisation and Synthesis in the Neonatal Rat Revisited. PLoS ONE, 4(12), e8241. doi:10.1371/journal.pone.0008241

Dunnett, S. B., Heuer, A., Lelos, M., Brooks, S. P., & Rosser, A. E. (2012). Bilateral striatal lesions disrupt performance in an operant delayed reinforcement task in rats. *Brain Research Bulletin, 88*, 251-260.

Elwakiel, M., Hageman, J. A., Wang, W., Szeto, I. M., van Goudover, J. B., Hettinga, K. A., & Schols, H. A. (2018). Human milk oligosaccharides in colostrum and mature milk of Chinese mothers: Lewis-positive secretor subgroups. *Journal of Agricultural and Food Chemistry, 66*(27), 7036-7043. https://doi.org/10.1021/acs.jafc.8b02021

Falsaperla, R., Fabiano, C., Magro, G., Di Rosa, G., Marino, S., & Mauceri, L. (2024). *Human milk oligosaccharides and their pivotal role in gut–brain axis modulation and neurologic development: A narrative review to decipher the multifaceted interplay*. Nutrients, 16(17), 3009. https://doi.org/10.3390/nu16173009

Fan, Y., McMath, A. L., & Donovan, S. (2023). Review on the impact of milk oligosaccharides on the brain and neurocognitive development in early life. *Nutrients, 15*, 1-37. https://doi.org/10.3390/nu15173743

Feldman, R., & Eidelman, A. I. (2003). Direct and indirect effects of breast milk on the neurobehavioral and cognitive development of premature infants. *Developmental Psychobiology, 43*, 109-119. https://doi.org/10.1002/dev.10126

Ferreira, A. L. L., Alves-Santos, N. H., Freitas-Costa, N. C., Santos, P. P., Batalha, M. A., Figueiredo, A. C., Yonemitsu, C., Manivong, N., Furst, A., & Bode, L. (2021). Associations between human milk oligosaccharides at 1 month and infant development throughout the first year of life in a Brazilian cohort. *Journal of Nutrition, 151*(12), 3543–3554. https://doi.org/10.1093/jn/nxab244

Fewou, S. N., Ramakrishnan, H., Büssow, H., Gieselmann, V., & Eckhardt, M. (2007). Down-regulation of polysialic acid is required for efficient myelin formation. *Journal of Biological Chemistry, 282*(22), 16700-16711. https://doi.org/10.1074/jbc.M610797200

Fleming, S. A., Chichlowski, M., Berg, B. M., Donovan, S. M., & Dilger, R. N. (2018). Dietary sialyllactose does not influence measures of recognition memory or diurnal activity in the young pig. *Nutrients, 10*, 1-12. https://doi.org/10.3390/nu10040395

Fleming, S. A., Mudd, A. T., Hauser, J., Yan, J., Metairon, S., Steiner, P., Donovan, S. M., & Dilger, R. N. (2020a). Human and bovine milk oligosaccharides elicit improved recognition memory concurrent with alterations in regional brain volumes and hippocampal mRNA expression. *Frontiers in Neuroscience, 14*, 1-13. https://doi.org/10.3389/fnins.2020.00770

Fleming, S. A., Mudd, A. T., Hauser, J., Yan, J., Metairon, S., Steiner, P., Donovan, S. M., & Dilger, R. N. (2020b). Dietary oligo fructose alone or in combination with 2′-fucosyllactose differentially improves recognition memory and hippocampal mRNA expression. *Nutrients, 12*, 1-17. https://doi.org/10.3390/nu12072131

Friard, O., & Gamba M. (2016). BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. *Methods in Ecology and Evolution, 7,* 1325-1330. doi: 10.1111/2041-210X.12584

Gabard-Durnam, L. J., Flannery, J., Goff, B., Gee, D. G., Humphreys, K. L., Telzer, E., Hare, T., & Tottenham, N. (2014). The development of human amygdala functional connectivity at rest from 4 to 23 years: A cross-sectional study. *NeuroImage, 95*, 193–207. https://doi.org/10.1016/j.neuroimage.2014.03.038

Gandhi, S. P., Cang, J., & Stryker, M. F. (2005). An eye-opening experience. *Nature Neuroscience, 8*(1), 9-10. https://doi.org/10.1038/nn0105-9

Gascon, E., Vutskits, L., & Kiss, J. Z. (2007). Polysialic acid-neural cell adhesion molecule in brain plasticity: From synapses to integration of new neurons. *Brain Research Reviews, 56*(1), 101-118. https://doi.org/10.1016/j.brainresrev.2007.05.014

Ghashghaei, H. T., & Barbas, H. (2002). Pathways for emotion: Interactions of prefrontal and anterior temporal pathways in the amygdala of the rhesus monkey. *Neuroscience, 115*(4), 1261–1279. https://doi.org/10.1016/s0306-4522(02)00446-3

Gieling, E. T., Nordquist, R. E., & van der Staay, F. J. (2011). Assessing learning and memory in pigs. *Animal Cognition, 14*, 151-173. https://doi.org/10.1007/s10071-010-0364-3

Gila-Diaz, A., Arribas, S. M., Algara, A., Martín-Cabrejas, M. A., López de Pablo, Á. L., Sáenz de Pipaón, M., & Ramiro-Cortijo, D. (2019). A review of bioactive factors in human breastmilk: A focus on prematurity. *Nutrients, 11*(6), 1307. https://doi.org/10.3390/nu11061307

Goehring, K. C., Marriage, B. J., Oliver, J. S., Wilder, J. A., Barrett, E. G., & Buck, R. H. (2016). Similar to those who are breastfed, infants fed a formula containing 2′-fucosyllactose have lower inflammatory cytokines in a randomized controlled trial. *Journal of Nutrition, 146*(11), 2559-2566. https://doi.org/10.3945/jn.116.236919

Guan, W., Cao, J. W., Liu, L. Y., Zhao, Z. H., Fu, Y., & Yu, Y. C. (2017). Eye opening differentially modulates inhibitory synaptic transmission in the developing visual cortex. *eLife, 6*, e32337. https://doi.org/10.7554/eLife.32337

Hauser, J., Pisa, E., Arias Vásquez, A., Tomasi, F., Traversa, A., Chiodi, V., … Macrì, S. (2021). Sialylated human milk oligosaccharides program cognitive development through a non-genomic transmission mode. *Molecular Psychiatry, 26*(7), 2854–2871. https://doi.org/10.1038/s41380-021-01054-9

Hegar, B., Wibowo, Y., Basrowi, R. W., Ranuh, R. G., Sudarmo, S. M., Munasir, Z., … Vandenplas, Y. (2019). The role of two human milk oligosaccharides, 2′-fucosyllactose and lacto-N-neotetraose, in infant nutrition. *Pediatric Gastroenterology, Hepatology & Nutrition, 22*(4), 330-340. https://doi.org/10.5223/pghn.2019.22.4.330

Herba, C. M., Roza, S., Govaert, P., Hofman, A., Jaddoe, V., Verhulst, F. C., & Tiemeier, H. (2013). Breastfeeding and early brain development: The Generation R study. *Maternal & Child Nutrition, 9*(3), 332-349. https://doi.org/10.1111/mcn.12015

Heo, S., Diering, G. H., Na, C. H., et al. (2018). Identification of long-lived synaptic proteins by proteomic analysis of synaptosome protein turnover. *Proceedings of the National Academy of Sciences, 115*(16), E3827–E3836. https://doi.org/10.1073/pnas.1720956115

Higgins, G. A., & Silenieks, L. B. (2017). Rodent test of attention and impulsivity: The 5-choice serial reaction time task. *Current Protocols in Pharmacology, 78*, 5.49.1-5.49.34. https://doi.org/10.1002/cpph.27

Hirschmugl, B., Brandl, W., Csapo, B., van Poppel, M., Köfeler, H., Desoye, G., Wadsack, C., & Jantscher-Krenn, E. (2019). Evidence of Human Milk Oligosaccharides in Cord Blood and Maternal-tp-Fetal Transport across the Placenta. *Nutrients, 11*(11), 2640. doi: 10.3390/nu11112640

Hoeflinger, J. L., Davis, S. R., Chow, J., & Miller, M. J. (2015). In vitro impact of human milk oligosaccharides on *Enterobacteriaceae* growth. *Journal of Agricultural and Food Chemistry, 63*(12), 3295-3302. https://doi.org/10.1021/jf505721p

Horta, B., Loret de Mola, C., & Victora, C. (2015). Breastfeeding and intelligence: A systematic review and meta-analysis. *Acta Paediatrica, 104*, 14-29. https://doi.org/10.1111/apa.13139

Hung, T.-W., Wu, K.-J., Wang, Y.-S., Bae, E.-K., Song, Y., Yoon, J., & Yu, S.-J. (2021). Human milk oligosaccharide 2'-fucosyllactose induces neuroprotection from intracerebral hemorrhage stroke. *International Journal of Molecular Sciences, 22*(18), 9881. https://doi.org/10.3390/ijms22189881

Jacobi, S. K., Yatsunenko, T., Li, D., Dasgupta, S., Yu, R. K., Berg, B. M., … Odle, J. (2016). Dietary isomers of sialyllactose increase ganglioside sialic acid concentrations in the corpus callosum and cerebellum and modulate the colonic microbiota of formula-fed piglets. *Journal of Nutrition, 146*(2), 200-208. https://doi.org/10.3945/jn.115.220152

Jantscher-Krenn, E., von Schirnding, L., Trötzmüller, M., Köfeler, H., Kurtovic, U., Fluhr, H., Mûller, A., & Bagci, S. (2022). Human Milk Oligosaccharides Are Present in Amniotic Fluid and Show Specific Patterns Dependent on Gestational Age. *Nutrients, 14*(10), 2065. doi: 10.3390/nu14102065

Jorgensen, J. M., Young, R., Ashorn, P., Ashorn, U., Chaima, D., Davis, J. C., … Maleta, K. (2021). Associations of human milk oligosaccharides and bioactive proteins with infant growth and development among Malawian mother-infant dyads. *American Journal of Clinical Nutrition, 113*(1), 209–220. https://doi.org/10.1093/ajcn/nqaa271

Khelfaoui, H., Ibaceta-Gonzalez, C., & Angulo, M. C. (2024). Functional myelin in cognition and neurodevelopmental disorders. *Cellular and Molecular Life Sciences, 81*, 181. https://doi.org/10.1007/s00018-024-05222-2

Kiryk, A., Janusz, A., Zglinicki, B., Turkes, E., Knapska, E., Konopka, W., Lipp, H. P., & Kaczmarek, L. (2020). IntelliCage as a tool for measuring mouse behaviour – 20 years perspective. *Behavioural Brain Research, 388*, 112620. https://doi.org/10.1016/j.bbr.2020.112620

Kramer, M. S., Aboud, F., Mironova, E., Vanilovich, I., Platt, R. W., Matush, L., Igumnov, S., Fombonne, E., Bogdanovich, N., Ducruet, T., Collet, J. P., Chalmers, B., Godnett, E., Davidovsky, S., Skugarevsky, O., Trofimovich, O., Kozlova, L., & Shapiro, S. (2008). Breastfeeding and child cognitive development: New evidence from a large randomized trial. *Archives of General Psychiatry, 65*(5), 578-584. https://doi.org/10.1001/archpsyc.65.5.578

Kuipers, S.D., Schroeder, J.E., & Trentani, A. (2015). Neurobiology of Aging, 36(1à, 365-379. Doi: https://doi.org/10.1016/j.neurobiolaging.2014.07.033

Kuntz, S., Kunz, C., Borsch, C., Vazquez, E., Buck, R., Reutzel, M., Eckert, G. P., & Rudloff, S. (2019). Metabolic fate and distribution of 2'-fucosyllactose: Direct influence on gut microbial activity but not on brain. *Molecular Nutrition & Food Research, 63*, 1-8. https://doi.org/10.1002/mnfr.201900035

Kunz, C., Rudloff, S., Baier, W., Klein, N., & Strobel, S. (2000). Oligosaccharides in human milk: Structural, functional, and metabolic aspects. *Annual Review of Nutrition, 20*, 699-722. https://doi.org/10.1146/annurev.nutr.20.1.699

Kunz, C., Meyer, C., Collado, M. C., Geiger, L., Garcia-Mantrana, I., Bertua-Rios, B., Martinez-Costa, C., Borsch, C., & Rudloff, S. (2017). Influence of gestational age, secretor, and Lewis blood group status on the oligosaccharide content of human milk. *Journal of Pediatric Gastroenterology and Nutrition, 64*, 789-798. https://doi.org/10.1097/MPG.0000000000001402

Lawson, M., O'Neill, I., Kujawska, M., Gowrinadh Javvadi, S., Wijeyesekera, A., & Flegg, Z. (2019). Breast milk-derived human milk oligosaccharides promote Bifidobacterium interactions within a single ecosystem. *The ISME Journal, 14*(2), 635-648. https://doi.org/10.1038/s41396-019-0553-2

Le Doare, K., Holder, B., Bassett, A., & Pannaraj, P. (2018). Mother's milk: A purposeful contribution to the development of the infant microbiota and immunity. *Frontiers in Immunology, 9*, 1-10. https://doi.org/10.3389/fimmu.2018.00361

Lees, H., Swann, J., Poucher, S. M., Nicholson, J. K., Holmes, E., Wilson, I. D., & Marchesi, J. R. (2014). Age and microenvironment outweigh genetic influence on the Zucker rat microbiome. *PLoS One, 9*, 1-11. https://doi.org/10.1371/journal.pone.0100916

Leuner, B., Glasper, E.R., & Gould, E. (2009). Thymidine analog methods for studies of adult neurogenesis are not equally sensitive. Journal of Comparative Neurology, 517 (), 123-133. Doi: https://doi.org/10.1002/cne.22107

Li, X., Wang, R., Wang, Y., & Zhang, Y. (2025). *Exploring human milk oligosaccharides (HMOs): Mechanisms linking gut function to cognitive development in human and pig physiology*. Frontiers in Nutrition. (Advance online publication).

Lis-Kuberka, J., & Orczyk-Pawilowicz, M. (2019). Sialylated oligosaccharides and glycoconjugates of human milk: The impact on infant and newborn protection, development, and well-being. *Nutrients, 11*(306), 1-23. https://doi.org/10.3390/nu11020306

Liu, S., Mao, Y., Wang, J., Tian, F., Hill, D.R., Xiong, X., Li, X., Zhao, Y., Wang, S., (2023). Lactational and geographical variation in the concentration of six oligosaccharides in Chinese breast milk: a multicentre study over 13 months postpartum. Front. Nutr., 10. Doi: 10.3389/fnut.2023.1267287

Lueptow, L.M. (017). Novel Object Recognition Test for the Investigation of Learning and Memory in Mice. *J Vis Exp, 30*(126), 55718. Doi: 10.3791/55718

Lynn, D. A., & Brown, G. R. (2010). The ontogeny of anxiety-like behavior in rats from adolescence to adulthood. *Developmental psychobiology, 52*(8), 731–739. https://doi.org/10.1002/dev.20468

Mathisen, P. M., Johnson, J. M., & Kawczak, J. A. (1997). Stabilization of myelin mRNAs as measured in a brain slice system. *Neuroscience Research, 50*(6). https://doi.org/10.1002/(SICI)1097-4547(19971215)50:6<1030::AID-JNR14>3.0.CO;2-8

McHugh, M. L. (2013). The chi-square test of independence. *Biochemia Medica, 23*(2), 143–149. https://doi.org/10.11613/bm.2013.018

McVeagh, P., & Miller, J. B. (2008). Human milk oligosaccharides: Only the breast. *Journal of Paediatrics and Child Health, 33*(4), 281-286. https://doi.org/10.1111/j.1440-1754.1997.tb01601.x

Meyer, D., Bonhoeffer, T., & Scheuss, V. (2014). Balance and stability of synaptic structures during synaptic plasticity. *Neuron, 82*, 430-443. https://doi.org/10.1016/j.neuron.2014.02.031

Miranda, M., Morici, J. F., Zanoni, M. B., & Bekinschtein, P. (2019). Brain-derived neurotrophic factor: A key molecule for memory in the healthy and the pathological brain. *Frontiers in Cellular Neuroscience, 13*, 1-25. https://doi.org/10.3389/fncel.2019.00363

Moossavi, S., Sepehri, S., Robertson, B., Bode, L., Goruk, S., Field, C. J., Lix, L. M., de Souza, R. J., Becker, A. B., Mandhane, P. J., Turvey, S. E., Subbarao, P., Moraes, T. J., Lefebvre, D. L., Sears, M. R., Khafipour, E., & Azad, M. B. (2019). Composition and variation of the human milk microbiota are influenced by maternal early-life factors. *Cell Host & Microbe, 25*, 324-335. https://doi.org/10.1016/j.chom.2019.01.01

Mountford, C., Quadrelli, S., Lin, A., & Ramadan, S. (2015). Six fucose-α(1-2) sugars and α-fucose assigned in the human brain using in vivo two-dimensional MRS. *NMR in Biomedicine, 28*(3), 291-296. https://doi.org/10.1002/nbm.3239

Mudd, A. T., & Dilger, R. N. (2017). Early-life nutrition and neurodevelopment: Use of the piglet as a translational model. *Advances in Nutrition: An International Review Journal, 8*, 92-104. https://doi.org/10.3945/an.116.013243

Mudd, A. T., Fleming, S. A., Labhart, B., Chichlowski, M., Berg, B. M., Donovan, S. M., & Dilger, R. N. (2017). Dietary sialyllactose influences sialic acid concentrations in the prefrontal cortex and magnetic resonance imaging measures in the corpus callosum of young pigs. *Nutrients, 9*, 1297. https://doi.org/10.3390/nu9111297

Muller, D., Wang, C., Skibo, G., Toni, N., Cremer, H., & Calaora, V. (1996). PSA–NCAM is required for activity-induced synaptic plasticity. *Neuron, 17*(3), 413-422. https://doi.org/10.1016/s0896-6273(00)80174-9

Munblit, D., Peroni, D. G., Boix-Amoros, A., Hsu, P. S., Van't Land, B., Gay, M. C. L., Kolotilina, A., Skevaki, C., Boyle, R. J., Collado, M. C., Garssen, J., Geddes, D. T., Nanan, R., Slupsky, C., Wegienka, G., Kozyrskyj, A., & Warner, J. O. (2017). Human milk and allergic diseases: An unsolved puzzle. *Nutrients, 9*, 1-43. https://doi.org/10.3390/nu9080894

Munni, S. K., Alam, M. M., Rahman, M. M., & Park, J. H. (2025). *2'-Fucosyllactose mitigates cognitive deficits in Alzheimer's disease model mice by improving synaptic plasticity and reducing neuroinflammation*. Frontiers in Pharmacology, 10, 1598030. https://doi.org/10.3389/fphar.2025.1598030

Murrey, H. E., Gama, C. I., Kalovidouris, S. A., Luo, W., Driggers, E. M., Porton, B., & Hsieh-Wilson, B. (2006). Protein fucosylation regulates synapsin Ia/Ib expression and neuronal morphology in primary hippocampal neurons. *Proceedings of the National Academy of Sciences, 103*(1), 21-26. https://doi.org/10.1073/pnas.0503381102

Murrey, H. E., & Hsieh-Wilson, L. C. (2008). The chemical neurobiology of carbohydrates. *Chemical Reviews, 108*, 1708-1731. https://doi.org/10.1021

Nolan, L. S., Parks, O. B., & Good, M. (2019). A review of the immunomodulating components of maternal breast milk and protection against necrotizing enterocolitis. *Nutrients, 12*, 1-14. https://doi.org/10.3390/nu12010014

Nyaradi, A., Li, J., Hickling, S., Foster, J., & Oddy, W. H. (2013). The role of nutrition in children's neurocognitive development, from pregnancy through childhood. *Frontiers in Human Neuroscience.* https://doi.org/10.3389/fnhum.2013.00097

Obelitz-Ryom, K., Bering, S. B., Overgaard, S. H., Eskildsen, S. F., Ringgaard, S., Olesen, J. L., Skovgaard, K., Pankratova, S., Wang, B., & Brunse, A. (2019). Bovine milk oligosaccharides with sialyllactose improve cognition in preterm pigs. *Nutrients, 11*, 1335. https://doi.org/10.3390/nu11061335

Oliveros, E., Ramirez, M., Vazquez, E., Barranco, A., Gruart, A., Delgado-Garcia, J. M., Buck, R., Rueda, R., & Martin, M. J. (2016). Oral supplementation of 2'-fucosyllactose during lactation improves memory and learning in rats. *The Journal of Nutritional Biochemistry, 31*, 20–27. https://doi.org/10.1016/j.jnutbio.2015.12.014

Oliveros, E., Vázquez, E., Barranco, A., Ramírez, M., Gruart, A., Delgado-García, J., Buck, R., Rueda, R., & Martín, M. (2018). Sialic acid and sialylated oligosaccharide supplementation during lactation improves learning and memory in rats. *Nutrients, 10(10)*, 1519. https://doi.org/10.3390/nu10101519

Oliveros, E., Martín, M., Torres-Espínola, F., Segura-Moreno, M., Ramírez, M., Santos, A., Buck, R., Rueda, R., Escudero, M., & Catena, A. (2021). Human milk levels of 2-fucosyllactose and 6-sialyllactose are positively associated with infant neurodevelopment and are not impacted by maternal BMI or diabetic status. *Journal of Nutrition & Food Sciences, 4(2)*, 100024. https://doi.org/10.1016/j.jnfs.2021.100024

Ong, R. L., & Yu, R. K. (1984). Interaction of ganglioside GM1 and myelin basic protein studied by carbon-13 and proton nuclear magnetic resonance spectroscopy. *Journal of Neuroscience Research, 12(2-3)*, 377-393. https://doi.org/10.1002/jnr.490120223

Oomen, C. A., Bekinschtein, P., Kent, B., Saksida, L., & Bussey, T. (2014). Adult hippocampal neurogenesis and its role in cognition. *Wiley Interdisciplinary Reviews: Cognitive Science, 5(5)*, 573-587. https://doi.org/10.1002/wcs.1304

Oomen, C. A., Hvoslef-Eide, M., Heath, C. J., Mar, A. C., Horner, A. E., Bussey, T. J., & Saksida, L. M. (2013). The touchscreen operant platform for testing working memory and pattern separation in rats and mice. *Nature Protocols, 8*, 2006-2021. https://doi.org/10.1038/nprot.2013.124

Paganini, D., Uyoga, M., Kortman, G. A. M., Boekhorst, J., Schneeberger, S., Karanja, S., Hennet, T., & Zimmerman, M. B. (2019). Maternal human milk oligosaccharide profile modulates the impact of an intervention with iron and galacto-oligosaccharides in Kenyan infants. *Nutrients, 11*, 1-17. https://doi.org/10.3390/nu11112596

Pallant, J. (2020). *SPSS survival manual: A step by step guide to data analysis using IBM SPSS* (7th ed.). Open University Press.

Pisa, E., Martire, A., Chiodi, V., Traversa, A., Caputo, V., Hauser, J., & Macri, S. (2021). Exposure to 3'-sialyllactose-poor milk during lactation impairs cognitive capabilities in adulthood. *Nutrients, 13(12)*, 4191. https://doi.org/10.3390/nu13124191

Pisa, E., Traversa, A., Caputo, V., Ottomana, A. M., Hauser, J., & Macrì, S. (2023). Long-term consequences of reduced availability and compensatory supplementation of sialylated HMOs on cognitive capabilities. *Frontiers in Cellular Neuroscience, 17*. https://doi.org/10.3389/fncel.2023.1091890

Poton, W. L., Soares, A. L. G., de Oliveira, E. R. A., & Gonçalves, H. (2018). Breastfeeding and behavior disorders among children and adolescents: A systematic review. *Revista de Saúde Pública, 52*, 1-17. https://doi.org/10.11606/S1518-8787.2018052000439

Pressler, R., & Auvin, S. (2013). Comparison of brain maturation among species: An example in translational research suggesting the possible use of bumetanide in newborns. *Frontiers in Neurology, 4*, 1-4. https://doi.org/10.3389/fneur.2013.00036

Prematunga, R. K. (2012). Correlational analysis. *Australian Critical Care, 25(3)*, 195–199. https://doi.org/10.1016/j.aucc.2012.02.003

Quirk, G. J., & Beer, J. S. (2006). Prefrontal involvement in the regulation of emotion: Convergence of rat and human studies. *Current Opinion in Neurobiology, 16(6)*, 723–727. https://doi.org/10.1016/j.conb.2006.07.004

Nolan, L. S., Parks, O. B., & Good, M. (2019). A review of the immunomodulating components of maternal breast milk and protection against necrotizing enterocolitis. *Nutrients, 12*, 1-14. https://doi.org/10.3390/nu12010014

Nyaradi, A., Li, J., Hickling, S., Foster, J., & Oddy, W. H. (2013). The role of nutrition in children's neurocognitive development, from pregnancy through childhood. *Frontiers in Human Neuroscience.* https://doi.org/10.3389/fnhum.2013.00097

Obelitz-Ryom, K., Bering, S. B., Overgaard, S. H., Eskildsen, S. F., Ringgaard, S., Olesen, J. L., Skovgaard, K., Pankratova, S., Wang, B., & Brunse, A. (2019). Bovine milk oligosaccharides with sialyllactose improve cognition in preterm pigs. *Nutrients, 11*, 1335. https://doi.org/10.3390/nu11061335

Oliveros, E., Ramirez, M., Vazquez, E., Barranco, A., Gruart, A., Delgado-Garcia, J. M., Buck, R., Rueda, R., & Martin, M. J. (2016). Oral supplementation of 2'-fucosyllactose during lactation improves memory and learning in rats. *The Journal of Nutritional Biochemistry, 31*, 20–27. https://doi.org/10.1016/j.jnutbio.2015.12.014

Oliveros, E., Vázquez, E., Barranco, A., Ramírez, M., Gruart, A., Delgado-García, J., Buck, R., Rueda, R., & Martín, M. (2018). Sialic acid and sialylated oligosaccharide supplementation during lactation improves learning and memory in rats. *Nutrients, 10(10)*, 1519. https://doi.org/10.3390/nu10101519

Oliveros, E., Martín, M., Torres-Espínola, F., Segura-Moreno, M., Ramírez, M., Santos, A., Buck, R., Rueda, R., Escudero, M., & Catena, A. (2021). Human milk levels of 2-fucosyllactose and 6-sialyllactose are positively associated with infant neurodevelopment and are not impacted by maternal BMI or diabetic status. *Journal of Nutrition & Food Sciences, 4(2)*, 100024. https://doi.org/10.1016/j.jnfs.2021.100024

Ong, R. L., & Yu, R. K. (1984). Interaction of ganglioside GM1 and myelin basic protein studied by carbon-13 and proton nuclear magnetic resonance spectroscopy. *Journal of Neuroscience Research, 12(2-3)*, 377-393. https://doi.org/10.1002/jnr.490120223

Oomen, C. A., Bekinschtein, P., Kent, B., Saksida, L., & Bussey, T. (2014). Adult hippocampal neurogenesis and its role in cognition. *Wiley Interdisciplinary Reviews: Cognitive Science, 5(5)*, 573-587. https://doi.org/10.1002/wcs.1304

Oomen, C. A., Hvoslef-Eide, M., Heath, C. J., Mar, A. C., Horner, A. E., Bussey, T. J., & Saksida, L. M. (2013). The touchscreen operant platform for testing working memory and pattern separation in rats and mice. *Nature Protocols, 8*, 2006-2021. https://doi.org/10.1038/nprot.2013.124

Paganini, D., Uyoga, M., Kortman, G. A. M., Boekhorst, J., Schneeberger, S., Karanja, S., Hennet, T., & Zimmerman, M. B. (2019). Maternal human milk oligosaccharide profile modulates the impact of an intervention with iron and galacto-oligosaccharides in Kenyan infants. *Nutrients, 11*, 1-17. https://doi.org/10.3390/nu11112596

Pallant, J. (2020). *SPSS survival manual: A step by step guide to data analysis using IBM SPSS* (7th ed.). Open University Press.

Pisa, E., Martire, A., Chiodi, V., Traversa, A., Caputo, V., Hauser, J., & Macri, S. (2021). Exposure to 3'-sialyllactose-poor milk during lactation impairs cognitive capabilities in adulthood. *Nutrients, 13(12)*, 4191. https://doi.org/10.3390/nu13124191

Pisa, E., Traversa, A., Caputo, V., Ottomana, A. M., Hauser, J., & Macrì, S. (2023). Long-term consequences of reduced availability and compensatory supplementation of sialylated HMOs on cognitive capabilities. *Frontiers in Cellular Neuroscience, 17*. https://doi.org/10.3389/fncel.2023.1091890

Poton, W. L., Soares, A. L. G., de Oliveira, E. R. A., & Gonçalves, H. (2018). Breastfeeding and behavior disorders among children and adolescents: A systematic review. *Revista de Saúde Pública, 52*, 1-17. https://doi.org/10.11606/S1518-8787.2018052000439

Pressler, R., & Auvin, S. (2013). Comparison of brain maturation among species: An example in translational research suggesting the possible use of bumetanide in newborns. *Frontiers in Neurology, 4*, 1-4. https://doi.org/10.3389/fneur.2013.00036

Prematunga, R. K. (2012). Correlational analysis. *Australian Critical Care, 25(3)*, 195–199. https://doi.org/10.1016/j.aucc.2012.02.003

Quirk, G. J., & Beer, J. S. (2006). Prefrontal involvement in the regulation of emotion: Convergence of rat and human studies. *Current Opinion in Neurobiology, 16(6)*, 723–727. https://doi.org/10.1016/j.conb.2006.07.004

Radlowski, E.C., Conrad, M.S., Lezmi, S., Dilger, R.N., Sutton, B., Larsen, R., Johnson, R.W. (2014). A Neonatal Piglet Model for Investigating Brain and Cognitive Development in Small for Gestational Age Human Infants. *PLoS One, 9*, 1-12. doi: 10.1371/journal.pone.0091951

Rajani, P. S., Seppo, A. E., Järvinen, K.M. (2018). Immunologically Active Components in Human Milk and Development of Atopic Disease, With Emphasis on Food Allergy, in the Pediatric Population. *Front Pediatr. 6*, 1-13. doi: 10.3389/fped.2018.00218

Rajhans, P., Mainardi, F., Austin, S., Sprenger, N., Deoni, S., Hauser, J., & Schneider, N. (2023). The Role of Human Milk Oligosaccharides in Myelination, Socio-Emotional and Language Development: Observational Data from Breast-Fed Infants in the United States of America. *Nutrients, 15*, 4624 doi: https://doi.org/10.3390/nu15214624

Ramani, S., Stewart, C.J., Laucirica, D.R., Ajami, N.J., Robertson, B., Autran, C.A., Shinge, D., Rani, S., Anandan, S., Hu, L., Ferreon, J.C., Kuruvilla, K.A., Petrosino, J.F., Prasad, B.V.V., Bode, L., Kang, G., Estes, M.K. (2018). Human milk oligosaccharides, milk microbiome and infant gut microbiome modulate neonatal rotavirus infection. *Nature, 9*, 1-12. doi: 10.1038/s41467-018-07476-4

Rozé, J.-C., Hartweg, M., Simon, L., Billard, H., Chen, Y., Austin, S., Boscher, C., Moyon, T., Darmaun, D., & Rodenas, C. L. G. (2022). Human milk oligosaccharides in breast milk and 2-year outcome in preterm infants: An exploratory analysis. *Clinical Nutrition, 41*(8), 1896–1905. https://doi.org/10.1016/j.clnu.2022.04.021

Sahay, A., Wilson, D.A., & Hen, R (2011). Pattern separation: a common function for new neurons in hippocampus and olfactory bulb. *Neuron, 70*(4), 582-588. doi: 10.1016/j.neuron.2011.05.012.

Sahay, A., Scobie, K.N., Hill, A.S., O'Carroll, C.M., Kheirbek, M.A., Burghardt, N.S., Fenton, A.A., Dranovsky, A., Hen, R.. (2011). Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. *Nature, 472*(7344), 466-470. doi: 10.1038/nature09817

Sánchez, C.L., Cubero, J., Sánchez, J., Franco, L., Rodriguez, A.B., Rivero, M. (2013). Evolution of the circadian profile of human milk amino acids during breastfeeding. *J Appl Biomed. 11*, 59-70. doi:10.2478/v10136-012-0020-0

Semple, B.D., Blomgren, K., Gimlin, K., Ferriero, D.M., Noble-Haeusslein, L.J. (2013). Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. *Prog Neurobiol., 0*, 1-16. doi: 10.1016/j.pneurobio.2013.04.001

Schnaar, R.L., Gerardy-Schahn, R., & Hildebrandt, H. (2014). Sialic acids in the brain: gangliosides and polysialic acid in nervous system development, stability, disease and regeneration. *Physiol Rev., 94(2)*, 461-518. doi: 10.1152/physrev.00033.2013.

Schneider, M., Al-Shareffi, E., & Haltiwanger, R. S. (2017). Biological functions of fucose in mammals. *Glycobiology, 27*(7), 601-618. doi:10.1093/glycob/cwx047

Scott, G.A., Terstege, D.G., Roebuck, A.J. et al. (2021) Adult neurogenesis mediates forgetting of multiple types of memory in the rat, *Molecular Brain, 14*(1): doi: https://doi.org/10.1186/s13041-021-00808-4

Shumake, J., Barrett, D.W., Lane, M.A., Wittke, A.J. (2014). Behavioral effects of bovine lactoferrin administration during postnatal development of rats. *BioMetals, 27*, 1039-1055. Doi: 10.1007/s10534-014-9735-6

Silvers, J. A., Insel, C., Powers, A., Franz, P., Helion, C., Martin, R., Weber, J., Mischel, W., Casey, B. J., & Ochsner, K. N. (2017). The transition from childhood to adolescence is marked by a general decrease in amygdala reactivity and an affect-specific ventral-to-dorsal shift in medial prefrontal recruitment. *Developmental cognitive neuroscience*, 25, 128–137. https://doi.org/10.1016/j.dcn.2016.06.005

Sipione, S., Monyror, J., Galleguillos, D., Steinberg, N., & Kadam, V. (2020). Gangliosides in the Brain: Physiology, Pathophysiology and Therapeutic Applications. *Front. Neurosci*, 14. https://doi.org/10.3389/fnins.2020.572965

Song, K., Takahashi, S., Sakurai, Y. (2020). Reinforcement schedules differentially affect learning in neuronal operant conditioning in rats. *Neuroscience Research*, 153, 62-67. doi: 10.1016/j.neures.2019.04.003

Soyyılmaz, B., Miks, M.H., Röhrig, C.H., Matwiejuk, M., Meszaros-Matwiejuk, A., Vigsnæs, L.K. (2021). The Mean of Milk: A Review of Human Milk Oligosaccharide Concentrations throughout Lactation. Nutrients, 13(8), 2737, doi: https://doi.org/10.3390/nu13082737

Steenhout, P., Sperisen, P., Martin, F. P., Sprenger, N., Wernimont, S., Pecquet, S., Berger; B. (2016). Term infant formula supplemented with human milk oligosaccharides (2'fucosyllactose and lacto-N-neotetraose) shifts stool microbiota and metabolic signatures closer to that of breastfed infants. *FASEB J.,* 30, 275.7. doi: 10.1096/fasebj.30.1_supplement.275.7

Tarr, A. J., Galley, J. D., Fisher, S. E., Chichlowski, M., Berg, B. M., & Bailey, M. T. (2015). The prebiotics 3'sialyllactose and 6'sialyllactose diminish stressor-induced anxiety-like behavior and colonic microbiota alterations: Evidence for effects on the gut–brain axis. *Brain, Behavior, and Immunity*, 50, 166–177. https://doi.org/10.1016/j.bbi.2015.06.025

ten Bruggencate, S., Bovee-Oudenhoven, I., Feitsma, A., van Hoffen, E., & Schoterman, M. (2014). Functional role and mechanisms of sialyllactose and other sialylated milk oligosaccharides. *Nutrition Reviews, 72*(6), 377-389. doi: 10.1111/nure.12106

Thongaram, T., Hoeflinger, J., Chow, J., & Miller, M. (2017). Human milk oligosaccharide consumption by probiotic and human-associated bifidobacteria and lactobacilli. *Journal Of Dairy Science, 100*(10), 7825-7833. doi: 10.3168/jds.2017-12753

Thurl, S., Munzert, M., Henker, J., Boehm, G., Müller-Werner, B., Jelinek, J., Stahl, B. (2010). Variation of human milk oligosaccharides in relation to milk groups and lactational periods. *Br J Nutr.*, 104, 1261-1271. doi:10.1017/S0007114510002072

Tonon, K.M., de Morais, M.B., Abrão, A.C.F.V., Miranda, A., & Morais, T.B. (2019). Maternal and Infant Factors Associated with Human Milk Oligosaccharides Concentrations According to Secretor and Lewis Phenotypes. *Nutrients*, *11*, 1358. Doi:10.3390/nu11061358

Tosh, N., Quadrelli, S., Galloway, G., & Mountford, C. (2019). Two new fucose-α (1-2)-glycans assigned in the healthy human brain taking the number to seven. *Scientific Reports, 9*(1), 18806. https://doi.org/10.1038/s41598-019-54933-1

Totten, S. M., Zivkovic, A. M., Wu, S., Nguyen, U. T., Freeman, S. L., Ruhaak, L. R., Darboe, M. K., German, J. B., Prentice, A. M., & Lebrilla, C. B. (2012). Comprehensive profiles of human milk oligosaccharides yield highly sensitive and specific markers for determining secretor status in lactating mothers. *Journal of Proteome Research, 11*(12), 6124–6133. https://doi.org/10.1021/pr300769g

Tottenham, N. (2017). The brain's emotional development. *Cerebrum: The Dana Forum on Brain Science, 2017*, cer-08-17.

Townsend, S. (2019). Human milk oligosaccharides: Defense against pathogens. *Breastfeeding Medicine, 14*(S1), S-5-S-6. https://doi.org/10.1089/bfm.2019.0039

Tuplin, E. W. N., Chleilat, F., Alukic, E., & Reimer, R. A. (2021). The effects of human milk oligosaccharide supplementation during critical periods of development on the mesolimbic dopamine system. *Neuroscience, 459*, 166–178.

Urashima, T., Saito, T., Nakamura, T., & Messer, M. (2001). Oligosaccharides of milk and colostrum in non-human mammals. *Glycoconjugate Journal, 18*(5), 357–371. https://doi.org/10.1023/a:1014881913541

Urashima, T., Sato, S., Hirabayashi, J., & Asakuma, S. (2025). *Recent advances in the science of human milk oligosaccharides*. Current Opinion in Food Science. (In press).

Vandenplas, Y., Berger, B., Carnielli, V., Ksiazyk, J., Lagström, H., Sanchez Luna, M., et al. (2018). Human milk oligosaccharides: 2'-fucosyllactose (2'-FL) and lacto-N-neotetraose (LNnT) in infant formula. *Nutrients, 10*(9), 1161. https://doi.org/10.3390/nu10091161

Varki, A., & Kornfeld, S. (2022). Historical background and overview. In A. Varki, R. D. Cummings, J. D. Esko, et al. (Eds.), *Essentials of Glycobiology* (4th ed.). Cold Spring Harbor Laboratory Press. https://www.ncbi.nlm.nih.gov/books/NBK579927/ https://doi.org/10.1101/glycobiology.4e.1

van Goethem, N. P., van Hagen, B. T. J., & Prickaerts, J. (2018). Assessing spatial pattern separation in rodents using the object pattern separation task. *Nature Protocols, 13*(8), 1763–1792. https://doi.org/10.1038/s41596-018-0013-x

Vandenplas, Y., Berger, B., Carnielli, V. P., Ksiazyk, J., Lagström, H., Luna, M. S., Migacheva, N., Mosselmans, J. M., Picaud, J. C., Possner, M., Singhal, A., & Wabitsch, M. (2018). Human milk oligosaccharides: 2′-fucosyllactose (2′-FL) and lacto-n-neotetraose (LNnT) in infant formula. *Nutrients, 10*(1), 1–12. https://doi.org/10.3390/nu10091161

Vázquez, E., Barranco, A., Ramírez, M., Gruart, A., Delgado-García, J. M., Martínez-Lara, E., Blanco, S., Martín, M. J., Castanys, E., Buck, R., Prieto, P., & Rueda, R. (2015). Effects of a human milk oligosaccharide, 2′-fucosyllactose, on hippocampal long-term potentiation and learning capabilities in rodents. *The Journal of Nutritional Biochemistry, 26*(5), 455–465. https://doi.org/10.1016/j.jnutbio.2014.11.016

Vazquez, E., Barranco, A., Ramirez, M., Gruart, A., Delgado-Garcia, J. M., Jimenez, M. L., Buck, R., & Rueda, R. (2016). Dietary 2'-fucosyllactose enhances operant conditioning and long-term potentiation via gut-brain communication through the vagus nerve in rodents. *PLoS One, 11*(11), e0166070. https://doi.org/10.1371/journal.pone.0166070

Vorhees, C. V., & Williams, M. T. (2014). Assessing spatial learning and memory in rodents. *ILAR Journal, 55*, 310–332. https://doi.org/10.1093/ilar/ilu013

Vukojevic, V., Mastrandreas, P., Arnold, A., Peter, F., Kolassa, I. T., Wilker, S., Elbert, T., de Quervain, D. J., Papassotiropoulos, A., & Stetak, A. (2020). Evolutionary conserved role of neural cell adhesion molecule-1 in memory. *Translational Psychiatry, 10*(1), 217. https://doi.org/10.1038/s41398-020-00899-y

Wallace, T. L., Ballard, T. M., & Glavis-Bloom, C. (2015). Animal paradigms to assess cognition with translation to humans. In *Handbook of Experimental Pharmacology* (Vol. 228, pp. 27–57). Springer. https://doi.org/10.1007/978-3-319-16522-6_2

Wang, B., Yu, B., Karim, M., Hu, H., Sun, Y., & McGreevy, P. et al. (2007). Dietary sialic acid supplementation improves learning and memory in piglets. *The American Journal of Clinical Nutrition, 85*(2), 561–569. https://doi.org/10.1093/ajcn/85.2.561

Wang, B. (2009). Sialic acid is an essential nutrient for brain development and cognition. *The Annual Review of Nutrition, 29*, 177–222. https://doi.org/10.1146/annurev.nutr.28.061807.155515

Wang, B. (2012). Molecular mechanism underlying sialic acid as an essential nutrient for brain development and cognition. *Advances in Nutrition, 3*(3), 465S–472S. https://doi.org/10.3945/an.111.001966

Wang, H. J., Hua, C. Z., Ruan, L. L., Hong, L. Q., Sheng, S. Q., & Shang, S. Q. (2017). Sialic acid and iron content in breastmilk of Chinese lactating women. *Indian Pediatrics, 54*, 1029–1031. https://doi.org/10.1007/s13312-017-1206-z

Weledji, E. P., & Assob, J. C. (2014). The ubiquitous neural cell adhesion molecule (N-CAM). *Annals of Medicine and Surgery, 3*, 77–81. https://doi.org/10.1016/j.amsu.2014.06.014

Wichmann, E., Enders, J., Heine, W., & Aßfalg, R. (2024). *Biological effects of combinations of structurally diverse human milk oligosaccharides*. Frontiers in Pediatrics, 12, 1439612. https://doi.org/10.3389/fped.2024.1439612

Wiera, G., Nowak, D., van Hove, I., Dziegiel, P., Moons, L., & Mozrzymas, J. W. (2017). Mechanisms of NMDA receptor- and voltage-gated L-type calcium channel-dependent hippocampal LTP critically rely on proteolysis that is mediated by distinct metalloproteinases. *The Journal of Neuroscience, 37*, 1240–1256. https://doi.org/10.1523/JNEUROSCI.2170-16.2016

Willemsen, Y., Beijers, R., Gu, F., Vasquez, A. A., Schols, H. A., & de Weerth, C. (2023). Fucosylated human milk oligosaccharides during the first 12 postnatal weeks are associated with better executive functions in toddlers. *Nutrients, 15*(6), 1463. https://doi.org/10.3390/nu15061463

Wilson, D. I., Langston, R. F., Schlesiger, M. I., Wagner, M., Watanabe, S., & Ainge, J. A. (2013). Lateral entorhinal cortex is critical for novel object-context recognition. Hippocampus, 23(5), 352–366. https://doi.org/10.1002/hipo.22095

Wise, A., Robertson, B., Choudhury, B., Rautava, S., Isolauri, E., Salminen, S., & Bode, L. (2018). Infants are exposed to human milk oligosaccharides already in utero. *Frontiers in Pediatrics, 6*, 270. https://doi.org/10.3389/fped.2018.00270

World Health Organization. (2020, May 6). Breastfeeding. Retrieved from https://www.who.int/health-topics

World Health Organization. (2024, 22 juni). *Breastfeeding*. World Health Organization. https://www.who.int/health-topics/breastfeeding#tab=tab_1

Xin, W., & Chan, J. R. (2020). Myelin plasticity: Sculpting circuits in learning and memory. *Nature Reviews Neuroscience, 21*, 682–694. https://doi.org/10.1038/s41583-020-00379-8

Xu, C., Efimova, O., Jiang, X., Petrova, M., Vinarskaya, A. K., Kolosov, P., Aseyev, N., Koshkareva, K., Ierusalimsky, V. N., Balaban, P. M., & Khaitovich, P. (2019). Identification of immediate early genes in the nervous system of snail *Helix lucorum*. *eNeuro, 6*(3). https://doi.org/10.1523/ENEURO.0416-18.2019

Xu, J., Li, Z., Wang, F., Huang, Y., & Chen, L. (2025). *Structural diversity, composition variation and neurodevelopmental-promoting effects of human milk oligosaccharides: A systematic review*. Carbohydrate Polymers, 368, 124229. https://doi.org/10.1016/j.carbpol.2025.124229

Yirmiya, R., & Goshen, I. (2011). Immune modulation of learning, memory, neural plasticity, and neurogenesis. *Brain, Behavior, and Immunity, 25*(2), 181–213. https://doi.org/10.1016/j.bbi.2010.08.014

Yu, Z. T., Nanthakumar, N. N., & Newburg, D. S. (2016). The human milk oligosaccharide 2'-fucosyllactose quenches *Campylobacter jejuni*-induced inflammation in human epithelial cells HEp-2 and HT-29 and in mouse intestinal mucosa. *The Journal of Nutrition, 146*, 1980–1990. https://doi.org/10.3945/jn.116.230706

Zeis Axio Observer for Life Science research (2020). ZEISS Group: Available at: https://www.zeiss.com/microscopy/en/products/light-microscopes/widefield-microscopes/axio-observer-for-life-science-research.html (Accessed 13 June 2024)

6

Curriculum Vitae

Sylvia Docq was born on July 7th, 1986 in Sint Truiden, a municipality in Limburg, Belgium. After graduating from secondary school in 2005, she first attended the Art Science programme at the Koninklijke Universiteit Leuven, though through her studies found herself to be more interested in the mental state of the artists and the wellspring of their creativity, rather than the history of the art itself. Therefore, she changed studies upon moving to the Netherlands and attended Tilburg University to obtain a bachelor in Psychology, graduating with honours in 2014. Afterwards, she attended the Research Master in Cognitive Neuroscience at the Radboud University in Nijmegen, which she graduated with honours in 2019. Throughout her studies she interned in multiple labs. The first lab was the EEG lab lead by dr van Boxtel at Tilburg University, where she studied the Event Related Desynchronisation of the alpha band in a Posner Spatial Cueing task in humans, and interned as a research assistant in a study concerning thermoregulation under social exclusion lead by Dr. Ijzerman. During her Masters, she assisted during the Neuroanatomy course and was involved in the study on the role of 5-HT in maternal care and the offspring's development of neuropsychiatric disorders. In 2019, she embarked on her PhD, and was involved in the study of how fucosylated and sialylated HMOs may contribute to infant neurodevelopment, cognitive development and emotional maturation in rodents, which led to the present PhD dissertation.

List of publications

Sun, M., Brivio, P., Shan, L. Docq, S., Heltzel, L. C. M. W., Smiths, C.A.J., Middelman, A., Vrooman, R., Spoelder, M., Verheij, M.M.M., Buitelaar, J.K;, Boillot, M., Calabrese, F., Homberg, J. R. & Hanswijk, S., I. (2024). Offspring's own serotonin transporter genotype, independently from the maternal one, increases anxiety and depression like behavior and alters neuroplasticity markers in rats. Journal of Affective Disorders, 350, 89-101. http://doi.org/10.1016/j.jad.2024.01.114

Docq, S., Spoelder, M., Wang, W., & Homberg, J.R. (2020). The protective and long-lasting effects of human milk oligosaccharides on cognition in mammals. Nutrients, 12(11), 3572. https://doi/org/10.3390/nu12113572

Portfolio

Courses

Courses & Workshops		ECTs
Graduate School Introduction Day	Donders Graduate School	7
Graduate School Day	Donders Graduate School	7
Scientific Integrity Course	Donders Graduate School	7
Graduate School Day 2	Donders Graduate School	7

Lectures and other

Conferences	Year
Dutch Neuroscience Meeting	2019
Donders Discussions Nijmegen	2019
Teaching	**Year**
Supervision of 1 Bachelor of Science student	2021-2022
Supervision of 2 Master of Science students	2020-2022
Supervision of 1 Laboratory Education student	2020-2021

Research data management statement

The research described in this thesis has been carried out under the research data management policy of the Donders Institute for Brain, Cognition and Behaviour. Research Data Management occurred according to the FAIR principles.

Ethical Approval

All included animal studies, described in chapters 3 and 4, were conducted under the strict ethical approval granted by the CCD (AVD1030020198833). All rodent experiments were performed in accordance with national guidelines and the European Union Directive 2010/63/EU, which ensures the ethical use of animals in research. Specific attention was given to the 3Rs principles: refinement, reduction, and replacement of animal use where possible, with particular efforts made to minimize animal suffering and reduction of the number of animals used. All research protocols are archived under the stewardship of Prof. J.R. Homberg (Judith.Homberg@radboudumc.nl). All research protocols are centrally stored under the stewardship of prof J.R. Homberg.

FAIR

This research was conducted in line with the Donders Institute for Brain, Cognition and Behavior's research data management policy and adhered to the FAIR (Findable, Accessible, Interoperable, and Reusable) principles. Upon final publication, all data will be deposited in the Radboud data Repository as Data Sharing Collections and made available to others upon reasonable request to the third party requesting access. All data archives as such remain available for 10 years after publication.

For all data, long lived file formats have been used which enable their potential re-use in the future. All collected data has been structured in a standardized way further detailed in accomplanying text files, including; 1) Experimental design, 2) Variables included, 3) Format of raw data/method of aquisition. Both chapters 3 and 4 can be found on the Donders Repository (DAC: di.dcmn.DAC_4180000.25_453).

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Information page Donders Graduate School

Donders Graduate School

For a successful research Institute, it is vital to train the next generation of scientists. To achieve this goal, the Donders Institute for Brain, Cognition and Behaviour established the Donders Graduate School in 2009. The mission of the Donders Graduate School is to guide our graduates to become skilled academics who are equipped for a wide range of professions. To achieve this, we do our utmost to ensure that our PhD candidates receive support and supervision of the highest quality.

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such as teachers in high school, or as lecturers in higher education. Others continue in a wide range of positions, such as policy advisors, project managers, consultants, data scientists, web- or software developers, business owners, regulatory affairs specialists, engineers, managers, or IT architects. As such, the career paths of Donders PhD graduates span a broad range of sectors and professions, but the common factor is that they almost all have become successful professionals.

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