Effects of laboratory housing conditions on neurobiology of energy balance in mice

Karapetsas, Giorgio

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Chapter 2

Neurobiology of postnatal overfeeding by litter size reduction in rodents; considerations and perspectives

G. Karapetsas [1], J. Bolding [1], S. van Heijningen [1], J. Komdeur [1], L. Schipper [1,2], G. Van Dijk [1]

[1] GELIFES, Groningen Institute for Evolutionary Life Sciences, University of Groningen, The Netherlands

[2] Danone Nutricia Research, Utrecht, The Netherlands

Abstract

Early postnatal overfeeding has been suggested to be one of the factors that contribute to the increasing rates of obesity among children and adults. One frequently applied method to model postnatal overfeeding in rodents is reducing the normal litter size during the lactation phase, and to subsequently study the resultant energy balance changes and underlying behavioral, physiological, and neuroendocrine mechanisms resulting from this model. By reducing the number of littermates, individual pups ingest more milk and are likely to develop overweight, (pre)diabetes, insulin and leptin resistance. Interestingly, these changes often take place when offspring are kept in postweaning healthy nutritional conditions and may be further exacerbated in unhealthy high fat conditions. Early life hyperinsulinemia and hyperleptinemia resulting from litter size reduction are likely to affect the arcuate and paraventricular nucleus of the hypothalamus, which regulate food intake and energy expenditure. Changes in the development of these neural circuits are capable of leading to hyperphagia and/or reduced metabolic rate in rodents raised in small litters. However, some aspects should be taken into account that could lead to a high level of variation between studies. Most important ones are strain differences, number of pups assigned to control litter size, and litter-sex distribution, which all have effects on the pups and possible health outcomes.

Background

It has become evident that inadequate nutrition during the first 1000 days of life can increase the risk of the development of obesity and the risk for non-communicable diseases (NCDs) later in life, during childhood and the adult stage (Mameli, Mazzantini & Zuccotti, 2016). During pregnancy, hyperalimentation of the mother leads to increased body weight of the offspring at delivery which is carried on at least through childhood (Tie et Al., 2014). On the other hand, reduced nutritional supply or protein restriction during pregnancy leads to intrauterine growth restriction of the fetus and lower body weight at delivery, but leads to catch up after birth (Morrison et al., 2010). Both aforementioned situations have been mentioned as risk factors for exaggerated adipose tissue development and cardiometabolic complication later in life (Fall & Kumaran, 2019; Agarwal et al., 2018). A second window of influence on the long-term regulation of body weight and energy balance is the lactation phase. For mammals, maternal milk is the sole source of nutrition right after birth. However, in the human situation many children are unable to receive maternal milk, and infant milk formula (IMF) via bottle feeding is provided as an alternative. IMF is not providing exactly the same macro and micro nutrients as human milk does, as human milk quantity and composition varies over the course of lactation (meeting infant requirements), and its consistency also depends on the maternal diet. IMF, however, has a standard composition (Emmet & Rogers, 1997; Codini et Al., 2020; EU Commission Directive, 2006), which may contribute to the observed differences between breastfed and formula fed infants in early growth trajectories. In fact, IMF has been shown to increase the risk for childhood and later in life obesity compared to breast-fed infants (Gale et al., 2012). Apart from the IMF consistency that differs from breast milk, bottle feeding might increase the risk of postnatal overfeeding (PNOF) (Appleton et Al., 2018). Newborn infants generally possess the ability to regulate their energy intake according to their nutritional needs (Adair, 1984). Bottle feeding, however, may lead to a tendency to override this natural self-regulation of infants. Examples of these feeding practices are parents having the infant to finish the bottle, the tendency to supplement above the recommended quantity based on the concern that the infant is not getting enough nutrition or to increase its sleep duration, and the use of bottle feeding to calm and soothe infants or to reward certain behaviors (Baughcum et al., 1998; Clark et al., 2007; Appleton et Al., 2018). The resultant effect of bottle feeding is that it can increase early life weight trajectories (Weng et al., 2012), which is associated with susceptibility to overweight, obesity and related comorbidities later in life

(Hopkins, Steer, Northstone, & Emmett, 2015; Singhal et al., 2010). However, the observed differences between formula and breastfed infants are mostly obtained in observational studies. Case-controlled investigations of the mechanisms underlying obesity development by gestational hyperalimentation and PNOF in humans is inherently difficult to perform. For this reason, animal studies provide an alternative. In particular rodents have been used to model PNOF and study its (long term) consequences on metabolic health and obesity development. In rodents PNOF can be induced by decreasing the size of the litter of the dam during the lactation period. The concept of litter size manipulation in rodents was pioneered by Kennedy in 1957 (Kennedy, 1957), who argued that downscaling the normal litter size right after birth would increase milk energy transfer from mother to the individual pup, leading to increased weight gain and development of the offspring during the lactation phase. This method has since then been used in numerous studies, among others those that have studied the early neurobiological determinants of energy balance regulation, and derangements hereof. The aim of the present paper is to revisit the reported effects of PNOF by litter size reduction in rodents on neurobiological control of energy balance regulation and fuel homeostasis, and to discuss some of the implications and caveats.

The rodent small litter size model to investigate postnatal overfeeding

Litter size reduction as a model of PNOF is currently often applied in obesity research, which may be related in part to its simple yet effective nature. For rats and mice, a substantial number of the delivered pups are typically removed from the litter on postnatal day (PND) 2 or 3, creating a smaller sized litter (SL) compared to normal sized litters (NL) for the remainder of the lactation period. SL often consist of 3 or 4 pups per litter while NL ranges between 6 or 10 pups/litter, depending on the typical average litter size of the species and strain used (Parra-Vargas et al., 2020). It is important to realize, however, that rodents are altricial animals, meaning that the developmental status of rodents at birth may be considered equivalent to that of a human fetus during the 3rd trimester of pregnancy (Dearden & Ozanne, 2015). For this reason, rodent PNOF by SL manipulation may be argued to model human hyperalimentation in the combined late prenatal (i.e., intrauterine) and postnatal lactation phase.

Milk energy output from dam to offspring can be estimated based on litter characteristics in relation to litter energy expenditure assessment and milk digestibility (Zhao et al., 2013). While dams typically reduce their own intake when nursing SL compared to NL, and energy transfer to the entire SL is equally reduced, energy transfer to individual SL pups is higher compared to that of individual NL pups during the lactation phase (Cunha et al., 2009). In addition, a typical increase in fat and energy content in milk from SL dams has been observed (Fiorotto et Al., 1991; Xavier et Al., 2019). As a result of the increased energy supply, individual SL offspring show increased weight gain during the lactation phase, which is particularly the resultant of an increase of fat mass, specifically of visceral white adipose tissue (WAT) (Bernardo et Al., 2016; Rodrigues et Al., 2011; Dai et Al., 2018; Hou et Al., 2011). These effects persist into adolescence (Hou et Al., 2011), young (Halah et Al., 2018) and adulthood (e.g. more than 12 weeks old), and this is often observed together with various metabolic derangements (Conceição et Al., 2013; Conceição et Al., 2011; Sánchez-García, Del Bosque-Plata & Hong, 2018; Dai et Al., 2018; Hou et Al., 2011; Ji et Al., 2014; Cunha et Al., 2009). Importantly, the effects of PNOF on weight gain and increased fat mass are consistent among studies, and observed when offspring is kept on normal (relatively healthy) rodent chow (Cunha et Al., 2009). In general, effects of PNOF may be further exacerbated by postnatal exposure to high calorie diets (Ji et al., 2014). SL animals show sustained hyperphagia in many (Cunha et Al., 2009; Conceição et Al., 2013; Rodrigues et Al., 2011) but not in all studies (Dai et Al., 2018; Halah et Al., 2018; Hou et Al., 2011), suggesting that the changes in body weight and fat mass in SL rodents may not be dependent only on alterations in energy intake, but also energy expenditure may be affected (Zhu, Eclarinal, Baker, Li, & Waterland, 2016). Indeed, energy expenditure was found to be decreased in SL at weaning and at adult age (Dai et al., 2018; Li et al., 2013). SL has been reported to reduce deposition and activity of interscapular brown adipose tissue (BAT) (Xiao et al., 2007, de Almeida et al., 2013), which may be among the underlying mechanisms of reduced energy expenditure found in SL offspring.

Litter size reduction and derangements in fuel homeostasis.

The effects of PNOF by SL to increased adipose tissue accumulation are sometimes found to be associated with hyperinsulinemia (potentially as a result of insulin resistance) and hyperglycemia in young SL rodents (Bernardo et al., 2016;

Rodrigues et al., 2011; Bei et al., 2015; Plagemann et al, 1992) and in adult SL rodents (Kappeler et al 2009; Bei et al., 2015; De Almeida et al., 2013; Cunha et al., 2009; Hou et al., 2011; Sánchez-García, Del Bosque-Plata & Hong, 2018). However, there are also studies that did not demonstrate any changes in glucose homeostasis in SL rodents versus NL rodents (Rodrigues et al., 2007; Rodrigues et al., 2011), which is probably the due to the fact SL rodents in those studies were fed a standard chow diet after weaning. This is relevant since a common approach to study derangements in insulin and glucose homeostasis is to subject rodents to high fat (HF) diet-induced obesity (DIO), leading to insulin resistance and glucose unresponsiveness per sé (Wang & Liao, 2012). Indeed, one study revealed that glucose tolerance was unaffected at weaning and in early adulthood by SL manipulation, but it was present in 6 weeks old rats only when these animals were fed a HF diet or in 10 and 16 weeks old rats fed either a standard diet or a HF diet (Ji et al., 2014). These findings suggest that the development of glucose intolerance can be exacerbated by HF feeding early in life, but may also develop later in life also in low fat conditions. The use of glucose tolerance tests (GTT) has been applied in adult SL rodents and showed glucose intolerance in rats at 13 weeks (Dai et al., 2018), 14 weeks (Bei et al., 2015), 16 weeks (Hou et al., 2011), 25 weeks (Rodrigues et al., 2011), and 1 year old rats (Cunha et al., 2009).

Hyperinsulinemia by SL manipulation may be the result of permanent physiological changes that alter glucose uptake in pancreatic beta cells, that in response secrete insulin. This process is driven by pancreatic glucose transporter (GLUT-2) that regulates the uptake of glucose by the pancreas (Thorens, 2015). Interestingly, the expression of GLUT-2 was increased in pancreatic islets of SL manipulated rats, explaining the increased insulin secretion (Cunha et al., 2009). Additionally, the endocrine pancreas of SL manipulated rats presented permanent defects in glucose-stimulated insulin secretion, as it was shown by differential expression of 10 key regulatory genes both at PND 26 and PND 100 (Waterland & Garza, 2002). Besides the pancreas, also glucose uptake in adipose tissue appears to be affected by SL manipulation. For example, isolated adipocytes from SL rats showed impaired glucose uptake in the presence of insulin (Aubert, Suquet, & Lemonnier, 1980). These effects are accompanied by in-vivo reduced GLUT-4 translocation and expression, suggesting that glucose uptake was decreased (Rodrigues et al., 2007). Similarly, protein and gene expression of Irs-1 and Glut-4, were decreased in 16 weeks old rats, suggesting that insulin signaling and glucose uptake were impaired in adipose tissue (Bei et al., 2015). Importantly, impaired insulin signaling was found also in the liver of 25 weeks old rats, where protein expression of IRβ, phospho-IRS-1, IRS-1, PI3K and Akt1 were decreased (Conceição et al., 2013). It is possible that the impaired insulin signaling in the pancreas, adipose tissue, and liver contribute to the development and insulin resistance in PNOF rodents.

Besides abnormalities in glucose homeostasis, SL manipulation is also able to affect lipid homeostasis, since circulating triglycerides (TG), total cholesterol (TC) and high-density lipoprotein-c (HDL-C) were increased in young SL mice and rats (Bernardo et al., 2016, Rodrigues et al., 2011). Lipid homeostasis seem to be affected also in adult rodents, as TG were increased also in 13 weeks old (Dai et al., 2018), 32 weeks old rats (Sánchez-García, Del Bosque-Plata & Hong, 2018) and together with free-fatty acids (FFA) in 16 weeks old rats (Bei et al., 2015). However, two studies failed to find changes in circulating lipids in young and adult rats' offspring (Cunha et al., 2009; Hou et al., 2011), but also in this case, the studies listed so far subjected SL rodents to low fat conditions and HF dietary regimens seem to aggravate lipid homeostasis in SL rodents (Ji et al., 2014). The mechanisms contributing to the changes in lipid homeostasis by SL manipulation are likely the result of increased hepatic lipogenesis and reduced hepatic fat oxidation, as liver gene expression of Acetyl-CoA carboxylase and sterol-regulatory element binding protein-1c (ACC and SREBP1c; i.e., committed steps towards hepatic lipogenesis) were increased at weaning. At early adulthood and later adulthood, the hepatic expression levels of peroxisome proliferator-activated receptor α (PPARα; i.e., a committed step for fat oxidation) was decreased in early adulthood leading to hepatic steatosis, particular when the SL rodents were subjected to a HF diet after weaning (Ji et al., 2014). Disturbances in lipid (and glucose) homeostasis and insulin action are intricately related (Samuel, Petersen & Shulman, 2010), but it is difficult to disentangle the causality between them.

While insulin resistance may be the common denominator linking hyperinsulinemia and glucose intolerance (Cavaghan, Ehrmann & Polonsky, 2000), insulin resistance and visceral obesity at young age is often causally related with activation of the immune system (Singer & Lumeng, 2017). Kayser et al observed that SL mice (relative to NL mice) have increased visceral fat deposition, which at weaning did not yet show overt signs of inflammation (like crown-like structures, TNF upregulation etc) (Kayser, Goran & Bouret, 2015). However, these SL mice at weaning did have increased expression of toll-like receptor 4 (TLR4), which is known to trigger innate immunity (Takeda & Akira, 2004), and can bind fatty acids leading to insulin resistance (Li et al, 2020). When exposed to a HF diet after weaning, SL mice had equal visceral fat accumulation as NL mice did, however, the SL HF exposed mice had higher levels of visceral tissue inflammation than NL mice on a HF diet, and this was also corroborated with stronger impairments in glucose tolerance and the expression of fatty liver syndrome (Singer & Lumeng, 2017).

In summary, PNOF by litter size reduction causes disturbances in fuel homeostasis when rodents are subjected to a LF diet, however, these outcomes were not always consistent. Exposing PNOF offspring by SL to post-weaning HF diets may exacerbate differences in fuel homeostasis between NL and SL manipulations. The various processes underlying these changes are still a matter of debate, but may be orchestrated at the level of the central nervous system (CNS). Indeed, the CNS is known to control peripheral fuel homeostasis through several behavioral, physiological and neuroendocrine/autonomic routes (van Dijk et al., 2003; Fliers et al., 2003; Russo et al., 2021), and it may therefore be possible that PNOF by SL manipulation exerts many of these effects by changes in neuronal circuitry at an early age. The next paragraphs will elaborate on these specific mechanisms.

Neurobiology of energy balance and fuel homeostasis

Since the mid twentieth century, the hypothalamus has been known to play a key role in the regulation of energy balance and fuel homeostasis (Anand & Brobeck, 1951). While attention was initially focused on the ventromedial and lateral nuclei serving a dual role in hunger and satiety (Stellar, 1954), focus shifted towards neurocircuitry, in particular the one originating from neuronal cell bodies in the arcuate hypothalamic area (ARH) and its vast projections to other hypothalamic and extra-hypothalamic regions (Elmquist, Elias & Saper, 1999**).** The discovery of leptin in 1994 was a hallmark for insight into the CNS control of energy balance (Zhang et al., 1994), and also how leptin could interact with specific neuronal cell body populations in the ARH to achieve this effect. Among the ARH populations are the so-called pro-proopiomelanocortin (POMC) neurons, which increase their activity in response to elevated circulating leptin, which in turn causes a decrease in food intake and increased energy expenditure (Kwon, Kim & Kim, 2016). Another subpopulation of ARH neurons co-express neuropeptide Y (NPY) and agouti-related protein (AgRP). In situations of food scarcity, leptin levels decline, which enhances the activity of NPY/AgRP neurons, that in turn stimulate appetite and reduce energy expenditure (Schwartz et al., 2000). Besides energy balance regulation, central leptin action through interplay with ARH neurons has been shown to affect peripheral glucose and lipid homeostasis through largely undiscovered routes (Schwarz et al., 1996; Diéguez et al., 2011).

Studies using mice that lack leptin (i.e., due to recessive homozygous mutation in the OB gene; Lep^{ob}/Lep^{ob}) have been instrumental in uncovering the neurobiology

of leptin (Pelleymounter et al., 1995). Leptin deficient mice display hyperphagia, obesity and related metabolic derangements (Lindström, 2007). Injection of leptin peripherally as well as centrally causes restoration of (many of) these characteristics, underscoring the importance of leptin in energy balance regulation and fuel homeostasis (Stephens et al., 1995; Pelleymounter et al., 1995; Campfield et al., 1995). The structural formation of the hypothalamic circuitry takes place during early postnatal life in rodents and leptin deficient mice show profound abnormalities in the densities of axonal projections from the ARH to other nuclei, which endure during their entire lifetime (Kamitakahara et al., 2018). Interestingly, ARH axonal outgrowth in Lep^{ob}/Lep^{ob} mice could be rescued by leptin injection between P4 and P12, but not by injections during adulthood (Bouret, Draper, & Simerly, 2004). This shows that leptin signaling specifically during the early postnatal period is an important mechanism targeting the formation of hypothalamic circuitry, and it may suggest that the (long term) metabolic effects of early life nutritional insults including SL may be mediated by altered postnatal leptin signaling. This seems likely since SL pups presented hyperleptinemia as early as during lactation (Sominsky et al., 2017; Rodrigues et al., 2011).

Leptin-mediated hypothalamic development and sensitivity

Increased leptin levels in PNOF rodents by SL manipulation have been shown to be associated with early life leptin resistance. Specifically, Davidowa and Plagemann using single unit electrophysiological recordings showed reduced neuronal activity to leptin in NL rats (n=10/litter), whereas those raised in SL (n=3/litter) were unresponsive to leptin when they were between 5 and 11 weeks old (Davidowa & Plagemann, 2000). In another electrophysiology study, Roberts and colleagues demonstrated that POMC neurons in SL mice (n=3/litter) became leptin-resistant during lactation which persisted into adulthood, a phenomenon that was not observed in NL (n=7-9/litter) (Roberts et al., 2019). Furthermore, SL manipulation caused an increase in the excitatory synaptic transmission and a decrease in inhibitory transmission of ARH LepR-expressing neurons in pubertal mice, suggesting that SL manipulation can exert robust effects on ARH neurons activity too (Zampieri et al., 2020). Finally, AgRP and NPY fiber density in the ARH was found to be increased by SL manipulation in 12 days old rats, which reversed at adulthood to lower NPY fiber density. No effects were observed on fiber density of POMC neurons (Sominsky et al., 2017).

In addition, also protein expression of key regulatory genes involved in leptin signaling in the hypothalamus have been affected by SL manipulation. Leptin acts by binding the leptin receptor (Ob-Rb) and this interaction induces a signaling cascade that involve Janus tyrosine kinase 2 (JAK2) and STAT3, that as a result stimulate the suppression of cytokine signalling-3 (SOCS-3) (Kwon, Kim & Kim, 2016). SL manipulation in rats did not affect hypothalamic expression of this pathway at weaning, but a decreased JAK2 protein expression and increased SOCS3 were detected at PND180, suggesting hypothalamic leptin resistance at adulthood (Rodrigues et al., 2011). In addition, SL mice displayed hypothalamic leptin resistance in the ARH as showed by a lower activation of signal transducer and activator of transcriptor-3, STAT3 (Glavas et al., 2010). In conclusion, the changes described so far indicate that SL manipulation is able to render ARH neurons less responsive to leptin by judging the smaller changes in expression or transcription of key regulatory proteins involved in leptin signaling. These changes could be involved in the control of energy intake and energy expenditure and could contribute to the development of obese phenotypes as a result of PNOF.

The mechanism that connects postnatal hyperleptinemia and leptin resistance may be related to the fact that early elevated hypothalamic leptin levels stimulate local astrocyte proliferation (Rottkamp et al, 2015). Astrocytes can fulfill multiple functions, including provision of metabolic support (Prebil et al., 2011), but can also undergo changes in which they start to secrete inflammatory cytokines (Colombo & Farina, 2016). PNOF by SL manipulation has indeed been shown to upregulate hypothalamic pro-inflammatory profiles, relative to NL manipulation (Ziko et al., 2014). In vitro studies using neonatal glial cell cultures showed that increased saturated (but not unsaturated) fatty acid levels are potent stimulators of cytokine release (Gupta et al., 2012). Because fatty acids are elevated in response to early neonatal hyperalimentation (Bei et al., 2015), it may be speculated that this route connects hypothalamic leptin via astrocytes – fatty acid interactions to leptin resistance.

In addition to leptin, also ghrelin appears to have profound neurodevelopmental effects. Ghrelin is a peptide released from the stomach into the circulation and able to affect CNS neurons mainly through interplay with the growth hormone secretagogue receptor 1a (Muccioli et al., 2007). Ghrelin levels are highest before feeding and lowest right after feeding (Cummings et al., 2001), and ghrelin has potent orexigenic effects in adulthood by actions in the hypothalamus (Wren et al., 2001). Whereas neonatal leptin levels peak the first and the third weeks of life as part of a leptin surge (i.e., which may be an important mechanism to stimulate the formation of ARH projections in neonates) (Ahima, Prabakaran & Flier, 1998), a subsequent surge

of ghrelin has been postulated to switch off this leptin mediated ARH outgrowth (Steculorum et al., 2015). Because ghrelin levels are reduced by PNOF as a result of SL manipulation, the leptin-mediated sprouting of ARH neurons is prolonged and becomes derailed (Collden et al, 2014), with long-term effects including increased body weight, visceral fat, disturbed fuel homeostasis and decreased leptin sensitivity (Steculorum et al., 2015).

Insulin-sensitive hypothalamic development

Similar to leptin, insulin exerts its action also in the CNS and in particular it acts on insulin receptors (IR) and starts a downstream pathway resulting in the downregulation of NPY/AgRP and upregulation of POMC (Simerly, 2008). Interestingly, insulin appears to have neurotrophic effects on the hypothalamus as well (Plagemann, 2008). Perinatal high levels of insulin and glucose are known to cause alterations in hypothalamic insulin sensing and actions (Steculorum, Vogt, & Brüning, 2013). The exact mechanism is still unclear and whether PNOF can affect this pathway is still not known. However, insulin treatment of brain slices of medial ARH showed that the inhibition of these neurons was significantly reduced in both juvenile and adult SL rats (Davidowa & Plagemann, 2007), suggesting that insulin may play a synergistic role with leptin in programming ARH neurons regulating energy intake.

Besides IRs, insulin can bind to another kind of receptor, namely insulin-like growth factor receptors (IGFR). These receptors are normally ligated by insulin-like growth factors (IGF), but because of the close relation of insulin with these growth factors leptin is also able to bind to IGFRs. Different isoforms of IGFRs exist, but in the brain the A isoform is predominantly expressed which has a higher affinity for IGF-2 than for other IGFs. This particular IGF is important for overall neural proliferation (Schubert et al., 2003). Since IGFRs and IRs can heterodimerize there seems to be some cross-over in signalling and biological effects of the two ligands (Kleinridders, Ferris, Cai, & Kahn, 2014). This could be a possible explanation for how insulin is able to affect neurodevelopment, but evidence is scant in this respect. The litter size reduction model is known to cause insulin resistance at later ages. However, even during postnatal development an indication of hypothalamic resistance to elevated central insulin levels has been found in rodents (Plagemann, et al., 1999a), meaning insulin would not be able to exercise its function on the hypothalamus during the critical period of hormone-dependent neurodevelopment in the ARH.

Epigenetic modulations

Besides the effects of "hormone-mediated" and graded changes in the expression levels of neuropeptides controlling energy balance and fuel homeostasis, an additional mode of control is that certain genes can be switched on or off by epigenetic mechanisms as a result of chromatin modifications like DNA methylation or histone modifications (Obri & Claret, 2019). For example, DNA methylation on a promotor site of a gene leads to silencing because the binding of transcription factors is disrupted (Kouzarides, 2007). This may be the case also for PNOF, as Plagemann et al. found that SL manipulation caused hypermethylation (and thus silencing) of the promotor of hypothalamic insulin receptor protein, potentially underlying predisposition to metabolic derailments throughout life (Plagemann et al., 2010). In addition, Plageman et al. also found that the SL manipulation caused hypermethylation of the hypothalamic gene promoter of POMC, within the two Sp1-related binding sequences (Sp1, NF-kappaB) which are essential for the mediation of leptin and insulin effects on POMC expression. Consequently, POMC expression lacked upregulation, despite hyperleptinaemia and hyperinsulinaemia in the SL rats (Plageman, et al., 2009). Interestingly, Li et al. reported that these changes did not persist throughout the lifetime of their mice. Additionally, they showed methylation changes in genes associated with hypothalamic neurodevelopment and function (*Aqp14*, *Nolz1*, *Gadd45b*). These epigenetic modulations were found to be sex-specific since only male mice showed changes in gene expression (Li, et al., 2013). Unfortunately, the studies investigated DNA methylation in the entire hypothalamus and not for specific nuclei associated with energy homeostasis and food intake, which would give a better understanding of the effect of PNOF. Nonetheless, these studies show that PNOF, by means of litter size manipulation causes epigenetic modulations, which could also explain the disrupted energy homeostasis and increased body weight. While this is not the scope of this review, it may very well be the case that SL manipulation, in addition to hypothalamic changes, will also affect the mesolimbic reward system. Indeed, a recent study by Rosetti et al. showed that perinatal feeding of a cafeteria diet (i.e., which is another way of overfeeding) caused profound epigenetic modifications in the offspring as early as embryonic day 21 and PN 10 in several genes encoding for factors implicated in the reward system (Rossetti et al., 2020).

Source of variation in the reduced litter size model

In a recent literature review by Parra-Vargas and colleagues, awareness was raised on the profound influence of litter size of rats and mice on neonatal growth and adult metabolic health and the authors strongly advocate for explicit reporting of this potentially confounding variable in scientific articles describing obesity and metabolic research models using rodents (Parra-Vargas et al., 2020). Another important aspect to take into consideration when using SL manipulation as a model for PNOF is that the "normal" litter size is dependent on several factors, including the strain of rodents (table 1) and maternal age (Patel et al., 2017). While it is a common and accepted strategy to decrease the number of pups per litter to 3 or 4 to achieve postnatal overfeeding, the choice of a control litter size appears to vary between studies. For mice, the litter size at birth generally does not exceed 10 pups, with few exceptions for some strains (Silver, 1995). For C57BL6 mice, one of the most commonly used strains in obesity and metabolic disease research, the average size of the litter at birth is around 6 (see table 1). A strain of mice that has been used as well is the Swiss Webster strain that shows susceptibility to diabetes and capability of overnutrition-induced obesity (Glavas et al., 2010; Liu et al., 2013) and shows an average litter size of 11 pups (see refs in table 1). On the other hand, rats are known to give birth to more pups compared to mice (Evans, 1986). For this reason, the quantification of a control standard litter is debatable, difficult to define and may be strictly dependent on the strain of rodents used. The issue that can arise is that if a strain has a low average litter size (as for example the C57BL6J), and control groups of 8-10 pups are chosen, researchers incur the risk of comparing PNOF to postnatal undernutrition. In litters larger than average (large litters; LL), milk competition between pups would be increased leading to a (relatively) postnatal underfeeding model. Somatic growth is stunted in LL mice and catch-up growth is variable, either leaving the mice with lower body weights than normal throughout their life (Kappeler, et al., 2009; Aubert, Suquet, & Lemonnier, 1980), or that it leads to catch-up growth later (Debarba et al., 2020).

In these cases, it can be speculated that if litter larger than average litter size is used as control against a SL litter, the apparent significant higher body weight of PNOF mice could in fact be partly due to their control group being retarded in growth, or that actually no difference is found because the two extremes lead to the same phenotype. Variation between studies may arise also for different reasons. For example, PNOF reduces competition over milk availability and this can affect maternal affective behaviour (Enes-Marques & Giusti-Paiva, 2018; Priestnall, 1972), pups' behaviour (Enes-Marques & Giusti-Paiva, 2018) and as a result it may be speculated this can affect energy balance regulation.

Another aspect that may be an additional source of variation is the distribution of male and female offspring within the litter (sex ratio). Litters consisting of only male offspring are not uncommon, and this is often accepted, as there is no evidence that litter sex ratio affects body weight at weaning and/or later in life (Curley & Rock, 2008; Hao, Huang, Nielsen, & Kosten, 2011). However, significant changes in behaviour associated with epigenetic modulations as a consequence of litter sex ratio have been reported. It has been hypothesized that these changes may be explained by maternal bias and/or sibling effect. Rodent mothers seem to have a bias toward directing more nursing care behaviour towards males. For instance, dams showed more licking towards males (Hao et al., 2011) and more frequent nursing behaviours compared to all female litters or mixed litters (Laviola & Terranova, 1998). These types of maternal behaviours and in particular licking and nursing behaviour are necessary for pup development (Bridges, 2015). It has been demonstrated that the amount of licking is negatively correlated with DNA methylation levels of the promotor of the *Nr3c1* gene (Weaver et al., 2004). Higher amounts of licking and lower amounts of DNA methylation on this promotor result in higher RNA levels of the glucocorticoid receptor, suggesting this can affect stress reactivity (Meaney, 2001). Similarly, effects of litter sex ratio on the methylation of the *Nr3c1* promotor site has also been found, suggesting these changes can affect early life stress-responsiveness (Kosten, Huang, & Nielsen, 2014). In line with this, C57BL6J female mice, who were reared with mothers that exhibited low frequencies of pup licking showed more anxiety-like behaviour than males (Pedersen, Vadlamudi, Boccia, & Moy, 2011). These findings show how litter sex ratio may be important in regulating pups' behaviour and how maternal behaviour can change as a result of litter sex ratio. In addition, also sibling effects (i.e. playing and social behaviour among siblings) can play a role in this respect. Observations from litters with predominantly female mice showed more social behaviour than litters with mainly male mice. However, litters with a sex-ratio of 1:1 exceeded the amount of exploration and social behaviour (Laviola & Alleva, 1995) and were significantly less fearful (Laviola & Terranova, 1998). In conclusion, whereas litter sex ratio does not appear to affect metabolism and body composition directly, it is likely that maternal nursing behaviour and sibling playing and social behaviour may play a role in affecting the offspring behaviour. These effects should be carefully evaluated when PNOF is applied.

Finally, another potential source of variation may arise in litter manipulation models that also apply randomization of pups from different litters (i.e. random redistribution of newborn pups to either their biological or non-biological dams) is that behavior of the dam towards her own vs fostered pups may be different. For instance, dams from the C57BL6J strain exhibit significantly larger amounts of pup licking and other pup-directed behaviours like nursing towards fostered pups (Curley & Rock, 2008; Van Der Veen et al., 2008). In line with the previously explained effect of pup licking on anxiety-like behaviour, non-fostered pups emitted more faecal boli during open field exploration. However, these effects were specific for the C57BL6J strain as 129S dams showed no sensitivity to this fostering effect and consequently there was no difference in anxiety-like behaviour. Interestingly, fostered C57BL6J pups were found to weigh slightly less than their non-fostered peers but this was not found to be significant (Van Der Veen et al., 2008). Moreover, cross fostering may result in differences in metabolic health and behavior in adulthood mediated by other mechanisms than maternal care and behavior (Bartolomucci et al, 2011). To prevent this source of variation pups may not be randomized at all (Ye et al., 2012; Schreiner et al., 2017) or all offspring can be fostered to non-biological dams. Overall, these findings indicate that fostering effects should be carefully taken into account when using SL manipulation as a model for PNOF. In conclusion, the use of different strains, different litter sizes as control, litter gender composition and the use of fostered versus non-fostered pups can result in an increased variation among studies and become a possible moderator of the outcomes.

Strain	Litter size
Balb/c	6-7
DBA2 hybrid	7
C57BL6J	6
C57BL6N	6-8
FVB/N	8
ICR	11.5
NMRI	10
Swiss (SJL/J)	11
129/SvPas	6.4

Table 1. List of average natural litter sizes of strains used by studies ("Animal Models for Research, Taconic Biosciences"; The Jackson Laboratory; Janvier Labs; Ferretti et al., 2011; Shin et al., 2017; Kappeler et al., 2009; Finlay et al., 2015).

Concluding remarks and perspectives.

SL manipulation is often used as a model for PNOF and to model some aspects of the etiology of (human) obesity and associated derangements. Mice and rats raised in SL are hyperphagic and become heavier than control rodents as early as during lactation, with these effects persisting into adulthood. These changes are often associated with early life hyperleptinemia and hyperinsulinemia and by disturbances in fuel homeostasis, traits that are all associated with obesity and these may be sparked an augmented inflammatory state. The mechanisms that may play a role in the development of insulin resistance and hyperinsulinemia are likely to be in the pancreas, adipose tissue and the liver, where glucose transport is deranged due to heightened inflammatory activity. Hyperleptinemia is a consequence of the increased fat mass deposition shown by SL rodents. Early life hyperleptinemia and hyperinsulinemia likely affect the development of the ARH, leading to hyperphagia and body weight gain and associated disturbances in fuel homeostasis. Rodent models are useful to study these effects, however litter size manipulation should be thoroughly evaluated. In fact, the optimal normal litter size is preferably the same size as the natural litter size of the chosen rodent strain. When selecting a rodent strain their natural litter size should be taken into account as it should be large enough to be able to create a small litter. Regarding litter sex composition it is best to stay as close to natural conditions as possible and choose for a mixed sex litter. And finally, pups should preferably not be randomized, but should all be placed with a foster dam to avoid bias. While this review on PNOF through SL manipulation was intended for improvement of our understanding of the aetiology of human obesity and its comorbidities from a clinical perspective, it may also help to understand the mechanisms that are driving these processes. The predictive adaptive response (PAR) hypothesis has been postulated to explain these latter mechanisms (Gluckman, Hanson and Spencer, 2005). The PAR hypothesis highlights the role of the early-life developmental period of young lending itself to late life plasticity. PAR predicts that the developmental period affects several components of an individual's development that will be advantageous later in life. This means that offspring which are developing in an environment with specific challenges, will become adapted better to those challenges later in life (Gallowat and Ettorson 2007; van den Heuvel et al. 2013). Therefore, individuals born at a low nutritional plane may do well when they experience later in life a low nutritional plane, but may do poorly when the forecast does not materialize (Nettle et al. 2013, Baig et al. 2011). The prototypical example is children born during the Dutch famine at the end of the second world war, who appeared to be at high risk of developing obesity and several inflammatory and cardiometabolic derangements at adulthood (Ravelli, Stein & Susser, 1976). The bases of these changes are poorly understood, but may find their origin in evolutionary prioritization of investments in visceral adipose tissue over other (adipose) tissues because of its importance in immune functioning (West-Eberhard, 2019). Visceral adiposity has indeed been shown to be of key importance to shield off the body from bacterial toxins (Desruisseaux et al., 2007). While this mechanism has been explained to indicate that catch-up growth associated with increased visceral adiposity compartmentalization occurs following fetal malnutrition (by adaptive programming), it may be argued that postnatal overnutrition after birth causes changes that tie into some of the same mechanisms as seen with postnatal undernutrition (Habbout et al., 2013). The rodent homologue of this is to stunt fetal growth by offering the dam a low protein diet during gestation, which results in low birth weight followed by catch-up growth in the postnatal period (Ozanne et al., 2004). Raising rodents in large litters (LL) also produces stunted growth after birth, but can - in some conditions - be related to the post-weaning hyperleptinemia and hypothalamic inflammation, which seemed quite comparable to that seen in rodents raised SL (Debarba et al., 2020). However, LL rearing also showed conflicting results in other studies, namely that it protected against obesity and leptin and insulin resistance in the offspring when exposed to nutritional overload later in life (Patterson et al, 2010). The basis of these discrepancies may rely on how the inflammatory system responds to early life nutritional cues, and how strong these relations are embedded in the (epi)genome as adaptive strategies to deal with prospective challenges.

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