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

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Chapter 1

General introduction

Developmental plasticity, energy balance regulation, and obesity

In the last decades, the incidence of obesity and overweight worldwide has dramatically risen. In 2016, 1,9 billion adults and 340 million children were overweight worldwide (World Health Organization 2017) and this is considered an important risk factor for the development of non-communicable diseases (NCDs) (WHO | Noncommunicable diseases 2017) such as cardiovascular diseases and diabetes. Together with the well-known risks that contribute to the global epidemic of obesity such as unbalanced diet and physical inactivity, epidemiological and animal studies have shown that the interaction between genes and fetal and postnatal environment play a role as well (Taylor and Poston 2007). Therefore, it has become evident that nutritional environment of the developing fetus and the offspring not only serves tremendous physiological growth and rapid functional development of metabolic organs of the child, but also influences the susceptibility of an individual to obesity and NCDs in adulthood (Koletzko et al. 2012; Martin-gronert and Ozanne 2013; Wrottesley, Lamper & Pisa 2015). The capability of animals, including humans, to respond to environmental cues during early life probably originated from evolved adaptive mechanisms with functional relevance for the survival of the organism. This concept, named the Predictive Adaptive Response (PAR) hypothesis, states that the perinatal environment triggers anatomical and functional adaptations that would benefit the survival of an individual in the current and anticipated adult environment (Gluckman, Hanson and Spencer, 2005). However, these adaptations may actually be ineffective, or even counterproductive, when the adult environment is different than what was anticipated during development (i.e. mismatch) (Bateson, Gluckman, and Hanson 2014). This indicates that each organism with a given genotype has the ability to shape the phenotype depending on the environmental circumstance. The prototypical example is provided by individuals born after the Dutch hunger winter which took place in the western part of the Netherlands towards the end of the second world war. Children with low birthweight (due to the fact that mothers were starving during pregnancy) had increased susceptibility later in life to obesity, cardiometabolic and inflammatory diseases compared to children born around the same time, but did not have low birthweight (Ravelli, Stein, and Susser 1976). These effects are explained to indicate that these children were primed for a life of low nutritional plane, but they were instead exposed to a life of high nutritional plane after the second world war. However, it remains to be elucidated how and to what extent developmental plasticity allows individuals to cope with environmental switches that are experienced after birth. To this end, the postnatal phase offers a second PAR window where animals could potentially strategically forecast future environmental conditions and undergo strategic phenotypic changes governed by their early-life conditions to adapt to future environments (Nettle, Frankenhuis, Rickard, 2013). Importantly, phenotypic changes in response to early life environmental influences are likely mediated by early maternal care and energy transfer (Sauce et al., 2017; Guidotti et al., 2013; Stefanidis & Spencer, 2012) and the associated development of the (neuro)endocrine and metabolic system (Monaghan, 2008; Criscuolo et al., 2008; Bouret, 2012) are known to affect energy balance regulation later in life (Remmers & Delemarre-van de Waal, 20011). Obviously, studying these mechanisms will improve our understanding how derangements in energy balance (such as in obesity) can develop. The scope of this thesis is to explore how (early life) social and environmental factors in preclinical (obesity) research can affect the development of obesity in mice. Specifically, the role that developmental plasticity plays in regulating phenotypic development of an individual in response to early life social and environmental conditions will be investigated.

Mouse models to study energy balance regulation and obesity

Prospective and retrospective cohort studies are of tremendous value in investigating specific environmental conditions and later in life risk for obesity. Due to the biological similarity with humans and for ethical, practical and financial reasons, the use of mouse models provides a useful and indispensable tool to study the causal mechanisms of developmental programming of obesity (Speakman et al., 2008). Specifically, mice are particularly useful in this respect for their relative short gestation period and the fact that their environments can be easily manipulated in a controlled way. For these reasons, multiple mouse models to study the mechanisms responsible for obesity development have been deployed (Seki et al. 2012). Since social and environmental factors (like in humans) are known to strongly affect energy balance regulation and growth in mice, these factors should also to be taken into account. The number of mice present in a litter or a cage and the temperature at which mice are kept can strongly affect growth and metabolic health, and impair the translational value of mouse models to humans and be a possible moderator of reported outcomes (Schipper et al., 2018; Parra-Vargas et al., 2020; Speakman and Keijer, 2013). Therefore, it is vital that we learn more about these social and environmental factors and how these can affect growth, sustainable health and reported outcomes.

The litter size reduction model

In humans, postnatal overfeeding (PNOF) has been proposed to accelerate growth trajectories early in life and thereby increase the risk of overweight, obesity and related comorbidities at later life stages (Hopkins et al. 2015; Singhal et al. 2010). The sole source of nutrition for a newborn infant is human milk. However, not all infants have access to breastfeeding due to medical or other reasons. These infants rely on infant milk formula (IMF) as a source of nutrition. One concerning aspect is that bottle feeding is often associated with a "tendency to overfeed" that could override the natural self-regulation of energy intake according to the infants' needs (Adair, 1984). For example, the tendency of parents to require infants to finish the bottle out of the fear that the infants are not getting enough nutrition or to increase sleep duration following larger intakes are considered overfeeding practices (Baughcum et al., 1998; Clark et al., 2007; Appleton et al. 2018). Among the various models to investigate PNOF and the resultant development of obesity, one of the most used and well accepted models is the litter size reduction model in rodents. This model relies on the fact that differences in the number of pups within a litter during the lactation phase may change the amount of milk transfer from mothers to individual offspring, thereby influencing directly the growth trajectory of the offspring (Habbout et al. 2013). As such, PNOF is mimicked by decreasing the number of pups in a litter (small litter, generally three or four pups per litter) and compared to normal size litters (generally from six to twelve pups, depending on species and strain). The resultant of this paradigm is that overfed offspring develop robust phenotypes characterized by increased body weight and adiposity, insulin resistance (Plagemann et al. 1999; Davidowa and Plagemann 2007) and leptin resistance later in life (Schmidt et al. 2001). Importantly, changes in circulating leptin and insulin levels as a result of PNOF are already observed during the lactation phase (Bernardo, 2016; Sominsky, 2017). Such alterations can affect early hypothalamic development of energy balance circuits leading to (mal)adaptive responses and metabolic derangements later in life (Bouret 2012; Plagemann 2008). Similar to the human situation, increased energy intake is generally recognised as the underlying mechanisms for (later in life) metabolic derangements observed in pups raised in small litters. However, there may also be other factors in the rodent small litter rearing model contributing to, and possibly amplifying the (adult) metabolic derangements, but these may be generally overlooked. Huddling is an adaptive strategy applied by rodents to minimize heat loss and thereby saving energy that is required for growth (Gilbert et al. 2010). Therefore, a different number of pups huddling in a litter can affect heat loss and energy expenditure. Both within and between litter differences seem to play a role in regulating energy balance. Indeed, locomotor activity and behaviours directed towards the middle of the (huddling) litter may be affected by litter size but also by variability in body size among pups from the same litter (Bautista et al. 2010). Studying ultimate and proximate mechanisms underlying phenotypic changes resulting from PNOF will allow us to develop and improve preventive strategies aimed at tackling postnatal overfeeding at the root of the problem. This would allow a better translation of rodent models of PNOF to the human situation.

Postweaning social (housing) factors

Next to the number of pups placed in a litter before weaning, also the number of rodents housed in a cage from weaning on can have an effect on the physiology and on (long-term) energy balance regulation (Schipper et al. 2018). Typically, rodents in the laboratory are weaned at three weeks of age, and are housed thereafter either individually or socially (i.e. in groups of two or more rodents per cage). Although it is strongly recommended to house rodents in groups in order to meet the social needs of these species (Council 2011), individual housing is still widely applied. Individual housing is favoured over social housing for practical reasons: to avoid aggression among cage-mates (especially in male rodents), to allow for the presence of exteriorised devices (Kappel, Hawkins, and Mendl 2017) and to allow differentiation among individuals when measuring individual parameters such as food intake and energy expenditure (Tschöp et al. 2012). A recent systematic review and meta-analysis by our group concluded that individually housed rodents at standard room temperature (≈21°C) had higher visceral fat mass and food intake compared to socially housed rodents, and these alterations were independent of changes in body weight (Schipper et al. 2018). We hypothesized that these changes were mediated by chronic emotional stress, due to the lack of social cues, and to mild chronic cold stress, as standard room temperature is below thermoneutrality for mice and individually housed rodents cannot engage in social thermoregulation (Schipper et al. 2018). In particular adolescence is a period of vulnerability to isolation stress in rodents (McCormick and Mathews 2007). Moreover, given that the majority of the neuroendocrine systems involved in regulating energy balance develop during this period (Grove et al. 2005), suggests that individual housing at weaning may have consequences on the programming of energy balance (and derangements herein) as well. Indeed, in a follow-up study we showed that postweaning individual housing compared to social housing at room temperature reduced adolescent growth rate and increased (adult) adiposity in male mice, which was accompanied by increased

energy expenditure and energy intake (Schipper et al. 2020). These results indicate that researchers should be more aware of the potential consequences of social housing factors early in life, as these factors contribute to (later in life) energy balance regulation. Interestingly, the majority of published studies that focus on the effects of social housing factors on energy balance regulation in mice include male subjects but female subjects are not well represented (Schipper et al. 2018). Therefore, a better understanding of how postweaning social factors affect growth and energy balance regulation in female subjects is required.

In addition to social versus individual housing, there is another "social" factor that may affect energy balance regulation in socially housed animals specifically: the social hierarchical structure in which individuals live can shape individual plasticity. For example, changes in social environment affect food intake and subsequent growth rate and body mass in fish (Reed et al. 2019) and mammals (Dantzer et al. 2013). Changes in body size among individuals living in the same group may be the result of developmental plasticity adjusting the offspring phenotypes to match the environment they experience and increase the chances of future survival. Hypothetically, larger or equal size subordinates may be perceived by dominants as competitors, and reducing growth rate may be a counter-adaptation employed by subordinates in order not to be harassed or evicted by dominants. On the contrary, individuals may increase growth rate to become large and attain a dominant position in the group. For example, subordinate Lake Tanganyika cichlid adjust their size to remain smaller than dominants and thereby decrease the threat of eviction from the territory (Heg, Bender & Hamilton, 2004). Interestingly, meerkats living in groups can adjust their growth rate in response to the body mass of their closest competitors irrespective of variation in food availability to the group (Huchard et al. 2016). These examples illustrate how the social hierarchical structure in which individuals live can shape individual plasticity and therefore the establishment of social hierarchies in group-housed rodents should be properly evaluated. In particular, potential differences in body size, growth rate, body composition and metabolic health as a result of social hierarchical structures can increase within-cage variance. Most of the studies investigating the effects of social hierarchy on energy balance considered only male animals kept in large groups (Lee, Yang & Curley, 2018) or in mixed male/female colonies (Tamashiro et al., 2007). Therefore, a better understanding of social hierarchy establishment in smaller groups is important too. Moreover, female rodents are thought not to form social hierarchies (Tamashiro et al. 2004) and thus studies on the establishment of social hierarchical structures in female rodents are underreported. Despite this, recent evidence suggests that female mice form hierarchies, especially when housed in large groups (n=12) (Williamson et al. 2019). For this reason, a better understanding of how social hierarchical structures in both sexes could affect energy balance is needed to improve preclinical research.

Environmental temperature in relation to (social) housing

In the last years, questions have been raised about which is the best temperature to house mice in order to best mimic human physiology and disease (Fischer, Cannon, and Nedergaard 2018; Keijer, Li, and Speakman 2019; Speakman and Keijer 2013). Existing research recognizes that housing mice at standard room temperatures (i.e. around 21°C) induces a mild cold stress challenge that can potentially limit the translational value of mouse models to mimic human physiology and disease, (Ganeshan and Chawla 2017; Karp 2012). In fact, mice are usually housed at room temperature in order to meet the thermal comfort of animal husbandry staff, but at these temperatures over one-third of their energy expenditure is required to maintain core body temperature (Speakman and Keijer 2013; Reitman, 2018). This depends on the mice' high surface-to-volume ratio that translates into greater heat loss, compared to the lower surface-to-volume ratio of humans that also use clothing for better insulation (Reitman, 2018). This means that humans spend little energy to keep warm at standard room temperature and have the possibility to insulate them with warm clothing, whereas mice do not, and therefore it has been argued that mice should be housed closer to, or even at their thermoneutral zone (i.e. when they experience no cold stress) to better model the human situation (Ganeshan & Chawla, 2017; Reitman, 2018). However, when mice are housed in groups of two or more, they typically huddle and decrease their collective surface area allowing them to conserve heat better at standard room temperatures (Harshaw and Alberts, 2012). Importantly, this strategy (also called social thermoregulation) is an adaptive and well-preserved strategy to maximize fitness in group-living endotherms (Gilbert et al. 2010). Therefore, at standard laboratory conditions, socially housed rodents may experience less or no mild chronic cold stress compared to individually housed rodents (Speakman and Keijer 2013). Whether this saving strategy can reduce thermal stress and affect growth in post-weaned mice is yet to be established. In a recent study, we found that postweaning individual housing increased the expression of uncoupling protein-1 (UCP-1) in the brown adipose tissue (BAT) at adolescence and in the inguinal adipose (iWAT) tissue at adulthood, which suggest increased thermoregulation relative to social housing at 21°C (Schipper et al. 2020). As

discussed before, these findings were associated with reduced adolescent growth and increased adult adiposity. Taken together, these findings suggest that environmental temperature may interact with the lack of social thermoregulation in individually housed mice to affect growth and obesity development. Therefore, it is important to dissociate the effects of social thermoregulation and the lack of social cues in affecting energy balance regulation between individual and socially housed mice. A strategy to better study this is to compare growth and obesity development in individually vs socially housed mice both at standard vivarium temperature and at thermoneutrality.

Studying feeding behaviour in mice to improve preclinical research into energy balance regulation and obesity

In a simplistic way, obesity can be seen as the resultant of energy intake overriding energy expenditure, leading to a positive energy balance and weight gain (Hill, Wyatt & Peters, 2012). However, next to energy intake and energy expenditure, feeding behaviour plays a role in obesity development as well (Wardle 2007). Therefore, mice have been widely deployed for the assessment of feeding behaviour through the use of meal pattern analyses (Ellacott et al. 2010). Meal pattern analyses are employed to study the mechanisms that control food intake regulation, using continuous monitoring of food intake. Mice, like humans, eat their food interspaced by time intervals (intermeal intervals) of different durations. However, contrary to humans, they are nocturnal species, and eat the majority of their daily food intake during the dark phase. Moreover, meals during the dark phase are typically separated by shorter intermeal intervals than during the light phase, where longer intermeal intervals are observed (Strubbe and Dijk 2002). A persistent problem with published studies on meal pattern analysis in mice is the lack of consistency in what is considered a meal and how much time should elapse between two feeding bouts to consider these bouts separately or clustered together as a single meal. This is important because feeding bouts interspaced by short intervals may be considered short pauses of food intake within a single meal and longer ones may be regarded as true intermeal intervals separating meals. A common practice in mouse studies on meal pattern analysis is that the researcher determines arbitrarily a minimum intermeal-interval that is used for meal definition, without properly assessing how meal related parameters can be affected by this (Demaria-Pesce and Nicolaïdis 1998). Moreover, and maybe even worse, meal criteria to define a meal may also simply be copied from other publications (Tolkamp et al. 2011). This practice is widely used and current methodologies to obtain a minimum intermeal-interval in mouse studies are scarce (Rathod, Di, and Id 2021), since the majority of these have been deployed in rats and not in mice (Castonguay, Kaiser, and Stern 1986; Zorrilla et al. 2005). Therefore, the choice of a robust and appropriate minimum intermeal-interval to define meals in mice is of importance. One of the most used methodology for meal definition is the log survivor analysis, in which the log transformed cumulated frequency of the number of behavioural events (like feeding bouts) over time is plotted as a function of the minimal time interval that separates these events (Slater and Lester 1982). However, this methodology has been used mainly in rats and not in mice. Moreover, it has a major limitation, as a minimum intermeal-interval for meal definition has to be chosen by visual inspection, making this method subject to different interpretations of assessors. In addition to the criterion or the methodology chosen to determine what is a meal, there are other current practices in feeding behaviour research that should be carefully evaluated. For example, researchers tend to perform meal pattern analysis during the dark phase omitting the whole or small parts of the light phase (Goebel-Stengel et al., 2012; Zorrilla et al., 2005), failing to address data from the light phase that may be relevant to consider for a more complete understanding of feeding behaviour. This would be especially relevant when dietary or pharmacological challenges affect circadian rhythms (Kohsaka et al. 2007) and selective evaluation of data may potentially prevent the detection of specific feeding events taking place outside the selected time frame, but that could be relevant to feeding behaviour. Another practice that is often applied is the exclusion of meals falling below a certain size, as these are assumed to be noise in the registration not due to mice initiating or consuming a meal (Treesukosol and Moran 2016). The relevance of the exclusion of these small meals should be also carefully evaluated. Therefore, a better understanding of how current practices affect study outcomes is needed to improve the study of feeding behaviour in mouse models.

Aim of the thesis

The main aim of the thesis is to study the effects of various social and environmental factors on growth and energy balance regulation. Firstly, the effects of (early-life) social and environmental factors on energy balance regulation have been examined. Secondly, a new methodology for feeding behaviour analysis in mice and current practices generally applied by researchers in mouse feeding behaviour studies have been studied.

Chapter 2 describes a study in which the neuroendocrine mechanisms resulting from the litter size reduction model have been investigated in a literature review. In particular, focus has been given to the leptin and insulin system and how these could affect the programming of the hypothalamus, which regulate food intake and energy expenditure. Potential limitations, aspects to consider and the adaptiveness/maladaptiveness of this model is also discussed. **Chapter 3**, describes a study in male mice investigating whether the metabolic effects of postweaning individual housing relative to social housing, as previously reported by our group, are ameliorated by increasing environmental temperature. In addition, social factors such as the establishment of social hierarchy was explored in socially housed littermates. **Chapter 4** describes a study in which the effects of postweaning individual housing were studied on growth and obesity development in female mice, relative to social housing. Furthermore, an exploratory analysis was conducted in the socially housed animals, to study whether social hierarchies in females could influence energy balance regulation.

In **chapter 5**, a novel methodology was developed and validated to assess meal definition in mice, using intermeal intervals as a function of averaged meal size that were automatically detected by an R package. Furthermore, meal pattern analysis performed through this methodology was compared with meal pattern analyses performed using standard arbitrary chosen minimum intermeal intervals. Finally, the relevance and the contribution of current practices generally applied by researchers in mouse feeding behaviour studies were revised.

Finally, the data presented in this thesis is summarized and evaluated in **chapter 6**.

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