DIFFERENTIAL SUSCEPTIBILITY
to rearing conditions in mice

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DIFFERENTIËLE GEVOELIGHEID

voor opgroei-omstandigheden in muizen

(met een samenvatting in het Nederlands)

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CHAPTER 1

General introduction

1. The relevance of the rearing environment in human development

Every newborn mammal depends on parental care for survival and development. Essential elements of early development such as nutrition, warmth and protection are provided by mammalian mothers in most species, receiving help from fathers in around 6% of mammals that exhibit biparental care[1,2]. Crucially important for survival, parental care determines the fate of offspring in terms of long-term alterations in neurodevelopment and behavior. Variations in parental care are among the most important sources of environmental variation experienced by offspring and elicit long-lasting effects in development and behavior, even across generations[3].

1.1 Parental care in humans

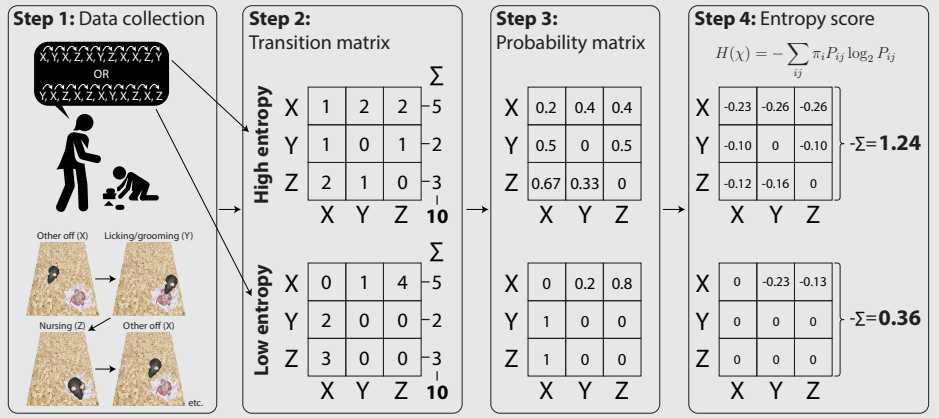
Humans, giving biparental care in most cultures[4], spend a tremendous amount of energy into caring for their offspring. Human childhood lasts exceptionally long and children are highly dependent on adult caregiving[5]. Moreover, this parental investment usually takes place in a complex society with large communities consisting of many males and females[6]. These, together with other unique or unusual human characteristics such as the relatively short time between siblings, have been argued to result in the evolution of the complex and intensive parenting and family formation observed in our species[7].

The efforts by human parents to raise their children cost lots of energy but are clearly important in evolutionary terms. However, even at a shorter timescale, i.e. within generations, parenting style is important: different parents parents raise their children differently, concurrent with differences in child development and behavior. In order to elucidate the parenting characteristics that affect development, the variations in parenting have been a topic of extensive research over the past decades. Multiple dimensions of parenting (such as acceptance versus rejection, warmth versus hostility, autonomy versus control etc.) have been studied[8]. Originally, these dimensions have been categorized into two main dimensions labelled parental support and parental control[9] or parental responsiveness and parental demandingness[10], also termed warmth and control.

However, after characterisation of these early domains, researchers began studying a wider range of parenting characteristics that also involved patterns of parent-child interactions and the direct environment created by the parents. They noticed the importance of cognitive stimulation[11] and rituals[12]. This led to the addition of another main parenting dimension to the other domains (warmth and control). This dimension regained new attention in recent years: Structure, the degree of predictability and consistency in the environment of a child appears to be another important route through which parents affect development of their offspring. Using an innovative approach to quantify this rate of unpredictability in parental care, recent studies have further confirmed the importance of this dimension[13,14].

In recent years, Baram and colleagues have used a mathematical formula derived from physics that describes entropy, i.e. chaos, to estimate the rate of unpredictability in parental care experienced by offspring[13,14]. The concept entails

Box 1



The concept of entropy in the assessment of unpredictability in parental behavior is based upon measuring the sequential transitions from one behavior to the next. In order to quantify this, behavioral data on human or rodent subjects is collected with great temporal resolution (step 1). Then, the number of times each possible transition is observed is scored in a transition matrix (step 2). From this, a probability matrix can be calculated, depicting the likelihood that one behavior predicts the next behavior for each possible transition (step 3). Using the formula, an overall entropy rate is calculated (step 4), in which higher numbers correspond to more chaos, i.e. higher unpredictability. Theoretical examples of two different observations are depicted. The top row represents a higher rate of entropy than the bottom row. Notice that in this example the absolute values of each behavior are identical, whereas the entropy values differ.

that the overall pattern of transitions between different parental behaviors could vary between parents that exhibit similar or even identical absolute levels of these behaviors. Parents that exhibit greater unpredictability in the transitions from one parental behavior to the other however would provide their children with lower scores on the 'structure' dimension. This unpredictability can be observed in the social, emotional and physical domain, but application of the mathematical estimation is restricted to a sequential behavioral approach (see Box 1). An advantage of this mathematical approach is that it can be used across different species such as humans and rodents. However, human-specific environmental unpredictability derived from elements such as frequent moving, tidiness of the house and parental divorce can be assessed using recently developed questionnaires[15] or by observing the home situation[16].

Together, differences on the warmth, control and structure dimensions describe the so-called parenting style deployed by different parents[8]. Since it is unlikely that the parenting style is defined by these three dimensions exclusively, other studies should be conducted to elucidate other contributing dimensions[8]. It is important to note that one parenting style is not necessarily better than the other. It is merely different, as are the resulting developmental trajectories of the offspring. Yet, extreme deviations from 'normal' parenting styles can be catastrophic for offspring development.

1.2 Early-life adversity in humans

Fortunately, the majority of human parents spare no effort into providing the best rearing environment for their children. However, other children suffer from the detrimental effects of poor parenting. The prevalence rates of child maltreatment such as sexual, emotional and physical abuse, as well as physical and emotional neglect are estimated to range between 12-36% worldwide[17]. Children exposed to these practices experience severely increased levels of stress early in life, drastically impacting (neuro-)development[18,19].

There is now comprehensive evidence that childhood maltreatment - in particular factors associated with maladaptive family functioning - is a major contributing factor to later-life psychopathology[20]. For instance, the population attributable risk for depression is accounted for 54% by adverse childhood experiences[21]. Naturally, the neurobiology underlying this substantial effect has been a topic of extensive research and discussing it at length exceeds the scope of this thesis. Some robust effects include: reduction of the volume of several brain regions such as the hippocampus[22] and the anterior cingulate cortex[23], alteration of developmental trajectories of fibre tracts[24] and affecting the processing of sensory information linked to stressful experiences[25,26]. Indeed, the underlying neurobiology of individuals with adverse childhood experiences appears to be fundamentally different from those who did not experience maltreatment[27]. In fact, neurobiological alterations contributing to psychopathology could be more consistent across, rather than within psychiatric disorders. Although these neurobiological alterations likely originated from adaptations to the environment that enhanced survival and reproductive success[27], they might have been at the expense of mental and probably also physical health in the long-run.

1.3 Genetic susceptibility to the (early-life) environment: differential susceptibility

The extent to which these environmental influences affect an individual both psychologically and physically differs for each person. An individual's genetic make-up appears to be important in determining the direction and magnitude of the effects early-life adversity exerts[28,29]. Although the initial study by Caspi and colleagues received criticism[30], the principle is generally accepted and many different genes have since then been associated with increased vulnerability to the early-life environment in relation to psychopathologies such as depression[31] and schizophrenia[32]. In particular genes involved in the serotonergic[33], dopaminergic[34] and stress system[35,36] have been shown to moderate the effects of (early-life) adversity. The mechanisms through which these alterations occur are a topic of extensive research and likely involve epigenetic mechanisms[31,37]. This concept of vulnerable versus resilient genotypes in the face of (early-life) adversity has been proposed in the dual-risk[38] and diathesis-stress models[39] (see Box 2 for an explanation).

However, if vulnerability genes would only aggravate the outcome of early-life stress without affecting development under 'normal' or supportive circumstances, evolutionary pressure would likely have removed these genes from the population. Researchers therefore proposed a different concept in which the same individuals

Box 2

Theoretical framework of this thesis

Throughout the introduction, experimental chapters and discussion of this thesis, several theories and hypothesis will be addressed that form the foundation of this work. Some of these theories are mutually exclusive, others are not. Here, the main theories will be described briefly.

Dual-risk/diathesis stress (figure 1)

This model proposes that some individuals are at increased risk for developing negative consequences as a result of adverse (rearing) conditions. However, when reared in neutral or even positive circumstances, these individuals are indistinguishable from non-susceptible individuals[38,39].

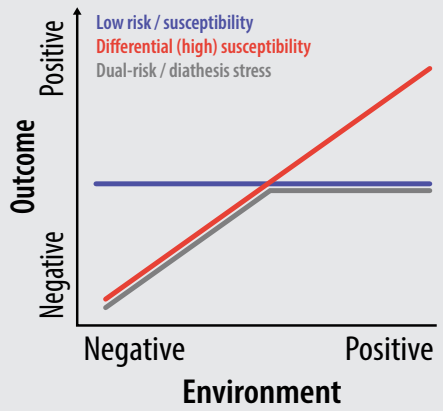


Figure 1. Graphical representation of the dual-risk/diathesis stress and differential susceptibility theories.

Differential susceptibility (figure 1)

The differential susceptibility theory proposes that individuals who are more vulnerable to the negative consequences of (early-life) adversity also benefit more from the positive effects of (early-life) enrichment. Hence, there is a cross-over for better and for worse effect.

Life history theory

According to the life history theory, individuals also adapt the pace of development to the rearing environment77. Uncertain and stressful conditions would accelerate maturation as part of a reproduction strategy in order to reproduce rapidly. Positive rearing conditions on the other hand would allow for ample time to develop optimally, slowing down the pace of (sexual) development.

The three-hit concept of vulnerability and resilience

The three-hit concept of vulnerability and resilience proposes that individuals adapt to stressors throughout their lives, only resulting in negative consequences once multiple hits accumulate in the same individual87. It requires three hits: 1) genetic predisposition, 2) early-life environment and 3) later-life environment, and also points to the predictive adaptation by an individual to optimally cope with the environment.

Match/mismatch hypothesis

Also focusing on the adaptation to (early-life) adversity is the match/mismatch hypothesis. This hypothesis states that individuals adapt to the rearing environment in order to function optimally under circumstances similar to the rearing environment[88]. If the rearing and later-life environment match, this adaptation is successful and helps the individual to thrive under these circumstances. If however the later-life environment is different from the rearing environment, these individuals perform suboptimally compared to well-matched individuals.

who suffer more from adversity would also benefit most from supportive conditions[40]. Termed differential susceptibility, this model would provide an explanation for the existence of "vulnerability genes" in the population, regarding them as "susceptibility genes" instead. Different genetics also affect an individual's temperament and physiology, which have been linked to enhanced susceptibility for better and for worse themselves[41]. For instance, the effects of an attachment-based intervention were most profound in highly reactive children[42]. In addition, Boyce and co-workers provided the first evidence that psychobiological stress reactivity itself can be a marker of differential susceptibility to different rearing environments by studying respiratory illnesses in high or low stress reactive children[43]. This effect has also been observed in highly stress reactive mice[44].

Innovative efforts to study the genetics of differential susceptibility have been executed and are still ongoing. Similar networks highlighted by studies into genetic vulnerability to early-life adversity – serotonergic, dopaminergic and stress-related – appear to be important for enhancing susceptibility to positive and supportive rearing environments. In particular the short variant of the serotonin transporter, 5-HTTLPR, and the 7-repeat variant of the dopamine receptor D4 received considerable attention and have been shown to display differential susceptibility characteristics on a meta-analytic level[45–47]. However, studies on the oxytocin receptor[48] and mineralocorticoid receptor[49] also highlight other potential candidate genes for future studies. Elucidating the exact mechanisms through which these receptors contribute to enhanced susceptibility for better and for worse is important for a more thorough understanding and applicability of the theory, yet requires more detailed, larger and preferably experimental studies. The research in this thesis focuses on two interesting candidate genes to study in the light of differential susceptibility. It should be noted that a candidate gene approach has several limitations compared to human studies in which an additive role of several susceptibility genes has been shown[50]. However, the animal work described in this thesis allows for experimental intervention studies without too many confounders. It should therefore be regarded as proof-of-concept without pretending to model exactly what is observed in humans.

1.4 Candidate genes in differential susceptibility

1.4.1 Mineralocorticoid receptor

The mineralocorticoid receptor (MR) is a high affinity receptor for cortisol (humans) or corticosterone (rodents). MR is mainly expressed in corticolimbic areas such as the hippocampus, amygdala and medial prefrontal cortex[51]. These regions also express the lower-affinity glucocorticoid receptor (GR), which is expressed more ubiquitously (than MR), throughout the brain[52]. When balanced, GR interacts with MR to mediate an adaptive and functional response to stress, thereby preparing the body to cope with future stressors. However, an imbalance between MR and GR has been hypothesized to result in disproportionate responses to stress and consequently increased vulnerability to psychopathology[53,54].

Some genetic variations in the mineralocorticoid receptor gene –particularly those resulting in a loss-of-function- have been associated with increased susceptibility to the negative consequences of early-life adversity in humans[55,56]. In addition, beneficial effects of a supportive environment appear to be enhanced by MR variants[49]. Given the increased susceptibility to environmental factors in children with high stress reactivity[57], the MR gene appears to be an interesting target for differential susceptibility research in more controlled animal models.

1.4.2 Dopamine receptor D4

The dopamine receptor D4 (DRD4) is a D2-like receptor expressed predominantly in GABAergic and pyramidal neurons in the prefrontal cortex and hippocampus[58]. In humans, a variable number tandem repeat ranging between 2 and 11 repeats exists on exon III of the DRD4 gene[59]. The most common allele of this polymorphism contains 4 repeats and is observed in 65% of the population, whereas 20% carries the 7-repeat (7R) version[59]. This DRD4-7R has been associated with lower gene expression[60] and efficacy of the DRD4 protein[61]. In addition, this variant fails to form heterodimers with the short isoform of the D2 receptor[62]. 7R-carriers are at elevated risk to develop externalizing problems such as ADHD[63], in particular following parental insensitivity[64] or chronic stress[34].

However, 7R-carrying children showed lower externalizing behaviors compared to control levels when raised by sensitive mothers[64]. In addition, interventions aimed at enhancing positive parenting resulted in most profound improvements in 7R-carrying children[65]. There is now growing experimental evidence that the DRD4-7R allele is associated with differential susceptibility[46,66].

2. Testing differential susceptibility in rodents

To enable the transition from solely testing the existence of differential susceptibility into a more detailed mechanistic approach, human subjects are not suited for obvious ethical reasons. As further discussed in Chapter 2 of this thesis, animal models offer several unique advantages over experiments in humans to elucidate more causal mechanisms. Using animal models, researchers have more control over the environment and genetics of the subjects studied. Moreover, underlying neurobiology can be studied in much greater spatiotemporal detail. We therefore aimed to utilize these advantages in order to test differential susceptibility in a more controlled experimental setting.

2.1 Manipulating the early-life environment

To test differential susceptibility in mice, the early-life environment should be manipulated so that it models both negative and positive rearing conditions, compared to the standard situation. In the research presented in this thesis, we chronically induced early-life adversity by exposing a mouse dam with her litter to a limited amount of nesting and bedding material. This condition upregulates the entropy of maternal care displayed by the dams, and increased levels of stress experienced by the offspring[14,67,68]. To model positive and supportive rearing conditions, we co-housed two lactating dams with both litters to form a communal nest. Communal nesting upregulates nest presence by the dams[69,70] and was reported to enhance sociability in offspring[71].

2.2 Manipulating the genetics

Inbred mice, all having identical genetics, were used in order to test the specific

contribution of two genes that were manipulated in these animals. The two receptors discussed above, MR and Drd4, were heterozygously knocked-out in the forebrain of mice to mimic reduced gene expression observed in humans carrying susceptible MR haplotypes or the DRD4-7R. Heterozygous knock-out of MR and Drd4 was induced by breeding wild-type mouse dams with heterozygous knock-out fathers[72,73]. This ensured that maternal care of the dams in the F0 generation would not be affected by the genetic manipulation itself. Moreover, this breeding strategy resulted in the birth of wild-type control litter mates that experienced identical rearing conditions without carrying the genetic manipulation.

2.3 Outcome parameters

To measure the effects of different rearing conditions and the (interaction with) genetic background, our experiments focused on developmental and adult parameters linked to sociability. Social behavior is well-known to be affected by early-life experiences[74,75] and genetics[76] and is crucial for social animals such as mice and humans.

2.3.1 Sexual development

A key moment in the development of mammals is puberty, a period of rapid neurobiological and physical changes that appears to be highly malleable by external factors. According to the life history theory, individuals can adapt to their rearing environment by either accelerating or delaying puberty onset (Belsky, Steinberg, & Draper, 1991, see also Box 2). Adversity would lead to acceleration of maturation as part of a reproductive strategy, since the future is uncertain and reproduction should happen as fast as possible. This comes at a cost; brain networks mature faster than normal, possibly leading to aberrations that are less relevant in a hasty life, but become problematic when the future turns out to be longer than anticipated. Accelerated sexual maturation has indeed been observed in female humans[78] and rodents[79], but the picture is ambiguous and less well-studied in males[80]. Moreover, the impact of genetic susceptibility to the effects of rearing conditions on sexual maturation has not been studied thus far.

2.3.2 Intergenerational transmission: maternal care

A crucial consequence of maternal care is that it has the potential to affect maternal care of the offspring towards the next generation, as hypothesized by Belsky in 1991[77]. Rats that were reared by dams that spend more time licking/grooming their offspring also spend more time licking/grooming their own offspring when adults themselves independent of genetic background[3]. The limited nesting and bedding model modifies maternal care of the offspring negatively[81], whereas communal nesting results in improved maternal care[82]. Maternal care therefore appears to be highly important in contributing to a feed-forward loop in social capacities over generations; offspring reared by poor mothers become poor mothers themselves rearing poor mothers etc., whereas positively reared individuals give rise to better mothers. Studying whether genetics contribute to increased susceptibility to these early-environmental factors might help to break this negative feed-forward loop and induce a positive one.

2.3.3 Cognition

Many different aspects of cognition such as anxiety, attention, inhibition, flexibility and learning form the foundation of the sociability of an individual[83]. Early-life adversity (ELA) has been shown to impact many of these cognitive parameters; ELA negatively affects non-stressful spatial learning[84], interacts with genetics to alter anxiety[29] and enhances freezing behavior during "safe" episodes in a fear conditioning paradigm[85]. Overall, early-life adversity has been shown to result in alterations in non-stressful and stressful learning in line with the match-mismatch hypothesis (Bonapersona et al., 2019, see Box 2 for an explanation of this hypothesis). The role of genetics in the match-mismatch hypothesis is unknown, as are the effects of positive rearing conditions on most of these cognitive parameters. We therefore used male offspring from our experiments to test measures of anxiety and non-stressful and stressful learning to study which aspects of cognition are most likely to show differential susceptibility characteristics.

3. Outline of this thesis

The aim of the research presented in this thesis was to establish an animal model for studying differential susceptibility in a controlled setting. Maternal care of mouse dams was manipulated by uninterrupted exposure to different housing conditions in order to model negative, neutral or positive early postnatal rearing conditions for the offspring. The offspring consisted of control animals and genetically manipulated siblings that expressed decreased levels of the mineralocorticoid receptor or dopamine receptor D4. To induce early-life adversity, mouse dams were provided with reduced amounts of nesting and bedding material. Positive rearing conditions were induced by communal nesting of two lactating dams that share care-giving behavior of the pups. This condition also directly affects (maternal) behavior of the mouse dams, which we aimed to study in more detail in the final chapter of this thesis. As part of the consortium on individual development (CID), the work in this thesis focuses on sexual development and behavioral parameters linked to sociability of male and female offspring. We thereby aimed to contribute to the overall question this consortium addresses: 'Why some children thrive, and others don't'

Chapter 2 reviews literature on animal models for studying the effects of parental care on offspring development. The added value of animal models is discussed, highlighting how animal models contribute to enhanced understanding of underlying neurobiology. Some critical notes with regard to translational validity and recommendations for future studies are discussed.

Chapter 3 describes the effects of manipulation of the MR gene and different rearing conditions on sexual development and maternal care towards the next generation in female mice. Heterozygous MR knock-out offspring was exposed to a limited nesting, standard or communal nesting rearing condition and monitored on puberty onset and adult maternal care. Extensive maternal observations were scored to assess the effects of the environmental manipulations in concert with genetic background on maternal care.

We applied a very similar approach in **chapter 4**, where we studied the effect of a heterozygous knock-out of the Drd4 receptor under positive, negative or neutral rearing conditions on the same outcome measures. In this study, the duration of exposure to the different rearing conditions was prolonged with 6 days compared to the study described in chapter 3.

Chapter 5 describes the effects of the genetic and environmental manipulations in adult male siblings of the female mice used in chapter 3 and 4. In this chapter, the effects on anxiety, spatial learning and fear conditioning are presented, next to the MR expression in several brain regions assessed by western blot analysis.

One of the most intriguing behaviors observed during the experiments conducted in this thesis was a 'retrieval of the other dam to the nest' in the communal nesting condition. In **chapter 6** we developed a pipeline for studying the communal nesting model 24/7 using Raspberry Pi devices and Deeplabcut tracking software. We aimed to answer some initial questions regarding this retrieval behavior, while simultaneously providing more insights into this understudied model for early social enrichment.

Chapter 7 provides a discussion in which I will summarize the main findings of this thesis with its implications and limitations. The results will be discussed within the context of the existing literature and the theoretical frameworks the work in this thesis was based upon.

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CHAPTER 2

The added value of rodent models in studying parental influence on offspring development: opportunities, limitations and future perspectives

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Highlights

- Rodent models of parental influence on offspring development offer high level of control
- They allow specific timing of both environmental and pharmacological interventions
- In these models, neurobiology can be studied from network to cell to genes
- Improved reporting of methodological details and meta-analyses are needed

Abstract

Over the past decades, the influence of parental care on offspring development has been a topic of extensive research in both human and animal models. Rodent models offer several unique advantages over human studies, allowing for higher levels of environmental control, exploration of interventions, genetic control and examination of underlying neurobiological mechanisms in greater spatiotemporal detail. Although exploitation of these opportunities has led to increased understanding of the neurobiological mechanisms underlying susceptibility to the early-life environment, translation of results to human parenting and child development appears to be challenging. Attuning animal models to the human situation and application of novel structural and functional techniques is therefore of crucial importance to reduce the gap between rodent and human research.

Introduction

Parental care is of vital importance for newborn mammals, including humans, enhancing both survival and development. It is now widely accepted that alterations in parenting during critical early-life periods contribute to long-lasting developmental effects and vulnerability to psychopathology in offspring[1,2]. Animal models offer unique opportunities to study the neurobiology underlying susceptibility to early-life rearing conditions, given the evolutionary conserved mechanisms involved[3]. Moreover, analogue developmental phases create the possibility to relate rodent age to human age[4,5], although this requires careful interpretation. Against this background, many animal studies of the detrimental effects of adverse early-life experiences have been undertaken, particularly in rodents[6–8]. Rodent models used to study these effects hold a certain face, construct and predictive validity[9]. Yet, future studies could benefit from further integration of human and rodent studies[10,11].

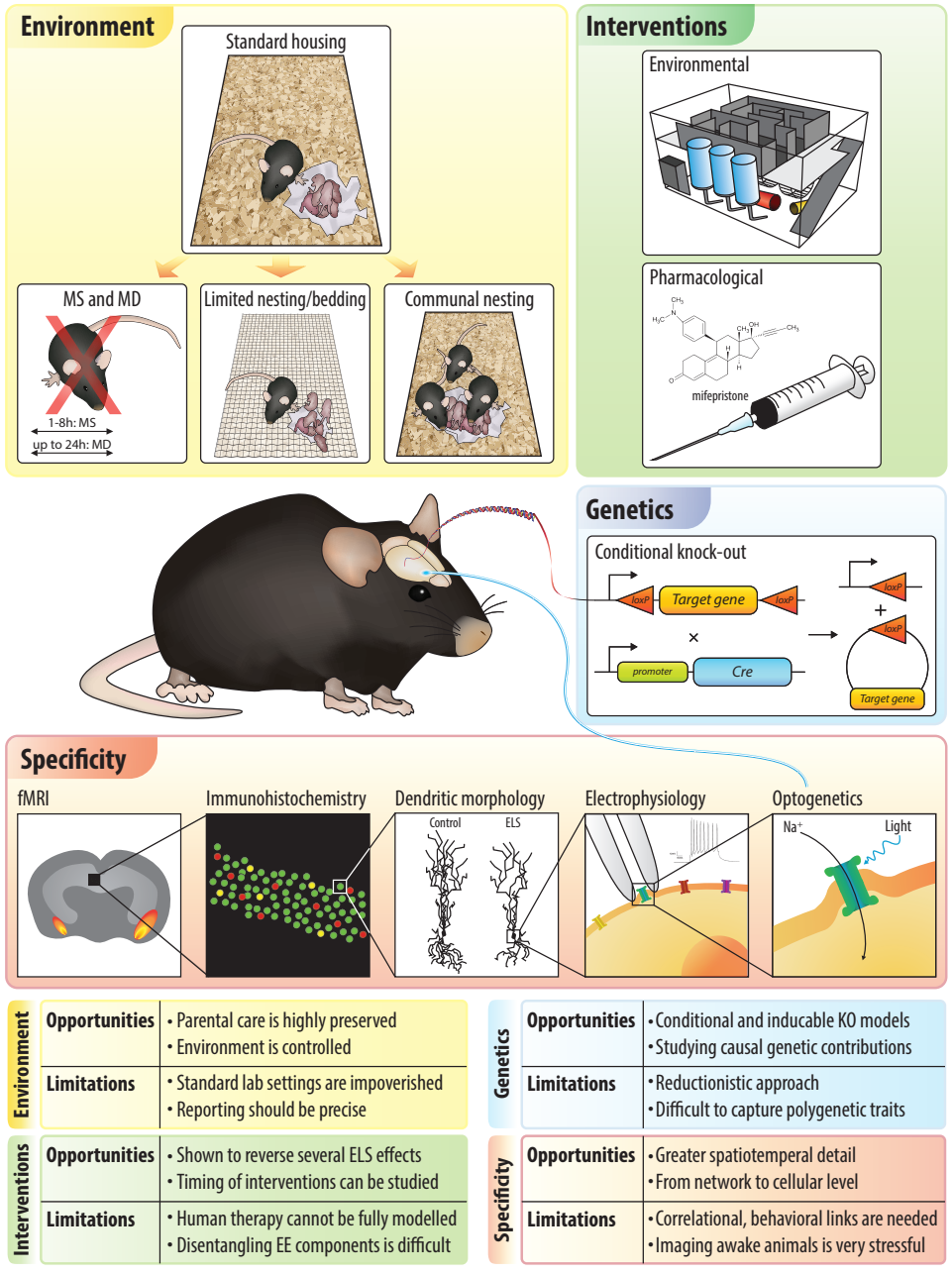
Here, we will discuss contributions of rodent studies to understanding the influence of parental care on offspring development, focusing on four main advantages of animal models: I-A higher level of environmental control; II-Controlled interventions; III-Manipulation of genetic background; IV-Revealing underlying neurobiological mechanisms. For each of these, we will briefly discuss research strategies, some key findings, limitations and suggestions for future research.

Environmental control

Although human studies support an important role of the early-life environment on child development, the complexity of different environmental conditions hampers dissociation of the various contributing factors. In rodent models, substantial environmental control allows for stepwise manipulations. Until weaning around 3 weeks of age, mouse and rat pups spend their life in the nest and their early-life environment is almost exclusively determined by the mother. Therefore, assessment and/or alteration of maternal care -consisting of several well-defined behaviors such as nest-building, licking/grooming, pup retrieval and nursing- are common approaches to study the influence of early-life environment on offspring development[12••].

The first studies in the field were conducted by Seymour Levine and Victor Denenberg, showing maternal mediation of early life manipulation on the offspring's stress responsiveness in adulthood[7]. Subsequently, numerous studies showed lasting effects of maternal separation (1-8h/day) and maternal deprivation (up to 24h) on the developing hypothalamic-pituitary-adrenal (HPA) axis and adult behavior of the offspring[6,13,14]. Importantly, duration, frequency, and timing[13,15••], as well as (social) environment during separation –e.g. homecage and contact with littermates- influence outcome, underlining the importance of the context in which early life stress takes place[15••].

Meaney and coworkers elegantly demonstrated the importance of quality of maternal care. They showed that naturally occurring variations in licking and grooming (LG) are related to HPA axis development, paralleling changes seen in deprived versus non-deprived rats[12••]. Moreover, not only *between-*, but also *within-*



← FIGURE 1 **Opportunities for studying parental influence on offspring development in rodent models**
Schematic representation summarizing the four domains in which rodent models offer unique advantages for studying parental influences on offspring development compared to human studies. For each aspect, some advantages and points of attention are provided. This figure is not extensive, but illustrates possibilities. MS: maternal separation, MD: maternal deprivation, ELS: early-life stress, fMRI: functional magnetic resonance imaging.

litter variations in received licking/grooming levels predict later-life behavior and neurobiology[12••,16]. These findings are of translational interest, as human parental investment can also vary between children[17]; noteworthy, rodents have large litters (often culled to 6-8 pups), which differs from multiple single births in humans. These rodent studies on natural variations in parental care have led to increased appreciation of the importance of assessing maternal care levels in deprivation/separation experiments.

Providing limited nesting and bedding material is a method to chronically expose dams and pups to adverse environmental conditions. This condition results in fragmented and unpredictable dam-pup interactions, highly relevant for modeling the often chronic adverse rearing conditions in humans[8]. Infant attachment to the mother can also be manipulated in rodents by coupling maternal odour to receiving a shock[18]. Pups maintain their preference for this maternal shock-odour, modelling abusive attachment. Rodent offspring reared in both of these conditions exhibited upregulated corticosterone levels and developed long-term cognitive, emotional and neuroendocrine abnormalities[8,18] similar to animals that received low levels of LG (Low-LG) or were maternally deprived.

Importantly, early-life stress (ELS) effects represent adaptations to the environment, rather than negative consequences of early-life adversity per se. This view is highlighted in the match/mismatch theory, stating that ELS-induced changes program an individual for optimal performance under similar conditions later in life[19,20]. Accordingly, in cognitive tasks, Low-LG or maternally deprived rats outperformed control animals after moderate stress, although severely stressed animals remained negatively affected in other aspects of brain function, especially in combination with a vulnerable genetic background[21].

A factor lacking in many animal studies is the contribution of paternal care[22], observed in the majority of human cultures. Indeed, human studies indicate the importance of paternal engagement in psychological development[23]. In biparental rodent species, e.g. prairie voles and California mice, paternal deprivation studies have highlighted the importance of paternal care for sex-specific developmental effects in offspring[24]. Although the vast number of genetic techniques used in mice (see genetics section) are not yet available in these species, promising developments are made[25•]. Most mammalian species, including rats and mice, are uniparental and males of these species can be infanticidal. But infanticide by males can be avoided and paternal care can be induced in rats by prolonged exposure of fathers to foster pups[26•] and in mice by post-copulatory cohabitation with a female during gestation and parturition[27], yielding paternal retrieval of pups guided by the mother[28]. Interestingly, father early-life trauma has been shown to affect behavior

in male adult offspring via sperm RNAs[29•].

Summarizing, many approaches have been used to elucidate the long-term effects of parental -predominantly maternal- care on offspring development, exploiting the possibility of high environmental control in rodent studies. However, a drawback of attempting to completely control the environment is the risk of providing impoverished living conditions, devoid of a minimum of external stimuli. Mice that were deprived from normal husbandry provide a striking example of detrimental effects of insufficient stimulation during development[7], but even standard lab settings likely represent impoverished rearing conditions[30]. This underlines the advantages of more naturalistic settings. For instance, co-housing lactating females allows communal nests, with upregulated maternal care levels, enhanced growth rates in pups and increased social competence and resilience to social stress in adult offspring[31•]. In addition, interaction with non-kin caretakers and peers may increase translational value of rodent studies. Hence, approximating naturalistic settings may help to improve the predictive validity of future animal studies[32].

Controlled interventions

Ultimately, the goal of studying early-life environment in relation to developmental disorders is to improve mental and physical health of affected individuals. Preventing ELS is generally difficult in humans, as poor rearing conditions often remain hidden[33]. Moreover, a number of symptoms arise during adolescence[34], years after early-life adversity started. It is therefore of crucial importance to dissect potential windows of interventions throughout development. This can be done in a controlled setting in rodents, after experimentally inducing ELS.

Post-weaning environmental enrichment (EE) is a non-invasive method shown to counteract certain detrimental effects of ELS, notably on adult stress responsivity[35], cognitive function[36], and hippocampal development[37]. For rodents, EE encompasses housing in a larger cage with more cagemates, a shelter, and increased cognitive and physical activity[38]. Thus, providing the appropriate environmental stimuli needed for healthy psychological and physiological development in the peripubertal period might partially reverse ELS effects. Still, disentangling the social, locomotor, cognitive and sensory aspects of EE in reversing developmental deficits is challenging. It has been argued that physical activity in EE is responsible for most effects[39], but this might depend on outcome measures[40•]. Currently existing variations in design, timing and parameters in EE models indicate the need of a meta-analysis to delineate the contributions of different EE components on a range of developmental outcomes.

Pharmacological treatment is a different approach to explore time-windows and possibilities to improve development following ELS. Altered methylation patterns in the hippocampus after ELS are observed in both rodents[12••] and humans[41]. Interfering with the epigenetic methylation process in low-LG offspring[12••], even in adulthood[42], proved to be effective in reversing low-LG effects on hippocampal expression of the glucocorticoid receptor (GR). Also, brief treatment with the GR-antagonist mifepristone during adulthood or adolescence has been shown to counteract some[43,44•,45], but not all[46] effects of maternal separation. Other

neurobiological systems have been targeted too in an attempt to reverse ELS effects, e.g. using antidepressants[47]. When effective, these brief treatments appear to rapidly 'reset' the stress system disturbed after ELS.

In short, although animal intervention models lack the possibility to mimic important aspects of human therapy including verbal instructions, placebo effects and compliance, they demonstrate the promising possibility to reverse several ELS effects by later-life interventions. Future animal studies could help to further fine-tune sensitive periods for intervention.

Genetic control

Detrimental effects of ELS are particularly evident in genetically susceptible individuals[48], underlining the importance of genetic variation in regulating individual responses to the early-life environment. Human evidence suggests a role for several candidate genes involved in the serotonergic system, HPA-axis and neurotrophin system in regulating vulnerability to early-life adversity[49]. Although currently available genetic profiling techniques enable examination of the effects of natural genetic variations in humans, studying causal contributions of specific genes by targeted deletion or overexpression is restricted to animals. Conventional (overall) and conditional (region-specific and inducible) knock-out (KO) models have been created to test the consequences of altered gene expression. Of note, testing the influence of genes of interest one-by-one is a highly reductionist approach, which does not capture the likely contribution of a multitude of risk genes contributing to ELS susceptibility, each with a very small effect size[50].

Conventional KO animals confirm an important role of the HPA-axis in moderating ELS effects, showing that corticotropin releasing hormone receptor-1 (CRHr1) mediates the corticosterone response following maternal deprivation in mice[51]. Forebrain-specific deletion and overexpression of CRHr1 further specified this receptor's role in cognitive deficits and anxiety-related behavior[52]. Studies focusing on the putative protective role of Mineralocorticoid Receptor (MR) overexpression are ongoing (e.g. [53]).

Animals with a deletion of the serotonin transporter (5-HTT) gene, an important modulator of ELS effects in humans, have also been used to study gene-by-environment interactions. Heterozygous 5-HTT KO *mice* are more vulnerable to negative consequences of reduced maternal care, via molecular mechanisms involving the neurotrophin system[54]. Yet, heterozygous 5-HTT KO *rats* show improved adult stress coping following maternal separation via methylation of the *Crf* gene[55]. These studies suggest a complex network in which candidate genes of the serotonergic system, HPA-axis and neurotrophic system -identified in human studies- together elicit the observed effects. Moreover, the improvements observed in 5-HTT KO rats indicate that developmental effects of genetic polymorphisms in response to ELS are not restricted to detrimental effects per se, in line with the match/mismatch theory.

In addition, human studies suggest that genetic variation could contribute to increased environmental susceptibility 'for better *and* for worse', i.e. differential susceptibility[56,57]. This has been studied in particular for the (loss-of-function) DRD4-7 repeat allele, in which carriers exposed to ELS are more prone to develop

disorders such as ADHD in chaotic environments[58]. At the same time, children carrying this allele are more likely to benefit from an intervention creating a more predictable, rewarding and sensitive environment[59]. For a thorough understanding of the neurobiological mechanisms underlying differential susceptibility in rodents, animals should be exposed to both adverse and stimulating rearing environments and subsequently tested for developmental progress.

Revealing the underlying neurobiology

In humans, neurobiological processes underlying adaptations to early-life environment can be studied e.g. with EEG, MRI or post-mortem dissection. For example, differences in functional connectivity following early-life adversity have been studied in relation to thalamic connectivity[60] and emotion regulatory networks[61]. Most of these techniques are also available for animals, although in contrast to humans, rodent imaging studies are predominantly conducted in anesthetized animals. Recent advancements enable imaging studies in awake animals[62], also applied in ELS studies[63]. However, stress associated with restraining in awake animals can interfere with outcome measures.

Overlapping methodology contributes to direct comparisons between the human and rodent brain; yet, more detailed knowledge on neurobiological changes can presently only be obtained in animal models. This is best illustrated by extensive work on the hippocampus, a brain area consistently affected by ELS[6,64,65]. Hippocampal neuronal cell proliferation and neurogenesis after ELS was found to be increased during adolescence and decreased in adult male animals. At the cellular level, ELS reduced neuronal complexity, as shown by alterations in mossy fiber density and granule cell dendritic morphology. In addition, GR, MR and IL-6 receptor expression, as well as AMPA, NMDA and GABA receptor function and subunit expression were all associated with reductions in maternal care[64,65]. Finally, electrophysiological recordings revealed that moderate to severe ELS usually suppresses the ability to induce synaptic plasticity in the adult hippocampus[66]. A similar focus on other brain regions might shed light on the ensemble of cellular changes responsible for ELS effects.

Linking these cellular measurements directly to behavioral observations will be of critical importance for the translational potential to humans[20]. Promising future directions include sophisticated optogenetic tools, in which light-sensitive ion channels are expressed in targeted neurons, which allows the activation or repression of specific neuronal populations by exposure to a light pulse. This technique enables in vivo examination of causal relations between stimulation, activity of neuronal subpopulations, and behavior at any point in time and has begun to delineate the precise underlying mechanisms of parental care in rodents[26•,67•] and, when applied to offspring, may help to characterize the molecular pathways involved in adaptations to early-life conditions.

Concluding remarks

Despite some limitations, rodent studies offer excellent gene-by-environment control, interventional opportunities and greater spatiotemporal detail in the examination of ELS effects on brain development compared to human studies. Cross-species effects of ELS point to converging mechanisms[3], and human and animal studies both benefit from integrating developments in the respective fields. Obviously, a direct translation from the animal to the human situation and vice versa is impossible; species specific (evolutionary) needs should always be kept in mind, and we should think in endophenotypes rather than modelling human disease[10]. Yet, future studies can and should address some of the shortcomings that currently hamper translational value. Firstly, rodent studies are systematically underpowered, partly because numbers of animals are based on effect sizes, often overestimated because of publication bias[68]. This is hindering reproducibility and stresses the need of reliable effect size estimations. Moreover, improved reporting of procedural details, group size and effect sizes, now often omitted in rodent studies[15••], should facilitate meta-analytic work, often used in human studies, but remarkably absent in the rodent literature.

In addition, it is important to fine-tune techniques used in both humans and animals, allowing direct comparisons between species while complementing human findings with results from experimental approaches that are unique to animal studies. Studies of early-life adversity effects on amygdala-prefrontal connectivity illustrate the power of this approach[69••]. Here, human experiments were driven by animal studies showing accelerated maturation of amygdala[70] and mPFC neurons[71] after ELS. Similarly, accelerated amygdala-mPFC connectivity maturation was found in previously institutionalized children, in a cortisol-dependent manner[69••].

In conclusion, animal models allow for detailed investigation of the mechanisms through which differences in parental care lead to alterations in offspring's brain development. With some improvements and application of novel techniques, our understanding of parental influence on offspring development will greatly expand.

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CHAPTER 3

The effects of different rearing conditions on sexual maturation and maternal care in heterozygous mineralocorticoid receptor knockout mice

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Highlights

- Different conditions affect unpredictability and fragmentation of maternal care
- Rearing conditions alter pubertal timing in male, but not in female mice
- Heterozygous mineralocorticoid receptor knock-out (MRKO) affects maternal care
- Basal corticosterone levels are increased in early-life stressed HE MRKO mice
- Differential susceptibility was not observed with MRKO mice

Abstract

Sexual and social development is affected by a complex interplay between genetic makeup and the early-life rearing environment. While many rodent studies focused primarily on the detrimental effects of early-life stress, human literature suggests that genetic susceptibility may not be restricted to negative environments; it may also enhance the beneficial effects of positive rearing conditions. To examine this interaction in a controlled setting, heterozygous mineralocorticoid receptor knockout ($MR^{+/-}$) mice and control litter mates were exposed to a limited nesting/bedding (LN, impoverished), standard nesting (SN, control) or communal nesting (CN, enriched) paradigm from postnatal day 2-9 (P2-P9). Offspring was monitored for puberty onset between P24-P36 and, in females, maternal care-giving (i.e. as F1) during adulthood, after which basal corticosterone was measured. Different home-cage environments resulted in profound differences in received maternal care and offspring body weight. In male offspring, LN resulted in delayed puberty onset that was mediated by body weight and unpredictability of maternal care received during early development. In female offspring, rearing condition did not significantly alter sexual maturation and had little effect on their own maternal care-giving behavior. Genotype did affect maternal care: female $MR^{+/-}$ offspring exhibited a less active nursing style and upregulated fragmentation during adulthood, irrespective of early life conditions. Basal corticosterone levels were highest in $MR^{+/-}$ mice with a background of LN. Overall, we found a gene-by-environment interaction with respect to basal corticosterone levels, but not for sexual maturation or maternal behavior.

1 Introduction

The early-life rearing environment of mammals, including parental care, critically contributes to development and functioning later in life. For instance, aberrations in maternal care affect a wide variety of behaviors in offspring, including cognitive and social abilities[1–3]. While early-life adversity has detrimental consequences in some individuals, others appear to be more resilient. Genetic factors have been proposed to (at least partly) underlie vulnerability to early-life stress in both humans[4,5] and rodents[6,7]. However, genetic susceptibility may not be restricted to negative environments; it may also enhance the beneficial effects of positive rearing conditions. Evidence in humans supports this for better *and* for worse concept, dubbed the 'differential susceptibility theory'[8,9], although different patterns have been observed[10]. To better understand differential susceptibility at a neurobiological level, we can use the unique advantages of animal models, including superior control over environment and genetic background[11,12].

1.1 The MR gene in differential susceptibility

A potential gene conveying differential susceptibility characteristics is the mineralocorticoid receptor (MR) gene. The MR is a high affinity receptor for cortisol (in humans) and corticosterone (in rodents) that is predominantly expressed in limbic-cortical areas such as the hippocampus, amygdala and medial prefrontal cortex; areas that additionally express the lower-affinity glucocorticoid receptor (GR)[13,14]. MR and GR interact to mediate an adaptive response to stress; an imbalance between the two is hypothesized to increase the risk of developing psychopathology[13,15]. In humans, MR haplotypes have been shown to sex-specifically moderate the effects of childhood maltreatment on (sub-clinical signs of) depression[16]. Moreover, the Iso/Val genotype of MR affects amygdala reactivity in individuals who experienced childhood neglect[17]. In rodents, MR overexpression was found to mitigate the cognitive impairment seen after early-life adversity[18]. Gene-by-(early)-environment interactions have also been found for GR[19] and its co-regulator FKBP5[20,21]. These findings highlight the importance of hypothalamic-pituitary-adrenal (HPA) axis activity in regulating the long-term effects of early-life adversity. Since biological sensitivity to stress has been proposed as a differential susceptibility marker[22], this network appears to be a promising target to test differential susceptibility in mice, particularly one of its key elements, the MR gene. This is further supported by the fact that in humans, an MR SNP in children has been implicated in the effect of sensitive parenting on attachment security[23].

1.2 Models for impoverished or enriched environments

The effects of different rearing environments on offspring development have been studied extensively using rodent models. Exposure to an impoverished environment, in which the dam has limited access to bedding and nesting material[24], results in fragmentation and increased unpredictability of maternal care[25]. This condition upregulates corticosterone levels in both the dam and offspring, affecting a wide variety of developmental outcomes during adulthood (see[26] for a review of this model). On the other side of the spectrum, early social enrichment can be modelled by utilizing the naturally occurring tendency of mice to

form communal nests[27]. Co-housing two or more lactating dams results in shared, upregulated care-giving behavior and facilitates peer interactions among pups[28]. Mice reared in this condition display enhanced sociability and exhibit markers of increased neuronal plasticity[29]. Experiments directly comparing the developmental effects of these two different rearing environments have not been conducted to date.

1.3 Timing of puberty onset as outcome

According to life history theory, timing of puberty onset is affected by rearing conditions as part of a reproductive strategy[30]. In females, early-life stress (ELS) has been shown to accelerate sexual maturation in both humans[31,32] and rodents[33,34]. Conversely, positive family relations were linked to a delay in puberty onset in humans[35]. Conflicting results were reported for males, where ELS either had no effect (rats[36] and humans[37]) or delayed puberty onset in rodent models of ELS[34,38]. Deviations in the timing of puberty are linked to various mental health problems including anxiety, depression and social disorders in both girls[39] and boys[40]. Although the direction of effects is not always clear[41], these effects highlight the importance of pubertal timing in development.

1.4 Intergenerational transmission

There is substantial evidence that maternal care, with its vital role in regulating social and reproductive behavior, may be transmitted across generations, altering maternal care of offspring. For instance, natural variations in received levels of licking/grooming during early postnatal rat development predict maternal behavior in adults[42], possibly through epigenetic mechanisms[43,44]. Changes in maternal care evoked by prenatal immune activation of the dam similarly transfer to the next generation[45]. Transgenerational effects of maternal care have also been studied using the limited bedding/nesting[46] and communal nesting[47] models, where animals reared in these conditions later in life display aberrant or improved maternal behaviors, respectively.

Overall, the aim of this study was to examine susceptibility of heterozygous MR knock-out ($MR^{+/-}$) mice to both negative and positive rearing environments, i.e. the limited bedding/nesting and a modified communal nesting model respectively. $MR^{+/-}$ mice were used to mimic the reduced functionality of MR in susceptible human haplotypes (e.g.,[48]) while maintaining translationally relevant MR levels. As outcome measures we tested i) puberty onset, as a key developmental readout; and ii) maternal care (as well as basal corticosterone level) in female offspring, to monitor transgenerational effects. In line with the life history theory and previous findings in rodents, we hypothesized puberty onset to be accelerated in limited nesting/bedding reared female animals and delayed in female mice that were exposed to the communal nesting paradigm. Available literature in males is too limited to predict male puberty onset. Maternal care of adult females was hypothesized to be poor in ELS mice, while being improved in CN reared animals. Finally, in line with the differential susceptibility theory, these effects were hypothesized to be stronger in heterozygous mineralocorticoid receptor knock-out mice.

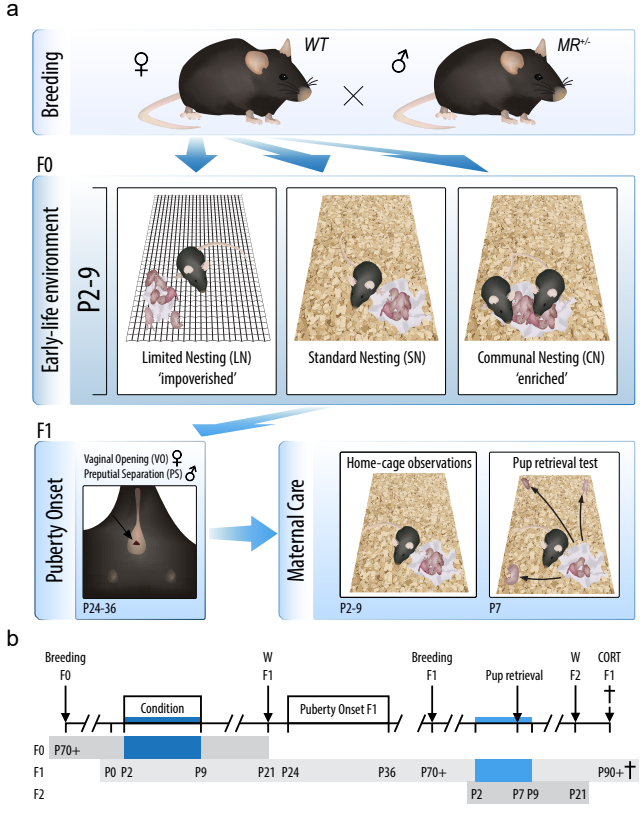

FIGURE 1 **Outline of the experiments**
(**a**) Study design and (**b**) timeline of the experiment. A wild-type female was paired with a heterozygous MR+/- male to obtain mixed litters. Experimental time points for each generation of mice are depicted. W = weaning. P = postnatal day. Blue lines indicate periods of home cage maternal care observations.

2 Materials & Methods

2.1 Animals and housing

All mice were bred in our own animal facilities. Wild-type (wt) C57BL/6JOlaHsd females were originally obtained from Harlan France and bred in-house for at least two generations before experiments. Forebrain-specific MR knock-out animals were generated by using the Cre/loxP-recombination system[49]. The F0 wt C57BL/6 females were bred with male $MR^{flox/flox\ -\ CaMKIIa\text{-}Cre/wt}$ mice, generating heterozygous forebrain-specific MR knock-out ($MR^{+/-}$) F1 offspring and control litter mates. Dam and litter were placed in either a limited nesting/bedding, control or communal housing condition, between postnatal day (P) 2 and 9. The F1 offspring (♂: n=112, ♀: n=128) of 38 breedings were tested on puberty onset. The female offspring were monitored as adults for maternal care (see figure 1 for a timeline of the experiment). Puberty onset and maternal care measures of F1 animals were obtained by a trained experimenter blind to rearing condition and genotype of the animals. Animals were housed under a reversed 12:12 hour light/dark cycle (lights off 08:00 h, temperature 21-22 °C, humidity 40-60 %) with *ad libitum* access to water and food. All experiments were performed in accordance with the EC council directive (86/609/EEC) and approved by the Central Authority for Scientific Procedures on Animals in the Netherlands (CCD approval AVD115002016644).

2.2 Breeding conditions F0

For the breeding of F0, two females were paired with a male for 4 days. Females were then co-housed until approximately one week prior to parturition. After separation, each dam was placed in a type II short Macrolon cage (21.5 x 16 cm) with a filter top and provided with a cotton Nestlet (5 x 5 cm, Technilab-BMI, Someren, The Netherlands) as nesting material. Each day at 09:00 h, animals were checked for litters, assigning the day prior to first appearance of a litter as date of birth (P0). At P2, all litters were weighed and culled or cross fostered to 6-7 pups. A maximum of 1 pup per litter was added from a different litter if a litter contained 5 pups and a minimum of 2 pups of each sex was included in each litter. Litters were randomly allocated to one of three conditions; limited nesting/bedding (LN), standard nesting (SN) or communal nesting (CN). The LN condition was performed as described earlier [24]. In short, a limited amount of sawdust bedding was provided, covered by a stainless steel wired mesh. In addition, half the regular amount of nesting material was available to the dam. In the SN condition, the dam had access to a standard amount of sawdust and nesting material. The CN paradigm consisted of co-housing the experimental dam (and her genetically heterogeneous F1 litter) with another wt dam (and wt litter) in a type II regular Macrolon cage (32 x 16 cm). This other wt dam was marked using ear punches and her pups were marked with a non-toxic, non-scenting surgical marker (ArcRoyal, Ireland) on P2 and P6 to ensure correct allocation of the pups to their mother at the end of communal housing at P9. At P9, all pups were weighed and all nests returned to standard nesting conditions until weaning at P21. All cages were cleaned once between P9 and weaning. At weaning, offspring was weighed and ear punched to facilitate individual recognition and allow for genotyping.

2.3 Maternal care observations F0

During exposure to different rearing conditions, maternal behavior of the dams was monitored using instantaneous sampling[50]. From P2-P9, maternal observations were performed three times a day, each for 75 minutes. The first observation took place at the end of the light phase (between 06:00 and 07:30 am), the second in the middle of the dark phase (between 12:00 and 14:00 pm) and the third at the end of the dark phase (between 16:30 and 18:30 pm). Dark phase observations were carried out in red light conditions. Within each observation period the behavior of each dam was scored every 3 minutes, resulting in 25 observations per period and 75 observations per day. The behaviors were identified as: arched-back nursing (ABN), passive nursing, licking/grooming pups (LG), nest building, self-grooming on nest, feeding and self-grooming off nest. If a behavior was observed that was not covered by one of these categories, only the location of the dam (on or off nest) was scored. Observations were scored using Pocket Observer 3.3 software (Noldus, The Netherlands) on a Samsung Galaxy Note 4 smartphone and analyzed using Observer XT 10.5 (Noldus, The Netherlands).

Maternal care was evaluated using three separate approaches. First, individual maternal behaviors were analyzed using the percentage of time the specific maternal behavior was shown. For each behavior, both development over postnatal days (pooling the 3 observations per day) and circadian rhythmicity during the day (pooling the 6 days) were assessed. Second, previous studies confirmed an important role of unpredictability and fragmentation of maternal care in pup development. Unpredictability was defined as the overall entropy rate of maternal care and calculated as described earlier[25]. In short, the entropy rate summarizes the probabilities that certain behaviors predict the transition to specific subsequent behaviors. The entropy rate can be regarded as a measure of unpredictability in which higher rates indicate higher unpredictability of behavior. Because transitions between off-nest behaviors might be regarded irrelevant for the pups, a separate entropy rate was calculated combining all off-nest behaviors into one category. Third, fragmentation of maternal behavior[24] was calculated by the average number of transitions from and to the nest. For the communal nesting condition in F0 dams, maternal care was calculated by averaging measures of both dams.

2.4 Puberty onset F1

To determine the effects of rearing conditions and heterozygous KO of MR on sexual maturation of F1 offspring, puberty onset in both males and females was determined. Female mice were examined daily from P24-P36 on vaginal opening as an external measure of puberty onset[51]. In males, daily examination of preputial separation from P27-P32 was used to determine puberty onset. Mice were restrained with one hand, while gently attempting to manually retract the prepuce[52]. Preputial separation was defined as the potential to fully retract the prepuce and expose the glans penis.

2.5 Maternal care F1

After P70, breeding of F1 females with a wild-type male was performed as described for F0. F2 offspring was culled to 6 pups per litter and weighed at P2, P9, P15 and P21, in parallel with transfer to clean cages on these days. All F1 dams were placed in standard nesting conditions. Observations of maternal care-giving behavior were done as in F0 from P2-P9. To challenge maternal responsiveness, a pup retrieval test was performed at P7 between 10:00 -12:00. The dam and pups were briefly removed from the home cage and sawdust bedding was leveled, leaving the nest site intact. In three corners distant from the nest a pup was placed, counterbalancing sex ratio and location of the pups across trials. The dam was replaced in the nest facing a wall and retrieval behavior was recorded for 5 minutes and analyzed for retrieval latencies of all three pups using Observer XT 10.5 (Noldus, The Netherlands). After testing, the three remaining pups were returned to the nest. All dams successfully retrieved all pups within 5 minutes.

2.6 Plasma corticosterone levels F1

After weaning of F2 litters, F1 dams (n = 5-8/group) were decapitated between 13:00 and 17:00 and trunk blood was collected on ice in heparin containing tubes (Sarstedt, The Netherlands). To prevent effects of cage disturbance in remaining mice, a maximum of two animals per cage was used and simultaneously decapitated by two experimenters. Effort was made to distribute all experimental groups evenly across the sampling period. Blood was centrifuged for 10 minutes (13000 rpm) at 4 °C and plasma was stored at -20 °C until corticosterone measurements using a

radioimmunoassay kit (MP Biomedicals, The Netherlands; sensitivity 3 ng/ml).

2.7 Statistical analysis

All data are expressed as mean ± SEM and SPSS 23 (IBM) was used for analysis. Outlying values, defined as deviating >3.29 SD from the mean, were winsorized[53]. A total of 3 data points in 2 variables were winsorized. The complex samples module of SPSS was used to account for litter effects in F1 animals. Because no effect sizes are provided in this module, these are not reported. Overall ANOVA statistics and eta squared effect sizes (η^2), the explained variance as proportion of the total variance in the model, are presented in the text; post-hoc comparisons with a Tukey HSD (main effects) or Sidak (interaction) correction are depicted in the figures.

F0 maternal care was analyzed using one-way or repeated measures ANOVAs with breeding condition as the between-subject factor and postnatal day or observation (1 day and 2 night observations) as within-subject factors. A Greenhouse-Geisser correction was used for repeated measures ANOVAs. To prevent major impact of the disturbance at postnatal day 2 caused by introduction to a novel environment, this day was excluded from analysis. For F1 maternal care no interactions of genotype or condition with postnatal day or observation were found, therefore overall levels were analyzed using a two-way ANOVA. Early life experience (condition) and genotype were included as between-subject factors and all other F1 variables were similarly analyzed. As an overall index for active parenting, a principal component analysis was conducted using frequent (>2% of time) on-nest behaviors: arched-back nursing, passive nursing, licking/grooming and self-grooming on nest. The eigenvalue was 1.68, 42% of variance was explained by the first factor and all variables loaded >0.40, with negative load for passive nursing. The resulting PCA factor was used as an index of active parenting in F1 dams.

Pearson correlations were used for associations between puberty onset and body weight/entropy and between corticosterone and entropy in F1. Mediation analysis with rearing condition as a multicategorical independent variable, body weight and received entropy as mediators, and pubertal timing as outcome was performed using the PROCESS v3 macro for SPSS[54]. To determine significance of mediation, 95% confidence intervals were calculated and deemed significant if they did not straddle zero.

3 Results

3.1 Maternal Care F0

3.1.1 Individual maternal behaviors

Exposure to different early-life rearing conditions affected several aspects of maternal behavior. Maternal behavior over different postnatal days is depicted in the left panels of fig. 2a-f and fig S1. Nesting condition affected arched-back nursing (ABN) ($F(2, 35) = 8.17$, $p = .001$, $\eta^2 = .32$, fig 2a). Post-hoc analysis showed that neither CN nor LN dams were different from standard housed animals, but dams in these two conditions differed from each other, with increased levels of ABN in the LN condition at the start of the week. (PND*condition interaction ($F(8.1, 141.7) = 2.95$, $p = .004$, $\eta^2 = .09$). A similar interaction for passive nursing (PND*condition: $F(8.8, 154.7) = 3.00$, $p = .003$, $\eta^2 = .14$, fig 2b) revealed that CN dams increased passive nursing behavior also specifically during the first part of the rearing period. Accordingly, the total time spent on nursing behavior, i.e. the sum of ABN and passive nursing, was unaffected by condition ($F(2, 35) = 0.42$, $p = .66$, $\eta^2 = .02$, fig 2c).

Condition did not alter licking/grooming behavior towards pups ($F(2, 35) = 1.32$, $p = .28$, $\eta^2 = .07$, fig 2d), but did affect the time spent on the nest ($F(2, 35) = 7.95$, $p = .001$, $\eta^2 = .31$, fig 2e). Post-hoc testing revealed that LN dams spent more time

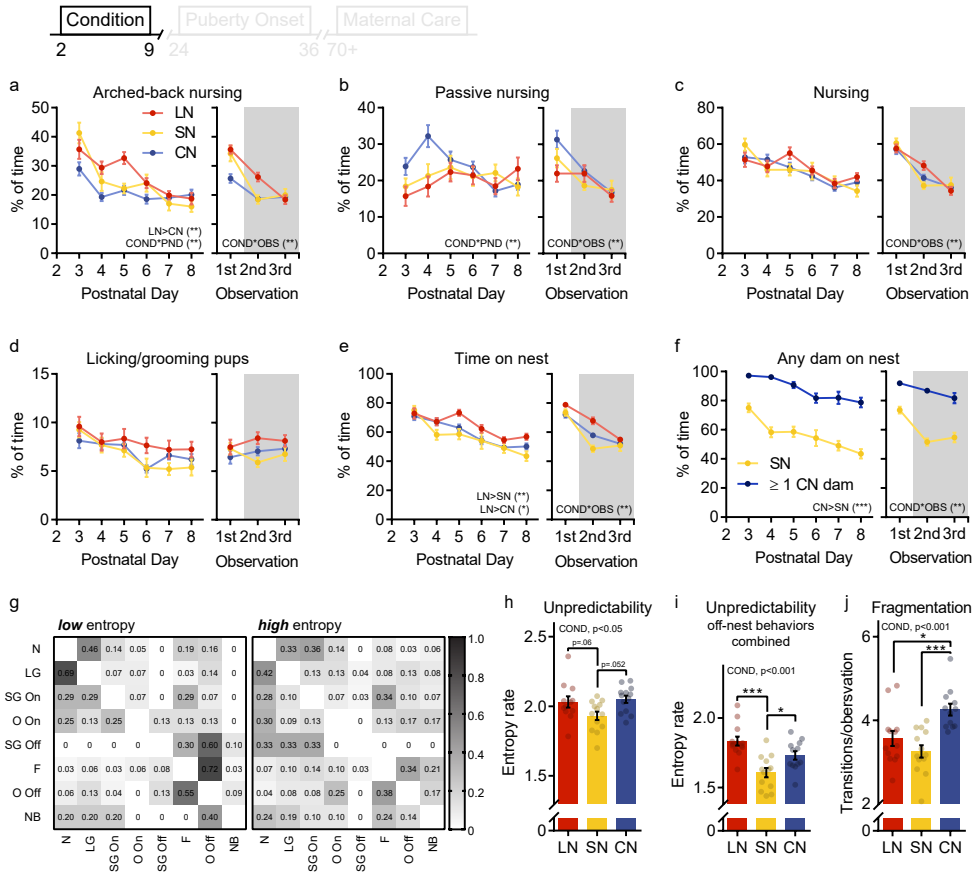

FIGURE 2 **Efffect of different housing conditions on maternal care**
(**a**) Arched-back nursing, (**b**) passive nursing, (**c**) nursing, (**d**) LG and (**e**,**f**) time on nest for limited nesting (red, n=13), standard nesting (yellow, n=13) and communal nesting (green, n=12) dams, depicted over postnatal days (left) and time of the day (right). The shaded area indicates the dark phase of the LD cycle. Data in f represents the time on nest by at least one dam from the litters perspective. (**g**) Example probability matrices of low (left) and high (right) entropy dams. N = nursing, LG = licking/grooming, SG On = self-grooming on nest, O On = other on nest, SG Off = self-grooming off nest, F = feeding, O Off = other off nest, NB = nest building. (**h**) Unpredictability of maternal care as calculated from the matrices in (**g**) and (**i**) unpredictability of maternal care when all off-nest behaviors were combined. (**j**) Fragmentation (on/off nest transitions) of maternal behavior. Each dot represents one dam and the average of two dams in the CN condition. Asterisks indicate interactions or post-hoc comparisons. *p < 0.05, **p < 0.01, ***p < 0.001.

on the nest, partly because they performed more self-grooming on-nest (F(2, 35) = 27.32, p < .001, η^2 = .61, fig S1b) than off-nest (F(2, 35) = 20.18, p < .001, η^2 = 0.54, fig S1c). However, regarding the percentage of time that the nest site had a dam present, the CN condition resulted in higher occupancy compared to the SN condition (F(1, 23) = 135.96, p < .001, η^2 = .86), reaching nearly permanent levels at postnatal day 3 and 4 (fig 2f).

3.1.2 Circadian rhythmicity

Differences in circadian rhythmicity of maternal care (fig 2a-f and fig S1, right panels, collapsed over days) were found for the following behaviors: ABN (F(3.5, 61.4) = 4.65, p = .004, η^2 = .10), passive nursing (F(3.9, 68.5) = 3.76, p = .008, η^2 = .10), total nursing behavior (F(3.7, 64.0) = 3.06, p = .026, η^2 = .05) time on nest (F(3.6, 2.6) = 4.13, p = .007, η^2 = .06), any dam on nest (F(1.4, 63.5) = 22.16, p = .007, η^2 = 0.13) and feeding (F(3.3, 57.8) = 3.71, p = .014, η_p^2 = .09). For all these behaviors, SN dams exhibited similar levels during both observations in the dark phase, whereas in particular LN mice showed a delay in displaying this dark-phase behavioral profile.

3.1.3 Unpredictability and fragmentation

The entropy rate, representing unpredictability of behavior, was significantly affected by condition (F(2, 35) = 3.95, p = .028, η^2 = .18, fig 2h), although the increase in both LN and CN animals compared to the SN condition did not reach significance after Tukey correction. When all off-nest behaviors were combined to better represent the amount of unpredictability received by the pups, the entropy rate of LN dams was higher compared to SN dams, whereas CN dams exhibited higher entropy rates compared to SN (F(2, 35) = 12.43, p < .001, η^2 = .42, fig 2i). In CN dams, an increase in transitions from and to the nest site revealed that maternal behavior of individual dams was more fragmented compared to the SN and LN condition (F(2, 35) = 10.20, p < .001, η^2 = .37, fig 2j). However, no difference

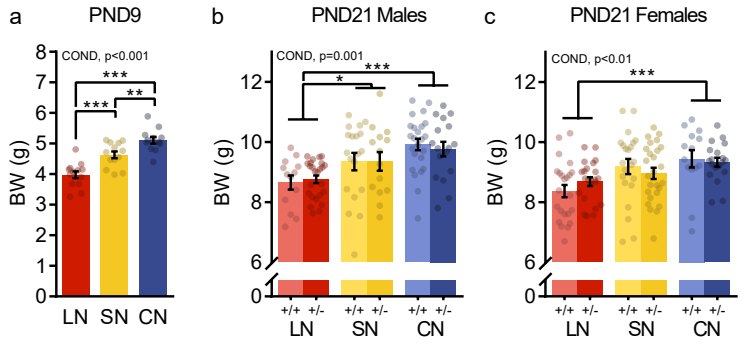

FIGURE 3 **Effects of different rearing conditions on body weight of male and female offspring** (**a**) Body weight at PND 9, each dot represents the average of one litter (LN: n=13, SN: n=13, CN: n=12). (**b**) Body weight at weaning - males. (**c**) Body weight at weaning - females. Each dot represents an individual. +/+: control, +/-: heterozygous MRKO. Group size: ♂: LN +/+: n=13, LN +/-: n=25, SN +/+: n=19, SN +/-: n=14, CN +/+: n=24, CN +/-: n=17; ♀:: LN +/+: n=23, LN +/-: n=22, SN +/+: n=22, SN +/-: n=26, CN +/+: n=14, CN +/-: n=19) . *p < 0.05, **p < 0.01, ***p < 0.001.

between LN and SN was found.

Overall, compared to SN dams, LN resulted in more time spent on the nest not engaging in pup directed behaviors, altered circadian rhythmicity and increased unpredictability of maternal care. CN dams exhibited increased unpredictability and fragmentation of maternal care, but the presence of two dams resulted in very high nest occupancy.

3.2 Body weight F1

Before being exposed to different rearing conditions at P2, litter weight was comparable over experimental groups (F(2, 35) = 1.92, p = .16, η^2 = .10). At P9, body weight was affected by condition (F(2, 35) = 25.76, p < .001, η^2 = .60, fig 3a). LN litters weighed less than SN litters, whereas CN animals showed an increased body weight compared to both LN and SN (fig 3a). The overall effect of condition remained significant at weaning, both in males (F(2, 35) = 9.25, p = .001, fig 3b) and females (F(2, 36) = 5.78, p = .007, fig 3c), although post-hoc testing revealed that some differences between groups (♂: SN vs. CN, ♀: SN vs. LN and SN vs. CN) were no longer significant. The $MR^{+/-}$ genotype did not interact with condition in the prediction of body weight at weaning (♂: F(2, 35) = 0.13, p = .88; ♀: F(2, 36) = 0.78, p = .47) and no main effect of genotype was found (♂: F(1, 36) = 0.004, p = .95; ♀: F(1, 37) = 0.010, p = .92).

3.3 Puberty onset F1

3.3.1 Males

Preputial separation was affected by condition (F(2, 35) = 5.94, p = .006, fig 4a). Compared to SN and CN animals, puberty onset was delayed in LN mice. There was no effect of $MR^{+/-}$ (F(1, 36) = 0.07, p = .79) and no condition*genotype interaction was found (F(2, 35) = 0.63, p = .54). Body weight at weaning was negatively correlated with puberty onset (r = -0.56, p < .001, fig 4b), whereas received entropy levels during the first week of life positively correlated with pubertal timing (r = 0.29, p = .002, fig 4c). Entropy did not predict body weight of offspring at weaning (r = 0.11, p = .25), indicating independence of these two factors. Mediation modelling with condition as the independent variable (fig 4d) revealed that the effects of condition on puberty onset were mediated by body weight at weaning for both the LN vs. SN contrast (95%CI = [.09, .72]) and CN vs. SN contrast (95%CI = [-.68, -.01]). In other words, the delay in puberty onset found in LN animals was mediated through a reduction in BW gain, and the acceleration in CN mice was mediated through increased body weight at weaning. In addition, both contrasts also showed a significant mediation by entropy rates received during the early-life environment (LN vs. SN: 95%CI = [.25, .82]; CN vs. SN: 95%CI = [.29, .80]). Here, both LN and CN reared mice experienced elevated entropy levels compared to SN animals, contributing to a relative delay in puberty onset counteracting the effects of increased body weight in CN reared animals.

3.3.2 Females

Rearing conditions had no significant effect on the timing of vaginal opening in females (F(2, 36) = 1.10, p = .34, fig 4e). Similar to males, no genotype effect (F(1, 37) = 0.90, p =.35) or condition*genotype interaction was observed (F(2, 36) = 1.09,

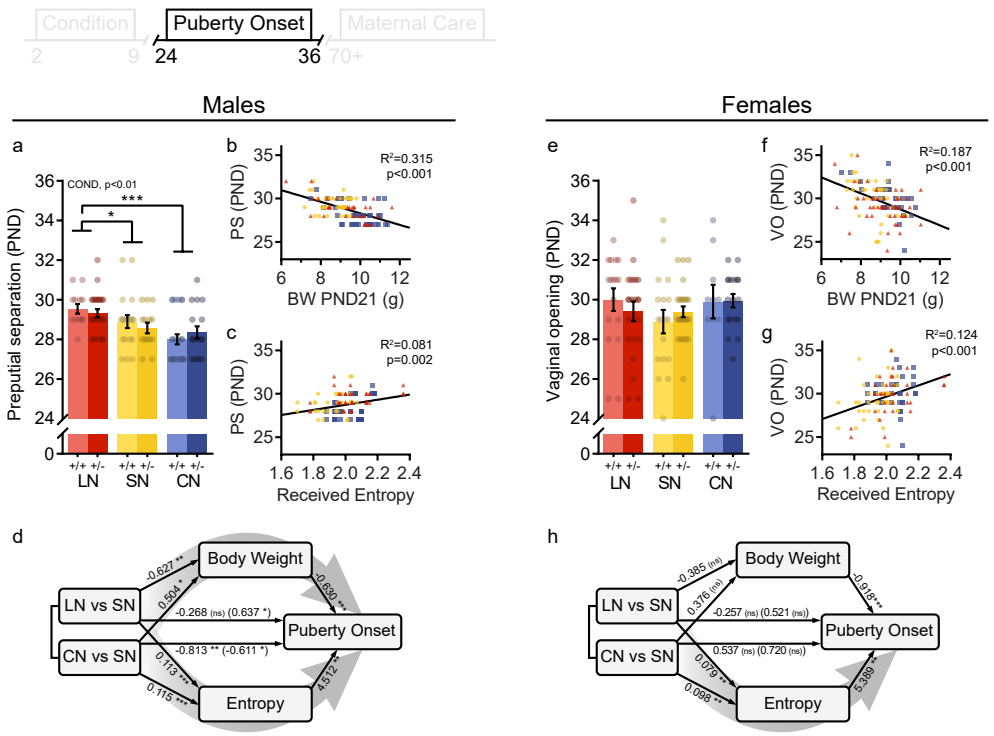

FIGURE 4 **Effects of different rearing conditions on puberty onset of male and female offspring**
(**a,e**) Puberty onset in male (preputial separation, left) and female (vaginal opening, right) mice. (**b,f**) BW at weaning and (**c,g**) received entropy rates during rearing correlated with puberty onset in both males and females. (**d,h**) Graphical representation of mediation models. +/+: control, +/-: heterozygous MRKO. Numbers represent estimated model coefficients. Grey arrows indicate a significant mediation pathway in males and a significant indirect effect in females. *p < 0.05, ***p < 0.001.

p = .35). Yet, similar correlations between BW at weaning and puberty onset (r = -0.43, p < .001, fig 4f) and received entropy and pubertal onset (r = 0.35, p < .001, fig 4g) were found. As in males, entropy scores and body weight were unrelated (r = -0.06, p = .52). Although a direct effect of rearing condition on puberty onset was absent, the mediation model revealed a significant indirect effect of rearing condition via entropy rate on vaginal opening (LN vs. SN: 95%CI = [0.14, 0.72]; CN vs. SN: 95%CI = [0.18, 0.96]), whereas no indirect effect of rearing condition via body weight on puberty onset was found (LN vs. SN: 95%CI = [-0.03, 0.80]; CN vs. SN: 95%CI = [-0.81, 0.06], fig 4h).

3.4 Maternal Care F1

The principal component analysis of parenting activity in (adult female) mice that were exposed to different rearing conditions early in life revealed that $MR^{+/-}$ mice had a less active parenting style compared to $MR^{+/+}$ controls (F(1, 70) = 17.93, p < .001, fig 5a), irrespective of rearing condition. Considering individual maternal behaviors, $MR^{+/-}$ was related to a different nursing style compared to wild-type

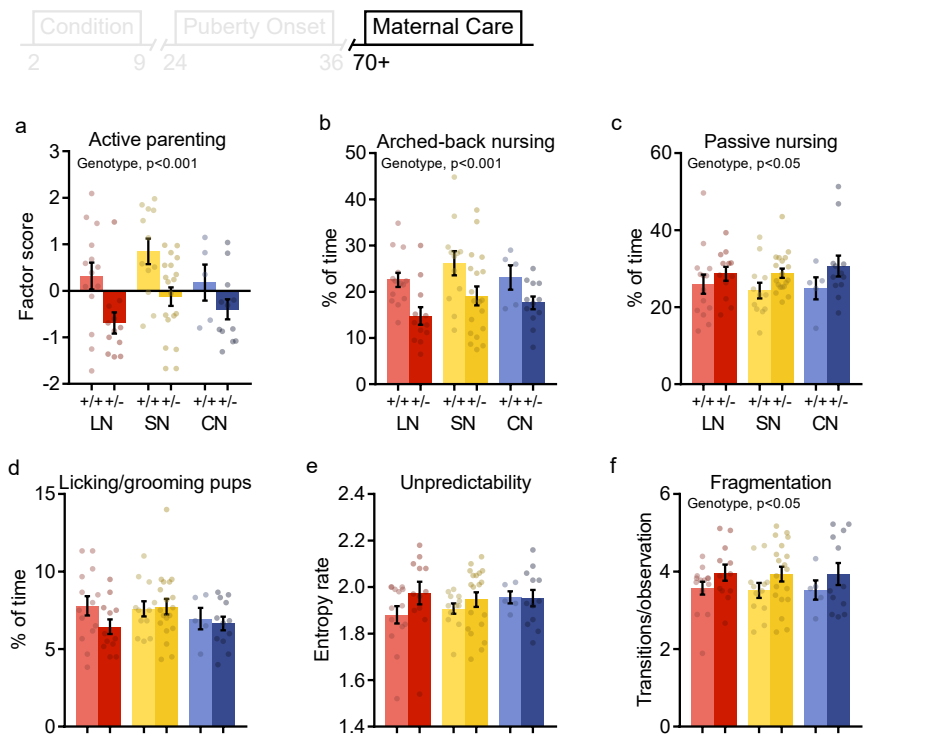

FIGURE 5 **Effects of different rearing conditions on maternal care in female offspring**
(**a**) Factor score for parenting quality as calculated from a principal component analysis (see methods section for details). Overall (**b**) Arched-back nursing, (**c**) passive nursing and (**d**) licking grooming levels. (**e**) Unpredictability and (**f**) fragmentation (on/off nest transitions) of maternal behavior. +/+: control, +/-: heterozygous MRKO. Group size: LN +/+: n=14, LN +/-: n=12, SN +/+: n=12, SN +/-: n=19, CN +/+: n=5, CN +/-: n=12). Statistics indicate main effects.

control litter mates (fig 5b-c): they spent less time on arched-back nursing (F(1, 68) = 13.47, p < .001) but showed more passive nursing (F(1, 68) = 5.35, p = .02), resulting in an equal overall amount of time spent on nursing (F(1, 68) = 1.19, p = .28, fig S2a). Licking/grooming the pups (fig 5d) and time spent on the nest (fig S2b) were unaffected by rearing condition, genotype or the interaction between rearing condition and genotype. In the pup retrieval test at P7, latency to retrieve all pups was similar across all experimental groups (F(2, 70) = 1.25, p = .29, fig S2c). The unpredictability of maternal behavior, measured as entropy rate (fig 5e), was not affected by rearing condition (F(2, 68) = 0.26, p = .77) nor by $MR^{+/-}$ (F(1,68) = 1.76, p = .19). A main effect of genotype on fragmentation of maternal care was found (F(1, 68) = 4.44, p = .039, fig 5f), with more fragmentation in $MR^{+/-}$ dams. No interaction between genotype and rearing conditions was observed for any of the maternal behaviors.

3.5 Corticosterone F1

We observed a significant genotype*condition interaction effect on adult basal

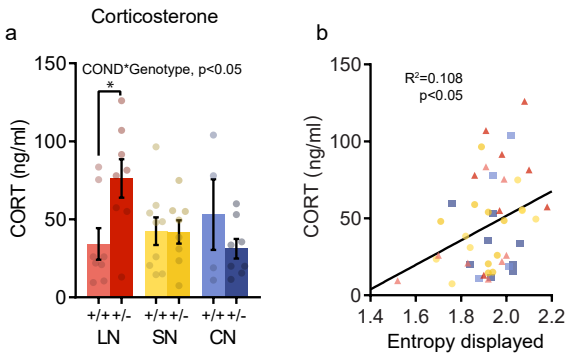

FIGURE 6 **Effects of different rearing conditions on corticosterone levels in female offspring**
(**a**) Basal corticosterone levels. (**b**) Unpredictability of maternal care positively correlated with basal corticosterone levels. +/+: control, +/-: heterozygous MRKO. Group size: LN +/+: n=8, LN +/-: n=8, SN +/+: n=8, SN +/-: n=8, CN +/+: n=4, CN +/-: n=8). **p < 0.01. SN +/-: n=19, CN +/+: n=5, CN +/-: n=12). Statistics indicate main effects.

corticosterone levels (in females) ($F(2,39) = 4.49$, $p = .018$, $\eta_p^2 = .18$), in the absence of a main effect of condition ($F(2,39) = 1.07$, $p = .352$, $\eta^2 = .04$) or genotype ($F(1,39) = 0.55$, $p = .464$, $\eta_p^2 = 0.01$). In particular $MR^{+/-}$ mice reared in a LN condition exhibited elevated plasma corticosterone levels, whereas all other groups showed similar concentrations (fig 6a). In addition, plasma corticosterone levels positively correlated with entropy rates of maternal care displayed by these F1 dams ($r = 0.33$, $p = .028$, fig 6b).

4 Discussion

The aim of this study was to establish an animal model that allowed detailed and controlled experiments on differential susceptibility, with emphasis on puberty onset and next generation maternal care as outcome measures. The applied paradigms, experimental manipulations of the rearing condition with limited bedding/nesting and communal nesting, both evoked alterations in several aspects of maternal care of F0 dams. Moreover, F1 mice reared in these conditions showed differences in body weight gain, where LN showed a decrease and CN an increase compared to SN animals. These effects, together with the rate of unpredictability in maternal care experienced during early development, mediated the delayed puberty onset found in LN males. However, rearing condition did not alter timing of puberty in females. In adult female offspring, heterozygous knock out of the mineralocorticoid receptor resulted in a less active parenting style and increased fragmentation of maternal behavior. Rearing conditions did not interact with genotype in the prediction of maternal behavior. However, a gene-by-environment interaction was found for basal corticosterone levels in adult females, where specifically $MR^{+/-}$ mice that had experienced early-life stress showed increased concentrations, although the typical cross-over differential susceptibility characteristics could not be observed. Finally, neither $MR^{+/+}$ nor $MR^{+/-}$ mice reared in a communal nesting environment differed significantly from SN reared animals in timing of puberty onset, maternal care or corticosterone levels.

4.1 Our models for impoverished or enriched environments

Similar to previous studies, the LN condition was related to decreased offspring body weight[24,26] and increased unpredictability of maternal behavior[25], although our

results indicate a more pronounced effect for on-nest unpredictability specifically. While the main focus of these earlier studies had been on the unpredictability and fragmentation of maternal care in the LN model, our results indicate that absolute levels of specific maternal behaviors were also affected. This discrepancy could be explained by the differences in timing of maternal observations. To our knowledge, this is the first study that observed effects of LN halfway through the dark phase, whereas other studies focused predominantly on the light phase or later part of the dark phase. LN dams deviated from SN mice in particular during the first dark phase observation, without affecting light phase and late dark phase behavior. Hence, it is possible that LN evokes alterations in maternal care specific to certain parts of the circadian rhythm. Because it has been shown that manipulation of circadian rhythmicity of dams affects pup development[55], aberrant circadian rhythmicity in behavior of LN dams may add to the range of alterations through which the limited bedding/nesting model exerts its effects. This would require more in-depth investigation, monitoring behavior over the entire 24h period of the day during the first postnatal week.

Interestingly, like in the LN model, we found an increase in on-nest entropy rates and fragmentation of dams in the CN condition, measures that have not been studied in communal nesting dams before. Although entropy rate could be indicative of poor maternal care in a single dam setting[25] it can be expected that in a setting with multiple nest-sharing dams the behavior of each dam is influenced by the other. This may lead to more on/off nest transitions to regulate temperature (hence, more fragmented care) and interrupted behavior (more unpredictability) on the level of the dam. However, maternal care received by the pups is not determined by unpredictability and fragmentation of the individual mothers, but rather by the overall pattern of two dams combined. Therefore, it remains to be elucidated whether the increased unpredictability of individual dams during communal nesting encodes a negative rearing environment similar to the single dam setting.

Apart from an increase in bodyweight at P9, which was normalized at weaning, we did not find differences between the communal and standard reared animals. This lack of effect of early life enrichment on the social read-outs might be partly related to the specific protocol applied. In order to synchronize the duration of communal nesting to the limited nesting/bedding model[24,26], animals were exposed to the communal nesting condition from P2-P9. Moreover, to facilitate individual characterization of maternal care in each dam, two -rather than three- dams were used. Studies with the communal nesting paradigm so far predominantly used three dams and litters in a cage, from birth till weaning[28,29,47]. By limiting exposure to P2-P9 in our model we restricted the effects of communal nesting to maternal care alterations while peer interactions, an important component of the paradigm, may not yet have developed. In addition, neural development of specific brain regions occurs at different periods in time[56]. The development of brain networks relevant for sexual maturation and maternal care may have been unaffected by our communal nesting model. Therefore, matching the exposure time window of communal nesting to the limited nesting model may have resulted in an enrichment condition too subtle to elicit positive effects.

4.2 Timing of puberty onset as outcome

In contrast to the acceleration hypothesis of life history, but in line with previous reports in rats[34,38], early-life stress resulted in delayed pubertal timing in males. Somewhat surprisingly, LN did not affect puberty onset in female mice, whereas others have found an acceleration in rats[33,34]. Due to the importance of body weight and leptin in mediating puberty onset in both rodents[57] and humans[58], it has been suggested that female resilience and male susceptibility to the effects of maternal separation on body weight may explain the sex-specific effects found in rats[34]. Our mediation analysis confirms an important role of body weight on puberty onset in males, while extending the model by also taking the unpredictability of received maternal care into account. The data reported here support a similar correlation between body weight at weaning and puberty onset for both male and female mice, whereas others have found this effect in male rats only[34]. This suggests a species difference in the importance of body weight in regulating puberty onset and highlights the challenges of studying puberty onset in early-life rodent models that inevitably affect body weight gain during early development. Therefore, these studies should always include body weight effects in the interpretation of results. Nevertheless, the finding that higher levels of unpredictability in maternal care experienced during early development were linked to delayed puberty onset contradicts the acceleration hypothesis of life history, at least in mice and with the presently used models.

4.3 The MR gene in maternal care

The less active parenting style and increased fragmentation of maternal behavior in $MR^{+/-}$ F1 dams suggests a broader role of MR in regulating complex patterns of social behavior. Although MR has been studied predominantly in relation to learning and memory[18,49,59], its role in regulating emotion and social behavior is now increasingly supported[60–63]. In humans, an MR SNP in children was found to moderate the effect of sensitive and insensitive parenting on attachment security[23]. Because parenthood elicits a wide variety of challenges[64] and continuous adaptation to novel situations, the stress coping characteristics of MR may be involved in regulating parental behavior as well. Although the results presented here suggest a role of MR in regulating maternal care in mice, involvement of MR in regulating the parental aspect of human parent-offspring interactions remains elusive.

4.4 Gene-environment interaction

Genetic variation in the mineralocorticoid gene has been shown to interact with early environmental factors in disorders such as depression[65,66] and addiction[67]. Although we did not find a gene-early environment interaction effect on behavioral measures such as maternal care, the increased basal corticosterone levels in $MR^{+/-}$ mice that experienced early-life adversity do support interaction effects. According to the three-hit concept of vulnerability[7], a genetic predisposition interacts with the early-life environment to program an individual into an adaptive phenotype. However, a third hit later in life is needed to evoke a maladaptive response. In our study, this suggests that early-life adversity programs particularly $MR^{+/-}$ mice to develop increased basal corticosterone levels, but a strong third hit –lacking in our experiment- would be required to reveal the behavioral consequences.

4.5 Differential susceptibility

We aimed to study differential susceptibility in a translationally relevant way, by manipulating the living conditions of dams and pups with a background of heterozygous, rather than complete, MR knock out. Previous studies taking a similar approach have highlighted the validity of animal studies in this field[3]. Moreover, recent animal work on differential susceptibility in socially monogamous prairie voles also indicates that differential susceptibility is not restricted to humans[68,69]. Although we did not find evidence to support the differential susceptibility theory with respect to the mineralocorticoid receptor and its role as susceptibility factor in the association between environmental factors and sexual maturation and maternal behavior, other candidate genes and behavioral domains remain to be investigated in controlled experimental settings. Promising candidates for future studies might be related to the dopamine-system or make use of polygenic susceptibility scores[9,70]. This would expand further on the insights presented here and enhance our understanding of the neurobiological processes related to the differential susceptibility hypothesis.

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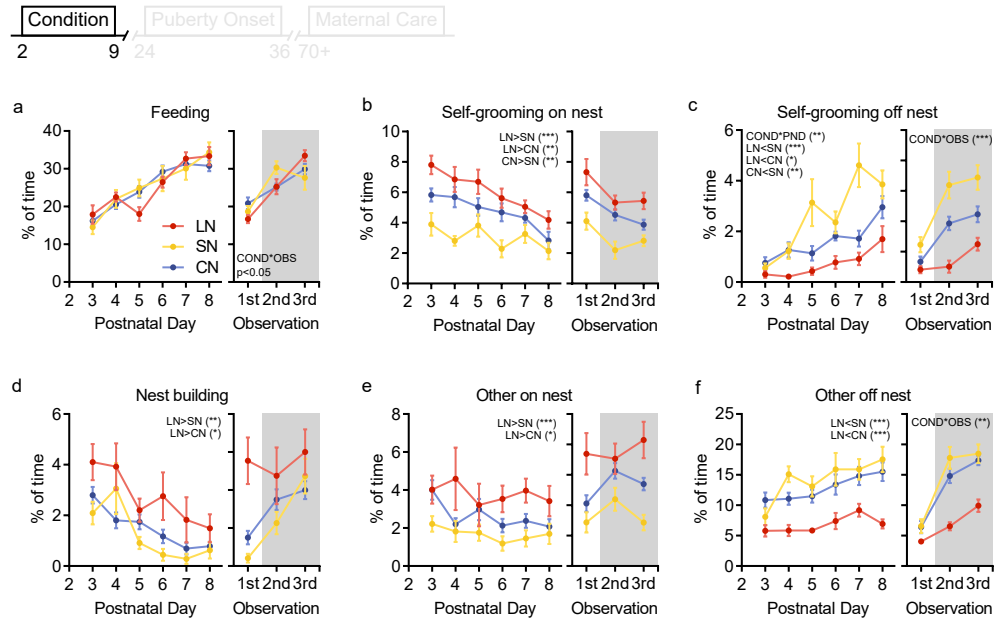

FIGURE S1 **Effect of different housing conditions on maternal care**
(**a**) Feeding, (**b**) self-grooming on nest, (**c**) self-grooming off nest, (**d**) nest building, (**e**) other on nest and (**f**) other off nest behavior for limited nesting (red, n=13), standard nesting (yellow, n=13) and communal nesting (green, n=12) dams, depicted over postnatal days (left) and time of the day (right). The shaded area indicates the dark phase of the LD cycle. Statistics indicate main effects or interactions. *p < 0.05, **p < 0.01, ***p < 0.001.

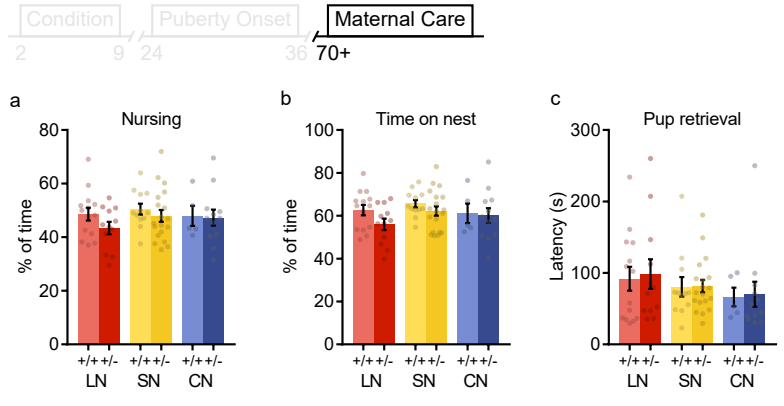

FIGURE S2 **Effects of different rearing conditions on maternal care in female offspring**
(**a**) Total nursing behavior, (**b**) time on nest and (**c**) pup retrieval latencies of F1 dams.
+/+: control, +/-: heterozygous MRKO. Group size: LN +/+: n=14, LN +/-: n=12, SN +/+: n=12, SN +/-: n=19, CN +/+: n=5, CN +/-: n=12).

CHAPTER 4

Maternal care of heterozygous Dopamine Receptor D4 knockout mice: Differential susceptibility to early-life rearing conditions

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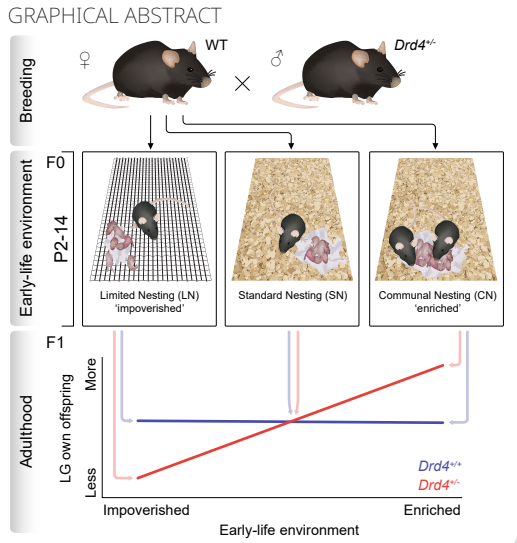
Highlights

- Dams housed in impoverished and enriched conditions clearly differ in maternal care
- Rearing environment affects puberty onset partly through changes in body weight
- Rearing environment altered maternal care towards the next generation
- Only licking/grooming of pups was differentially affected in Drd4+/- mice
- F1 maternal care did not mimic received care during early-life conditions

GRAPHICAL ABSTRACT



Abstract

The differential susceptibility hypothesis proposes that individuals who are more susceptible to the negative effects of adverse rearing conditions may also benefit more from enriched environments. Evidence derived from human experiments suggests the lower efficacy dopamine receptor D4 (*DRD4*) 7-repeat as a main factor in exhibiting these for better and for worse characteristics. However, human studies lack the genetic and environmental control offered by animal experiments, complicating assessment of causal relations. To study differential susceptibility in an animal model, we exposed *Drd4*$^{+/-}$ mice and control litter mates to a limited nesting/bedding (LN), standard nesting (SN) or communal nesting (CN) rearing environment from postnatal day (P) 2-14. Puberty onset was examined from P24-P36 and adult females were assessed on maternal care towards their own offspring. In both males and females, LN reared mice showed a delay in puberty onset that was partly mediated by a reduction in body weight at weaning, irrespective of *Drd4* genotype. During adulthood, LN reared females exhibited characteristics of poor maternal care, whereas dams reared in CN environments showed lower rates of unpredictability towards their own offspring. Differential susceptibility was observed only for licking/grooming levels of female offspring towards their litter; LN reared *Drd4*+/- mice exhibited the lowest and CN reared *Drd4*+/- mice the highest levels of licking/grooming. These results indicate that both genetic and early-environmental factors play an important role in shaping maternal care of the offspring for better and for worse.

1 Introduction

1.1 Differential susceptibility

Parental care is essential for survival and development of newborn mammals, including humans. Variations in parental care substantially contribute to the environmental variability experienced by offspring. Extensive evidence indicates that poor parental care can contribute to increased vulnerability to develop later-life psychopathology in humans and impaired cognitive performance in rodents[1,2]. This vulnerability crucially depends on a complex cross-talk between an individual's genetic makeup and rearing environment[3]. While the genetic background of some individuals is related to a vulnerable phenotype in the face of early-life adversity, others appear to be more resilient. Interestingly, individuals who are genetically more susceptible to the detrimental consequences of negative (rearing) conditions may also experience greater benefits from a positive and stimulating (rearing) environment[4,5]. This crossover effect *for better and for worse*, also called differential susceptibility, is supported by studies investigating the role of human allelic variation across a variety of susceptibility genes[6].

An example of such differentially susceptibility concerns the exon III 7-repeat polymorphism of the D2-like dopamine receptor D4 gene (*DRD4-7R*). In humans, this variant has been associated with reduced gene expression and efficiency[7,8] and acts as a susceptibility marker of dopamine-related genes[6]. Carriers of this variant have an increased risk of developing externalizing problems in relation to parental insensitivity[9] and chronic stress[10]. However, these individuals also benefitted most from enhanced positive parenting[11]. Meta-analytic evidence further supports an important role of dopamine-related genes in moderating susceptibility to both positive and negative rearing environments[12]. Of note, the DRD4 also plays a role in moderating parental care itself[13,14].

1.2 Rodent models of impoverished or enriched rearing environments

Studying differential susceptibility in humans is hampered by random genetic variability. Moreover, it is often difficult to randomly allocate individuals to specific environments while also taking genotype into account. Therefore, we set out to study the causal contribution of decreased *Drd4* functioning to differential susceptibility with a truly randomized experiment in rodents, allowing strict control for both genetic variation and environmental factors[15]. By using heterozygous dopamine receptor D4 knock-out (*Drd4*$^{+/-}$) mice, we aimed to mimic the reduced *DRD4* efficiency observed in human *DRD4-7R* allele carriers.

We selected two rodent models developed to chronically induce alterations in the quality and quantity of parental care received by offspring. First, limited availability of nesting and bedding (LN) material to a mouse dam was used to induce an adverse early life environment; this model increases unpredictability of maternal care received by the pups[16–18], leading to increased corticosterone levels in pups [19] and altered offspring development and behavior in adulthood[20,21]. Second, as beneficial and stimulating social rearing environment we selected a communal nesting (CN) condition, where two or more dams share care-giving behavior towards multiple litters[22]. In this condition, pups experience higher levels of nest occupancy by at least one dam[18,23] and can interact with peers as well as siblings.

Mice reared in communal nesting conditions exhibit various neurobiological and behavioral characteristics that are indicative of improved social competences[24].

1.3 Outcome parameters

In line with a previous study[18], we focused on timing of puberty onset, a key moment in development that is malleable by environmental influences as part of an adaptive reproductive strategy[25]. Although adverse rearing conditions in females are linked to accelerated pubertal onset in humans[26] and rats[27], such effects have not yet been observed in mice[18,28]. In human males, adverse rearing conditions had no effect on puberty onset[29], while puberty onset in male rodents was either unaffected or delayed[18,27,30]. However, rodent models of early-life adversity (ELA) invariably decrease body weight gain, which is an important mediator of puberty onset. Therefore, it is unclear whether the delayed puberty onset observed in ELA reared animals is the result of decreased body weight gain or whether a *relative* acceleration irrespective of body weight exists in rodents as well.

A second outcome was maternal care provided by female offspring. In addition to sexual maturation, the theory submitted by Belsky et al.[25] predicted that variations in early parental care would have the potential to alter adult parental care in humans. Preclinical rodent studies allow for feasible, controlled intergenerational studies on maternal care and, in line with the life history theory, extensive evidence suggests that alterations in maternal care may be transmitted across generations[31]. Variations in levels of licking/grooming (LG) behavior and arched-back nursing (ABN), core features of positive parenting in rodents, have been shown to affect corticosterone reactivity, hippocampal development and maternal care of the offspring[31]. In addition, the limited bedding/nesting model, which evokes changes in maternal care, results in aberrant patterns of maternal care of the offspring[32], whereas mice reared in a communal nesting condition display improved maternal behavior towards their own pups[33]. Taken together, these studies highlight the importance of maternal care for offspring development, as well as the potential of maternal care to be shaped by the early-life environment, contributing to the intergenerational transmission of social behavior.

In this study, we tested heterozygous *Drd4* knock-out (*Drd4*$^{+/-}$) mice and control litter mates on susceptibility to both adverse (LN) and enriched (CN) rearing environments to model differential susceptibility in mice. Animals were examined on i) puberty onset, to track early development, ii) their own maternal care towards the next generation as an indicator of transgenerational effects and iii) basal corticosterone levels, to investigate involvement of the hypothalamic-pituitary-adrenal-axis (HPA-axis) in differential susceptibility. Although puberty onset would be hypothesized to be accelerated in LN and delayed in CN reared animals according to life history theory, previous findings indicate that the opposite may be true in mice due to the strong effects of body weight. LN reared mice were hypothesized to display poor maternal care, whereas CN reared mice were hypothesized to show enhanced maternal care. To confirm differential susceptibility, these effects would have to be amplified in, or exclusive to, *Drd4*$^{+/-}$ mice.

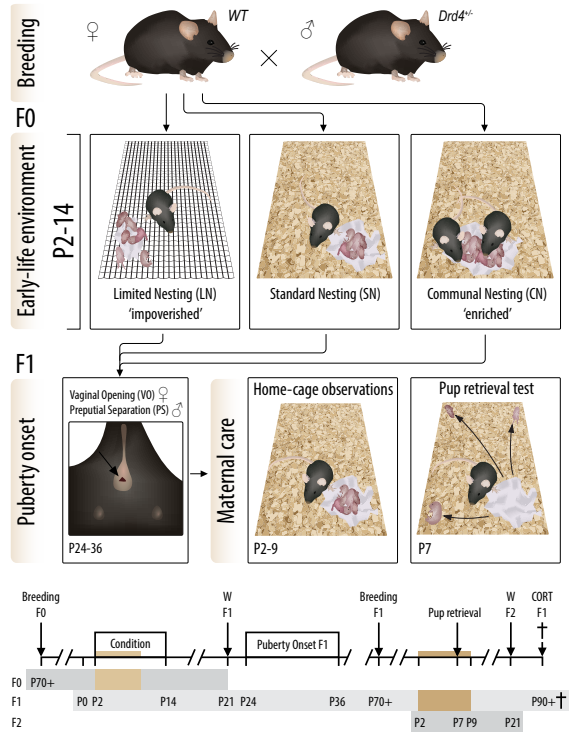


FIGURE 1 **Outline of the experiments**
Study design and timeline of the experiment. A wild-type female was paired with a *Drd4*$^{+/-}$ male to obtain litters of mixed genetic background. Experimental time points for each generation of mice are depicted. W = weaning. P = postnatal day. Colored bars indicate periods of home cage maternal care observations.

2 Materials & Methods

2.1 Animals & Housing

B6.129P2-*Drd4*^{tm1Dkg}/J (*Drd4*$^{+/-}$) mice[34] were originally obtained from the Jackson Laboratory (Bar Harbor, Maine, USA) and bred in-house with C57BL/6JOlaHsd (breeding colony, originally obtained from Harlan, France) mice for at least 4 generations before experiments started. All breeding was performed in our own animal facility. Wild-type (wt) female C57BL/6 mice were allowed to breed with male *Drd4*$^{+/-}$ mice to generate *Drd4*$^{+/-}$ F1 offspring and *Drd4*$^{+/+}$ control litter mates. *Drd4*$^{+/-}$ mice are viable, healthy and visually indistinguishable from control animals. Between postnatal day 2 and 14 (P2-14), dam and litter were exposed to a limited nesting/bedding (LN), standard (SN) or communal nesting (CN) condition. A total of 129 female and 116 male F1 offspring obtained from 40 breedings was used to assess puberty onset and, in females (n = 75), maternal care of this generation (see Fig 1. for a timeline of the experiment). Final numbers per experimental group are depicted in figure legends and specified per litter in supplementary Table 1. Puberty onset and F1 maternal care were scored by a trained experimenter blind to rearing condition and genotype of the animals. Mice had *ad libitum* access to water and chow and were housed on a reversed LD cycle (lights off 08:00 h, temperature 21-22 °C, humidity 40-60 %). All experiments were performed in accordance with the EC council directive (86/609/EEC) and approved by the Central Authority for Scientific Procedures on Animals in the Netherlands (CCD approval AVD115002016644).

2.2 Breeding conditions

Breeding was performed as described earlier[18]. In short, one male was paired with two females for 4 days, after which females were co-housed until approximately one week prior to birth. Pregnant dams were then housed in a type II short Macrolon cage (21.5 x 16 cm) with filter top and a Nestlet (5 x 5 cm, Technilab-BMI, Someren, The Netherlands) as nesting material. Nestlets are made from sterilized cotton fiber material that the dam can use to shred and form a nest site while still allowing for observation of maternal behavior. Daily inspection for the birth of litters was conducted at 09:00 h, assigning the day prior as P0. At P2, dam and litters were weighed and randomly assigned to the LN, SN or CN condition. All litters were culled (or cross-fostered if necessary) to 6-7 pups per litter, with a maximum addition of 1 pup per litter and a minimum of 2 pups of each sex in each litter.

The LN condition consisted of placing the dam and litter in a cage with limited bedding material, made inaccessible by a stainless steel wired mesh. In addition only half the regular amount of nesting material (Nestlet, 5 x 2.5 cm) was available. In the SN condition, standard amounts of bedding (±3 cm bedding) and nesting material (Nestlet, 5 x 5 cm) were available to the dam. The CN paradigm consisted of co-housing the experimental wt dam (and her genetically heterogeneous F1 litter) with another wt ear-punched dam (and wt litter) in a type II regular Macrolon cage (32×16 cm, 5 x 5 cm Nestlet and regular bedding). The pups of this second mother were marked with surgical marker at P2 and P7 (ArcRoyal, Ireland) to ensure correct allocation of the pups to their mother at the end of communal housing at P14. At P9 and P14, all dams and litters were weighed and provided with clean cages, adding a bit of used bedding material to maintain odor cues. From P14 until weaning at P21, animals were housed in standard nesting conditions. All mice were weighed at weaning and ear punches were obtained to facilitate individual recognition and genotype offspring.

2.3 Maternal care observations F0

An instantaneous sampling method[18] was used to score maternal behavior of the dams in different conditions. Three 75-minute scoring sessions were performed daily from P2-9 between 06:00-07:30 h (end light phase), 12:00-14:00 h (mid dark phase) and 16:30-18:30 h (end dark phase). Red light conditions were used to score during the dark phase sessions. Maternal behavior of each dam was scored every three minutes, leading to 25 observations per session and 75 observations per day for each dam. Maternal behaviors were classified as: arched-back nursing (ABN), passive nursing, licking/grooming pups (LG), nest building, self-grooming on nest, feeding and self-grooming off nest. For observations during which the behavior did not qualify for one of these categories, only on or off nest location of the dam was scored. A Samsung Galaxy Note 4 with Pocket Observer 3.3 software (Noldus, the Netherlands) was used for behavioral scoring, and data was analyzed using Observer XT 10.5 (Noldus, the Netherlands). Both dams in the communal nesting condition were scored separately, using average scores of each pair of dams as an indication of maternal behavior received by the litter.

Assessment of maternal care was performed by looking at i) frequencies of the various maternal behaviors, ii) unpredictability of maternal care and iii) fragmentation, using on/off nest transitions. First, percentage of time spent on the various maternal behaviors was calculated per day (pooling the 3 daily sessions) or circadian phase (pooling over 6 postnatal days) to assess the development over days and circadian rhythmicity of maternal care, respectively. Second, overall unpredictability of maternal behavior was evaluated using the entropy rate of transitions between different maternal behaviors [16]. The entropy rate is obtained by calculating the probabilities of certain maternal behaviors predicting specific subsequent behaviors, in which higher entropy rates are indicative of higher unpredictability. In addition, unpredictability of maternal care specifically on the nest site was calculated by pooling all off-nest behaviors to enhance representation of the unpredictability rate as experienced by the offspring. Third, the average number of transitions from and to the nest site per observation was used as an index of fragmentation of maternal care [19].

2.4 Puberty onset F1

As an external measure of puberty onset in males, mice were restrained and gently examined daily from P27-P33 (10:00 – 12:00 h) on the potential to fully retract the prepuce and expose the glans penis which was designated as puberty onset[35]. Female mice were scored daily from P24-P36 for vaginal opening, here taken as sign of puberty onset [36]. All mice were weighed at puberty onset.

2.5 Maternal care F1

During adulthood (>P70), female F1 mice were allowed to breed with a wild-type male as described for F0. All F2 litters were culled/cross-fostered to 6 pups and reared in standard nesting conditions. At P2, P9, P14 and P21, clean cages were provided and animals were weighed. Maternal care observations were performed as described for F0 maternal behavior. At P7 between 10:00-12:00 h, pup retrieval behavior was measured using a 5 minute pup retrieval test as described earlier[18]. If a dam did not retrieve all three pups within 5 minutes, a latency of 300 seconds was assigned.

2.6 Plasma corticosterone levels F1

To measure plasma corticosterone levels, all F1 dams were decapitated in random order between 13:00 – 17:00 h at least 3 weeks after weaning of F2 litters. Trunk blood was collected in heparin containing tubes (Sarstedt, The Netherlands) on ice and centrifuged for 10 minutes (13000 rpm) at 4 °C. Plasma was collected and stored at -20 °C until radioimmunoassay (MP Biomedicals, The Netherlands; sensitivity 3 ng/ml).

2.7 Statistical analysis

Data are expressed as mean ± SEM. Values deviating >3.29 SD from the mean were defined as outlying and winsorized accordingly[37]. The entropy rate of one F0 LN dam was winsorized. Data was analyzed using SPSS 23 (IBM) and litter effects in all F1 measures were accounted for using the SPSS complex samples module. However, no effect sizes are provided in this model. In other analyses, eta squared effect sizes (η^2), representing the explained variance relative to the total model

variance, are reported. Overall ANOVA statistics are presented in supplementary tables, Tukey HSD (main effects) or Sidak (interactions) corrected post-hoc comparisons are depicted in figures.

Greenhouse-Geisser corrected repeated measures ANOVAs with breeding condition as the between-subject factor and postnatal day or observation as within-subject factors were used to analyze F0 maternal behaviors. Maternal behaviors from two observation sessions at P2 were analyzed separately to dissociate acute

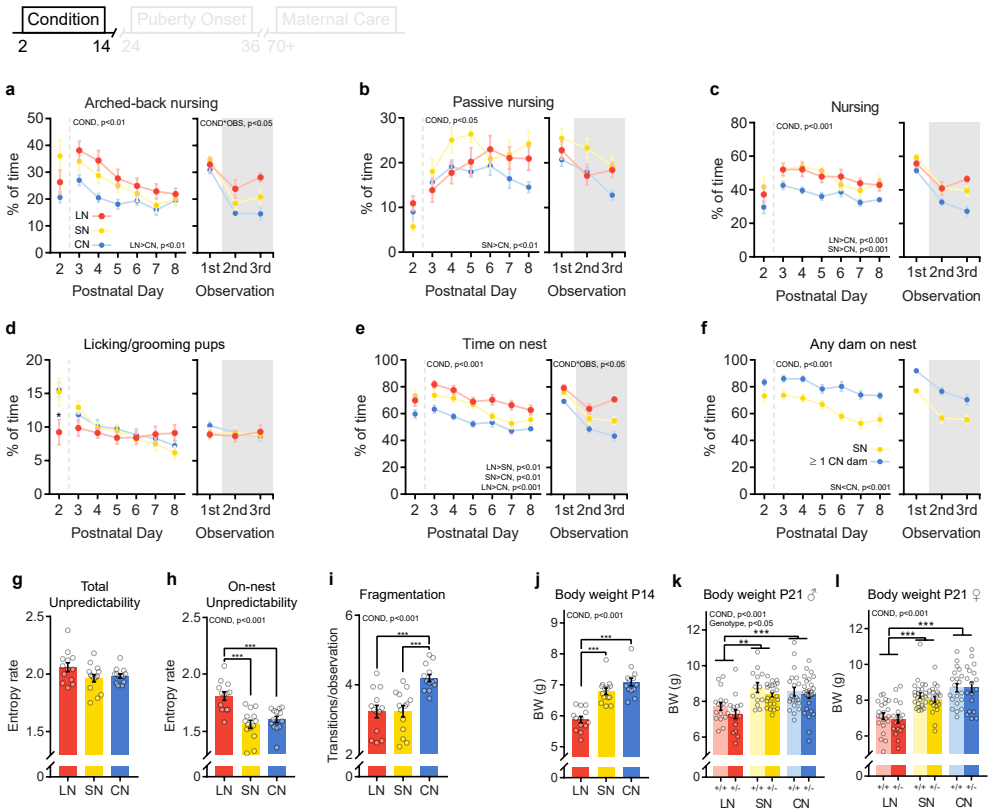


FIGURE 2 **Effect of different housing conditions on F0 maternal care and F1 body weight**
(**a**) Arched-back nursing, (**b**) passive nursing, (**c**) total nursing, (**d**) licking/grooming and (**e,f**) time on nest for limited nesting (red, n = 13), standard nesting (yellow, n = 14) and communal nesting (blue, n = 13) dams, depicted over postnatal days (left) and time of the day (right). The shaded area indicates the dark phase of the LD cycle. Data in f represents the time on nest by at least one dam from the litters perspective. (**g**) Unpredictability of all scored maternal behaviors and (**h**) unpredictability of maternal care when all off-nest behaviors were combined into one measure. (**i**) Fragmentation (on/off nest transitions) of maternal behavior. Each dot represents one dam and the average of two dams in the CN condition. (**j**) Offspring body weight averaged per litter at postnatal day 14. (**k**) Offspring body weight per individual at weaning for males and (**l**) females. +/+: control, +/-: heterozygous *Drd4*. Group size: ♂: LN +/+: n = 17, LN +/-: n = 16, SN +/+: n = 13, SN +/-: n = 23, CN +/+: n = 22, CN +/-: n = 27; ♀: LN +/+: n = 22, LN +/-: n = 17, SN +/+: n = 26, SN +/-: n = 22, CN +/+: n = 20, CN +/-: n = 18. ANOVA main effects are depicted in the top left of each figure. Post-hoc comparisons are depicted bottom right or by lines. COND = main effect of condition. COND*OBS = condition*observation interaction effect. Asterisks indicate interactions or post-hoc comparisons. *p < 0.05, **p < 0.01, ***p < 0.001.

effects of novel environment exposure from more chronic alterations in maternal care. P2 maternal behavior, entropy rates and fragmentation were analyzed using a one-way ANOVA with breeding condition as the between-subjects factor. Pup retrieval latencies of F1 dams were analyzed using cox regression, as this method is preferred if a subset of animals fails to complete a certain task[38]. All other F1 measures were analyzed using a two-way ANOVA including rearing condition and genotype as independent variables. Pearson correlations were used for correlational data. Mediation analysis was conducted using the PROCESS v3 SPSS macro[39], with rearing condition as a multicategorical independent variable and the SN group as the reference category. The day of puberty onset was used as dependent variable and body weight at weaning and received entropy rates as potential mediators. Significant mediation was assigned when 95% confidence intervals of mediation did not include zero.

3 Results

3.1 Maternal care by F0: care provided in an enriched or impoverished environment

The maternal care of mouse dams was affected by environmental condition (Fig. 2, supplementary table 1). Arched-back nursing (ABN) levels in LN dams were increased compared to CN dams (Fig. 2a), while passive nursing was decreased in CN dams compared to SN dams (Fig 2b). Taking the sum of ABN and passive nursing together, total nursing levels displayed by individual CN dams were decreased compared to LN and SN dams (Fig. 2c), but feeding behavior in the CN condition increased (Fig. S1a). Although environmental conditions did not affect licking/grooming behavior from P3-8, LG levels were affected more acutely at P2 (Fig. 2d). Post-hoc testing indicated that specifically pups in a LN setting were deprived from LG on this first day of novel environment exposure. Overall nest occupancy of LN dams was increased compared to SN and CN dams (Fig. 2e), but this was mostly due to an increase in the time LN dams were engaging in non pup directed behaviors on the nest site (self-grooming and other behavior, see Fig. S1).

Despite a reduction of nest occupancy by individual CN dams compared to both LN and SN mice, the nest site in the CN setting had higher levels of nest occupancy by at least one dam compared to the SN condition (Fig. 2f). Moreover, circadian rhythmicity of maternal behavior was altered by exposure to different conditions (Fig. 2e, right panel). The pattern of maternal care displayed towards the end of the dark phase (third observation time-point) was more comparable to the light phase (first observation time-point) in LN dams, whereas CN and SN dams displayed similar levels of maternal behaviors during both dark phase observations (second and third observation time-points). This pattern appeared to be consistent across different behaviors but reached significance for ABN, nest occupancy and off-nest behaviors.

The overall unpredictability of behavior displayed by dams was not significantly affected by environmental condition (Fig. 2g). However, unpredictability of behavior specifically on the nest site (on nest entropy rates) was altered (Fig. 2h). Post-hoc comparisons revealed that the LN dams displayed increased unpredictability of maternal care compared to the SN and CN dams. Nesting condition also affected

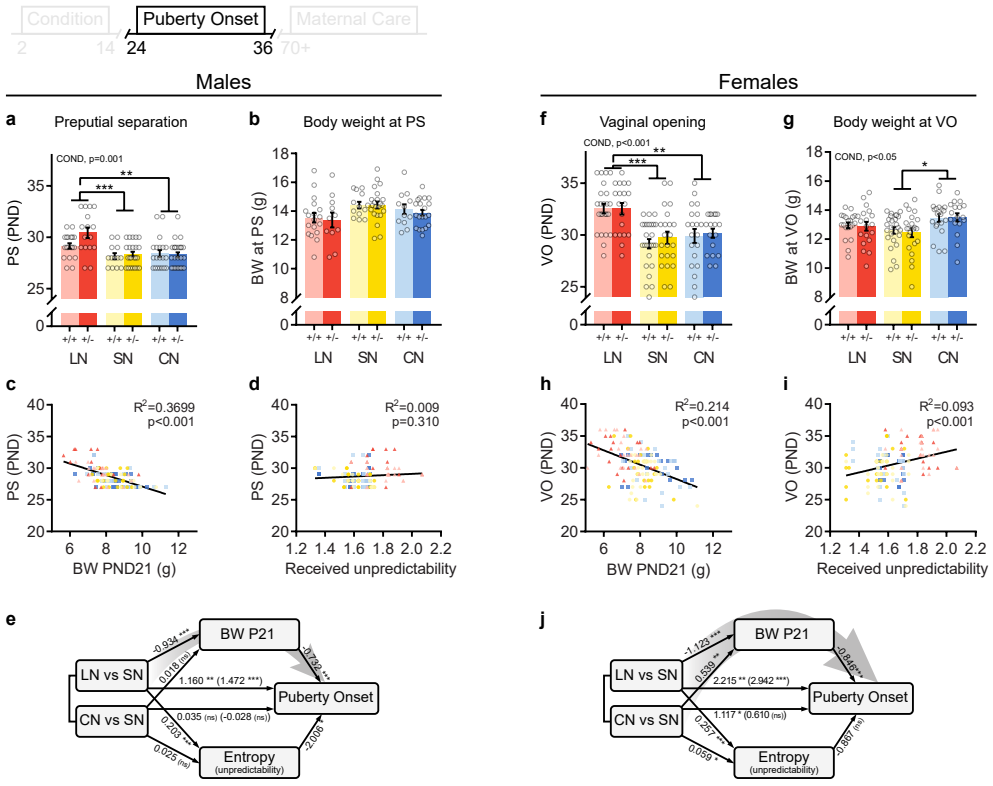


FIGURE 3 **Effects of different rearing conditions on sexual maturation in male and female offspring** (**a,f**) Puberty onset in male (preputial separation) and female (vaginal opening) mice. (**b,g**) Body weight at puberty onset. (**c,h**) Body weight at weaning negatively correlated with puberty onset in both males and females, whereas (**d,i**) received on-nest unpredictability rates during rearing positively correlated with puberty onset only in females. (**e,j**) Graphical representation of mediation models. Numbers represent estimated model coefficients, direct effects are depicted in parenthesis. Grey arrows indicate a significant mediation pathway. +/+: control, +/-: heterozygous *Drd4*. Asterisks indicate post hoc comparisons. *p < 0.05, **p < 0.01, ***p < 0.001.

fragmentation of maternal care, measured by the number of transitions from and to the nest site (Fig. 2i); CN dams exhibited increased fragmentation compared to SN and LN dams.

3.2 Effects of enriched or impoverished rearing conditions on F1

3.2.1 Effects of rearing conditions on early development

At P14, body weight of LN litters was decreased compared to SN and CN litters (Fig. 2j), an effect that remained at weaning in both males (Fig. 2k) and females (Fig. 2l). Puberty onset was also affected by rearing condition in both males (Fig. 3a) and females (Fig. 3f); LN reared animals displayed a delay in puberty onset compared to SN and CN reared mice. In females (Fig. 3g), but not males (Fig. 3b), body weight at puberty onset was increased in CN reared animals compared to SN and LN mice. In both males and females, body weight at weaning negatively correlated with puberty onset (Fig. 3c, h), whereas a positive correlation between received

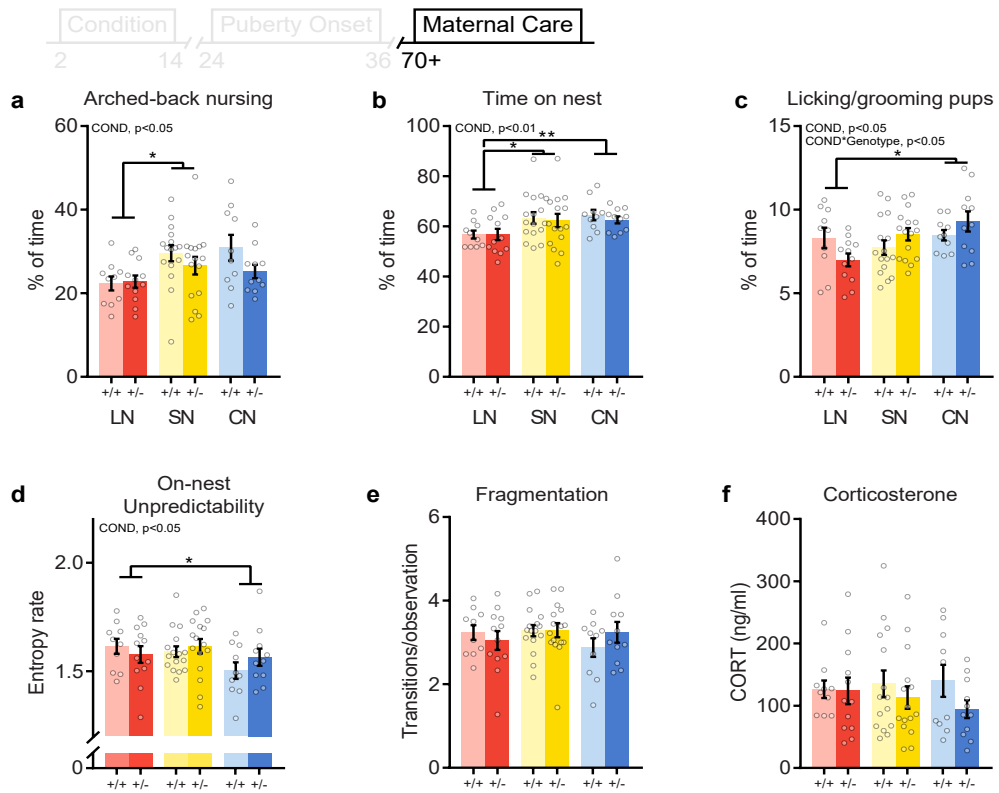


FIGURE 4 **Effects of different rearing conditions and *Drd4* genotype on maternal care and basal corticosterone levels in female F1 offspring**
Overall (P2-9) levels of (**a**) Arched-back nursing, (**b**) time on nest and (**c**) licking grooming exhibited by F1 female dams. (**d**) On-nest unpredictability and (**e**) fragmentation (on/off nest transitions) of maternal behavior. (**f**) Basal corticosterone levels. +/+: control, +/-: heterozygous *Drd4*. Group size: LN +/+: n = 10, LN +/-: n = 12, SN +/+: n = 16, SN +/-: n = 16, CN +/+: n = 10, CN +/-: n = 11). Asterisks indicate post hoc comparisons. *p < 0.05, **p < 0.01.

entropy rates during early development and puberty onset was only observed in females (Fig. 3d, i). Mediation analysis revealed that in males, the delayed puberty onset observed in LN reared mice was partly mediated by the reduced body weight at weaning (95%CI = [0.36, 1.17], Fig. 3e). In females, body weight at weaning was a significant mediator of puberty onset for both LN (95%CI = [0.36, 1.66], Fig. 3j) and CN reared animals (95%CI = [-0.96, -0.08]). However, entropy rates did not mediate the effects of rearing condition on puberty onset (LN: 95%CI = [-1.21, 0.83]; CN: 95%CI = [-0.29, 0.23]).

3.2.2 Maternal care by F1: effects of rearing conditions on later-life maternal care

Mice that were exposed to LN rearing conditions during early development displayed decreased levels of arched-back nursing (ABN) towards their own

offspring compared to SN reared animals (Fig. 4a). While passive nursing levels were not affected by rearing condition (Fig. S2a), total nursing behavior was decreased in LN reared mice compared to CN reared animals (Fig. S2b). In addition, the total time spent on the nest site was decreased in LN reared animals compared to both SN and CN reared mice (Fig. 4b). A main effect of rearing condition was also observed for the percentage of time dams spent licking/grooming their own pups, a key maternal behavior; LN reared dams spent less time licking/grooming than dams reared in a communal nesting environment (Fig. 4c).

While F0 dams did not differ in total entropy rate, the total entropy rate of F1 maternal behavior was decreased in CN reared mice compared to dams reared in a SN environment (Fig. S2c). In addition, CN reared dams displayed lower on-nest unpredictability rates compared to LN reared animals (Fig. 4c). Fragmentation of maternal care was not affected by early life condition. Thus, while CN animals were raised with more fragmented maternal care, they did not differ in this behavior themselves when allowed to breed in a standard nesting condition. Cox regression revealed that pup retrieval was unaffected by rearing condition (hazard ratio 95%CI = [0.72, 1.39], p = 0.986). Although P2 body weight of the next generation (F2) was decreased in offspring from a LN reared mother compared to offspring from SN and CN reared dams (Fig. S2e), this was normalized at weaning at P21 (Fig. S2f). Finally, basal levels of blood plasma corticosterone were not affected by rearing condition (Fig. 4f).

3.3 Effects of heterozygous *Drd4* knock-out on F1

In males, but not females, heterozygous knock-out of the dopamine receptor D4 (*Drd4*$^{+/-}$) resulted in a decreased body weight at weaning (Fig. 2k). *Drd4*$^{+/-}$ mice did not differ from *Drd4*$^{+/+}$ animals in any of the sexual maturation measures (Fig. 3). In addition, home-cage maternal care levels towards the next generation were unaffected by *Drd4* genotype (Fig. 4 and Fig. S2). However, maternal responsiveness, as measured by pup retrieval, was improved in *Drd4*$^{+/-}$ dams compared to *Drd4*$^{+/+}$ animals (Fig. S2d); *Drd4*$^{+/-}$ dams showed a higher completion rate in all rearing conditions (hazard ratio 95%CI = [1.03, 2.85], p = 0.040).

3.4 Moderation of rearing condition effects by *Drd4* genotype

Different rearing conditions did not interact with *Drd4* genotype to determine body weight at weaning (Fig. 2) or sexual maturation (Fig. 3). In addition, basal corticosterone levels and most measures of maternal care were not affected by a gene-early environment interaction (Fig. 4). However, an interaction effect was observed for the percentage of time dams spent licking/grooming their own offspring (Fig. 4c). In line with the differential susceptibility theory, *Drd4*$^{+/-}$ dams reared in the LN environment exhibited the lowest LG levels, whereas CN reared *Drd4*$^{+/-}$ mice spent the most time licking/grooming their own pups.

4 Discussion

In the present study, we examined the causal role of *Drd4* in differential susceptibility to the environment using a randomized experiment in rodents, allowing strict control for both genetic variation –using *Drd4*$^{+/-}$ mice- and early-life environmental factors. After extensive characterization of the effects of different

environmental conditions on maternal care, we observed a differential susceptibility effect only for licking/grooming levels of adult female offspring towards their own litter. LN and CN reared *Drd4*$^{+/-}$ mice exhibited the lowest and highest levels of licking/grooming, respectively. In addition, we demonstrated main effects of rearing conditions on sexual maturation and maternal care towards the next generation. Mice reared in a limited nesting/bedding environment displayed characteristics of poor mothering, whereas communal nesting during early development resulted in higher predictability of maternal care.

4.1 Modelling impoverished and enriched rearing environments

The pattern of F0 maternal care resulting from exposure to the LN condition was largely in line with earlier findings using this model[16–19]. While different pup-directed maternal behaviors remained relatively unaltered, the unpredictability of maternal behavior, particularly on the nest site, increased. In addition, pups in the LN condition were deprived from normal levels of licking/grooming upon first exposure to this condition on P2, whereas LG levels were similar to the SN and CN conditions from P3-P8. In contrast to other reports, but in line with previous findings from our lab[18], fragmentation of maternal care was similar to control conditions, a difference that could be due to the difference in timing of observations. In the present study, maternal behaviour was observed predominantly during the dark phase of the animals, whereas previous studies focused more on the light phase of the day/night cycle[16,19]. This difference in timing of observations is important as we observed, in line with earlier reports from our lab[18], a different circadian pattern in nest occupancy and ABN. LN dams exhibited altered circadian rhythmicity in maternal care, stressing the point that multiple time-points or continuous monitoring across the day-night should be examined to better grasp the implications of the LN condition.

Individual mouse dams adapted their maternal care to the communal nesting condition by decreasing nursing levels and increasing feeding behavior. Despite decreased nursing time per dam, offspring body weight was similar compared to SN reared animals. This could be explained in part by the observation that pups in the communal nesting condition have increased accessibility to at least one mouse dam, a hallmark of the early social enrichment provided by this model[24]. In addition, litters in the CN condition are of a larger litter size, likely requiring less energy per pup to regulate body temperature.

4.2 Rearing conditions affect sexual maturation

The delayed puberty onset observed in both male and female LN reared mice was mediated by a decrease in body weight gain at weaning. The importance of body weight and leptin in regulating puberty onset is well-known for both humans[40,41] and rodents[42]. We therefore also measured body weight at puberty onset for the adolescent mice that were raised in different early life conditions. The minimal differences in body weight at puberty onset suggest that, irrespective of early life background and subsequent body weight at weaning, the majority of mice postpone the onset of puberty until a certain body weight is reached. This is in contrast to a recent study where body weight at vaginal opening was increased in female mice that were reared in a LN condition from P2-9[28]. However, because

body weight at weaning of control groups is similar in both studies, this is unlikely to be a result of measurement differences. Future studies should therefore help to elucidate whether body weight at puberty onset is consistently affected by limited nesting rearing conditions.

In our study, only female mice reared in a CN setting showed increased body weight at puberty onset, indicating that these animals might exhibit, in line with the acceleration hypothesis, a *relative* delay in puberty onset, irrespective of bodyweight. It should be noted that early-life adversity not only affects body weight, it also alters adipose tissue, plasma leptin and leptin mRNA levels[43]. Therefore, the mediation of puberty onset following LN is more complex and should be studied in more detail than only examining body weight per se. Nevertheless, the lack of differences in body weight at puberty onset between LN and SN reared mice, in combination with the delayed puberty onset of female mice that experienced increased unpredictability during rearing are not in line with the acceleration hypothesis of life history earlier proposed in humans. This may point to species differences but could also signify the relevance of uncontrolled factors in humans (e.g. caloric intake) that are controlled for in the current design.

4.3 Rearing conditions affect later-life maternal care

Different rearing conditions have been shown to affect maternal care provided to the next generation in the LN[32] and CN[33] models. Although previous results from our lab showed no effects of either LN or CN from P2-9 on adult maternal behavior[18], the results presented here do support long-lasting effects of rearing condition on maternal care. This could be explained by the duration and timing of exposure to early-life rearing conditions (P2-P9 in previous study compared to P2-14 in the present study). Given the different trajectories in brain circuit development[44,45], the effects of early-life adversity, and potentially also enrichment, strongly depend on the critical period during which it occurs[46]. The importance of this critical or sensitive period is highlighted by a recent study showing that different windows of exposure to a combination of maternal separation with limited nesting differentially alter susceptibility to social defeat stress during adulthood[47]. By extending the exposure of pups to different rearing conditions the development of brain regions involved in the regulation of maternal care, like the MPOA and mPFC[48], may have been targeted more profoundly.

Extensive research from Meaney and co-workers have identified the pivotal beneficial role of arched-back nursing and licking/grooming behavior in rodent development[31,49,50]. Many studies investigating intergenerational transmission of maternal care observe a similar phenotype in the offspring and the mother[51,52]. Interestingly, the lower ABN and nest occupancy levels of LN reared female mice observed in our current study did not coincide with a lower ABN or nest presence of their own mother. On the contrary, female LN reared pups experienced *increased* levels of nest occupancy by the dam compared to the SN condition, but showed *lower* levels of nest occupancy when taking care of a litter themselves. Similarly, CN reared mice received comparable levels of unpredictability as standard reared mice, yet provided more predictable maternal behavior towards their own offspring. Finally, LN reared animals received increased on-nest unpredictability but showed similar on-nest entropy rates compared to SN reared dams. Thus, although the differences in maternal care of F1 dams presented here are not mimicking the phenotype of the mother, the quality of the early-life environment (poor vs. enriched) did affect the quality of F1 maternal care under standard breeding conditions.

4.4 *Drd4* genotype moderates the effects of rearing conditions

For licking/grooming behavior, the effects of rearing conditions were restricted to *Drd4*$^{+/-}$ animals, whereas rearing conditions had no effect on LG levels in wild-type animals. Using *Drd4* genotype as a susceptibility factor, this is supportive evidence for differential susceptibility in a controlled animal model. Interestingly, the alterations were observed across generations, a finding that requires significant effort to study in humans. Studies on differential susceptibility in humans focused predominantly on the effects of maternal care on child development, highlighting the increased susceptibility of *DRD4-7R* carrying children to parental sensitivity[53]. However, as these studies have not yet examined parental care of the next generation, the translational relevance of results presented here is yet to be studied.

Clearly, the exact mechanisms through which the early-life environment impacts on later-life behavior remain to be elucidated. Previous studies suggest an important role for the methylation of genes involved in the HPA-axis[54]. Human studies also link the *DRD4-7R* genotype to alterations in components of the HPA-axis. Gene-early environment effects have been observed for basal cortisol in children[53], as well as stress induced cortisol levels of young adults[55]. A prominent role for alterations in circulating basal corticosterone levels in adulthood is not supported by our data. However, stress reactivity was not assessed and could, at least in part, underlie the observed alterations in maternal care.

Other systems may also be critical in the mechanism underlying differential susceptibility. Recent studies using different molecular tools and mouse knock-in models have begun to unravel the exact function of the *DRD4-7R* in corticostriatal glutamatergic neurotransmission, enhancing our understanding of the *Drd4* receptor and susceptibility to the environment[56,57]. Other studies used a wide array of techniques to show the involvement of other dopamine receptors in mediating the social deficits observed after severe early-life stress[58]. At a meta-analytic level however, the effects of early-life adversity on the dopaminergic system appear limited, although significant for some parameters and areas[59]. It is important to note that none of the studies included in the meta-analysis examined *Drd4* as a potential target, highlighting the lack of preclinical evidence on the role of *Drd4* expression in mediating effects of adverse rearing conditions. The advances in our understanding of *Drd4* functioning at a molecular level and the role of other dopamine receptors in regulating susceptibility will help to guide future studies into the role of *DRD4*.

Finally, there is increasing awareness that most consequences of early-life rodent models have small effect sizes[21], which is also the case in our study. Although we have sizable group numbers compared to common practice in the field, we should take this into consideration and interpret the results with care. To increase

statistical power in future experiments, animal numbers should be adapted to realistically expected effect sizes and animal ethical committees should be aware of this[60]. Moreover, more meta-analyses in this field should be stimulated and can help in designing future studies[21].

Conclusion

The research presented here provides a translational approach to examine the contribution of the *Drd4* gene in differential susceptibility. While other preclinical studies on differential susceptibility in socially monogamous prairie voles focused on the role of *prenatal* stress in enhancing developmental plasticity to both adverse and supportive contexts[61,62], we show that adverse or enriched *postnatal* environments also interact with *genetic* factors in mice, for better and for worse. Future experiments should be targeted to test which neurobiological mechanisms are involved in mediating the effects of *DRD4* with regard to differential susceptibility.

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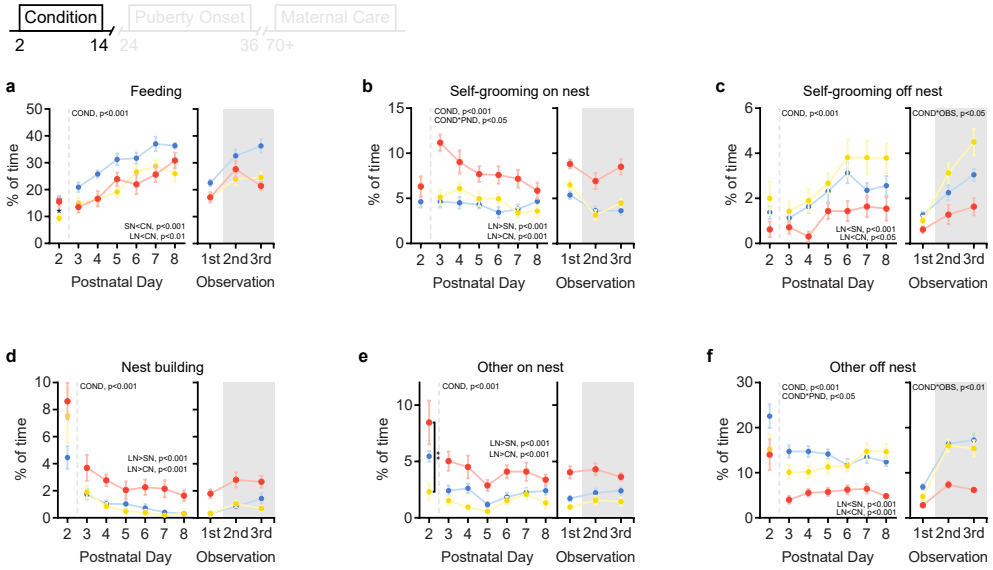

FIGURE S1 **Effect of different housing conditions on maternal care**
(**a**) Feeding, (**b**) self-grooming on nest, (**c**) self-grooming off nest, (**d**) nest building, (**e**) other on nest and (**f**) other off nest behavior for limited nesting (red, n = 13), standard nesting (yellow, n = 14) and communal nesting (blue, n = 13) dams, depicted over postnatal days (left) and time of the day (right). The shaded area indicates the dark phase of the LD cycle. ANOVA main effects are depicted in the top left of each figure. Post-hoc comparisons are depicted bottom right or by lines. COND = main effect of condition. COND*OBS = condition*observation interaction effect. COND*PND = condition*postnatal day interaction effect. *p < 0.05, **p < 0.01, ***p < 0.001.

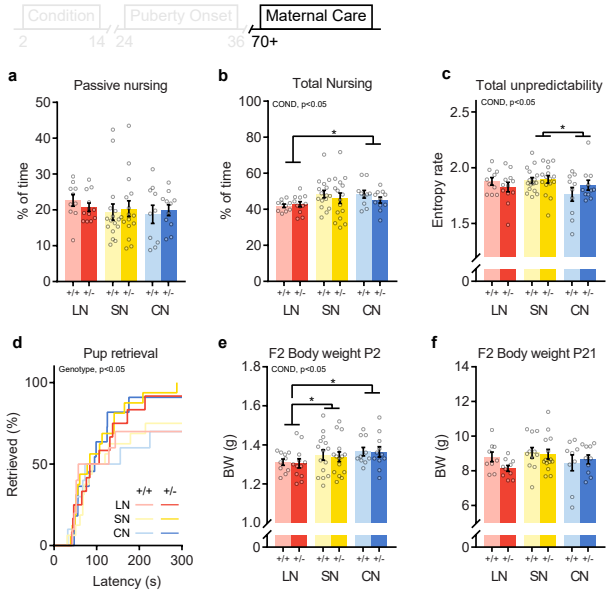

FIGURE S2 **Effects of different rearing conditions and *Drd4* genotype on F1 maternal care and F2 body weight**
(**a**) Passive nursing and (**b**) total nursing levels. (**c**) Total unpredictability rates. (**d**) Pup retrieval latencies and completion rates. (**e**) F2 offspring body weight at P2 and (**f**) P21. +/+: control, +/-: heterozygous *Drd4*. Group size: LN +/+: n = 10, LN +/-: n = 12, SN +/+: n = 16, SN +/-: n = 16, CN +/+: n = 10, CN +/-: n = 11). Asterisks indicate post hoc comparisons. *p < 0.05, **p < 0.01.

	Litter	Males +/+	Males +/-	Females (mothers) +/+	Females (mothers) +/-
Limited Nesting	1	0	3	0 (0)	1 (1)
	2	1	1	3 (2)	1 (0)
	3	2	1	2 (1)	2 (1)
	4	2	1	2 (0)	2 (1)
	5	1	1	2 (0)	2 (1)
	6	1	2	1 (1)	3 (2)
	7	1	1	1 (1)	1 (1)
	8	1	3	1 (1)	2 (1)
	9	2	0	2 (1)	1 (1)
	10	2	0	4 (1)	0 (0)
	11	2	1	2 (2)	1 (1)
	12	1	2	2 (0)	2 (1)
	13	3	1	2 (0)	1 (1)
Standard Nesting	1	1	0	1 (1)	4 (3)
	2	1	3	0 (0)	1 (0)
	3	3	0	2 (1)	2 (2)
	4	1	3	2 (0)	1 (1)
	5	1	3	0 (0)	1 (0)
	6	1	2	3 (3)	0 (0)
	7	0	2	1 (0)	3 (3)
	8	0	0	1 (1)	4 (3)
	9	1	3	3 (2)	0 (0)
	10	2	1	1 (1)	1 (1)
	11	1	0	2 (1)	4 (2)
	12	0	2	4 (1)	0 (0)
	13	1	1	3 (2)	1 (1)
	14	0	3	3 (3)	0 (0)
Communal Nesting	1	0	3	2 (1)	1 (1)
	2	2	0	2 (2)	3 (1)
	3	2	2	1 (1)	1 (0)
	4	2	2	2 (1)	1 (0)
	5	2	1	1 (1)	3 (3)
	6	1	3	3 (1)	0 (0)
	7	2	3	0 (0)	3 (3)
	8	1	3	0 (0)	3 (2)
	9	1	3	1 (1)	2 (0)
	10	3	2	1 (0)	0 (0)
	11	3	1	2 (0)	0 (0)
	12	1	2	3 (2)	1 (1)
	13	2	2	2 (0)	0 (0)

TABLE S1 **Number of animals per litter used in this study**
Numbers in parentheses indicate the number of females that succesfully raised a litter themselves. +/+: control, +/-: heterozygous *Drd4*.

	P2				P3-8				Observation*condition			
	F-statistic	p-value	η^2	Post-hoc	F-statistic	p-value	η^2	Post-hoc	F-statistic	p-value	η^2	Figure
Individual behaviors												
Arched-back nursing	F(2, 36) = 2.92	.067	.14		F(2, 36) = 5.69	**.007**	.24	LN>CN	F(3.69, 68.31) = 3.40	**.016**	.07	2a
Passive nursing	F(2, 36) = 2.32	.112	.11		F(2, 36) = 5.10	**.011**	.22	SN>CN	F(3.93, 41.20) = 0.97	.428	.04	2b
Total nursing	F(2, 36) = 1.58	.219	.08		F(2, 36) = 14.27	**<.001**	.44	LN=SN>CN	F(3.75, 69.33) = 2.28	.073	.05	2c
Licking/grooming	F(2, 36) = 4.32	**.021**	.19	LN<SN=CN	F(2, 36) = 0.13	.880	.01		F(3.98, 73.70) = 0.80	.528	.04	2d
Time on nest	F(2, 36) = 3.00	.062	.14		F(2, 36) = 25.95	**<.001**	.59	LN>SN>CN	F(3.51, 64.86) = 2.72	**.044**	.06	2e
Feeding	F(2, 36) = 6.17	**.005**	.26	LN>SN<CN	F(2, 36) = 12.36	**<.001**	.41	LN=SN<CN	F(3.65, 67.43) = 2.31	.073	.07	S1a
Self-grooming on nest	F(2, 36) = 1.44	.250	.07		F(2, 36) = 19.59	**<.001**	.52	LN>SN=CN	F(3.98, 73.68) = 2.00	.104	.06	S1b
Self-grooming off nest	F(2, 36) = 1.88	.168	.09		F(2, 36) = 13.89	**<.001**	.44	LN<SN=CN	F(3.88, 71.73) = 3.60	**.011**	.10	S1c
Nest building	F(2, 36) = 2.12	.135	.11		F(2, 36) = 21.10	**<.001**	.54	LN>SN=CN	F(3.10, 57.35) = 0.78	.512	.03	S1d
Other on nest	F(2, 36) = 6.27	**.005**	.26	LN>SN	F(2, 36) = 28.83	**<.001**	.62	LN>SN=CN	F(3.83, 70.90) = 0.69	.594	.03	S1e
Other off nest	F(2, 36) = 2.16	.130	.11		F(2, 36) = 52.59	**<.001**	.75	LN<SN=CN	F(3.13, 57.96) = 4.20	**.008**	.08	S1f
Communal nesting	T-statistic	p-value	Cohen's D									
Any dam on nest	t(24) = -1.92	.067	0.75		F(1, 24) = 69.53	**<.001**	.74		F(1.63, 40.67) = 0.38	.641	.01	2f
Unpredictability/fragmentation												
Overall unpredictability					F(2, 37) = 2.70	.081	.13					2g
On-nest unpredictability					F(2, 37) = 16.02	**<.001**	.46	LN>SN=CN				2h
Fragmentation					F(2, 37) = 13.08	**<.001**	.41	LN=SN<CN				2i

TABLE S2 **Statistical tests on the effects of different environmental conditions on F0 maternal care**
P-values in bold are considered statistically significant. P2 = postnatal day 2. P3-8 = postnatal day 3 till 8. η^2 = eta squared effect size.

	Condition			Genotype			Condition*Genotype		
	F-statistic	p-value	Post-hoc	F-statistic	p-value		F-statistic	p-value	Figure
Body weight									
P14									
Litters	F(2, 37) = 32.44	**<.001**	LN<SN=CN	unknown			unknown		2j
P21									
Males	F(2, 36) = 8.77	**.003**	LN<SN=CN	F(1, 38) = 4.97	**.032**		F(2, 36) = 0.39	.686	2k
Females	F(2, 37) = 20.60	**<.001**	LN<SN=CN	F(1, 38) = 0.45	.505		F(2, 37) = 0.15	.829	2l
Puberty onset									
Males									
Puberty onset	F(2, 36) = 10.33	**.001**	LN>SN=CN	F(1, 37) = 2.83	.101		F(2, 36) = 1.84	.121	3a
Body weight at puberty onset	F(2, 34) = 2.20	.382		F(1, 35) = 0.38	.544		F(2, 34) = 0.20	.777	3b
Females									
Puberty onset	F(2, 38) = 13.09	**.003**	LN>SN=CN	F(1, 39) = 0.52	.477		F(2, 38) = 0.28	.947	3f
Body weight at puberty onset	F(2, 38) = 4.29	**.010**	LN=SN<CN	F(1, 39) = 0.09	.767		F(2, 38) = 0.06	.930	3g
F1 maternal care									
Maternal behavior									
Arched-back nursing	F(2, 33) = 4.02	**.027**	LN<SN	F(1, 34) = 3.01	.092		F(2, 33) = 1.32	.281	4a
Passive nursing	F(2, 33) = 0.73	.475		F(1, 34) = 0.00	.958		F(2, 33) = 0.58	.630	S2a
Total nursing	F(2, 33) = 4.79	**.024**	LN<CN	F(1, 34) = 1.14	.293		F(2, 33) = 0.94	.366	S2b
Licking/grooming	F(2, 33) = 4.51	**.011**	LN<CN	F(1, 34) = 0.10	.758		F(2, 33) = 4.99	**.028**	4c
Time on nest	F(2, 33) = 7.40	**.002**	LN<SN=CN	F(1, 34) = 0.33	.571		F(2, 33) = 0.13	.845	4b
Feeding	F(2, 33) = 1.70	.165		F(1, 34) = 3.31	.078		F(2, 33) = 0.23	.845	-
Self-grooming on nest	F(2, 33) = 2.02	.146		F(1, 34) = 3.37	.075		F(2, 33) = 0.90	.331	-
Self-grooming off nest	F(2, 33) = 0.24	.763		F(1, 34) = 0.72	.401		F(2, 33) = 0.92	.424	-
Nest building	F(2, 33) = 1.03	.350		F(1, 34) = 2.13	.153		F(2, 33) = 1.09	.453	-
Other on nest	F(2, 33) = 0.42	.806		F(1, 34) = 1.53	.224		F(2, 33) = 2.52	.058	-
Other off nest	F(2, 33) = 2.88	.055		F(1, 34) = 0.47	.498		F(2, 33) = 0.11	.893	-
Unpredictability/fragmentation									
Total entropy	F(2, 33) = 3.20	**.032**	SN>CN	F(1, 34) = 0.12	.733		F(2, 33) = 0.72	.411	S2c
On-nest entropy	F(2, 33) = 3.62	**.044**	LN>CN	F(1, 34) = 0.31	.579		F(2, 33) = 0.85	.374	4d
Fragmentation	F(2, 33) = 1.08	.269		F(1, 34) = 0.12	.728		F(2, 33) = 0.55	.505	4e
F2 body weight									
P2	F(2, 33) = 5.48	**.012**	LN<SN=CN	F(1, 34) = 0.11	.746		F(2, 33) = 0.03	.964	S2e
P21	F(2, 30) = 2.36	.566	LN<SN=CN	F(1, 31) = 1.56	.221		F(2, 30) = 0.69	.630	S2f
Corticosterone	F(2, 32) = 0.10	.904		F(1, 33) = 2.04	.163		F(2, 32) = 0.55	.532	4f

TABLE S3 **Statistical tests on the effects of different rearing conditions on F1 outcome measures**
P-values in bold are considered statistically significant. P = postnatal day.

CHAPTER 5

Heterozygous knock-out of the Dopamine Receptor D4 or Mineralocorticoid Receptor does not alter susceptibility to impoverished or enriched rearing conditions in male mice: A study on anxiety, spatial memory and fear conditioning

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Abstract

While some individuals are genetically more vulnerable to the negative consequences of early-life adversity, these individuals may also benefit more from an enriched rearing environment. In particular genes involved in the dopaminergic (dopamine receptor D4; DRD4) and stress system (mineralocorticoid receptor; MR) could exhibit these cross-over interaction characteristics, called differential susceptibility. By utilizing genetic and environmental control offered by animal models, we aimed to examine differential susceptibility in relation to anxiety, spatial memory and fear conditioning. In two separate experimental series, we exposed heterozygous Drd4 or MR knock-out mice and control litter mates to either a limited nesting and bedding (LN), standard nesting (SN) or communal nesting condition (CN) from postnatal day 2-14 (Drd4 study) or 2-9 (MR study). In adulthood, we tested animals in an object in location task (OIL; Drd4 study), elevated plus maze (EPM; Drd4 study) and fear conditioning paradigm (FC; Drd4 & MR study), after which basal corticosterone levels (Drd4 & MR study) and hippocampal MR expression (MR study) were measured. Spatial memory and anxiety behavior were unaffected by rearing condition or genotype of the animals, although general activity in these tasks was consistently increased in LN reared mice. While rearing conditions did not affect fear conditioning performance, decreased expression of MR impaired the capacity of mice to discriminate between 'safe' and 'threatening' episodes and increased basal corticosterone levels. Throughout this study, no gene-environment interactions were observed, providing no evidence for the differential susceptibility theory with regard to the genes and behavioral domains explored here.

1 Introduction

Early postnatal development of mammals is shaped by the interplay between parental care and genetic characteristics of the infant. In humans, poor parental care contributes to increased vulnerability to the development of psychopathology during adulthood[1]. Moreover, some individuals are genetically more vulnerable to the negative consequences of early-life adversity, while others appear to be more resilient[2–4]. Animal models have shown comparable gene-environment interactions and are used to study the underlying neurobiological mechanisms[5–7]. However, gene-environment interactions may point to genetic susceptibility that, in addition to enhancing the negative consequences of early-life adversity, also contribute to increased benefits from an enriched and stimulating rearing environment[8]. This for better *and* for worse concept, termed 'differential susceptibility', is primarily supported by evidence derived from studies in human subjects[9].

Research on differential susceptibility genes has focused predominantly on serotonergic and dopaminergic gene polymorphisms, although other genetic profiles may result in enhanced susceptibility to the (rearing) environment as well[9]. Differential susceptibility supporting evidence concerning the dopaminergic system is mainly focused on the dopamine receptor D4 (*DRD4*), where an exon III 7-repeat polymorphism (*DRD4-7R*) acts as a susceptibility marker of dopaminergic genes. The *DRD4-7R* variant is associated with reduced gene expression and efficacy[10,11] and 7R-carrying individuals are at increased risk to develop externalizing problems after experiencing parental insensitivity[12] or chronic stress[13]. Interestingly, an intervention aimed to promote positive parenting and sensitive discipline was found to be more effective in 7R-carrying children compared to non-7R-carriers[14]. The notion that these individuals are more susceptible to both positive and negative rearing environments is further supported by meta-analytic evidence into the role of dopaminergic genes, although findings might be dependent on developmental window[15,16].

Genes involved in the regulation of the hypothalamic-pituitary-adrenal-axis (HPA-axis), such as the high affinity cortisol (humans) or corticosterone (rodents) mineralocorticoid receptor (*MR*) gene, have been implicated in moderating the effect of sensitive parenting on attachment security as well[17]. Genetic variation in the MR gene has been found to enhance the negative effects of childhood maltreatment[18] or neglect[19], illustrating a "double-hit" model of MR-by-(early)-environment interactions. Finally, stress responsivity itself has been proposed as a marker of differential susceptibility[20]. Therefore, the functional role of the mineralocorticoid receptor in moderating differential susceptibility to different rearing conditions appears to be a relevant target for further investigation.

Although human studies have been fundamental in highlighting the relevance and potential benefits of understanding differential susceptibility in more detail, the designs are hampered by issues of random genetic variability and difficulties to allocate individuals to controlled environments. Using rodent models that allow for randomized controlled experiments with control over genetic and environmental factors could help in elucidating the causal pathways through which differential susceptibility occurs[7]. Previous attempts to test the role of the serotonin transporter (5-HTT) in moderating effects of both adverse and enriched rearing environments

have been unsuccessful in observing crossover gene-by-environment interactions in mice[21]. We therefore aimed to study whether reduced expression of two other candidate genes, *Drd4 and MR*, would result in increased susceptibility to both the negative consequences of early-life adversity, as well as the beneficial effects of enriched rearing conditions.

Rodent models that alter the quality and quantity of maternal care have been widely used to model impoverished or enriched rearing conditions. Providing a lactating mouse dam with limited amounts of nesting and bedding material increases unpredictability and fragmentation of maternal care[22–24]. This results in upregulated stress levels in the pups[22], leading to a wide variety of alterations in neurocircuitry and adult behavior[25]. Amongst other things[25], detrimental effects of the limited bedding and nesting model have been observed for spatial memory[26], anxiety[27,28] (but see[29]) and fear learning[30]. Of note, it appears important to make a distinction between stressful learning and non-stressful learning to disentangle the effects of early-life adversity[31].

Early social enrichment, as opposed to adversity, can be modeled by co-housing two or more lactating dams and litters to form a communal nest[32]. In this condition, care-giving behavior is shared and upregulated, resulting in increased sociability of mice reared in communal nesting conditions[33,34].

In this study, we aimed to examine the effects of heterozygous genotype manipulations, mimicking the partial rather than complete loss of function in the human situation. In two separate experimental series we exposed both wildtype controls and heterozygous *Drd4* (*Drd4* study) and (*MR* study) animals respectively to either the limited nesting and bedding condition (LN), standard nesting (SN) or communal nesting paradigm (CN). In adulthood, we tested animals in an object in location task (OIL; *Drd4* study), elevated plus maze (EPM; *Drd4* study) and fear conditioning paradigm (FC; *Drd4* & *MR* study), to include measures of stressful and non-stressful learning, anxiety, and flexibility of behavior. Since fear learning is known to be controlled by both mineralocorticoid[35–38] and dopamine D4 receptors[39–41], this is a promising target to study in this context. Examining whether non-stressful learning, anxiety, and fear learning are affected by rearing conditions and genotype in a differential susceptibility fashion could provide important insights in the development and treatment of anxiety-related disorders following poor or benevolent early-life conditions.

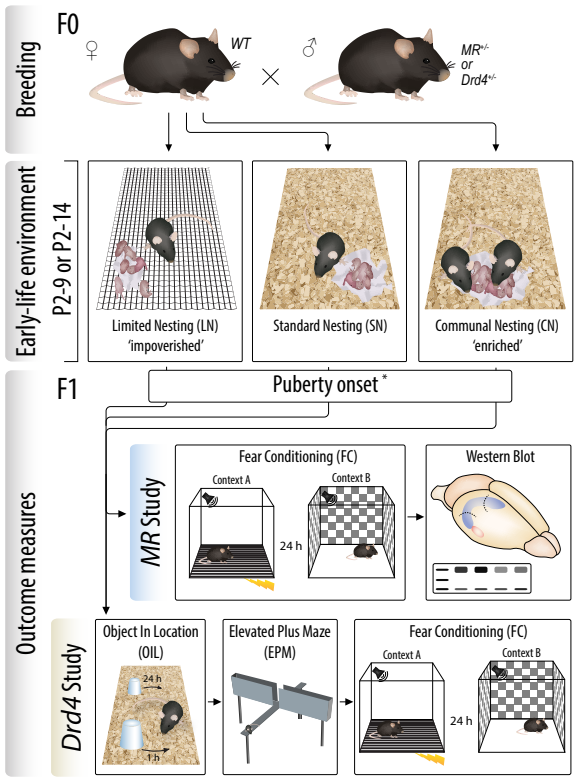

FIGURE 1 **Outline of the experiments**
Study design of the experiment. A wild-type female was paired with a $MR^{+/-}$ or $Drd4^{+/-}$ male to obtain litters of mixed genetic background. *The results of puberty onset measurements are published in Knop et al., 2019 & 2020.

2 Materials & Methods

2.1 Animals & Housing

Two heterozygous knock-out mice strains on a C57BL/6 background were used and bred in-house. First, forebrain-specific MR knock-out C57BL/6JOlaHsd mice were generated by means of a Cre/loxP recombinase approach[42]. A heterozygous $MR^{flox/flox\ -\ CaMKIIa\text{-}Cre/wt}$ male was crossed with a C57BL/6JOlaHsd (breeding colony, originally obtained from Harlan, France) wild-type (wt) female to generate heterozygous MR knock-out animals ($MR^{+/-}$) and control litter mates ($MR^{+/+}$). Second, heterozygous *Drd4* knock-out mice ($Drd4^{+/-}$) and control litter mates ($Drd4^{+/+}$) were generated by crossing a heterozygous B6.129P2-Drd4^{tm1Dkg}/J[43] male with a wt female. *Drd4* mice were originally obtained from the Jackson Laboratory (Bar Harbor, Maine, USA) and bred in-house with our C57BL/6JOlaHsd breeding colony for at least 4 generations before experiments started. Both strains of knock-out mice are viable, healthy and cannot be distinguished from control animals by basic appearance.

A total of 233 male mice (*MR* study: 112, *Drd4* study: 121) obtained from 76 breedings (*MR* study: 37, *Drd4* study: 39) were used for the experiments (see supplementary table 1 for number specifications). In two separate experimental series, the dam and litters were exposed to limited nesting/bedding (LN), standard nesting (SN) or communal nesting (CN) conditions from postnatal day (P) 2-9 (*MR* study) or P2-14 (*Drd4* study) respectively. The study design is depicted in Figure 1. After exposure to rearing condition, animals were monitored for sexual maturation by measuring puberty onset[44,45]. In adulthood, $Drd4^{+/-}$ and $Drd4^{+/+}$ animals were tested in the object relocation task (spatial learning), the elevated plus maze (anxiety) and fear conditioning task. $MR^{+/-}$ and $MR^{+/+}$ mice were tested only in the fear conditioning task, after which MR expression in regions of the hippocampus was measured. Basal corticosterone levels in blood plasma were measured in all mice. Throughout all experiments, animals were housed on a reversed LD cycle (light off 08:00-20:00, temperature 21-22 °C, humidity 40-60 %). Experiments were performed in accordance with the EC council directive (86/609/EEC) and approved by the Central Authority for Scientific Procedures on Animals in the Netherlands (CCD approval AVD115002016644).

2.2 Breeding conditions

To generate litters, one male (either *MR*$^{+/-}$ or *Drd4*$^{+/-}$) was paired with two wt females for 4 days. The two females were then separated from the male and co-housed until approximately one week prior to birth. Before parturition, pregnant dams were individually housed in filter-topped type II short Macrolon cages (21.5 x 16 cm). As nesting material, dams were provided with sterilized cotton fiber Nestlets (5 x 5 cm, Technilab-BMI, Someren, The Netherlands) that can be shredded by the dam to form an open nest site, thus allowing for maternal behaviour observations. All cages were monitored daily at 09:00 h for the birth of litters. If parturition was confirmed at this time, the day prior was assigned as P0. At P2, the dam and litter were weighed, culled/cross-fostered to 6-7 pups and randomly assigned to one of the three conditions (LN, SN or CN). The minimum number of pups in a litter to be included was 5, in which case only one pup was added. All litters contained pups of both sexes after cross-fostering.

Exposure to the specific rearing condition lasted from P2-9 in the *MR* study and prolonged to P2-14 in the *Drd4* study in order to include the developmental window that has been shown to affect stress susceptibility[46]. Dams in the LN condition were housed in a type II short Macrolon cage, where a limited amount of sawdust was made inaccessible to the dam by a stainless steel wired mesh. In addition, the amount of nesting material was halved (Nestlet, 5 x 2.5 cm). In the SN condition a regular amount of sawdust (±3 cm) and nesting material (Nestlet, 5 x 5 cm) were provided in a type II short Macrolon cage. In the CN condition, one wt dam (and genetically heterogeneous litter) was co-housed with another wt dam (and wt litter) in a regular type II Macrolon cage (32 x 16 cm, 5 x 5 Nestlet and regular bedding). To ensure correct allocation of the pups after CN housing, pups of the wt litter were marked with a non-scenting, non-toxic surgical marker (ArcRoyal, Ireland). All dams and litters were weighed at P9, P14 and P21. At P9 (*MR* study) or P14 (*Drd4* study), all animals returned to standard nesting conditions, which was maintained until weaning at P21. To facilitate individual recognition and genotyping of the animals, all offspring was ear-punched at weaning. During the diverging early-life rearing conditions, maternal care of the dams was extensively monitored. In addition, all offspring was monitored for puberty onset from P24-36 (see Knop *et al.* 2019 & 2020 for methodological details and results). The effects of these environmental and genetic manipulations on adult maternal care of mouse dams towards the next generation are described in Knop et al., 2019 (*MR* study[44]) and Knop et al., 2020 (*Drd4* study[45]). Here, we examine the effects on different aspects of cognition in males.

2.3 Object in location task (OIL)

In adulthood (> P70), male *Drd4*$^{+/-}$ and *Drd4*$^{+/+}$ mice were tested in an object-in-location task (OIL). All testing was performed between 13:00-17:00 h during the dark phase under red light conditions. Animals were exposed to daily handling during the three days before testing. On day 1 and 2 of the experiment, mice were transported to the experimental room and habituated to an arena (Macrolon type III cage, 43 x 27 cm, height 15 cm) for 5 minutes per day. On day 3, mice were trained by allowing them to freely explore two similar objects (set of marble containing glass cups) for 5 minutes. The objects were placed approximately 10 cm from the walls and 15 cm from each other. 1 hour after the training session, spatial memory retrieval was assessed for 5 minutes by relocating one of the two objects and allowing the animal to freely explore (1h retrieval). On day 4, 24 hours after training, the other object was relocated and memory retrieval was again assessed for 5 minutes (24h retrieval). Each animal was assigned to one testing cage throughout the experiment, leaving sawdust undisturbed. All phases were video recorded and analyzed for locomotor activity using Ethovision XT 11.5 (Noldus, the Netherlands), measured by the distance travelled in the arena. Moreover, sniffing behavior defined as the nose-point of the animal being <2 cm from the object, was analyzed. The discrimination index (DI) was calculated as: (time spent exploring the relocated object)/(time spent exploring the relocated object + time spent exploring the fixed object). An index greater than 0.5 indicates a preference for the relocated object and is interpreted as successful spatial memory retrieval.

2.4 Elevated plus maze (EPM)

At least two weeks after OIL testing, *Drd4*$^{+/-}$ and *Drd4*$^{+/+}$ animals were tested on anxiety behavior using an elevated plus maze (EPM) between 13:00-17:00 h under dim light conditions. The apparatus (height: 75 cm) consisted of two open and two enclosed arms with a length of 32 cm. Animals were placed in the center square of the maze facing an open arm and allowed to freely explore for 5 minutes. Between trials, the maze was thoroughly cleaned using an Anistel solution (0.5%; Tristel Solutions Ltd, UK). All trials were video recorded and the location and locomotor activity of the animals was tracked using Ethovision XT 11.5 (Noldus, The Netherlands). Time in the open or closed arm was defined as all body points (nose-point, center-point and tail-base) being in one of the arms, thereby excluding ambiguous locations where one of the body points was in the center square of the maze. The percentage of time spent in the open arm was calculated by: time spent in open arms/(time spent in open arms + time spent in closed arms). The anxiety index was calculated by: 1-[(time spent in open arms/total time in the maze)+(number of entries to the open arm/total exploration of the maze)/2], where the total time in the maze was 300 seconds and total exploration of the maze was defined as the sum of open and closed arm entries. A higher anxiety index is considered to correspond to more anxious animals[47].

2.5 Tone-cued fear conditioning

At least two weeks after EPM testing for *Drd4*$^{+/-}$ and *Drd4*$^{+/+}$ mice and >P80 for *MR*$^{+/-}$ and *MR*$^{+/+}$ mice, animals were tested in a tone-cued fear conditioning paradigm as described earlier [30]. In contrast to the study by Arp et al. where testing was performed in the light phase of the animals, in our study experiments were conducted in the dark phase (13:00 – 17:00 h). To test whether this could affect freezing behavior, we performed an experiment in which two groups of mice (n = 8 mice per group) were tested in different phases of their respective circadian rhythms. The dark phase group was housed under an 8:00 – 20:00 h lights off cycle, whereas the light phase group was housed under an 8:00 – 20:00 h lights on cycle. Testing took place between 14:00 – 16:00 in this experiment.

To assess tone-cued fear conditioning, mice were trained in context A (day

1); a standard operant fear conditioning chamber lacking distinct visual cues (30.5 × 24.2 × 21.0 cm; Med Associates Inc., USA). This chamber contained a house light, tone cue generator and a floor of stainless steel metal rods that was connected to a shock generator. Animals were allowed to freely explore the cage for 3 minutes (baseline), after which a 30 s tone was presented. During the last 2 seconds of the tone, animals received a foot shock (0.4 mA). After 30 seconds, mice were returned to their home cage. To assess fear memory retrieval 24 hours after training (day 2), animals were placed in context B; a different operant chamber with house light, tone cue generator, flat white floor and clear visual cues on the walls. Again, mice were allowed to freely explore this novel environment for 3 minutes, which was followed by six consecutive 30 second tone exposures without foot shock alternated with six 1-minute intervals. Mice were returned to their home cage after the final 1-minute interval. Behavior was video recorded and freezing behavior was scored using Observer XT 10.5 software (Noldus, The Netherlands) by a trained experimenter blind to rearing condition and genotype of the animals.

Freezing behavior was defined as no movement except for breathing and the percentage of time spent freezing was calculated for each episode of the paradigm. Fear generalization was analyzed using the percentage of time animals were freezing during the first 3 minutes of baseline exposure to context B before onset of the first tone. Fear memory retrieval was determined by analysis of freezing levels upon first tone exposure. Freezing levels during 6 consecutive tones or intervals were used to assess discrimination of 'threatening' (cue-on) vs. 'safe' (cue-off) episodes and fear extinction over time.

2.6 Plasma corticosterone levels

In both the *MR* (*MR*$^{+/-}$ and *MR*$^{+/-}$) and *Drd4* (*Drd4*$^{+/-}$ and *Drd4*$^{+/+}$) study, all animals were decapitated in random order between 13:00-14:00 h, in line with timing of behavioral experiments, at least 4 weeks after fear conditioning. Trunk blood was collected on ice in heparin containing tubes (Sarstedt, The Netherlands). Blood samples were centrifuged at 4°C for 10 minutes and plasma was stored at -20°C until radioimmunoassay (MP Biomedicals, The Netherlands; sensitivity 3 ng/ml).

2.7 Western Blotting

In addition to corticosterone measurements, MR protein levels in the ventral and dorsal hippocampus of *MR*$^{+/-}$ and *MR*$^{+/+}$ animals were measured using Western blot analysis. Dorsal (upper third of the dorsal part) and ventral (lower third of the ventral part) hippocampi of both hemispheres were dissected and collected in tubes on ice. Tissue was freshly homogenized using a homogenizer (IKA® T10 basic) in RIPA lysis buffer (1 M Tris, 1 M NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton, and 1 mM EDTA of pH 8). Samples were then centrifuged at 4°C for 20 minutes (15 682 rcf) and supernatant was aliquoted and stored at -80°C until Western blot analysis.

After denaturation at 95°C for 5 minutes, protein samples were separated on an 8% polyacrylamide-SDS gel. Loading of samples was semi-randomized so that each gel contained 6 samples, one of each experimental group. Protein was then transferred onto a nitrocellulose membrane (0.45 µm thickness, GE Healthcare Life Sciences, Amsterdam) for 1 hour at 100 V. The membrane was then shortly washed with Tris-buffered saline (TBS) and blocked for at least 10 minutes using a Supermix solution (0.05 mol/L Tris, 0.9% NaCl, 0.25% gelatin and 0.5% Triton X-100, pH 7.4) to reduce unspecific binding of antibodies. Membranes were incubated overnight with primary antibodies anti-MR (mouse monoclonal rMR1–18 1D526, dilution 1:500[48]) and anti-GAPDH (rabbit polyclonal GAPDH 14C10, 1:3000, Cell Signalling Technology® Inc., Santa Cruz, CA, USA). The next day, membranes were washed with TBS 1% Tween20 (TBS-T) for 3 x 10 minutes and incubated for 1 hour at room temperature with fluorescent secondary antibodies diluted in Supermix (anti-mouse IRdye800, 1:5000, anti-rabbit Cy5, 1:2000). After washing for 3 x 10 minutes with TBS-T, membranes were rinsed with H_2O and developed using Enhanced Chemiluminescent Western Blotting Substrate (Pierce)**.** Quantification was performed using ImageJ software by an experimenter blind to experimental group. MR expression data was normalized to GAPDH expression in all samples.

2.8 Statistical analysis

All data are presented as mean ± SEM. Data points that deviated >3.29 standard deviations from the mean were defined as outliers and winsorized accordingly[49]. The total distance travelled in the OIL task was winsorized for one individual. SPSS 23 (IBM) software was used for all analyses and the complex samples module was used to control for the dependent nature of sibling data. Because this type of analysis does not provide effect sizes, these are not reported. Effects of rearing condition and genotype were analyzed using a two-way ANOVA. Discrimination indexes of animals in the OIL task were tested against a 50% chance level using a one sample Student's t-test (see supplementary Table S1 for results). The fear conditioning data were analyzed using repeated measures ANOVAs. Fear conditioning behavior was analyzed for fear memory upon first exposure to the tone (baseline vs. first tone), freezing upon 6 consecutive tones (T1 through T6) or intervals (I1 through I6) and the discrimination of cue-on vs. cue-off episodes (tones vs. intervals). Overall ANOVA and t-test statistics are presented in supplementary tables, Tukey HSD (main effects) or Sidak (interactions) corrected post-hoc comparisons are depicted in figures.

3 Results

3.1 Object in location task (*Drd4* study)

Exploration of objects by *Drd4*$^{+/-}$ and *Drd4*$^{+/+}$ mice during the acquisition phase was not affected by rearing condition, *Drd4* genotype or a condition*genotype interaction (supplementary Table S4). However, because some animals spent little time exploring the objects, a threshold of 20 seconds of exploration in each of the phases (acquisition, 1 hour retrieval and 24 hour retrieval) was used to ensure that mice spent sufficient amounts of time at both objects for reliable acquisition and indications of memory retrieval[50]. A total of 18 mice (LN +/+: 4, LN +/-: 2, SN +/+: 2, SN +/-: 3, CN +/+: 2, CN +/-: 5) did not reach this criterion and were excluded from analysis**.**

Spatial memory retrieval after 1 hour was affected by rearing condition of the animals, but not by *Drd4* genotype or a condition*genotype interaction (Fig.

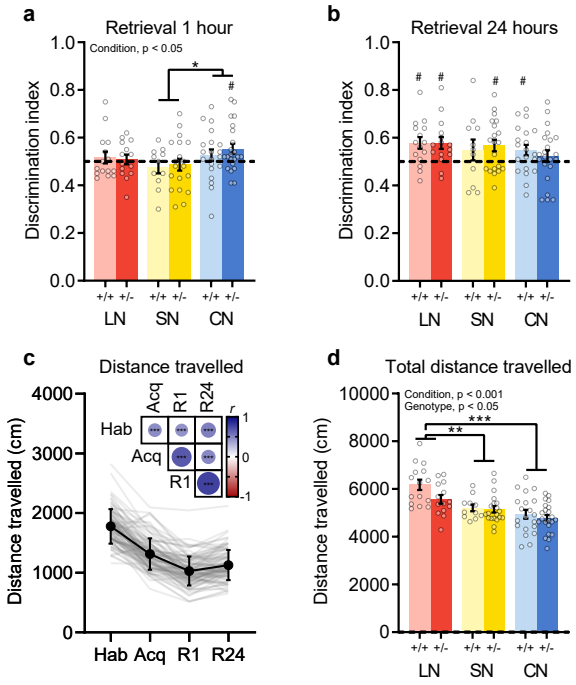

FIGURE 2 **Effects of different rearing conditions and *Drd4* genotype on spatial memory and locomotor activity in the Object in Location (OIL) task**
Discrimination indexes during the OIL (**a**) 1 hour and (**b**) 24 hours after acquisition. (**c**) Distances travelled during four phases of the OIL task (habituation, acquisition, retrieval after 1 hour and retrieval after 24 hours) strongly correlated with each other. Correlation matrix is depicted in the inlay. (**d**) Total distance travelled throughout the entire OIL task. +/+: control, +/-: heterozygous *Drd4*. Group size: LN +/+: n = 15, LN +/-: n = 15, SN +/+: n = 11, SN +/-: n = 20, CN +/+: n = 20, CN +/-: n = 22). Asterisks indicate post hoc comparisons. *p < 0.05, **p < 0.01, ***p < 0.001. # indicate a significant deviation from chance-level (0.5).

2a). Mice reared in a CN environment spent more time with the relocated object compared to SN mice. Only $CN^{+/-}$ mice spent significantly more time with the relocated object compared to the fixed object. All other groups did not exhibit a preference for one of the objects. Retrieval after 24 hours was unaffected by rearing condition, *Drd4* genotype or condition*genotype interaction (Fig. 2b). In contrast to retrieval after 1 hour, most groups were successful in discriminating the relocated object from the fixed object.

To assess general activity in the OIL test, the distance travelled during the experiment was analyzed. Because the distance travelled during the habituation, acquisition, and both retrieval phases highly correlated with each other (see inlay in Fig. 2c and supplementary Table S2), the total sum of all phases was used as a general measure of activity. Total distance travelled was affected by rearing condition and genotype of the animals (Fig. 2d). Post-hoc analysis revealed that mice reared in a LN environment travelled larger distances compared to both SN and CN reared mice. In addition, heterozygous knock-out of the *Drd4* receptor resulted in a decreased amount of distance travelled compared to $Drd4^{+/+}$ animals. Rearing condition did not interact with genotype of the animals in determining activity in the OIL task. Of note, inclusion of animals that were excluded in the analysis due to a lack of exploration time did not alter this activity pattern (data not shown).

3.2 Elevated plus maze (*Drd4* study)

Anxiety-like behavior of $Drd4^{+/-}$ and $Drd4^{+/+}$ mice, measured by the ratio of time spent in the open arms (Fig. 3a) and anxiety index (Fig. 3b), was not affected by

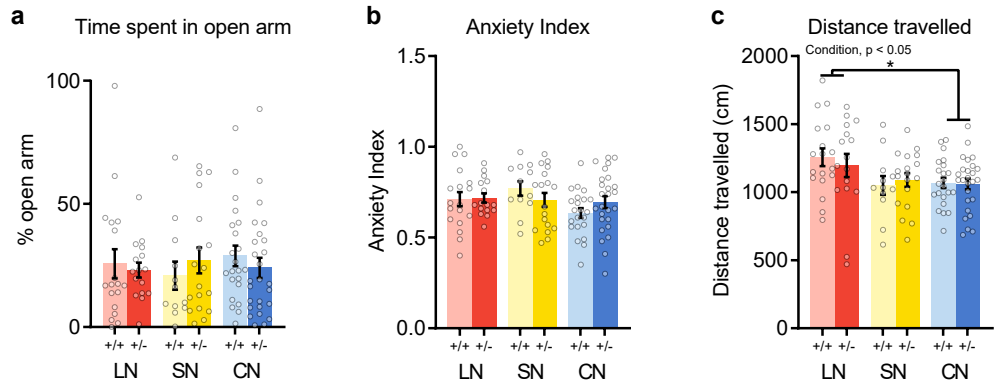

FIGURE 3 **Effects of different rearing conditions and *Drd4* genotype on anxiety in the Elevated Plus Maze (EPM)**
Anxiety as measured by (**a**) the % of time spent in the open arm and (**b**) anxiety index (see methods section for details on the calculation). (**c**) Distance travelled during the EPM. +/+: control, +/-: heterozygous *Drd4*. Group size: LN +/+: n = 18, LN +/-: n = 16, SN +/+: n = 12, SN +/-: n = 18, CN +/+: n = 22, CN +/-: n = 26). Asterisks indicate post hoc comparisons. *p < 0.05.

rearing condition, genotype or condition*genotype interaction. Similar to the OIL test, activity in the EPM was altered by rearing condition, but not genotype or condition*genotype interaction (Fig. 3c). Mice reared in LN conditions travelled greater distances compared to CN, but not SN animals.

3.3 Tone-cued fear conditioning (*Drd4* & *MR* study)

3.3.1 Circadian rhythm

Twenty-four hours after training in context A, freezing behavior of mice during baseline exploration of context B was not affected by the circadian phase (Fig. 4b). Moreover, both groups of mice, tested in either the dark or light phase of the circadian cycle, did not differ in freezing levels upon first tone exposure, during all tones or during all intervals (supplementary Table S4). As indicated by a significant time effect, freezing behavior declined during 6 consecutive tone exposures and 6 consecutive intervals (supplementary Table S4). However, this decline was not affected by circadian phase. The results of this experiment show that performance in this tone-cued fear conditioning paradigm is not affected by timing of the test in the relative circadian phase of mice.

3.3.2 Drd4 study

Freezing behavior during baseline exploration of context B (Fig 4c) was not affected by rearing condition, *Drd4* genotype, or genotype*condition. All groups learned to associate the tone with the shock equally, as indicated by a significant increase in freezing during the first tone exposure that was not affected by any of the independent variables (supplementary Table S3 & S4). Freezing behavior during 6 consecutive tones or intervals declined over time, indicating an extinction of fear for the tone, as well as a decrease in anxiety during the intervals within the test. These patterns were not affected by the independent variables. A significant increase in freezing behavior during tones compared to intervals

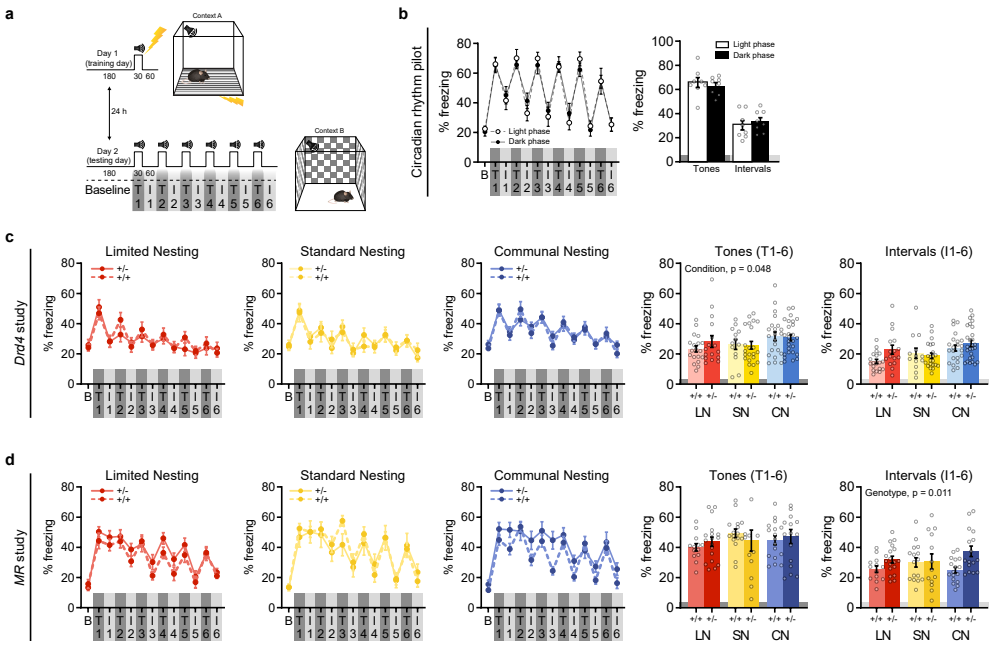

FIGURE 4 **Effects of circadian phase, different rearing conditions and genotype (*Drd4* or *MR*) on fear conditioning performance**
(**a**) Illustration of the fear conditioning paradigm. Duration of each phase is depicted in seconds. (**b**) Freezing behavior on the day of testing during tone and interval phases of mice (n = 8/group) tested in different episodes of the light/dark cycle throughout the test (line graph) and averaged for tones or intervals (bar graph). Freezing behavior on the day of testing during tone and interval episodes of (**c**) *Drd4*$^{+/+}$, *Drd4*$^{+/-}$, (**d**) *MR*$^{+/+}$ and *MR*$^{+/-}$ mice reared in different environments throughout the test (line graphs) and averaged for tones or intervals (bar graphs). +/+: control, +/-: heterozygous *Drd4* or *MR*. Group sizes for *Drd4* study: LN +/+: n = 19, LN +/-: n = 16, SN +/+: n = 13, SN +/-: n = 22, CN +/+: n = 22, CN +/-: n = 27. Group sizes for *MR* study: LN +/+: n = 13, LN +/-: n = 18, SN +/+: n = 16, SN +/-: n = 13, CN +/+: n = 18, CN +/-: n = 16. B = baseline, T = tone, I = interval.

without independent variable interactions showed that all groups were able to discriminate between the cue-on and cue-off episodes. Overall, these data suggest that rearing condition and *Drd4* genotype did not affect the capacity to learn this fear conditioning paradigm and all mice are equally flexible in adapting behavior to the situation.

3.3.3 MR study

Baseline freezing behavior of *MR*$^{+/-}$ and *MR*$^{+/+}$ mice in context B was not altered by rearing condition, *MR* genotype or condition*genotype interaction (Fig. 4d). All groups showed increased freezing levels upon exposure to the first tone compared to baseline levels, indicating successful conditioning to the tone. Freezing levels during this first tone exposure were not affected by rearing condition, *MR* genotype or condition*genotype interaction. Extinction of freezing behavior during the tones or intervals within the test was not affected by rearing condition or genotype of the animals.

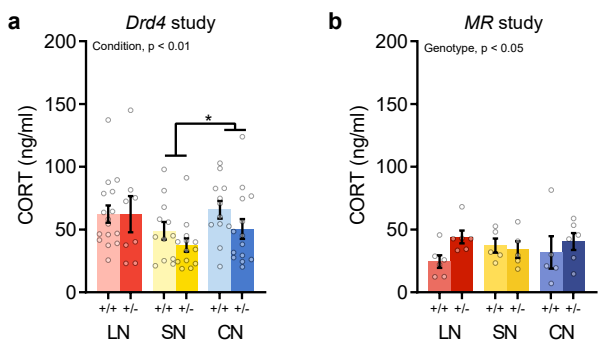

FIGURE 5 **Effects of different rearing conditions and *Drd4* or *MR* genotype on basal corticosterone levels**
Basal corticosterone levels of (**a**) *Drd4*$^{+/+}$, *Drd4*$^{+/-}$, (**b**) *MR*$^{+/+}$ and *MR*$^{+/-}$ mice reared in different environments. +/+: control, +/-: heterozygous *Drd4* or *MR*. Group sizes for *Drd4* study: LN +/+: n = 16, LN +/-: n = 8, SN +/+: n = 12, SN +/-: n = 13, CN +/+: n = 12, CN +/-: n = 14. Group sizes for *MR* study: LN +/+: n = 6, LN +/-: n = 6, SN +/+: n = 5, SN +/-: n = 5, CN +/+: n = 5, CN +/-: n = 6. *p < 0.05.

Although animals discriminated between cue-on and cue-off periods, heterozygous *MR* knock-out mice spent more time freezing during the cue-off episodes than wild-type controls. A significant cue*genotype interaction was observed: the effect was restricted to intervals, but freezing levels during tones were unaffected by *MR* genotype. Rearing condition did not affect freezing levels and did not interact with *MR* genotype to determine freezing behavior during the tones and intervals. Together, these data suggest that while all mice are capable of discriminating between 'threatening' (cue-on) and 'safe' (cue-off) episodes, *MR*$^{+/-}$ mice discriminate less.

3.4 Corticosterone

3.4.1 Drd4 study

Rearing condition significantly affected basal corticosterone levels in *Drd4*$^{+/-}$ and *Drd4*$^{+/+}$ mice (Fig. 5a). Post-hoc analysis revealed an increase in CN reared animals compared to SN, but not LN, mice. Genotype did not alter basal corticosterone concentrations nor interact with rearing condition. Moreover, corticosterone levels did not correlate with any of the behaviors observed.

3.4.2 MR study

No effects of rearing condition were observed in *MR*$^{+/-}$ and *MR*$^{+/+}$ mice (Fig. 5b). However, *MR*$^{+/-}$ mice displayed elevated basal corticosterone levels compared to *MR*$^{+/+}$ controls. No condition*genotype interaction was found and corticosterone levels did not correlate with fear conditioning performance in these animals.

3.5 Western Blotting

MR$^{+/-}$ mice had reduced MR expression in the ventral and dorsal hippocampus compared to *MR*$^{+/+}$ controls (Fig. 6). However, no effects of rearing condition or condition*genotype interactions were observed. In addition, MR expression did not correlate to freezing levels in the fear conditioning paradigm.

4 Discussion

To assess differential susceptibility to rearing conditions in *Drd4*$^{+/-}$ and *MR*$^{+/-}$ individuals in a controlled animal study, we exposed male mice to impoverished, standard or enriched rearing conditions and examined several aspects of adult

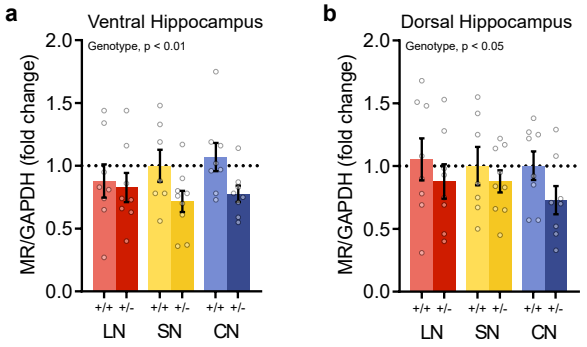

FIGURE 6 **Effects of different rearing conditions and *MR* genotype on MR protein expression**
MR expression levels in the (**a**) ventral and (**b**) dorsal hippocampus normalized for GAPDH expression. +/+: control, +/-: heterozygous *MR*. Group sizes: LN +/+: n = 8, LN +/-: n = 8, SN +/+: n = 7, SN +/-: n = 7, CN +/+: n = 8, CN +/-: n = 8.

behavior. While rearing conditions and genetic background exerted some modest, yet significant effects, other variables were unaffected by these manipulations. Moreover, gene-environment interactions were not supported by our data. Mice reared in impoverished conditions displayed increased locomotor activity, whereas prolonged communal nesting in the *Drd4* study resulted in increased basal corticosterone levels compared to standard nesting conditions. Fear learning was unaffected by rearing conditions, but $MR^{+/-}$ mice showed increased freezing levels during 'safe' periods. In addition, basal corticosterone levels were upregulated in these animals and, as expected, hippocampal MR expression reduced. Together, these results indicate that rearing condition and *Drd4* or *MR* genotype independently affected some, but not all, aspects of adult behavior and markers of the HPA-axis. However, they did not interact to alter spatial learning, anxiety, fear learning, flexibility in fear behavior, basal corticosterone levels or hippocampal MR expression. Altogether, we did not find support for differential susceptibility in male mice, at least for these genes and set of behaviors.

The lack of discrimination in the control group ($SN^{+/+}$) during both retrieval latencies in the OIL task could indicate a generic issue with the protocol or experimental setup. Therefore, the results presented here have to be interpreted with great caution. In contrast to the indications of improved short-term (1h) memory retrieval in CN reared mice during non-stressful conditions observed in this study, communal nesting has previously been reported to not affect spatial memory during stressful experimental conditions in a water maze[51]. It would be interesting to further explore the validity of this difference and the role of testing conditions in future studies. In addition, the lack of differences in discrimination indexes between LN and SN reared mice in our study are in contrast with some[52–54], but not all[55], previous reports. It has been suggested that a preference for the fixed object by some individuals in the OIL task could indicate a coping style rather than a failure to recognize the relocation[56]. However, the individual variation in our study appears to be limited and similar in different experimental groups. Therefore, our experimental approach likely did not result in different coping styles in the OIL task.

Anxiety in the elevated plus maze was not affected by rearing condition in this study. A meta-analysis with papers included till December 2017 (conducted with MaBapp[31], Fig. S1) shows that anxiety behavior is generally increased following LN.

However, this effect appears to be driven primarily by one study using the light/dark box to measure anxiety-like behavior, as exclusion of this paradigm decreased the CI of the effect size to straddle zero. Moreover, in line with our results, no increase in anxiety-like behavior is observed if animals are tested in the EPM (independent studies included in these meta-analyses can be found in Fig. S1). Our findings are not in line with studies reporting anxiogenic effects of a communal nesting rearing environment on EPM performance[57,58], although others have similarly reported no effects of CN in the EPM[59]. In our study we used a shorter CN exposure compared to other experiments (P2-14 in our study vs P0-21 in other studies), which may be an important factor in regulating anxiogenic effects of CN. However, this requires more in depth investigation. Finally, the lack of differences between $Drd4^{+/+}$ and $Drd4^{+/-}$ mice are in agreement with previous reports showing no effects of selective D_4 receptor antagonists on anxiety-like behavior[60,61]. In general, the results presented here provide no evidence in support of a gene-environment interaction between rearing conditions and *Drd4* genotype with regard to alterations in later anxiety-like behavior.

We observed a consistent increase in locomotor activity of LN reared mice during OIL and EPM tasks. Several studies reported no effect of LN on locomotor activity in the EPM and open field test[26,52,54,62,63] or even decreased distance travelled in the open field[64]. In contrast, others have similarly reported increased locomotor activity, specifically in mice reared by abusive-like kicking LN dams[29]. However, because we did not include abusive-like kicking observations in our study, we cannot test whether the increased locomotor activity is restricted to mice raised by abusive dams in our study as well. The studies that reported no effects or a decrease of LN on locomotor activity applied the LN condition from P2-9, whereas we exposed mice to the LN paradigm from P2-14 in this study. Therefore, it is possible that either the severity of the LN paradigm[29], or the duration of the period of exposure plays a role in explaining effects of LN on locomotor activity.

Given the evidence for the role of D_4 receptors in regulating fear memory formation and retrieval[39–41], the lack of differences in freezing levels between $Drd4^{+/+}$ and $Drd4^{+/-}$ mice was in contrast to our expectations. However, because D_4 antagonists dose-dependently alter fear conditioning behavior[40], heterozygous *Drd4* knock-out mice may express sufficient levels of the D_4 receptor to maintain normal freezing levels. Future studies using region-specific and dose-dependent manipulations of the D_4 receptor may elucidate the role of this receptor in regulating fear memory and the potential of early-environmental factors to alter this system.

The increase in freezing levels of $MR^{+/-}$ mice specifically during cue-off periods is in line with earlier studies using full MR knockout mice[35,37] and would mimic the phenotype of LN reared mice[30]. However, this effect of impoverished rearing conditions was not replicated in the *Drd4* and *MR* studies presented here. This difference is unlikely to be due to a discrepancy in timing of behavioral testing, as we demonstrate no effect of circadian phase on fear memory using this paradigm. Moreover, in contrast to previous reports from our lab[65], we observed no effects of LN on dorsal and ventral hippocampal MR expression. In addition, the reduction of MR expression in $MR^{+/-}$ mice was less pronounced in our study than the 50%

reduction observed in previous reports[65]. This limited reduction could be explained by the use of non-naïve animals in our study, as fear conditioning itself likely affects MR expression due to the stressful nature of the test[66,67]. Together, these results indicate that the LN model was unsuccessful in eliciting robust changes in fear conditioning performance or MR expression during adulthood, but provide additional evidence for the role of the mineralocorticoid receptor in response to stressful stimuli[68]. In summary, the dopamine receptor D_4 and mineralocorticoid receptor do not appear to interact with rearing conditions to affect fear learning.

It should be noted that freezing levels and discrimination between tones and intervals differed between the three fear conditioning experiments presented here. While there are numerous explanations for the variability in fear conditioning performance[69], many of the environmental aspects remained identical throughout this study. However, absolute freezing levels and the capacity to discriminate between 'safe' and 'threatening' episodes appeared to decline as testing load increased; mice in the circadian rhythm experiment that showed the highest freezing levels were left undisturbed from birth until testing, whereas *Drd4*$^{+/+}$ and *Drd4*$^{+/-}$ mice that showed the lowest freezing levels were tested extensively throughout their lives. Animals in the *Drd4* study received more bouts of handling, habituation and behavioral testing than mice in the other studies. Since extensive[70], but not limited[71] exposure to repeated handling results in full habituation to the procedure, mice in the *Drd4* study likely have entered the fear conditioning paradigm with lower stress levels compared to mice in the *MR*, and especially the circadian rhythm study. The impact of differences in handling and methodological procedures between laboratories may extend beyond the fear conditioning results. For instance, habituation procedures affect the time it takes for mice to reach inclusion criteria in a novel object recognition test[50]. Moreover, differences in manipulations to the rearing environment such as the use of pregnant-purchased dams for LN exposure affect behavioral outcomes[31]. In addition, exposure to multiple stressful 'hits' later in life appear to enhance effects of adverse rearing conditions (first 'hit)[31]. Together, the use of in-house bred primiparous females, strict environmental control and variations in handling experience all could have contributed to differences between the results presented here and reported elsewhere.

The alterations in basal corticosterone concentrations related to rearing condition in the *Drd4* study and genotype in the *MR* study do not appear to coincide with changes in adult behavior, as no correlations between CORT levels and behavioral parameters were observed. The mild increase in circulating corticosterone levels in CN reared animals compared to SN mice has not been observed previously[44,72], nor in the hippocampus specifically[73]. In addition, plasma corticosterone was higher in the *Drd4* study compared to the *MR* study, possibly reflecting a more general response to uncontrollable environmental factors or repeated testing in this study. Finally, increased plasma corticosterone concentrations in full MR knock-out mice have been reported[74], although others found no effects[35,37]. In females, this increase was restricted to LN reared mice[44]. Although the overall pattern appears similar of in males, the interaction effect of genotype with rearing condition did not reach statistical significance, possibly due to insufficient statistical power. Together, the results presented here suggest that basal corticosterone levels are only mildly altered by manipulations of the rearing environment or MR genotype. However, since stress responsivity is sex-specifically altered by LN and heterozygous MR knock-out[65], studying the effects of CN and *Drd4* manipulations on stress responsivity is required to further elucidate the role of the HPA-axis.

In sum, rearing condition and *Drd4* or *MR* genotype do not appear to interact with regard to the behavioral domains explored here. Naturally, we cannot exclude that other rearing conditions, behavioural domains, genes and developmental windows might lend support to the theory of differential susceptibility. We showed that manipulations of the early-life environment and genotype of mice exert some long-lasting effects but robust effects appeared to be lacking. This highlights the importance of publication of null findings, replications, and meta-analyses in this field of inquiry.

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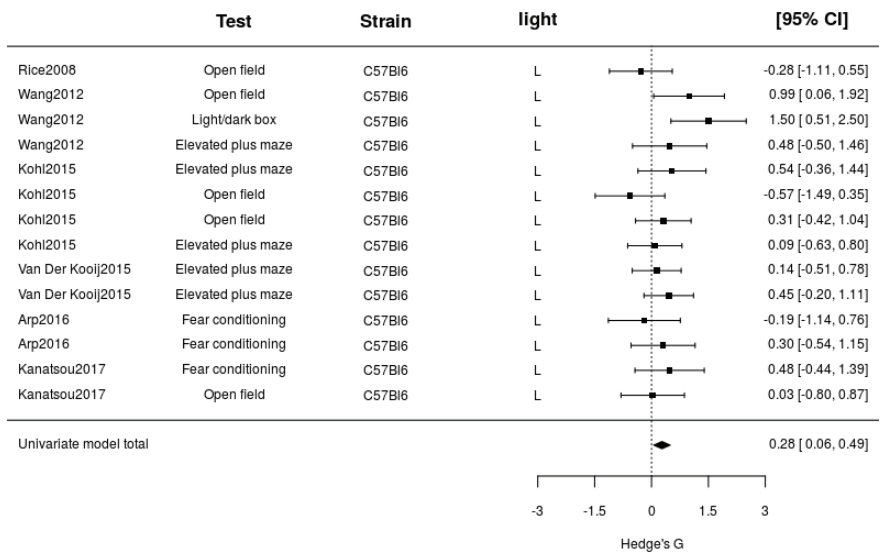

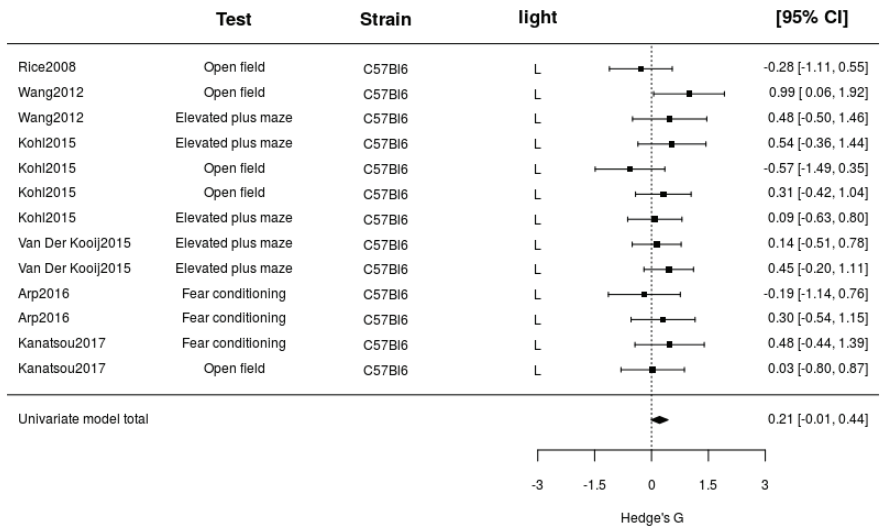

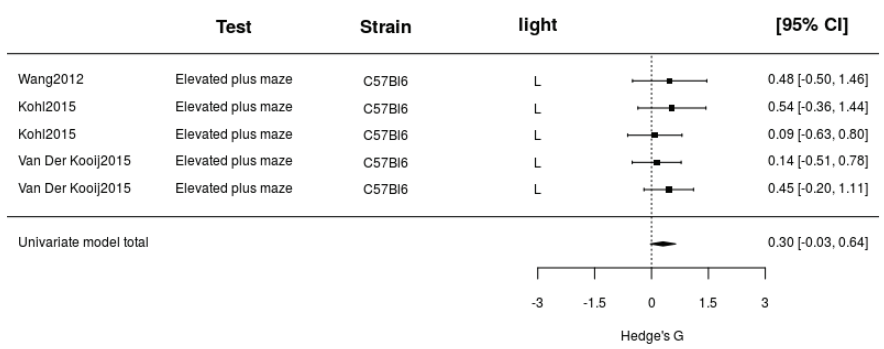

Included studies

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FIGURE S1 **Meta-analyses on anxiety-like behavior**
All anxiety-like behavior (top), anxiety-like behavior not measured using the light/dark box (center) and anxiety-like behavior measured exclusively in the elevated plus maze (down) following limited nesting and bedding rearing conditions in C57Bl/6 mice. LN significantly increases general anxiety-like behavior, but does not significantly affect EPM performance. Analyses conducted with MaBapp. Studies using male C57Bl/6 mice were included.

		MR study		*Drd4* study	
	Litter	+/+	+/-	+/+	+/-
Limited Nesting	1	0	3	0	3
	2	1	2	1	1
	3	1	2	2	1
	4	2	1	2	1
	5	1	1	1	1
	6	1	2	1	2
	7	0	2	1	1
	8	2	1	1	3
	9	1	2	2	0
	10	1	4	2	0
	11	0	3	2	1
	12	0	2	1	2
	13	3	0	3	1
	Total	**13**	**25**	**19**	**17**
Standard Nesting	1	3	1	1	0
	2	1	0	1	3
	3	3	1	3	0
	4	2	1	1	3
	5	2	1	1	3
	6	0	2	1	2
	7	1	1	0	2
	8	1	2	1	3
	9	0	2	2	1
	10	3	1	1	0
	11	1	2	0	2
	12	2	0	1	1
	13			0	3
	Total	**19**	**14**	**13**	**23**
Communal Nesting	1	1	2	0	3
	2	3	0	2	0
	3	1	2	2	2
	4	1	2	2	2
	5	3	1	2	1
	6	3	0	1	3
	7	1	3	2	3
	8	1	3	1	3
	9	1	3	1	3
	10	2	0	3	2
	11	4	1	3	1
	12	3	0	1	2
	13			2	2
	Total	**24**	**17**	**22**	**27**

TABLE S1 **Number of animals per litter used in this study**
+/+: control, +/-: heterozygous knock-out.

Condition	Genotype	Retrieval 1 hour t-statistic	Retrieval 1 hour p-value	Retrieval 24 hours t-statistic	Retrieval 24 hours p-value
LN	+/+	t(14) = 0.72	0.486	t(14) = 3.23	**0.006**
	+/-	t(14) = 0.57	0.576	t(14) = 3.07	**0.008**
SN	+/+	t(10) = 0.97	0.348	t(10) = 1.14	0.283
	+/-	t(19) = 0.36	0.724	t(19) = 2.76	**0.012**
CN	+/+	t(19) = 1.13	0.274	t(19) = 2.12	**0.047**
	+/-	t(21) = 2.49	**0.021**	t(21) = 0.99	0.334

	Hab	Acq	R1	R24
Hab		.446***	.422***	.495***
Acq	.446***		.648***	.646***
R1	.422***	.648***		.743***
R24	.495***	.646***	.743***	

TABLE S2 **Student's t-test against 50% chance level for discrimination index values in the object in location task of *Drd4*$^{+/+}$ and *Drd4*$^{+/-}$ mice (top, Figure 2). Correlation matrix with r-values of distance travelled in all four phases of the OIL task corresponding to the inlay in Figure 2c (bottom)**
+/+: control, +/-: heterozygous *Drd4*. P-values in bold are considered statistically significant. ***p < 0.001.

	Condition F-statistic	Condition p-value	Condition Post-hoc	Genotype F-statistic	Genotype p-value	Condition*Genotype F-statistic	Condition*Genotype p-value	Figure
Object in location								
Acquisition								
Exploration time	F(2, 37) = 1.92	.116		F(1, 38) = 0.89	.351	F(2, 37) = 0.47	.609	
Retrieval 1 hour								
Discrimination index	F(2, 35) = 3.50	**.023**	SN<CN	F(1, 36) = 0.003	.954	F(2, 35) = 0.48	.548	2a
Retrieval 24 hours								
Discrimination index	F(2, 35) = 1.54	.161		F(1, 36) = 0.01	.921	F(2, 35) = 0.24	.803	2b
All phases								
Distance travelled	F(2, 35) = 14.89	**<.001**	LN>SN=CN	F(1, 36) = 5.12	**.030**	F(2, 35) = 1.72	.196	2d
Elevated Plus Maze								
% in open arm	F(2, 37) = 0.14	.861		F(1, 38) = 0.01	.918	F(2, 37) = 0.66	.459	3a
Anxiety index	F(2, 37) = 1.91	.113		F(1, 38) = 0.00	.965	F(2, 37) = 2.29	.076	3b
Distance travelled	F(2, 37) = 2.91	**.047**	LN>CN	F(1, 38) = 0.04	.851	F(2, 37) = 0.28	.858	3c
Corticosterone								
Drd4 study								
Basal corticosterone	F(2, 28) = 6.56	**.003**		F(1, 29) = 1.67	.207	F(2, 28) = 0.33	.670	5a
MR study								
Basal corticosterone	F(2, 30) = 1.03	.371		F(1, 31) = 4.31	**.046**	F(2, 30) = 1.74	.411	5b
MR expression								
MR study								
Ventral hippocampus	F(2, 31) = 0.54	.533		F(1, 32) = 9.10	**.005**	F(1, 31) = 1.42	.335	6a
Dorsal hippocampus	F(2, 31) = 0.30	.713		F(1, 32) = 5.21	**.029**	F(1, 31) = 0.16	.840	6b

TABLE S3 **Statistical tests on the effects of different rearing conditions and *MR* or *Drd4* genotype on F1 different outcome measures**
P-values in bold are considered statistically significant.

	Time F-statistic	Time p-value	Time*Condition F-statistic	Time*Condition p-value	Time*Genotype F-statistic	Time*Genotype p-value	Time*Condition*Genotype F-statistic	Time*Condition*Genotype p-value	Figure
Fear Conditioning									
Circadian rhythm pilot									4b
Baseline vs. first tone	F(1, 14) = 133.34	**<.001**	F(1, 14) = 0.03	.869					
Freezing tones	F(5, 70) = 2.88	**.020**	F(5, 70) = 0.31	.906					
Freezing Intervals	F(5, 70) = 6.09	**<.001**	F(5, 70) = 0.44	.818					
Tones vs. Intervals	F(1, 14) = 150.67	**<.001**	F(1, 14) = 1.26	.281					
Drd4 Study									4c
Baseline vs. first tone	F(1, 113) = 154.18	**<.001**	F(2, 113) = 0.05	.954	F(1, 113) = 0.04	.836	F(2, 113) = 0.21	.815	
Freezing tones	F(5, 565) = 32.20	**<.001**	F(10, 565) = 1.53	.123	F(5, 565) = 0.86	.511	F(10, 565) = 0.70	.724	
Freezing intervals	F(5, 565) = 12.36	**<.001**	F(10, 565) = 1.36	.195	F(5, 565) = 0.32	.902	F(10, 565) = 1.03	.420	
Tones vs. Intervals	F(1, 113) = 104.95	**<.001**	F(2, 113) = 0.49	.613	F(1, 113) = 0.70	.406	F(2, 113) = 1.95	.147	
MR Study									4d
Baseline vs. first tone	F(1, 87) = 322.76	**<.001**	F(2, 87) = 0.25	.781	F(1, 87) = 0.25	.618	F(2, 87) = 1.13	.327	
Freezing tones	F(5, 435) = 8.32	**<.001**	F(10, 435) = 0.50	.892	F(5, 435) = 0.81	.540	F(10, 435) = 1.51	.134	
Freezing Intervals	F(5, 435) = 48.63	**<.001**	F(10, 435) = 1.11	.355	F(5, 435) = 0.47	.801	F(10, 435) = 0.77	.656	
Tones vs. Intervals	F(1, 87) = 120.36	**<.001**	F(2, 87) = 0.52	.594	F(1, 87) = 4.94	**.029**	F(2, 87) = 0.67	.516	

	Condition F-statistic	Condition p-value	Condition Post-hoc	Genotype F-statistic	Genotype p-value	Condition*Genotype F-statistic	Condition*Genotype p-value
Fear Conditioning							
Circadian rhythm pilot							
Baseline vs. first tone	F(1, 14) = 0.14	.717					
Freezing tones	F(1, 14) = 0.30	.594					
Freezing Intervals	F(1, 14) = 0.43	.524					
Tones vs. Intervals	F(1, 14) = 0.01	.943					
Drd4 Study							
Baseline vs. first tone	F(2, 113) = 0.01	.994		F(1, 113) = 0.39	.534	F(2, 113) = 0.11	.894
Freezing tones	F(2, 113) = 3.13	**.048**	ns	F(1, 113) = 0.13	.719	F(2, 113) = 0.42	.661
Freezing intervals	F(2, 113) = 2.89	.060		F(1, 113) = 0.12	.735	F(2, 113) = 0.41	.666
Tones vs. Intervals							
MR Study							
Baseline vs. first tone	F(2, 87) = 0.04	.961		F(1, 87) = 0.53	.467	F(2, 87) = 1.36	.262
Freezing tones	F(2, 87) = 0.87	.423		F(1, 87) = 0.02	.886	F(2, 87) = 0.65	.523
Freezing Intervals	F(2, 87) = 0.34	.716		F(1, 87) = 6.70	**.011**	F(2, 87) = 1.88	.159
Tones vs. Intervals	F(2, 87) = 0.71	.495		F(1, 87) = 1.80	.297	F(2, 87) = 1.23	.297

TABLE S4 **Statistical tests on the effects of circadian phase, different rearing conditions, and *MR* or *Drd4* genotype on F1 fear conditioning behavior**
Values in the time column indicate an effect of first tone exposure for the baseline vs. first tone measure and an discrimination between tones and intervals for the tones vs. intervals measure. For the freezing tones and freezing intervals, the time effect relates to the extinction of freezing behavior over 6 consecutive tones or intervals. P-values in bold are considered statistically significant.

CHAPTER 6

Retrieving the other mother to the nest in a communal nesting paradigm: unraveling a new type of social behavior

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Abstract

Communal nesting is the naturally occurring phenomenon where multiple mouse dams raise their litters together. This form of breeding –compared to singular nest rearing- increases nest occupancy by dams, and pups reared in this condition have more peers to interact with, likely contributing to the enhanced sociability observed in communally reared offspring. However, the social dynamics between dams in a communal nesting setting are poorly studied. We recently observed dams grabbing and fiercely dragging the partner dam back to the nest site. To explore the function and origin of this maternal retrieval behavior, we examined the role of litter size, dominance status and pup retrieval performance. We developed a cost-effective pipeline to continuously record home-cage videos of communally nesting dams with 6 or 12 pup litters using Raspberry Pi devices. Video recordings were then processed using DeepLabCut and relevant video sequences were collected using Python and manually scored. Maternal retrieval behavior occurred in almost all pairs of dams, although the frequency was highly variable. The majority of maternal retrieval was performed by the same dam of the pair (actor). After successful retrieval of the partner (recipient), the actor preferred to leave the nest site. Higher rates of maternal retrieval did not result in higher nest occupancy or increased body weight gain of pups or dams. Maternal retrieval was not affected by litter size. Dominant dams (dominance being determined with a tube test) were equally likely to be actors of maternal retrieval behavior as subordinate dams. Finally, actors did not differ in pup retrieval performance compared to recipients, arguing against the possibility that maternal retrieval behavior results from a generalized drive to retrieve. We conclude that maternal retrieval behavior may be the result of subtle social dynamics between dams, with a hitherto unknown function in communal nesting.

1 Introduction

The early-life environment is of crucial importance for survival and development of offspring. In mammals such as humans and rodents, parental care is among the most important sources of early-life environmental variation, thereby playing a critical role in offspring development. While parental care in humans is usually provided by both parents in most cultures[1], mice display exclusively maternal care under natural circumstances[2]. Yet, wild mice are often reared by multiple dams, as up to 80% of feral female mice rear their offspring in communal nests[3]. In this condition, a nest site is shared and dams take care of their offspring together, nursing all pups without discriminating between own and other pups[4,5].

Cooperative breeding, assistance of rearing offspring by non-parents, covers a variety of breeding systems[6]. Humans can be considered a cooperative breeding species too and this allowed our species to produce extremely costly and slowly maturing offspring[7]. The display of communal nursing is however quite rare for mammals and has raised interest over the past decades. Kin recognition does not induce discrimination in nursing towards individual pups, but might affect the choice of whom to form a communal nest with[5]. It has been suggested that communal nursing is costly behavior associated with communal nesting and non-adaptive by itself as the dams also nurse non-offspring[8]. Some researchers have argued that communal nesting increases pup survival through increased levels of nest defense[4]. Others have shown that communal nests of wild mice often include litters from multiple sires and that higher numbers of sires in a communal litter improve offspring survival, whereas higher numbers of dams do not[9]. Hence, these authors suggest that communal nesting creates paternity confusion and thereby reduces infanticide by males.

The effects of communal nesting are not restricted to increased pup survival, as development of pups reared in a communal nest is also different from pups reared in a singular nest setting. Pups reared in communal nests display characteristics of improved sociability later in life[10–13] that are likely mediated by differences in levels of neurotrophins such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF)[14]. These alterations might be explained by the complex social dynamics in the communal nesting (CN) condition. Nest occupancy by at least one dam is generally increased in the CN condition[11,15,16] and in addition to sibling interactions, pups in the CN condition have access to other peers[17]. However, dams in a communal nesting setting interact with each other too, an aspect that to our knowledge received no attention in the literature.

Indeed, while live scoring maternal behavior of communally nesting pairs, we observed fierce bouts of interactions between the dams unlike regular social behavior observed in non-nesting groups of mice (see supplementary video 1). We noticed how one dam would grab the other dam and retrieve or attempt to retrieve her back to the nest site. This behavior was observed infrequently but in many different cages and raised questions with regard to the origin and function of this maternal retrieval behavior. Does the behavior depend on the demand from the nest site? What happens after the retrieval, is it functionally relevant? Do both dams perform similar levels of maternal retrieval behavior or is there directionality? If so, does directionality depend on dominance status of the dam? Or are the dams so

focused on performing maternal behavior that they retrieve anything, even other dams, back to the nest site, i.e., is it related to pup retrieval performance? Finding answers to these questions may shed light on the complexity of social behavior in a communal nesting setting and may help to understand the factors contributing to the early social enrichment provided to pups in a communal nesting rearing environment.

In our earlier studies, maternal retrieval behavior was observed infrequently, making live scoring an impractical method of quantification. We therefore developed a pipeline to continuously monitor two dams in a communal nesting setting and facilitate feasible quantification of maternal retrieval behavior. In this study, we recorded the home cages of pairs of communally nesting dams continuously throughout the first postnatal week of the litter. We used open source DeepLabCut (DLC) tracking software to determine the location of the two dams. DLC is a markerless pose estimation program that uses transfer learning and deep neural networks to achieve reliable tracking performance in a variety of lighting conditions[18,19]. Using the tracking data, we then filtered out the video fragments containing potential maternal retrieval related events and used these for manual scoring.

Using this set-up, we studied the effect of litter size manipulation and dominance status on the frequency and directionality of maternal retrieval behavior. Behavior of dams taking care of a mixed litter containing 6 pups was compared to the behavior of dams with 12 pup litters. Both dams were tested on pup retrieval performance to study the relation between maternal retrieval and pup retrieval. Dominance of the pairs of dams was assessed using the tube dominance test[20] and maternal retrieval was compared between dominant and subordinate dams.

If maternal retrieval behavior is performed because of a high demand from the nest, we expect this behavior to be increased in cages with 12 pups compared to litters containing 6 pups. If maternal retrieval is merely a by-product of the intense drive of mothers to perform maternal behavior, dams performing more maternal retrieval are expected to be better pup retrievers. If the behavior is a personality trait, one dam might consistently exhibit more maternal retrieval than the other. This directionality could be related to dominance status of the dams. Our study is exploratory in nature and should therefore be regarded as a first step in elucidating potential avenues for future research.

2 Materials & Methods

2.1 Animals & Housing

Wild-type albino C57BL/6 mice were used in this study and bred in-house. The BL6 background was chosen to improve comparability to previous studies[11,16] while the white fur facilitated individual recognition by color marking. A total of 36 dams (18 pairs) were used for this study, divided in three batches (5, 5 and 8 cages, respectively). Animals were communally housed with either 6 or 12 pups in the litter; 6 pup litters always contained 3 pups from each sex, 12 pup litters contained at least 5 pups from one sex. Continuous home-cage video recordings were made from postnatal day (P) 2-9. At P6 and P7, a pup retrieval test was performed for both mothers in the home cage in turn. After weaning of the litters, a dominance tube test was conducted to assess relative dominance of the two dams. Throughout all experiments, regular chow and water was available ad libitum and animals were housed on a reversed LD cycle (light off 08:00-20:00, temperature 21-22 °C, humidity 40-60 %). All experiments were performed in accordance with the EC council directive (86/609/EEC) and approved by the Central Authority for Scientific Procedures on Animals in the Netherlands (CCD approval AVD115002016644)

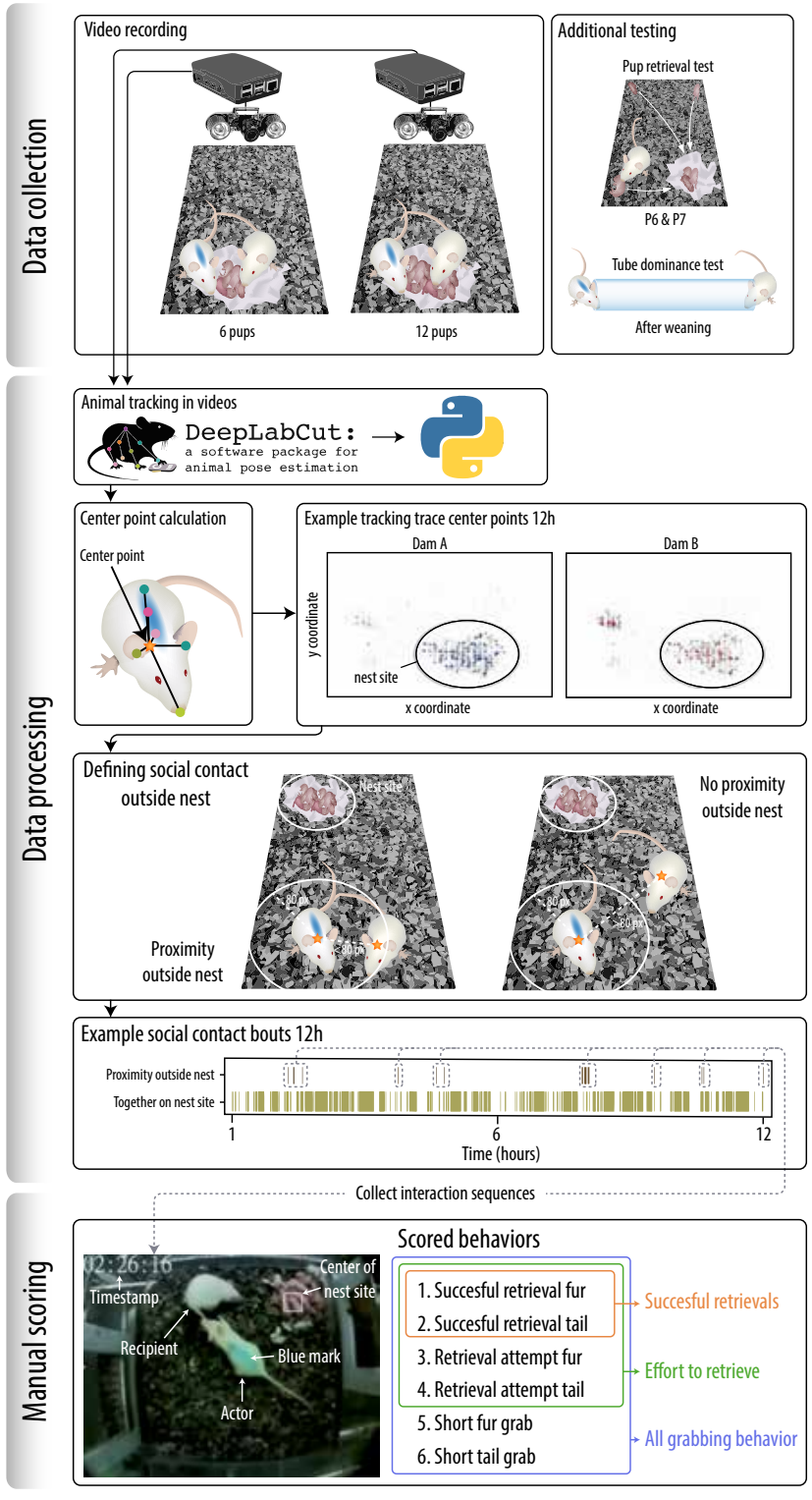
2.2 Breeding conditions

Litters were generated by pairing one male with two females for 4 days, after which the females were co-housed until approximately one week prior to giving birth. Dams were then housed in type II short Makrolon cages (21.5 x 16 cm) and provided with cotton fiber Nestlets (5 x 5 cm, Technilab-BMI, Someren, The Netherlands) that can be shredded to form an open nest site. Around parturition, all dams were checked daily at 09:00 h for the presence of litters and the day prior was assigned as P0 if a litter was born. Dam and litter were weighed at P2 and co-housed with another dam and litter from a different father that gave birth on the same day. Pairs of dams and litters were randomly assigned and culled to form a small (6 pups) or large (12 pups) nest. To facilitate individual recognition of the dam and pups, one dam and her litter were marked. Pups were marked using a non-scenting, non-toxic surgical marker (ArcRoyal, Ireland). Dams were marked by shaving a small patch of fur on the back of the dam (for dark phase recognition), and coloring the fur around the patch with a hair dye (for light phase recognition, Tish & Snooky's, Manic Panic, Rockabilly Blue). Of note, removal of fur was not performed in the first batch of animals, making it impossible to distinguish the two dams in infrared video recordings for this batch. The coloring procedure consisted of applying a small drop of dye on the back of the dam using a long cotton swab. The dam was then placed on the lid of her home-cage in order to distract her, thus preventing the dam from licking the dye. If the dam started grooming and licking the dye, she was distracted using a clean long cotton swab. After 10 minutes, excess dye was rinsed of with water and the dam was then briefly fixated and dried using paper towels. The non-marked dam received a sham procedure where no dye was applied.

After marking, dams and litters were co-housed in Plexiglas cages (30 x 30 cm) with one Nestlet for communal nesting. Earlier pilot studies revealed that an additional Nestlet for 12 pup communal nests was not used by the dams, indicating that one Nestlet was sufficient for optimal nest quality. The water bottle and food container were positioned at one side of the cage on the outside to prevent obstruction of view. Sawdust was painted black in order to increase contrast between the bottom of the cage and the white mice. To dye the sawdust, black acrylic paint was diluted with water (1:3 dilution) and carefully mixed with regular sawdust. The dyed sawdust was then spread out in a layer of ±2 cm and air dried until fully dry.

2.2 Home-cage video recording

Continuous video recordings of the home-cages were made from the start of entering the CN condition at P2 until P9. Each cage was equipped with a top view IR-CUT camera (SOS Solutions, The Netherlands) connected to a Raspberry Pi 3 device (SOS Solutions, The Netherlands). This type of camera records infrared



← FIGURE 1 **Study design and dataflow.**
Data collection: Litters containing 6 or 12 pups were recorded continuously using cameras connected to raspberry pi devices. A pup retrieval test was conducted at postnatal day (P) 6 and 7. After weaning of pups, a tube dominance test was performed to assess dominance status of pairs of dams.
Data processing: Animals were tracked using DeepLabCut, after which data was processed in Python. Center points of the two dams were calculated and used for localization of each dam in the cage. Frames of the videos in which social proximity outside the nest area was determined were then collected and used for manual scoring.
Manual scoring: Relevant sequences were manually scored for succesful retrievals, retrieval attempts and grabbing behavior. These behaviors were categorized into three domains: succesful retrievals, effort to retrieve and all grabbing behavior.

videos, as used for dark phase recordings, and color videos, used for light phase recordings. Raspberry Pi devices were programmed to switch from infrared to color recordings at the time of the light switch. This process required a reboot of the Raspberry Pi device, leading to the splitting up of video material into 12 hour long video files. Fish eye smartphone lenses (MikaMax) were mounted to the cameras in order to capture the entire cage. Video recordings were captured at a 640x480 pixel resolution at 15 frames per second (fps).

2.3 Selection of relevant video material

To select video sequences of interest for this study, continuous video recordings were processed using a series of automated steps. First, videos were downsampled (see 2.3.1), to increase processing speed. Step two consisted of using DeepLabCut to track the position of the two dams in the cage (2.3.2). Datafiles were then analyzed in Python to define relevant video sequences (2.3.3). Finally, these sequences were cut from the original video files to obtain potentially relevant video material for manual scoring of maternal retrieval (2.3.4). All Python code is available online (https://osf.io/9g2a8/).

2.3.1 Step 1: Downsampling

Original video files (640 x 480 pixels, 15 fps) were downsampled into video files of 320 x 240 pixels and 1 frame per second using the ffmpeg-python package in Python 3.7. Resolution was thus decreased 4 times and the frame rate was decreased 15 times, resulting in drastically decreased file sizes. This step was required to achieve reasonable processing speed in step 2, while maintaining acceptable image quality.

2.3.2 Step 2: DeepLabCut tracking

The downsampled videos were then used for automated animal tracking using the multiple animal module of DeepLabCut 2.2b7, an open source tracking software. To train the network, 120 frames from 10 different cages were selected manually with emphasis on off nest location of the dam(s). For each cage, a short fragment where at least one dam was off the nest site was selected from four different days throughout the entire week of recording. From these fragments, DLC selected 3 frames. Hence, 3x4x10=120 frames were labelled to train the network. This method of sampling frames was chosen to include high variability in training frames and sufficient frames containing dams located off the nest site, contributing to better performance of the model.

A total of 10 body points were labelled for each dam: nose point, left ear, right ear, shoulders, spine1, spine2, spine3, tailbase, left flank and right flank. If the view of certain body points was obstructed, such as by nesting material, no label was set, ensuring that DeepLabCut would not guess the location of invisible body points. Using these labelled frames, the model was trained with a maximum of 50 000 iterations, in line with the recommendations for multiple animal training on the DLC YouTube channel.

The model was then used to track the position of the two dams in the downsampled videos. Videos in which the body points were labelled were made and visually inspected for correct allocation of the body points. Tracking was deemed satisfactory (see sample video) and the x, y coordinates of each body point for each dam were imported in Python for further analysis.

2.3.3 Step 3: Define relevant video sequences

To determine the position of each dam in the cage, a center point was calculated from the x, y body points coordinates given by DLC. Each body point with a likelihood of >0.95 was included, removing body points where the model was unsure of correct allocation. This occurred predominantly when the dam was on the nest site and for instance the nose point and ears were obstructed by nesting material. Then, the mean x, y coordinates of remaining body points were used as an estimate of the true location of each dam.

For each frame, the position of each dam was determined in relation to the nest site. Since dams spend most (>50%) of their time on the nest site, the median x,y coordinates of each dam in each 12 hour video was calculated and the two points were averaged to calculate the center of the nest site. This automated approach was validated in the manual scoring step for each video. The nest area was then defined as a circle around this center point with a 150 pixel diameter (determined by optimization). Using the ellipse equation, it could be calculated whether a dam was in or outside the nest area within each timeframe.

Relative distance between the dams was then calculated using Pythagorean theorem. A relative distance of <80 pixels (~15 cm, found by optimization) was regarded as social proximity. Using this criterion, the frames in which the two dams were in close proximity outside of the nest area were determined. These frames were deemed of interest for this study and used for selecting video sequences for manual scoring.

2.3.4 Step 4: Collecting relevant video sequences

The frames of interest determined in the previous step were used to cut video sequences from the original video files (640 x 480 pixels, 15 fps) using the ffmpeg-python package in Python. A period of 10 seconds before and 50 seconds after the frame(s) of interest was chosen to observe behavior before and after the retrieval. To facilitate timing of the behavior and validation of nest site localization, output videos were provided with a time stamp and a square box at the automatically determined location of the nest site.

2.4 Manual scoring of maternal retrieval

Social behavior related to the retrieval of dams was then manually scored. Individual recognition of the mice in all cages was only possible during the light phase, so only light phase video recordings were analysed at this stage. After initial inspection of video material from 4 different cages, 3 behaviors were defined. A successful retrieval was defined as one dam grabbing and redirecting the other dam back to the nest site. A retrieval attempt was defined as one dam grabbing and redirecting the other dam, but failing to relocate her to the nest site. A short grab was defined as one dam grabbing the other dam and briefly pulling, but failing to relocate the other dam. These three behaviors occurred by either grabbing the fur or tail of the other dam, resulting in quantification of 6 different behaviors. If the grabbing dam (actor) let go of the other dam (recipient), this produced a new behavioural score. So, when a dam briefly grabbed the other dam, let go, and then performed a successful retrieval, this would be scored as two separate behaviors.

Behaviors were then categorized into three categories. Successful retrieval was defined as all successful retrieval by grabbing the fur or tail. The effort to retrieve was defined as all retrieval behavior (successful and attempted) with the clear intention of relocating the other dam back to the nest site. All grabbing behavior included the three types of behavior: shortly grabbing the other dam, retrieval attempts, and successful retrievals.

After successful retrieval, the behavior within the 50 seconds following the event was monitored. Four options were scored; 1) the actor and recipient both left the nest site, 2) the actor left the nest site while the recipient dam stayed, 3) the actor stayed on the nest site while the recipient dam left, 4) the actor and recipient both stayed on the nest site.

2.5 Pup retrieval test

A pup retrieval test was conducted at P6 and P7 between 10:00-12:00 h. Both dams were removed from the home cage for 5 minutes before the first pup retrieval test to ensure separation from the pups for both dams. Three pups, including pups from both dams, were placed in three corners of the home cage distant from the nest site while the remaining pups stayed in the nest site. After 5 minutes, one dam was placed back in the home cage facing the corner away from the pups. Retrieval behavior was video recorded for 5 minutes and analyzed using Observer XT 10.5 (Noldus, The Netherlands). The latency to retrieve all 3 pups back to the nest was scored. After the first pup retrieval test, the first dam was removed from the home cage and the second dam was tested using three different pups. The dam tested secondly on P6 was tested first on P7. The average latency of the two pup retrieval tests was used for analysis. If a dam failed to retrieve all three pups on either P6 or P7, this dam was considered unsuccessful in completing pup retrieval. 5 out of 36 dams did not retrieve all pups within 5 minutes on both days. After pup retrieval by the second dam, the first dam was returned to the home cage too.

2.6 Tube dominance test

After weaning of the pups, the dams remained co-housed in pairs and a tube dominance test was conducted in line with the protocol described by Fan et al. (2019). In this test, mice simultaneously enter opposite sides of a tube that allows only one mouse to walk through and is too small to turn around. The two mice then meet in the center of the tube, where a social confrontation determines

which mouse continues to walk forward and which mouse walks backwards to exit. The first is considered the dominant mouse, the latter the subordinate.

The protocol consists of three phases: 1) habituation to handling and the tube, 2) training to walk through the tube and 3) testing dominance. During the first phase, a transparent PVC tube (14 cm, Ø36,2 mm, PVCvoordeel.nl, The Netherlands) was introduced into the home cage. This tube had a slightly bigger diameter compared to the test tube, encouraging the mice to explore and walk through this object. In addition, mice were habituated to handling for 2 minutes twice a day for 5 consecutive days by scooping and releasing into the habituation tube.

A longer and thinner test tube (30cm, Ø28,4mm, PVCvoordeel.nl, The Netherlands) was placed in a type IV Makrolon cage (34 x 46cm) for the training phase. Each dam was placed (one at a time) in the set-up and allowed to freely explore for 1 minute before being gently guided towards the test tube using one or two gloved hands, encouraging the mouse to enter the tube. This process was repeated 10 times per day with a 20 seconds interval for each dam (5 entries from each side of the tube), on 3 consecutive days. By the end of the training phase, all mice readily walked through the tube directly upon release from the hand.

The testing phase started with a 1-minute exploration time of the box, after which the first trial started. Both dams were picked up and simultaneously released on opposite sides of the tube with the body already inside the tube. The trial ended when one dam got out of the tube with all 4 paws. Both mice were then allowed to freely move around for 20 seconds in the experimental set-up before the next trial started. Mice completed 5 trials per session (alternating starting sides of the tube) for 4 sessions on 2 separate days. The morning session took place between 10-12 am, the afternoon session between 14-16 and the mice were left undisturbed for at least 3 hours between sessions. If the mice did not meet in the center of the tube, this trial was excluded. The tube and box were carefully cleaned using a 70% ethanol solution and paper towels between different cages.

All trials were video recorded and analyzed using Observer XT 10.5 (Noldus, The Netherlands). The number of wins divided by the number of successful trials was used to calculate a dominance index. This index was then used to dichotomize dominance status of the dams, where a dominance index of >0.5 was considered dominant.

2.7 Statistical analysis

All data are expressed as mean ± SEM and statistical tests were performed using SPSS 27 (IBM). Ranges of data are depicted in brackets. Statistical significance was set at $p < 0.05$. To assess directionality of maternal retrieval related behavior, the percentage of the total number of observations in the cage was calculated for each dam. All grabbing behavior was chosen to determine which mouse was considered the actor (>50% of total events) or the recipient (<50% of total events). The grabbing style (fur or tail) was determined by calculating the percentage of fur grabs taking into account all grabbing behavior. Dams that exhibited less than 3 grabbing events (n=8) were excluded from this descriptive measure.

To determine the effects of litter size manipulation, the body weight gain of pups from P2-9, average time spent on the nest site by dams and maternal retrieval behavior were calculated for each cage (1 cage = 1 N). Body weight gain was analyzed using an independent samples t-test. The average time spent on the nest site was analyzed using a repeated measures ANOVA. Due to loss of video material, 10 out of 126 data points were imputed for this analysis using the replace missing values module in SPSS. To assess whether body weight gain of the pups was mediated by nest presence of the dams, a mediation analysis was conducted using the PROCESS v3.5 macro for SPSS[21]. Significant mediation was assigned when the 95% confidence interval did not include zero. Maternal retrieval behavior was positively skewed and analyzed using a Mann-Whitney U test.

To explore whether higher rates of maternal retrieval behavior provide functional advantages for dams or pups, cages were divided in low retrieving cages and high retrieving cages using a median split on successful retrievals. Average nest presence and body weight gain of pups and dams were compared between low retrieving cages and high retrieving cages with an independent t-test. To control for the effects of litter size on nest occupancy and body weight gain of pups and dams, Z scores were calculated separately for litters containing 6 or 12 pups.

Dams winning >50% of trials in the tube dominance test were considered dominant and dams winning <50% of trials were considered subordinate. Because of the dependent nature of the data in comparing pairs of dams, paired analyses were performed. A paired samples t-test was used to compare body weight of dominant versus subordinate dams. Maternal retrieval related behavior was analyzed using a Wilcoxon signed rank test. To analyze pup retrieval behavior of actors vs. recipients and dominant vs. subordinate dams, a cox regression analysis was used in GraphPad Prism 9.0.2. This test takes both latencies and completion rates of pup retrieval into account, analyzing retrieval performance as a survival curve[22].

3 Results

3.1 Maternal retrieval observations

From 1280 hours of original video material, a total of 438 events related to maternal retrieval behavior were observed during the light phase in all cages combined. High variability between cages was observed; some pairs of dams did not perform any maternal retrieval behavior, whereas other dams displayed the behavior readily (successful retrieval: 3.06 ± 0.96, [0, 18]; effort to retrieve: 13.22 ± 2.92, [0, 45]; all grabbing behavior: 24.33 ± 5.28, [0, 80]). Maternal retrieval behavior rarely occurred at the start of communal nesting at P2, but increased over time reaching the highest levels around P6 (Fig. 2a). Highest levels were observed in the beginning of the light phase, after which smaller peaks were observed every 3-4 hours (Fig. 2b). This pattern was also observed when only attempted retrieval or grabbing behavior were considered (supplementary Fig. S1a).

The majority of dam pairs displayed a strong directionality in maternal retrieval related behavior; in 7 out of 11 cages where successful maternal retrieval was observed, all events were performed by the same mouse. A similar pattern was observed for the effort to retrieve and all grabbing behavior, where one dam performed the majority of events in most cages (Fig. 2c). The deviation from a 50/50 distribution in most cages suggests that this behavior is not random but

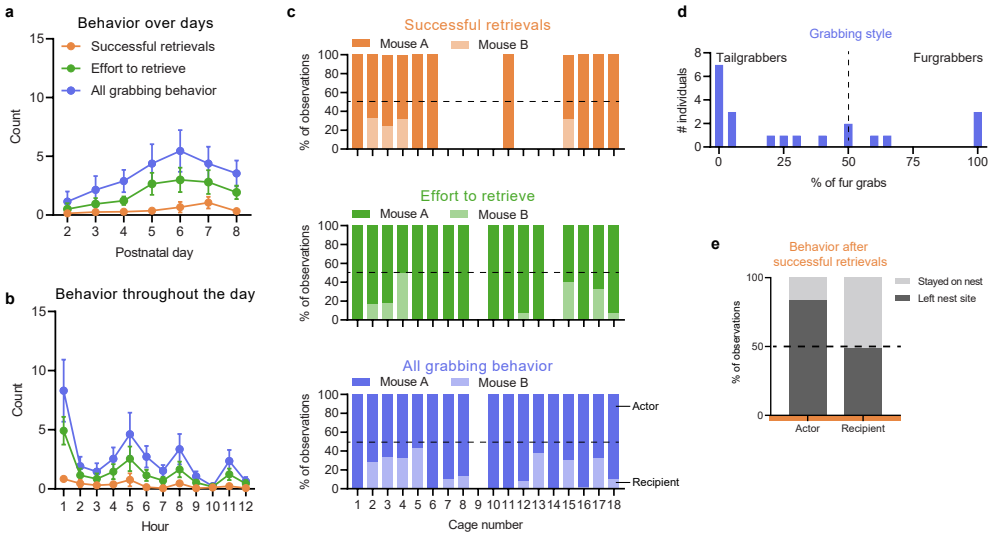


FIGURE 2 **Maternal retrieval related behavior during communal nesting.**
(**a**) Maternal retrieval related behavior over postnatal days and (**b**) daily rhythm (collaped over P2-8) during the light phase of the light-dark cycle. (**c**) Distribution of behavior in different domains of maternal retrieval between pairs of dams. One dam performed the majority of events (actor) over the other dam (recipient) in most cages. (**d**) Grabbing style: the percentage of observed fur grabs in all grabbing behavior. Individuals to the left side are considered tailgrabbers, whereas individuals on the right side are considered furgrabbers. (**e**) Behavior after succesful retrievals. Actors generally left the nest site after retrieving the recipient, wheres recipients did not show a preference for staying or leaving the nest site.

rather a stable characteristic from one dam to the other. In addition, the body point of grabbing appeared to be a consistent behavior, as 13 out of 21 dams that performed more than three grabbing events did so by grabbing the other dam (almost) exclusively by the fur (n=3) or tail (n=10, Fig. 2d).

A total of 55 successful retrieval events were observed. In 84% of these cases, the dam performing the retrieval (actor) left the nest site within 50 seconds after the retrieval (Fig. 2e). The recipient dam was equally likely to stay on or leave the nest, i.e. left the nest site in 49% of events.

3.2 The effect of litter size on communal nesting dynamics

Pups in litters of 12 pups gained less body weight than pups reared in litters of 6 pups ($t(16) = 2.39$, $p = .03$, $\eta^2 = 0.26$, Fig. 3a). However, the body weight gain of large litters was similar to single housed dams (supplementary Fig. S1b), indicating healthy pup growth in large litters and larger than normal pup growth in small CN reared litters. Dams rearing a large litter spent less time on the nest site compared to dams in the small litter condition ($F(1, 16) = 4.98$, $p = .04$, $\eta^2 = 0.24$, supplementary figure Fig. S1c). This resulted in a lower nest occupancy of larger litters, meaning there was less often at least one mother present on the nest ($F(1, 16) = 5.45$, $p = .03$, $\eta^2 = 0.25$, Fig. 3b). However, mediation analysis revealed that the reduction in body weight observed in the pups was not significantly mediated by the decreased nest occupancy of larger litters (95% CI = [-0.19, 0.40]).

Litter size did not alter maternal retrieval behavior (Fig. 3c). Successful retrievals ($U = 38.00$, $p = .82$, $\eta^2 = 0.003$), effort to retrieve ($U = 36.50$, $p = .72$, $\eta^2 = 0.007$) and all grabbing behavior ($U = 37.00$, $p = .76$, $\eta^2 = 0.006$) were not affected by condition.

3.3 Potential benefits of maternal retrieval behavior

Nest occupancy in cages with high maternal retrieval observations was not increased compared to cages with low retrieving observations ($t(16) = 0.069$, $p = 0.95$, $\eta^2 = .000$, Fig. 4a). In addition, pups reared in cages where high levels of maternal retrieval were observed did not gain more body weight compared to pups reared in cages with low retrieval observations ($t(16) = 1.105$, $p = 0.29$, $\eta^2 = .071$, Fig. 4b). Dams themselves also did not differ in body weight gain over the first postnatal week ($t(16) = 0.693$, $p = 0.50$, $\eta^2 = .029$, Fig. 4c). These results suggest that maternal retrieval behavior did not promote nest occupancy, pup growth or dam body weight.

3.4 The effect of dominance status on retrieval behavior

The tube dominance test revealed clear dominance in most of the pairs of dams (Fig. 5a). All trials were won by the same dam in 9 out of 18 pairs and in 4 other pairs the subordinate dam only won one trial. Tube dominance was not related to differences in body weight of the dominant and subordinate dams ($t(34) = 0.77$, $p = .45$, $\eta^2 = 0.017$, Fig. 5b). In addition, the preference for grabbing the tail or fur of the other dam was not an indication of dominance status (see supplementary Fig. S1d).

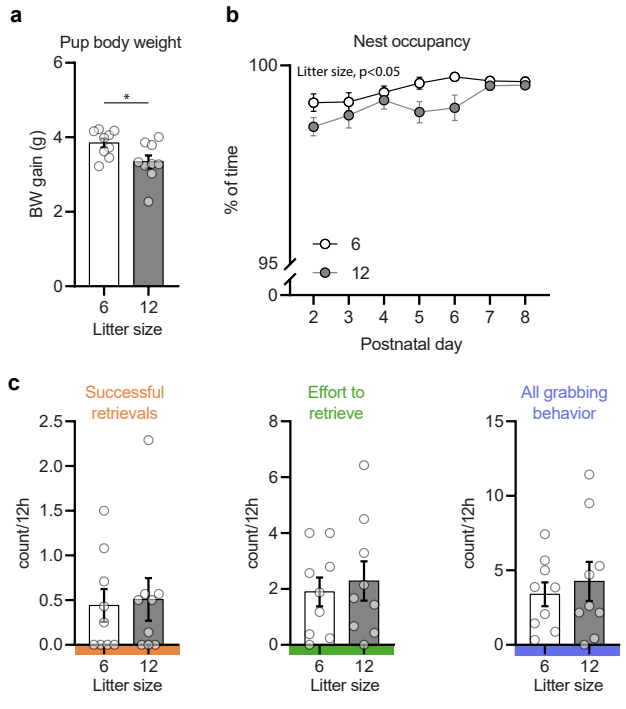


FIGURE 3 **Effects of litter size on communal nesting characteristics.**
(**a**) Body weight gain of pups from P2-9. (**b**) Nest occupancy of the nest site over postnatal days. (**c**) Succesful retrievals, effort to retrieve and all grabbing behavior in cages containing 6 or 12 pups. Data is averaged over all postnatal days and presented as a count per 12 hours. Each dot represents the average (body weight) or sum (retrieving behavior) per cage. Group size: n=9 for both conditions. *p < 0.05.

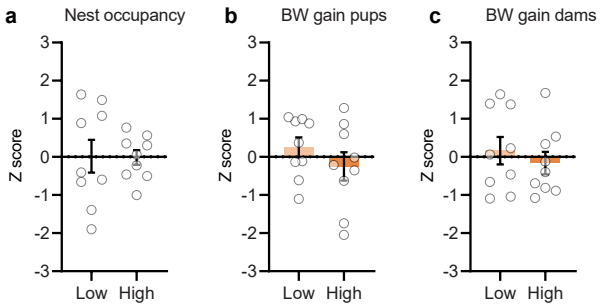


FIGURE 4 **Effects of low or high levels of succesful maternal retrievals on nest presence and body weight gain.**
(**a**) Nest occupancy, (**b**) body weight gain of pups from P2-9 and (**c**) body weight gain of dams from P2-9 for cages in which dams exhibited low (left bars) or high (right bars) levels of succesful maternal retrieval behavior. Each dot represents the average of one cage. Group size: Low: n = 9, High: n = 9.

When taking into account the most active maternal retrieving dams (dams mostly being the actor in a retrieval related behavior), 8 out of 17 were considered to be the dominant dam and 9 the subordinate in the tube test (Fig. 5c). This indicates that the dams that perform most of the maternal retrieval related behavior did not exhibit dominant behavior in the tube test. Moreover, the dominant dam in the tube test did not show more successful retrievals (Z = -0.845, p = .40), effort to retrieve (Z = -0.682, p = .50) or grabbing behavior (Z = -0.355, p = .72) during communal nesting compared to subordinate dams (Fig 5d-f left panels). However, the highest levels of these behaviors were consistently observed in dams that were most dominant in the tube test as well, i.e. those that won all or almost all of the trials in the tube test (Fig. 5d-f, right panels).

3.5 The relation between maternal retrieval and pup retrieval behavior

To explore the relation between maternal retrieval related behavior and pup retrieval performance, pup retrieval behavior of dams was analyzed. When comparing dominant dams to subordinate dams, survival curve analysis revealed that dominant dams were slightly inferior pup retrievers compared to subordinate dams ($\chi^2(1, N = 46) = 3.86$, p = .0496, Fig. 5g). They were slower in retrieving the pups and more dams failed to retrieve all the pups back to the nest site. Regarding maternal retrieval related behavior, no difference in pup retrieval behavior was observed between dams being predominantly actors versus recipient dams ($\chi^2(1, N = 34) = 1.30$, p = .25, Fig. 5h), indicating that dams that exhibit more maternal retrieval related behavior did not differ in pup retrieval performance from dams that show lower levels of this behavior. Together, these results provide no evidence to support the notion that maternal retrieval related behavior is related to pup retrieval behavior.

4 Discussion

The work presented here is the result of a preliminary effort to describe and understand a particular social behavior observed in communally nesting dams. Maternal retrieval behavior has not been described in literature before, and relations to the social enrichment provided by communal rearing have not been studied. In this study we show that maternal retrieval related behavior is infrequent and highly variable, yet occurs in the majority of dams that raise a litter

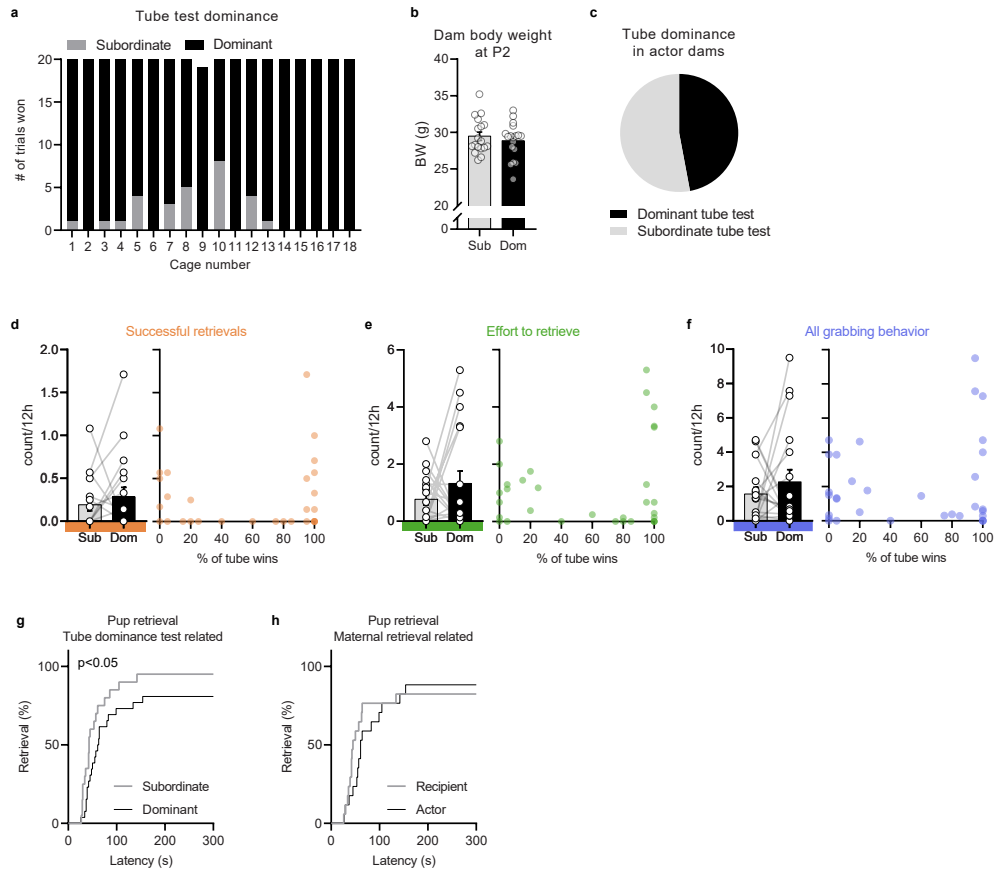


FIGURE 5 **Effects of dominance rank on maternal retrieval related behavior and pup retrieval performance.**
(**a**) Dominance rank assignment by tube test results, dams winning the majority of trials were considered dominant. (**b**) Body weight of dams at P2. (**c**) Distribution of dominant and subordinate dams in the dams that exhibited the strongest drive to retrieve during communal nesting. (**d**) Succesful retrieval, (**e**) effort to retrieve and (**f**) all grabbing behavior of subordinate and dominant dams (left panels). Each dot represents one individual, lines connect the two individuals belonging to the same cage. Data is averaged over all postnatal days and presented as a count per 12 hours. Right panels depict the levels of maternal retrieval related behavior expressed as a function of the rate of dominance (% of tube test wins). (**g**) Pup retrieval performance of dominant and subordinate dams according to the tube test. (**h**) Pup retrieval performance of actors and recipients of maternal retrieval during communal nesting.

together. The majority of maternal retrieval related behavior observed in one cage was usually performed by the same dam, and that dam generally left the nest site after successfully retrieving the partner back to the nest site. However, the data presented here provide no evidence in support of a role of litter size in triggering maternal retrieval and no apparent benefits of maternal retrieval for the offspring (nor the dam) have been observed: higher levels of maternal retrieval did not coincide with higher nest presence by the dams or increased body weight gain of pups or dams. In addition, dams that perform the majority of maternal retrieval in a cage (actors) are not necessarily dominant over the other dam (recipient). Finally,

actors do not perform differently in a pup retrieval test compared to recipients, likely refuting an alternative explanation for maternal retrieval to be a by-product of a strong drive to retrieve anything back to the nest site.

Studying an infrequent behavior such as maternal retrieval requires prolonged observation of the home cage. Until recently, these studies would be immensely time consuming, expensive and/or invasive to the animals. A tremendous development in easily accessible open source programs and techniques over the past years now allow for feasible, affordable, automatic and continuous behavioral observations for all sorts of purposes[23]. Raspberry pi devices offer low-cost and completely modifiable alternatives to commercially available set-ups[24–27]. In addition, tracking software such as DLC[18,19], but also other open source programs such as JAABA[28], have unprecedented accuracy in tracking of animals, outperforming commercially available programs and reaching human-level accuracy[29]. Although not utilized in this study, other programs can use these tracking datasets and apply machine-learning techniques to automatically quantify behavior[30]. Researchers can now start building a pipeline of dataflow that opens up new avenues of behavioral research that previously could not be explored.

However, while allowing for continuous quantification of various behaviors, fully automated experimental set-ups still require careful interpretation. Indeed, the initial observations leading to the experiments described here would likely not have been made if no live, 'manual' observations were conducted in the first place. In addition, the output of these models is only as good as the input provided by the experimenters[31]. Experimenters need to be well trained in scoring well-defined behaviors before applying deep behavior profiling; it should be used as a tool to assist researchers in finding answers rather than a method to produce statistically significant results itself.

Despite successful application of these tools in the present study, we have not been successful in finding conclusive answers with regard to the origin and function of maternal retrieval behavior. It appears unlikely to be affected by an increased demand from the nest site and no clear direct benefits for dams or pups have been observed in terms of body weight measures. Redirected pup retrieval behavior is also unlikely to be the driving force for maternal retrieval behavior, as actors and recipients of maternal retrieval did not differ in pup retrieval performance.

An important limitation of the current investigation is that fact that observations were confined to the light phase, where activity level is generally low, which most likely also holds true for maternal retrieving behaviour. Indirect evidence for the latter is supplied by the observation that the first bout of observations (after light was switched on) showed the highest levels of maternal retrieval behaviour which then subsided during the rest of the light phase. Future experiments in the dark phase of the animals and in varying quality of the environment such as a limited nesting and bedding setting[32] could help in further understanding maternal retrieval behavior. Is retrieval behavior restricted to other lactating dams or are non-lactating foster dams or inanimate objects also retrieved back to the nest site? Does the rate of retrieval differ between kin and non-kin pairs of dams? Do wild house mice exhibit this behavior under natural conditions? Is pup development, other than body weight, affected by increased rates of maternal retrieval? Finding answers to these outstanding questions will help to elucidate whether maternal retrieval serves a purpose in regulating the social dynamics observed in communal nesting conditions or whether this behavior is a potentially costly consequence of raising a litter together.

While maternal retrieval related behavior was generally performed by the same dam, this behavior was not related to dominance status of the dams. The tube test assay used to measure hierarchy in this study correlates well with other measures of dominance and shows great stability in dominance ranks over time[33]. However, most of the studies on dominance have been conducted in males, and female dominance and its relevance to other behavior is less well studied[34]. Recent studies show that female dominance is less linear, steep and despotic than in males[35]. In addition, dominance in this study was only assessed between pairs of dams and the position of each dam relative to other dams is unknown. The link between tube dominance and maternal retrieval behavior could therefore be subtler and may require larger sample sizes and more maternal retrieval data.

Our study indicated that dominance status might be related to pup retrieval behavior; subordinate dams outperformed dominant dams in the pup retrieval test, showing faster retrieval and higher completion rates compared to dominant dams. Since dominant animals show higher levels of exploratory behavior in novel environments[36–38], they could be more likely to better explore a novel situation such as the start of the pup retrieval test, although the pup retrieval test took place in the home cage. Subordinate dams may be less distracted by these novelties and remain more focused on retrieving the pups back to the nest site or experience greater stress from encountering pups outside the nest. In addition, dominance status of male mice affects oxytocin and vasopressin receptor densities[39], receptors that are of crucial importance for the regulation of maternal care and pup retrieval[40]. Future experiments could be designed to further explore the role of dominance status in maternal care.

In summary, we here explored a hitherto undescribed social behavior between two communally nesting mouse dams, i.e. maternal retrieving behavior, and investigated some potential avenues with regard to its origin and function. While the function of maternal retrieval is still unresolved, observations with regard to variability, directionality and grabbing style contradict an interpretation of maternal retrieval behavior to occur randomly. Associations with other characteristics of individual dams, dominance status and retrieval behaviour should be explored in more detail and may lead to new insights into the complex social dynamics between dams in communal nesting settings. This will help to understand which factors are important for cooperative breeding in mice and may shed new insights into cooperative breeding in our own species.

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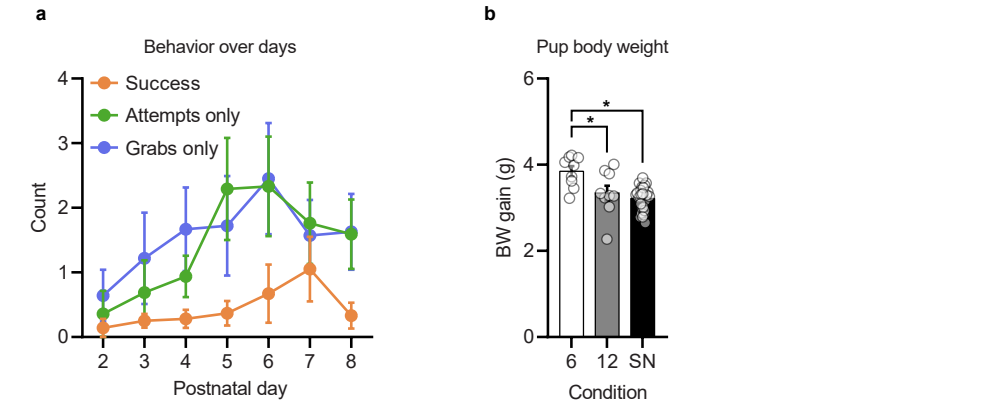


SUPPLEMENTARY FIGURE 1
(**a**) Maternal retrieval behavior over postnatal days, separated by each behavior individually. (**b**) Body weight gain of pups from P2-9, compared to standard reared litters (SN) from earlier studies. (**c**) Average time spent on the nest site by individual dams (**d**) Grabbing style of dams, separated by dominant and subordinate dams according to the tube test.

CHAPTER 7

General discussion

Summary of the main findings

The research presented in this thesis is the result of our efforts to study differential susceptibility in an animal model. By exposing heterozygous *MR* or *Drd4* knock-out mice and control litter mates to negative, neutral or positive rearing conditions, we aimed to create a study design for detecting *for better and for worse* gene-by-environment interactions while controlling for non-specific environmental and genetic variation. Here, I will provide an overview of the main results and discuss how our data fits with the existing theories and hypotheses proposed in the field of developmental psychology and psychopathology.

The core principle of this thesis is that experiments using animal models offer several unique advantages over studies using human subjects when studying offspring development. These advantages were reviewed in **chapter 2**, where we illustrated how animal models allow for: 1) strict control over the environment, 2) exploration of interventions, 3) manipulation of specific genes and 4) studying underlying neurobiological mechanisms in greater spatiotemporal detail. An additional advantage of animal models is the relatively short duration of generations, which allows a prospective multigenerational design over the course of months rather than decades. This element as well as the advantages indicated by 1), 2) and 3) apply to the current thesis. We aimed to set the stage for advantage 4), but that remained out of reach in this thesis. Although seemingly obvious, we noticed that these advantages do not reach their full potential in contributing to translational relevance of results for the human situation due to a gap between rodent and human research. We recommend improvements in (reporting of) statistical power of animal studies and closer alignment of human and animal studies, aiming to enhance the added value of animal models in studying the effects of parental care on offspring development.

Our first efforts of modelling genetic differential susceptibility in mice are described in **chapter 3**, where heterozygous mineralocorticoid receptor (*MR*) knock-out mice were exposed to either limited nesting/bedding (LN), standard nesting (SN) or communal nesting (CN) rearing conditions from postnatal day 2-9. Direct effects of these environmental manipulations on F0 maternal care were extensively monitored, largely replicating what has been described in literature. Important pup-directed maternal behaviors were generally unaffected, but LN dams showed less predictable maternal behavior on the nest site. Communal nesting resulted in upregulated nest presence by at least one dam of the pair, yet increased unpredictability and fragmentation of maternal behavior. Body weight was affected by these conditions in line with our aim to model impoverished and enriched rearing conditions; LN reared mice had lower body weight, whereas CN reared animals showed an increased body weight. Male offspring reared in the LN condition showed a delay in puberty onset, a relevant parameter to study with regard to the life history theory that has been developed based on human studies. This delay was mediated by a reduction in body weight and an increase in maternal care unpredictability experienced during early development. In contrast, puberty onset of female offspring was unaffected by rearing condition, although unpredictability of maternal care appeared to play a role in females too. During adulthood, maternal care of females towards their own offspring was unaffected by early-life rearing conditions. Genetic background however influenced care behavior; reduced expression of the mineralocorticoid receptor in $MR^{+/-}$ dams caused a less active parenting style and increased fragmentation. In addition, a gene-by-environment interaction was observed in basal corticosterone levels of these dams; $MR^{+/-}$ dams with a LN background displayed increased levels of corticosterone. Overall, the manipulations in this study did not provide supportive evidence for the mineralocorticoid receptor as a marker of differential susceptibility in mice, yet provided interesting avenues to continue differential susceptibility research in mice.

Chapter 4 describes a highly similar approach to studying differential susceptibility in mice, but changing the gene of interest to the dopamine receptor D4 (*Drd4*) and prolonging the duration of exposure to early-life rearing conditions to postnatal day 2-14. The alterations in F0 maternal care observed in chapter 3 were replicated in this study. In addition, we again observed a delay in puberty onset in LN reared male and female offspring that was mediated by a reduced body weight. However, in contrast to the MR study, body weight was not increased in CN reared offspring and received rates of maternal care unpredictability did not mediate puberty onset. Rearing conditions altered adult maternal care; LN reared dams spent less time arched-back nursing their offspring and exhibited decreased nest presence. CN reared dams showed lower rates of unpredictability compared to LN reared dams. In addition, licking/grooming (LG) behavior of the female offspring (once adult) was affected by a gene-by-environment interaction in line with the differential susceptibility theory. $Drd4^{+/-}$ dams reared in impoverished conditions showed the lowest LG levels, whereas communally reared $Drd4^{+/-}$ dams spent the most time licking/grooming their pups. These results demonstrate that both genetic and early-environmental factors are important and can interact with each other to affect later-life maternal behavior.

Male siblings of the animals used in chapter 3 and 4 were studied in **chapter 5**, describing the effects of early-life environmental and *MR* or *Drd4* genetic manipulations on spatial memory (object in location task), anxiety (elevated plus maze) and fear conditioning behavior (tone-cued fear conditioning). While spatial memory and anxiety behavior appeared not to be affected by rearing condition or genotype of the animals, LN reared mice appeared to exhibit higher levels of general activity in these tasks. Fear conditioning performance was unaffected by rearing conditioning in both experiments. However, decreased expression of the mineralocorticoid receptor impaired discrimination of cue-on and cue-off episodes in the paradigm. In addition, $MR^{+/-}$ mice showed increased levels of basal corticosterone. No gene-by-environment interactions were observed throughout these experiments, providing no evidence to support differential susceptibility for this combination of genes and cognitive domain outcomes.

In the experimental studies presented in this thesis, we observed an intriguing social behavior between dams in the communal nests, hitherto not described: dams would grab and fiercely drag the other dam back to the nest site, raising questions with regard to the origin and functionality of this maternal retrieval

behavior. In **chapter 6**, we therefore explored the role of litter size, dominance status of the dams and pup retrieval performance, in affecting maternal retrieval rates during the first postnatal week of communal nesting. We describe that maternal retrieval behavior during the light phase of the animals is infrequent, yet occurs in the vast majority of communally nesting pairs of dams. Most maternal retrieval behavior in a cage was performed by the same dam (actor), and the actor usually left the nest site after successful retrieval of the partner dam (recipient). Maternal retrieval rates were similar between pairs of dams taking care of 6 pups versus 12 pups, indicating that this behavior is unlikely to be the result of a varying demand from the nest site. Moreover, higher maternal retrieval rates did not result in increased nest occupancy, pup body weight gain or dam body weight gain, suggesting that no advantage of maternal retrieval can be elucidated with regard to these parameters. Dominance status of the dams, determined using a tube test, could not predict whether a dam would be the actor or recipient of maternal retrieval. Finally, actors did not differ from recipients in their pup retrieval performance, suggesting both retrieval behaviors are fundamentally different. Given the directionality of maternal retrieval, this behavior is likely a component of more subtle social dynamics between the dams.

Rodent models of early-life manipulations

The limited nesting and bedding model

The model used to induce early-life adversity (ELA) in this thesis, i.e. the limited nesting and bedding (LN) model, is a relatively recently developed paradigm[1]. Some previously developed models to induce ELA, such as maternal deprivation[2,3] and maternal separation[4,5], both rely on interventions through the experimenter. Another approach, studying natural variation in maternal care [6], only takes the quantity of maternal behavior - rather than fragmentation and unpredictability - into account when studying the effects of maternal care on offspring development. The key component of the LN model is that it induces erratic, fragmented maternal care chronically, without affecting absolute levels of maternal care and without interference of the experimenter[1,7,8]. Our findings in chapter 3 and 4 are largely in line with this, showing that absolute levels of pup-directed maternal behavior were unaffected by the exposure to the LN paradigm. In addition, our observations confirmed the unpredictability of maternal care to be increased in LN dams. However, fragmentation of maternal care, measured by the number of nest exits, was unaffected in our studies, while others observed an increased fragmentation in the LN condition[1]. More recent studies on the fragmentation of maternal care in a LN setting focus on the frequency and duration of licking/grooming bouts, showing that LG bouts of LN dams last shorter and are more frequent compared to SN dams[8]. Thus, the definition of fragmentation has changed between these studies and our approach is similar to only the first definition (nest exits). The way in which we collected data (frequent instantaneous sampling instead of continuous sampling) does not allow for quantification of LG bout duration and frequency. We therefore cannot compare our observations to the literature on this new measure of fragmentation and its importance for our read-out parameters remains unknown.

The LN condition induced only a mild phenotype in the offspring in most of our experiments. Body weight of the pups was consistently decreased, contributing to a delayed puberty onset in LN reared offspring. However, the LN condition did not affect maternal care of the offspring towards the next generation in chapter 3 and anxiety, neutral memory, fear conditioning performance and *MR* protein expression in hippocampus of male offspring was not affected, as described in chapter 5. Especially the lack of differences between LN and SN reared offspring in the fear conditioning paradigm was surprising, as previous studies using identical LN and fear conditioning protocols revealed a clear deficit of recognizing "safe" periods in LN reared mice[9]. We have not observed such an effect in two separate studies using relatively large sample sizes, and even experiments conducted in the same lab as the original observations could not replicate these results (unpublished data, personal correspondence). Inter-rater reliability of freezing behavior between the researcher conducting the latest experiments in Amsterdam and myself was excellent (>0.95), but was not compared to the original studies that were conducted by yet another experimenter. Moreover, this latter scientist was a woman, whereas the other experimenter and myself are both men. This could play a role, as male scientists themselves appear to stress out rodents[10]. While we will never know exactly why the results differ, it highlights the difficulties of replicating seemingly robust findings.

The lack of clear LN (and CN) effects in the *MR* study made us decide to prolong the window of early-life exposure from P2-9 to P2-14. This was predominantly based on a paper published around that time, showing that a later window of exposure to the LN model was more effective in inducing differences in susceptibility to social defeat stress during adulthood[11]. Since we were mostly interested in components of social behavior in our experiments, it made sense to better align the window of LN exposure to this work. In chapter 4, we indeed observed a weak, but significant, effect of the LN condition to impair later-life maternal care, in line with other studies[12].

The latter finding could be in line with the match-mismatch hypothesis. This hypothesis states that individuals adapt to the rearing environment in order to function optimally under cicrumstances similar to the rearing environment[13,14]. If the rearing and later-life environment match, this adaptation is succesful and helps the individual to thrive under these circumstances. If however the later-life environment is different from the rearing environment, these individuals perform suboptimally compared to well-matched individuals. Evidence in support of the match-mismatch has been observed on a meta-analytic level[15], although this work focused exclusively on the effects of early-life adversity. The decreased nest presence and arched-back nursing by LN reared dams reported in chapter 4 could indicate that these dams are performing poorly because they are nesting under standard rearing conditions and are not adapted to these settings. It would therefore be interesting to also test F1 maternal care under LN nesting conditions to study whether LN reared dams indeed outperform SN reared mice under these challenging nesting conditions. If so, maternal behavior would indeed be adaptive according to the match-mismatch hypothesis, but clearly more studies need to be conducted to explore whether this is the case.

Overall, LN exposure induced only subtle effects in our experiments, albeit consistently observed in the expected directions. Some considerations may shed a light on the subtle phenotype we observed. In our experiments we bred all litters in-house and males and females were housed separately in rooms that were different from the behavioral testing rooms under standard experimental conditions. Effort was made to prevent additional stressors at all times throughout experiments. In short, apart from the stress induced by the LN model, our animals were left completely undisturbed, potentially even leading a 'boring' life[16]. A meta-analysis by Bonapersona et al.[15] revealed that the effects sizes of ELA were dramatically increased when animals experienced multiple stressors during the study (such as transport of pregnant females, blood sampling, exposure to stressful tests, etc.) compared to studies, such as ours, in which no additional "hits" could be defined. Moreover, the duration of ELA exposure appears to be important for eliciting ELA effects, as the meta-analysis depicts an optimum at around 10 days of exposure, which is more comparable to the paradigm we applied in chapter 4.

While optimal exposure windows likely differ for various ELA paradigms, the importance of timing and duration of ELA models also depends on the behavioral domain of interest, as different brain regions mature during different developmental windows and at a different pace[17]. However, many studies follow a set protocol after initial publication of a new paradigm, and only later researchers dare to deviate, especially when results are disappointing. Indeed, most studies using the LN paradigm exposed animals from P2-9[18], in line with the original publication[1]. However, these studies predominantly studied hippocampal function and neurobiological endpoints [18]. Studies published on the effects of LN on social behavior have used a slightly different model in rats, but also a later exposure to the paradigm from P8-12[19,20]. Together, the timing, duration and intensity of LN exposure appears to be crucial for the effects it exerts [17] and our experiments might have been more successful in eliciting a robust phenotypical effect when these factors were better tuned to social behavior. When studying social behavior, I would recommend exploration of a more prolonged exposure to the early-life rearing conditions and/or exposure at a later developmental stage. This could result in alterations of the social dynamics between litter mates during the third postnatal week and affect brain development relevant for social behavior.

While restricting ELA exposure to specific moments in development is informative to study plasticity and resilience with regard to certain developmental windows, the restricted window of exposure might not be as translationally relevant when modelling poor early-life rearing conditions similar to the human situation. Early-life adversity in humans is, unless intervened with, often long-lasting and poor parental care usually coincides with other forms of early-life adversity[21]. Moreover, children often experience multiple forms of abuse and neglect combined[22,23]. It is therefore likely that most humans exposed to ELA experience adverse childhood experiences throughout multiple stages of development. If we aim to capture this in a rodent model, we should expose these mice to multiple, age-appropriate stressors ("hits"). By varying stressors, habituation to the stressors as found during repeated maternal separation[24] will not occur and unpredictability of the early-life environment is expected to be increased. While the LN model is certainly better than maternal deprivation and separation models in chronically exposing offspring to poor rearing conditions without repeated handling of the mice, dams do appear to habituate to this setting over time and nest exits by the dams decrease over time (Gallo *et al.* 2019 and unpublished data from our lab). A downside of prolonging ELA exposure and varying the origin of stressors over time is the impossibility to disentangle the causational role of each factor independently; it would also ask for many control groups. Still, when aiming to model ELA as translationally relevant as possible, such an approach is better at capturing the diverse and prolonged nature of ELA in humans and is much more likely to elicit robust effects in rodents.

The communal nesting model

The aspects on timing and duration of early-life exposure to LN discussed above apply to the communal nesting (CN) model too. Mice reared in the CN paradigm generally grow-up in this condition from birth till weaning[26]. To align CN exposure with the LN model, we exposed our animals to CN from P2-9 in the *MR* study, prolonging exposure to P2-14 in the *Drd4* study. Switching back to standard nesting conditions at P9 or P14 may have been a stressful event, by disrupting the environment, potentially counteracting any beneficial effects of the CN condition. In addition, communal nests in our experiments consisted of two dams and litters only, whereas other studies predominantly used three[27]. Throughout the work in this thesis, the phenotype of CN reared offspring was very subtle. Similar to earlier studies[28,29], communal nesting drastically increased nest occupancy by at least one dam to almost permanent levels on some days. Although pups reared in the CN paradigm gained more weight during early development, CN mice were indistinguishable from SN mice in most behavioral tests we conducted in adulthood. This could again be explained at least in part by the misalignment in exposure window and development of relevant brain regions. Moreover, pups reared in the CN paradigm till weaning experience peer interactions in addition to sibling interactions, a key feature of the CN paradigm that is likely to exert the largest effects when pups are socially more active in the third postnatal week. During the first 2 postnatal weeks pups' eyes are closed[30]; they rarely leave the nest site and social play has not yet emerged. Hence, because we stopped the CN condition by P9 or P14, the CN pups in our experiments lacked this extra element of CN rearing.

With the communal nesting paradigm we aimed to model early social enrichment in mice. In humans, this situation is perhaps best compared to child care by a grandparent, nanny or au pair, of which the effects on child development are complex and understudied[31]. However, in a communal nesting setting, the biological mother and foster mother are continuously available and the offspring has access to other peers. In this aspect CN rearing is comparable to child day-care, where peer interactions additionally may contribute to enhanced child development in some areas[32–34]. Care-takers in this setting alternate, offering more variable conditions compared to the CN setting. It is however very different from institutional care, where the biological mother is permanently absent and care-takers work in shifts. This situation is often characterised by fragmented care-giving, leading to poor developmental outcomes for children[35,36].

To study differential susceptibility in humans, beneficial rearing conditions are often induced by programmes aimed at promoting positive parenting[37,38]. The work in this thesis does not provide conclusive evidence with regard to the benefits of CN for parenting quality. Individual mothers did not differ in licking/grooming and entropy levels, but fragmentation increased and arched-back nursing (ABN) was decreased compared to standard nesting conditions. As discussed earlier, the importance of increased fragmentation as measured by the number of nest exits is unclear and it is likely a logical consequence of the social dynamics between two dams. The decreased ABN levels could be explained by the size of the litter, as it appeared to be nearly impossible to adopt a full arched-back posture over a large litter. Nest occupancy was indeed increased in the CN condition and two mothers, instead of one, were available to provide maternal care, but of course the number of pups was also doubled. Because the phenotype of CN reared animals, when subtly different from SN reared mice, was consistently altered in a beneficial direction, it seems plausible that the communal nesting condition did create a positive and enriched early environment. However, it presumably did not do so by directly affecting the maternal care of the dams. The experiments in chapter 6 also suggest that the complex social dynamics in the CN paradigm are likely a by-product of the circumstances, rather than a goal-directed alteration of behavior. In short, while communal nesting offers an interesting model to induce early social enrichment for rodent offspring, it may involve fundamentally different pathways from the enrichment provided in human studies in Western society. However, communal nesting may be more directly translated to care-taking dynamics in societies where larger networks of parents and other family members participate in raising children.

Limited bedding and nesting model vs. the communal nesting paradigm

To model differential susceptibility in mice, we chose two paradigms to manipulate the early-life environment. Both models rely on the behavior of the mother to be affected. However, both models may also affect thermoregulation in the nest; the metal grid in the LN cage allows for ample ventilation whereas the larger litter in the CN condition may help to retain heat. In addition, as discussed above, the CN model may exert its effects primarily through secondary effects of maternal presence, rather than differences in quality of maternal care. While human studies on differential susceptibility use the same continuous variable as a measure of the early-life environment[39–42], our study design resulted in a multi-categorical measure on the early-environment scale. Given the differences in aspects of maternal care that were affected by the LN (predictability) and CN (fragmentation and nest occupancy) models, it should be noted that our approach is different from comparing extremes on the same continuous scale.

Nevertheless, many read-out parameters in this thesis revealed a pattern where only the LN and CN group were statistically different from each other and the SN group appeared to exhibit an intermediate phenotype, in particular after prolonged changes in early-life exposure, from P2-14. We included the SN group in our experiments and analyses, allowing us to compare both environmental conditions to this control group. However, such a 2x3 design requires large samples or effect sizes to detect effects. The results presented in this thesis established that the LN and CN paradigms, when deviating from the SN condition, do so in the expected directions. Researchers performing future experiments could therefore choose to only include the LN and CN groups in the study. Alternatively, the SN can be included but only used for post-hoc analysis, retaining full statistical power to test differential susceptibility. Finally, if the experimental set-up allows, a recently developed approach to include prior data from literature or pilot studies can be applied for the control group to increase statistical power or decrease the number of animals used, on the assumption that experimental animals across experiments belong to the same population[43].

Genetic manipulations

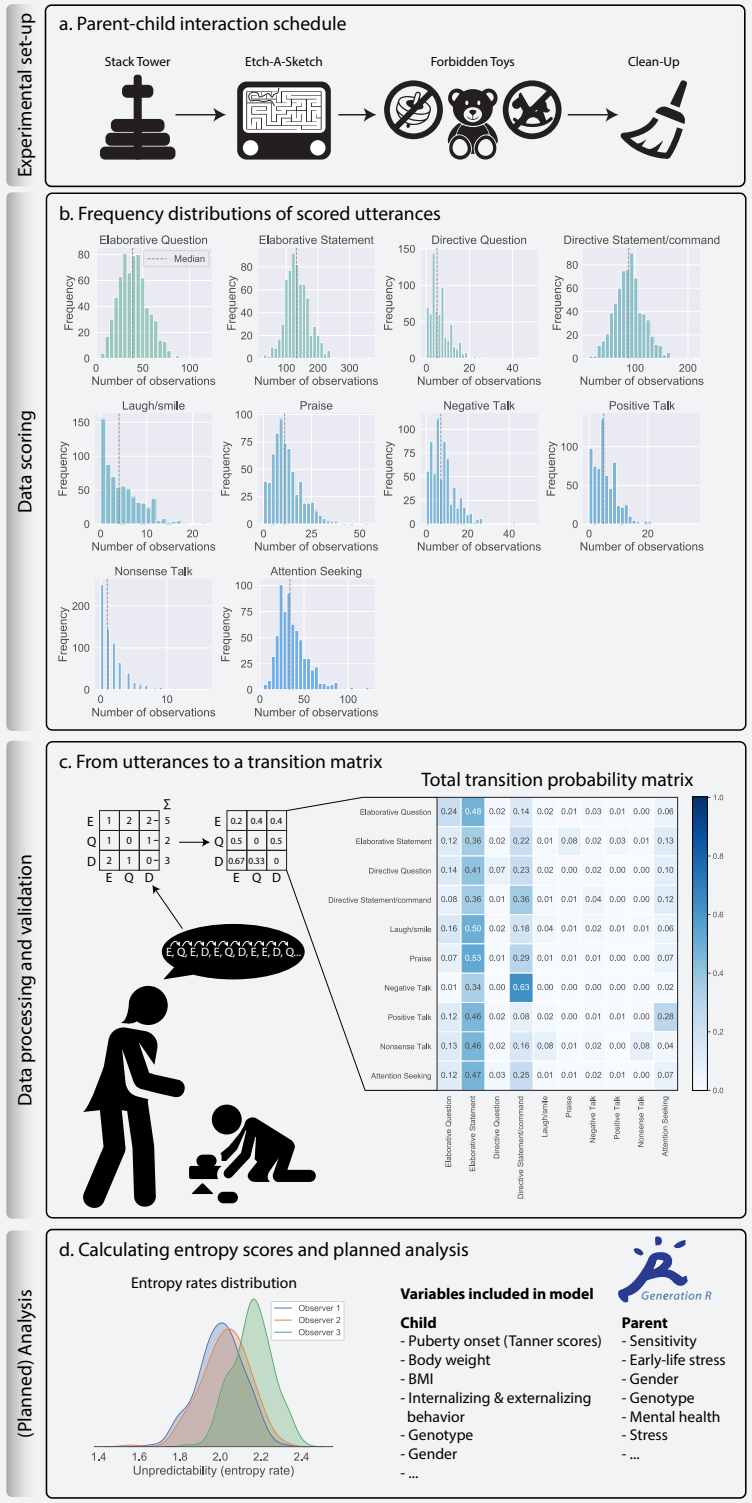
The work in this thesis used genetic manipulations in mice to model alterations in gene expression and function observed in haplotypes and polymorphisms of relevant human genotypes. To model the decreased gene expression or function observed in humans, rather than complete ablation, we used heterozygous knock-out of two candidate genes in order to enhance translational validity of our findings. However, some important considerations about this approach will be addressed here.

When using conventional knock-out animals, there tends to be an adaptation to this alteration in gene expression. Compensatory buffer mechanisms come into effect, leading to a cascade of pathways that confound the examination of the role of specific genes[44]. Although it is likely that compensatory mechanisms also occur in humans carrying genetic *MR* or *Drd4* variants, it is unknown whether compensatory mechanisms are comparable in rodents and humans. In this thesis, we did not study compensatory mechanisms by measuring expression levels of other genes –including effectors involved downstream of the receptors- in relevant neurobiological pathways, so the magnitude and direction of these confounders is unknown.

We indeed confirmed decreased protein expression of *MR* in the ventral and dorsal hippocampus of these animals, validating the knock-out approach for the mineralocorticoid receptor study. However, reduction was, in contrast to other reports[45], less than 50% in SN reared animals. The observed reduction of 20-30% is however comparable to the effects of different MR haplotypes on MR expression in humans[46], thus resulting in a translationally relevant reduced gene expression.

Although Drd4 expression was not studied in this thesis, earlier studies reported a 60% reduction of Drd4 in the frontal cortex of heterozygous knock-out mice[47]. However, the effects of the human dopamine receptor D4 7-repeat (7R) variant appear to be more specific than only the reduction of gene expression modeled in our experiments. Recent studies using a knock-in of the human 7R in mice showed that this variant prevents the formation of functional heterodimers with the D2 receptor[48], induces over-suppression of the NMDA receptor[49] and blunts metamphetamine-, but not cocaine-, induced release of dopamine[50]. Studies using this mouse line are now elucidating important neurobiological mechanisms through which the 7R variant may contribute to the increased ADHD susceptibility observed in 7R carriers. Future experiments into the differential susceptibility of these individuals may benefit from using 7R knock-in mice and could explore the

Box 1



Box 1 (continued)

Exploring a relation between entropy and puberty onset in humans

Extensive recordings of parent-child interactions have been made of a subset of genR participants to measure sensitive discipline. In these interactions, three year old children had to complete a series of tasks in the presence of the parent (experimental set-up). These tasks were too difficult for 3-year old children to complete and the parents were told that they could "give as much or as little instructions as they would like, but the children should accomplishthe task by themselves". Verbal communication of the parents was scored using several categories of utterances: elaborative questions/statements, directive questions statements, laugh/smile, praise, negative talk, positive talk, nonsense talk, inaudible and attention seeking (see data scoring section for distribution frequencies). Using a similar approach as used in chapter 3 and 4, we then calculated the probabilities of a transition from one utterance to the other (data processing and validation, left part). We first calculated a transition matrix for all participants combined to explore whether this approach would make sense (data processing and validation, right part). From this transition matrix we can see that for instance negative talk ("no" was always scored as negative talk) was followed by a directive statement/command in 63% of cases. This would be a sentence like: "no, you should pick it up" or "no, you should put it there" and sounds logical. However, negative talk was never followed by a praise for example, as indicated by the 0% of transitions from negative talk to praise. This makes sense, as a sentence like "no, good job!" sounds illogical. This screening of data and sanity check was encouraging, as the videos were not scored with this type of analysis in mind. Based on these results, we then calculated an entropy score for each parent-child pair separately. However, interrater reliablity was unsatisfactory, as 1 observer clearly deviated in the distribution of entropy scores compared to the other two raters (see planned analysis, left part). Nevertheless, data scored by the other two raters appeared useful for further analysis. We planned to use these entropy scores to explore its relation to sexual maturation measured in Tanner scores at the age of 13. The interest of the genR study to us is that the dataset also includes measures of BMI, genetic background, early-life background and internalizing and externalizing scores of the children at 9 years of age. We planned to build a comprehensive model including various relevant variables based on our animal experiments (body weight, early-life background, entropy) and human literature (genetics, other personality traits of children and parents) in order to directly compare the link between entropy and sexual maturation while correcting for multiple confounding factors (planned analysis, right part).

effects of different early-life rearing conditions on the neurobiological targets shown to be affected in earlier studies using these mice.

Nevertheless, this approach remains reductionistic, as it focuses only on the effects of one gene. It is becoming increasingly clear that a wider array of plasticity genes is usually more important for affecting differential susceptibility than one single gene. This has first been studied by Belsky and Beaver[51] and has since been further supported[52], showing that children carrying multiple susceptibility genes were the most responsive to the environment, for better and for worse. These studies relied on the cumulative scores of known plasticity genes, whereas another approach can be to compute polygenic susceptibility scores (PSS) based on genome-wide association study (GWAS) data, including thousands of SNPs in the calculation of PSS scores for each individual[53]. This approach has its own limitations and considerations[54] and it has therefore been argued that biologically informed risk scores are of major importance for correct prediction of outcomes[55]. This has also been used in rodent models[56], but requires extensive and coherent datasets. As scientists become increasingly aware of these challenges, further integration of existing datasets and novel computational solutions using animal model data will help to better capture the relevant pathways and mechanisms potentially leading to psychiatric disorders in humans[57].

Adding even more to the complexity, gene expression and function is clearly not restricted to only the effects of DNA sequences. Epigenetic mechanisms such as DNA methylation, histone modification, non-coding RNAs and many others can fine-tune gene expression throughout time and have been related to the negative consequences of early-life adversity[58] and positive effects of enrichment[59]. While this aspect has not been explored in this thesis, the mechanisms through which our early-life manipulations affected development and later-life behavior likely involved these mechanisms. However, in a design with genetically manipulated mice, it is difficult to elucidate cause and consequence, as some heritable genetics themselves have been shown to affect epigenetic profiles[60]. Recent insights further suggest that epigenetic patterns can be a consequence of, rather than a cause for, phenotypic variation[61]. Nevertheless, future studies on differential susceptibility could benefit from the expanding toolbox of –omics to highlight the important parts of the genome where gene expression is both negatively affected by early-life stress and positively altered by enriched rearing conditions.

Translational implications and translational efforts on entropy

Experiments in this thesis aimed to utlilize the advantages of animal models to test the differential susceptibility theory. Environmental factors were controlled and inbred mouse lines were used to eliminate genetic variation other than the genes of interest. The translational validity of the early-life manipulations and use of conventional (heterozygous) knock-out mice has been discussed above. Here, the translational relevance of some of the findings will be discussed.

In general, it is important to note that mouse parental care, usually consisting of only maternal care unless manipulated under strict experimental conditions[62,63], is different from human parental care, in which both parents participate in care-taking behavior in most cultures[64]. Other species may be more relevant to model this aspect[65], although they usually lack the potential to extensively manipulate genes.

With regard to the results in this thesis, puberty onset was the read-out parameter with most consistent effects of the early-life environment. According to the life history theory, individuals also adapt the pace of development to the rearing environment[66]. Uncertain and stressful conditions would accelerate maturation as part of a strategy to reproduce rapidly. Positive rearing conditions on the other hand would allow for ample time to develop optimally, slowing down the pace of (sexual) development. The acceleration of sexual maturation has been described in humans[67] and later in rats[68]. However, our findings consistently showed a *delayed* puberty onset following LN. We highlight the importance of body weight in mediating sexual development in mice, showing that the *reduced* body weight gain following LN contributes to the *delayed* puberty onset observed in these animals. Moreover, our results in chapter 4 suggest that the majority of mice postpone puberty onset until a certain body weight threshold is reached. These observations show that LN reared mice also did not reach puberty at an earlier body weight, suggesting that LN also did not result in a *relative* acceleration (i.e., lighter body weight at puberty onset) compared to SN reared mice.

While reduced body weight is also associated with delayed puberty onset in humans[69], the effects of ELA on puberty onset are opposite. Unfortunately, body weight has not been reported or included in the analysis of puberty onset following stress in humans[67]. However, other reports indicate that maternal stress, when affecting body weight of offspring, is related to an *enhanced* BMI in the offspring[70,71]. It would be interesting to study whether the accelerated puberty onset observed in humans that experienced ELA during early-development is (at least partly) mediated through an increase in body weight gain. This could be related to an increased intake of high caloric in individuals that experience early-life adversity[72]. Chronic stress in rodents also results in a preference for a high fat diet if given the choice[73]. However, this choice was lacking in our experiments, as all mice were fed normal chow. In order to increase translational validity of this aspect in rodent studies, unlimited access to high caloric food would be an interesting parameter to add in experiments on the effects of ELA on sexual maturation in rodents.

It is also possible that body weight is more important for determining puberty onset for a small organism as the mouse than for bigger organisms such as humans. A similar line of reasoning has been proposed as an explanation for the sex differences observed for susceptibility to different stressors[74]; female mice are more susceptible to cold stress, as they are lighter than males and deviations in temperature form a bigger threat to their smaller bodies (James P. Herman, presentation during the Neurobiology of Stress Workshop in Banff, 2019). It remains to be studied whether body weight is a stronger predictor for lighter animals such as mice compared to heavier species such as humans, where psychological aspects could be more important.

The mediation of puberty onset by received levels of entropy was less consistent, reaching significance in chapter 3 but not in chapter 4. The direction of this effect was however also in contrast to what would be expected if early-life adversity

accelerates sexual development. *Higher* levels of maternal care unpredictability (i.e. more stress) were related to a *delayed* puberty onset in mice. The translational validity of this finding is a subject for future study. Entropy of the early-life environment of humans can be measured using several approaches[75–77], but its link to sexual development has not been studied. Answering this question requires detailed information on the patterns of maternal care and many years of longitudinal data on development until at least the start of puberty in a subset of children/teenagers. We found such an opportunity in the generation R (genR) cohort study at Erasmus MC, that collects a rich body of data on developmental measures and early-life environment. In fact, we planned to conduct an analysis using genR data to explore whether the unpredictability of maternal care is also related to sexual maturation in humans. Maternal entropy scores were calculated based on observed language use during several different tasks completed during home visits (see box 1). We were planning to couple these observations to genetic background and puberty onset, but then the world was hit by the COVID-19 pandemic, making physically visiting Erasmus MC in order to locally conduct analyses on secured servers impossible. The approach we had planned is described in Box 1 and illustrates the potential power of this translational approach. It is a pity that this analysis has not been conducted, but I hope that the approach of estimating entropy scores in humans could be implemented in future studies in Generation R and other studies.

One of the major advantages of using an animal model is the short generation time which allows for studying maternal care of multiple generations, and thus intergenerational effects can be studied extensively. Although early-life manipulations affected later-life maternal care in chapter 4, these alterations were not replicating the maternal care offspring received. LN reared dams experienced increased entropy rates but unaltered arched-back nursing and even increased nest occupancy themselves, yet provided decreased ABN and nest occupancy to their own offspring while exhibiting normal entropy rates. This is important when studying maternal care for multiple generations in humans, highlighting that "what you get is what you give" is not the pattern observed in our studies. Longitudinal studies in humans[78] should therefore study a wide array of parental behaviors in order to capture the intergenerational effects of maternal care.

The less active parenting style and increased fragmentation of maternal care as observed in *MR*$^{+/-}$ mice in chapter 3 could be more straightforward to study in humans, as this effect was not affected by the early-life background. This would require data on MR haplotypes of the parent and measures on the parenting style. Given the role of the MR in regulating social behavior[79,80] and the stressful nature of parenthood[81,82], the mineralocorticoid receptor could very well play an important role in regulating the parenting style of humans too.

As discussed in chaper 2, another advantage of using animals models is the potential to study underlying neurobiological mechanisms in much greater spatiotemporal detail. However, the lack of a robust phenotype in our experiments discouraged us from exploring the underlying neurobiology in greater detail. We instead focused on finetuning the early-life models and testing more behavioral read-outs in males. Although neurobiological findings are most relevant when they are coupled to a behavioral phenotype, the increased basal CORT levels in LN reared *MR*$^{+/-}$ mice specifically (chapter 3) show that relevant circuitry could be affected without altering the behavior of interest. This could be interpreted to be in line with the accumulative stress hypothesis[83] double (three)-hit hypothesis[84], showing that two hits (LN and the genetic knock-out) result in alterations in circuitry, but a third hit is lacking and therefore no differences in phenotype are observed.

However, the CORT levels in this experiment were measured in the same individuals that were observed for maternal care, a rather challenging period in the lives of these animals. Interpretation of these results with regard to the 3-hit concept of vulnerability is therefore challenging, as it can be argued that these mice indeed experienced 3-hits (hit-1: *MR* knock-out, hit-2: LN rearing, hit-3: motherhood). Several reasons for the lack of a phenotype can therefore be argued: 1) the LN rearing was too mild, 2) motherhood should not be regarded as a hit, potentially because mice are not susceptible to environmental stressors during this period, 3) the 3-hit concept does not apply to the effects of MR and LN with regard to intergenerational transmission of maternal care. The final point could indicate that maternal care (and the other behaviors we studied) are not affected by basal CORT levels. Here, biological findings could drive future studies to explore which behaviors are affected. We could have opted for such an approach in general, conducting our experiments and analyzing a wide variety of neurobiological factors before studying a behavioral phenotype. However, we were specifically interested in the role of MR and DRD4 and their interaction with the early-life rearing environment in relation to social behaviors and intergenerational transmission of maternal care. Hence, we chose to study these behaviors first and only pursue neurobiological experiments once an interesting and robust behavioral phenotype emerged.

Read-out parameters on social behavior: suggestions for the future

As part of the Consortium on Individual Development (CID), we aimed to study behavioral read-outs related to social behavior and social competences. Rodent models allow for much faster intergenerational experiments on maternal care compared to humans, a unique opportunity that we utilized in this thesis. However, each study required two rounds of breeding and a ~50-60% success rate for breeding resulted in significant drop-out in the studies. We therefore started each study with a very large number of animals in order to obtain sufficient sample sizes in each of the six experimental groups. Due to the amount of work required for this type of study, we have not been able to explore all read-out parameters that could be relevant in relation to (the development of) social behavior.

A crucial step in the development of social behavior of rodents is the expression of social play, where the foundation for adult social behavior is formed[85,86]. Social play has been shown to be increased[87], decreased[88,89] or not affected[90] by early-life adversity, depending on the ELA model and social play paradigm used. Moreover, a direct link between within-litter variation of licking/grooming towards individual pups and social play behavior has been observed[91], highlighting the importance of maternal care in development of this behavior. Exposure to complex housing conditions during adolescence also affects social play behavior[88], indicating that

an enrichment such as communal nesting may also affect social play behavior. Although mice exhibit less social play than rats, they do exhibit social play from P15-25[92]. Given the role of maternal care, early-life adversity and adolescent enrichment on the expression of social play, it would be interesting to include this behavior as a read-out parameter for future studies on differential susceptibility.

Other potentially interesting aspects of social behavior include social recognition and discrimination as tested by the three-chamber social approach task[88,93] and a response to social stress in the social defeat[94] or resident-intruder test[95]. Both social discrimination[96] and social defeat susceptibility[11] have been shown to be affected by ELA. In addition, a key feature of the phenotype of CN reared animals is that they appear to be resilient to social, but not non-social, stressors[97]. Directly comparing the effects of early-life adversity and enrichment on social discrimination and social defeat stress while studying genetic differential susceptibility would provide valuable insights into the social characteristics of differential susceptibility.

Concluding remarks

Altogether, the work described in this thesis provides an initial attempt to study genetic differential susceptibility in a controlled setting using an animal model. While the effects of the early-life manipulations were mild, they were consistently observed in the expected directions when significant. I therefore conclude that the models are suited for this type of work, although they could be further fine-tuned in terms of duration, timing and severity of early life interventions. In addition, future studies should broaden the scope of genetic manipulations, studying multiple genes and genetic models that optimally mimic the human situation. If succesful, researchers can start to utilize other advantages animal models offer that we have not exploited to their full potential; 1) studying the underlying neurobiology of differential susceptibility in greater spatiotemporal detail, 2) studying within-litter variation in the early-life environment and its role in differential susceptibility. The latter is important, as within-litter variation of received licking-grooming levels correlates with rodent development[91] and offers a unique opportunity to examine the proposition that children within a family should vary in susceptibility to the (early-life) environment as an evolutionary strategy[98]. Future studies could more precisely target genetic susceptibility in different pups from a litter using techniques such as CRISPR/Cas9, and subsequently monitoring within-litter variation of maternal care could greatly benefit from deep learning tracking and automated behavioral scoring tools such as DeepLabCut (as applied in chapter 5) and SimBA. As the application of these techniques becomes more accesible to all researchers through open science networks, our understanding of the neurobiological underpinnings of differential susceptibility will expand.

The aim of this work was to study the differential susceptibility theory in a controlled setting using a mouse model. Based on the evidence provided in this thesis I conclude that:

1. Manipulations of the early-life rearing environment of mouse pups requires careful alignment of the timing and duration of exposure and the behavioral domain of interest in order to elicit robust effects.
2. In contrast to the acceleration hypothesis, puberty onset in mice is delayed following early-life adversity through a reduction in body weight (chapter 3 and 4) and potentially by increased unpredictability (chapter 3). The latter finding would be interesting to validate in humans, where the effects of early-life adversity on sexual maturation are opposite.
3. Both genetic and early-life environmental factors independently or interactively have the potential to shape offspring development and adult behavior, including maternal behavior of female offspring towards the next generation.
4. Differential susceptibility can be studied using mouse models, but requires further fine-tuning of rearing conditions and genetic tools in order to achieve more robust effects and optimally utilize the added value of rodent models.
5. The newly described maternal retrieval behavior during communal nesting is an intriguing and as of yet unresolved social behavior that deserves further investigation.

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ADDENDUM

Nederlandse samenvatting
Curriculum vitae
List of publications
Dankwoord

Nederlandse samenvatting

De omgeving waarin kinderen opgroeien is van cruciaal belang voor hun ontwikkeling en kan op de langere termijn van grote invloed zijn voor hun functioneren in de maatschappij. Deze omgeving wordt bij zoogdieren voor een heel groot deel bepaald door de ouderzorg die het nageslacht ontvangt. Slechte ouderzorg, bijvoorbeeld gekenmerkt door verwaarlozing of misbruik, zorgt voor grote hoeveelheden stress die kunnen bijdragen aan het ontstaan van psychische aandoeningen als depressie, angst en schizofrenie. Niet ieder kind heeft hier een even grote aanleg voor; sommige kinderen maken het relatief goed, terwijl anderen extra gevoelig zijn voor de nadelige gevolgen van stress. Deze gevoeligheid wordt deels door onze genen bepaald en blijft waarschijnlijk niet beperkt tot alleen de negatieve effecten van stress. De 'differential susceptibility' theorie stelt dat individuen die (genetisch) extra kwetsbaar zijn voor deze negatieve effecten ook extra zouden kunnen profiteren van de positieve effecten van een goede en stimulerende omgeving. Deze gevoelige individuen zouden kunnen worden vergeleken met orchideeën; ze zijn kwetsbaar voor veranderingen in de omgeving (weinig water, onvoldoende zonlicht), maar eenmaal in bloei vormen ze een prachtige bloem. De minder gevoelige individuen zouden vergeleken kunnen worden met paardenbloemen; ze hebben niet veel zorg nodig en gedijen in zeer uiteenlopende condities, maar ze zullen niet zo snel in een boeket van de bloemist worden opgenomen.

Om gevoelige individuen als orchideeën te laten ontwikkelen is het van belang te begrijpen hoe de genetische gevoeligheid precies tot stand komt. Welke neurobiologische processen liggen eraan ten grondslag en waarin kunnen deze individuen gestimuleerd worden? Deze vragen zijn lastig te beantwoorden in het onderzoek met mensen om verschillende ethische en praktische redenen. Diermodellen kunnen hierbij uitkomst bieden omdat ze bepaalde mogelijkheden bieden die in mensen niet mogelijk zijn. In hoofdstuk 2 van dit proefschrift staat beschreven hoe diermodellen kunnen bijdragen aan het bestuderen van de effecten van ouderzorg op de ontwikkeling van het nageslacht. 1) de omgeving kan gecontroleerd en gemanipuleerd worden om stress of stimulering te induceren, 2) interventies kunnen bestudeerd worden voordat deze in mensen worden getest, 3) de genetische achtergrond van individuen kan gecontroleerd en zeer nauwkeurig gemanipuleerd worden en 4) de onderliggende (neuro)biologische mechanismen kunnen op een gedetailleerde manier worden bestudeerd.

Het doel van dit proefschrift is om van deze voordelen gebruik te maken in het onderzoek naar de 'differential susceptibility' theorie. In genetisch gemodificeerde muizen onderzoeken we de effecten van een negatieve (stressvolle), standaard of positieve (verrijkte) omgeving vroeg in de ontwikkeling van muizenpups. Deze verschillende omgevingen zorgen voor veranderingen in de moederzorg en daarmee voor veranderde ontwikkeling van de pups. Negatieve opgroei-omstandigheden worden nagebootst door de moeder met haar pups in een kooi te plaatsen waarin geen zaagsel binnen bereik is en waarin slechts de helft van de normale hoeveelheid nestmateriaal aanwezig is (het limited nesting/bedding model). Deze omgeving zorgt voor een verhoogde onvoorspelbaarheid van de moederzorg waardoor de pups gestresst raken. In de positieve omgeving plaatsen we twee moeders met beide nestjes in dezelfde kooi waarbij de moeders een gezamenlijk nest vormen en ook voor elkaars pups gaan zorgen (het communal nesting model). Pups die opgroeien in een gezamenlijk nest hebben vaker beschikking over een moeder en ervaren ook sociale interacties met andere leeftijdsgenoten dan alleen broertjes en zusjes.

De muizen die in deze verschillende condities opgroeien zijn genetisch gezien allemaal identiek, behalve in 1 specifiek gen wat gemanipuleerd is. In dit proefschrift maken we gebruik van muizen met een gereduceerde expressie van de mineralocorticoid receptor, een van de twee receptoren van het stresshormoon cortisol (in mensen) of corticosteron (in muizen). Veranderingen in dit gen zorgen in mensen en muizen voor een verhoogde gevoeligheid voor stressvolle gebeurtenissen, maar het is onbekend of deze individuen ook gevoeliger zijn voor een positieve omgeving. Vervolgens bestuderen we ook muizen met een gereduceerde expressie van de dopamine receptor D4 (Drd4), een van de receptoren voor de signaalstof dopamine. Mensen met een bepaalde variant van deze receptor vertonen gevoeligheid voor ouderzorg die overeenkomt met de 'differential susceptibility' theorie, en dit gen vormt dus een voorname kandidaat om in een gecontroleerd diermodel te testen.

Nadat de genetisch gemanipuleerde pups in een van de drie condities opgroeien bestuderen we de seksuele ontwikkeling en bepaalde gedragingen die betrekking hebben op het (sociaal) functioneren op volwassen leeftijd. Volgens de 'life history' theorie zouden individuen die in een stressvolle omgeving opgroeien namelijk versneld ontwikkelen om sneller vruchtbaar te worden en voort te planten. Immers, in een stressvolle omgeving weet je nooit wanneer je laatste dagen geteld zijn dus je kunt evolutionair gezien beter zo snel mogelijk voortplanten, ook als dit ten koste gaat van een optimale ontwikkeling. Wij onderzoeken of gestresste muizen ook eerder de puberteit in gaan, of verrijking de ontwikkeling vertraagd en of genetische gevoeligheid hierbij een rol speelt. Op volwassen leeftijd bestuderen we vervolgens in vrouwelijke muizen hoe zij zelf voor hun eigen nageslacht zorgen. Door de korte generatietijd is in muizen goed te bestuderen of individuen die als pup bepaalde moederzorg hebben ontvangen dit ook of op een andere manier doorgeven aan hun eigen nageslacht. In mannelijke muizen onderzoeken we de invloed van de vroege omgeving en genetische gevoeligheid op cognitieve gedragingen als angst, ruimtelijk leren en stressvol leren. Deze gedragingen zijn van belang voor optimaal (sociaal) functioneren in de maatschappij en eerder onderzoek heeft aangetoond dat vroege levensomstandigheden en veranderde expressie van bepaalde genen hiervoor van belang kunnen zijn. Voor al deze aspecten (seksuele maturatie, moederzorg en cognitief gedrag) onderzoeken we dus of ze verslechterd zijn na stressvolle opgroei-omstandigheden, verbeterd zijn na verrijkte opgroei-omstandigheden en of deze effecten sterker of uitsluitend aanwezig zijn in genetisch gemanipuleerde muizen.

In hoofstuk 3 en 4 staat beschreven hoe het moedergedrag van muizen wordt beïnvloed door de condities waarin ze voor de pups moeten zorgen. Moeders in een limited nesting/bedding conditie zijn inderdaad onvoorspelbaarder in hun moederzorg, maar zorgen wel even veel voor hun pups. De pups die in deze conditie opgroeien zijn lichter en gaan hierdoor later de puberteit in. Ook gaan

pups die meer onvoorspelbaarheid in moederzorg ontvangen later de puberteit in. Beide aspecten zijn dus tegengesteld aan de 'life history' theorie. In communal nesting kooien is er vaker tenminste één moeder op het nest te vinden. Pups die hierin opgroeien zijn wat zwaarder, maar beginnen de puberteit op hetzelfde moment als de controledieren.

Vrouwelijke muizen die tussen postnatale dag 2-9 in de verschillende opgroei-omstandigheden zitten verschillen niet van elkaar in moedergedrag naar de volgende generatie (hoofdstuk 3). Wel zijn muizen met een gereduceerde expressie van de mineralocorticoid receptor slechtere moeders; ze hebben een minder actief type moederzorg en zijn daarbij gefragmenteerder. Muizen die tussen postnatale dag 2-14 blootgesteld worden aan het limited nesting/ bedding model zijn wel slechtere moeders; ze spenderen minder tijd op het nest (hoofdstuk 4). Verminderde expressie van de dopamine receptor D4 zorgt zelf niet voor veranderingen in de moederzorg, maar we zien wel een interactie tussen de opgroei-omstandigheden en genetische gevoeligheid op het gebied van het likken en verzorgen van de pups. Deze interactie komt overeen met de 'differential susceptibility' theorie; gestresste muizen met minder Drd4 doen dit het minste, terwijl verrijkte muizen met minder Drd4 hier het meeste van vertonen. Angst, ruimtelijk leren en stressvol leren in volwassen mannelijke muizen wordt niet beïnvloed door de opgroei-omstandigheden of genetische manipulaties (hoofdstuk 5).

In hoofdstuk 6 onderzoeken we een opvallend sociaal gedrag dat we observeerden bij de moeders in de communal nesting conditie. Een van de moeders pakt soms de andere moeder bij de vacht of staart om deze vervolgens terug naar het nest te trekken. We laten zien dat dit gedrag meestal door één van de twee moeders wordt gedaan, maar dat het niet wordt beïnvloed door de nestgrootte of dominantie van de moeders. Ook lijkt dit gedrag niet direct bij te dragen aan de verrijking in de communal nesting conditie.

In dit proefschrift hebben we de eerste stappen gezet om de 'differential susceptibility' theorie op een gecontroleerde manier te testen met behulp van een muismodel. De effecten van de veranderde opgroei-omstandigheden zijn mild, maar in de juiste richting. Ook laat ik zien dat opgroei-omstandigheden en genetische factoren afzonderlijk of interactief het gedrag van volwassen muizen kunnen beïnvloeden. Toekomstig onderzoek zou de timing en duur van de vroege condities beter af moeten stemmen op het gedrag van interesse om deze effecten nog duidelijker te maken. Dit zou de bruikbaarheid van een muismodel in het onderzoeken van 'differential susceptibility' vergroten, zodat er in de toekomst meer duidelijk wordt over de neurobiologische mechanismen die voor 'differential susceptibility' zorgen.

Curriculum Vitae

Jelle Knop was born on 15 December 1989 in Alkmaar, the Netherlands. He graduated high school in 2008 (OSG Huyenwaard) and enrolled in the bachelor Biology at the Rijksuniversiteit Groningen in 2009. He majored in Behavioral Neurosciences, and contributed to several research projects regarding the role of animal models in studying schizophrenia, social behavior and aggression. He received his bachelor degree in 2013. After being a board member of study association for life sciences and biology GLV Idun, he was admitted to the research topmaster Behavioral- and Cognitive Neurosciences at the Rijksuniversiteit Groningen. The first internship during this master program was under supervision of Dr. Bauke Buwalda and Prof. Dr. Sietse F. de Boer. He studied the link between aggression and decision-making in wild-type Groningen rats to explore the role of personality traits in coping mechanisms. During his second year, Jelle did his second internship at the Californian National Primate Research Center at University of California, Davis. Under supervision of Prof. Dr. Karen L. Bales, he assisted in studying the long-term effects of chronic intranasal oxytocin administration during early development in titi monkeys. In addition, he worked on developing a predator confrontation test to measure fear and anxiety behavior in titi monkeys. Jelle obtained his master degree cum laude in 2015, after which he continued his scientific career as PhD candidate under supervision of Prof. Dr. Marian Joëls, Prof. Dr. Marinus H. van IJzendoorn and daily supervisor Dr. Rixt van der Veen. He studied the differential susceptibility theory in a mouse model and the results are presented in this thesis.

List of publications

Knop, J., van IJzendoorn, M. H., Bakermans-Kranenburg, M. J., Joëls, M., & van der Veen, R. (2020). **Maternal care of heterozygous Dopamine Receptor D4 knockout mice: differential susceptibility to early-life rearing conditions.** *Genes, Brain and Behavior, 19(7)*, e12655.

Knop, J., van IJzendoorn, M. H., Bakermans-Kranenburg, M. J., Joëls, M., & van der Veen, R. (2019). **The effects of different rearing conditions on sexual maturation and maternal care in heterozygous mineralocorticoid receptor knockout mice.** *Hormones and behavior*, 112, 54-64.

Knop, J., Joëls, M., & van der Veen, R. (2017). **The added value of rodent models in studying parental influence on offspring development: opportunities, limitations and future perspectives.** *Current opinion in psychology*, 15, 174-181.

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[1]Lasse is een hond, geen kind. Dit ter verduidelijking dat ik niet stiekem in de tussentijd vader ben geworden, al scheelt het niet veel.