Sperm noncoding RNAs as mediators of paternal epigenetic inheritance modulating offspring affective and social behaviours

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Ég heima við sjóinn

Og á nóttunni

Þá kafa ég níður

Undir allar iður

Niður á hafsbotninn

Og ég lagt út akkerið

Hér vill ég vera

Hér á ég heima

The anchor song

Björk

Abstract

Background: Studies have shown that paternal environmental conditions prior to conception can influence the innate behaviours of their offspring, and the evolutionary impacts of such intergenerational effects are therefore of considerable interest. Epigenetic mechanisms have been shown to underlie this inheritance, as the microinjection of sperm small noncoding RNAs into fertilised oocytes induces reprogramming of the early embryo, which is thought to be responsible for the differences observed in adult phenotype. Our group previously showed in a mouse model of daily stress that glucocorticoid treatment of adult male breeders prior to conception leads to increased anxiety-related behaviours in male offspring, and this accompanies changes in the paternal sperm small noncoding RNA profile. Additionally, in a model of paternal running wheel voluntary exercise preconception, our group observed lower anxiety levels and a more robust fear extinction memory in the male offspring, as well as changes in paternal sperm small noncoding RNA expression.

Aims: In this study, we aimed to understand the transgenerational effects of paternal stress exposure on the social behaviour of the male progeny and its potential influence on reproductive success by analysing its effects on social reward, male attractiveness and social dominance. We also assessed the paternal sperm long noncoding RNA profile following glucocorticoid treatment or running wheel voluntary exercise. We used CaptureSeq, a sequencing technique that is more sensitive than the ones used in other studies in the field. We next sought to determine the role of sperm long noncoding RNAs by microinjecting them into fertilised oocytes.

Results: We report that paternal corticosterone-treatment was associated with increased display of subordination towards other male mice. Those mice were unexpectedly more attractive to female mice while expressing reduced levels of the key rodent pheromone Darcin, contrary to its conventional role in driving female attraction. Furthermore, no overt differences of the prefrontal cortex transcriptome were found in the offspring, implying that peripheral mechanisms are likely contributing to the phenotypic differences. No transgenerational differences were observed. Paternal corticosterone exposure led to dysregulation of sperm long noncoding RNA expression, which encompassed lncRNAs, circular RNAs and transposable elements. Although they have poor functional annotation, bioinformatic approaches indicated their expression in the brain, as well as their potential in regulating brain function. Running wheel exercise led to hundreds of downregulated lncRNAs, as well as transposable elements, and bioinformatic strategies predicted their function in biological processes, such as cell adhesion. Lastly, we separated and isolated the sperm long noncoding RNA population after glucocorticoid exposure and performed microinjections into fertilised oocytes. We

observed that the resulting adult offspring had lower body weight and altered behavioural responses in the light-dark box and Porsolt swim test.

Conclusion: Our findings highlight the potential of paternal stress to affect intergenerational (mal)adaptive responses. They also provide insights into the potential biology of long noncoding RNAs and highlight that efforts to annotate their function are highly necessary for the understanding of the mechanisms underlying the epigenetic inheritance. We are also the first to show that voluntary exercise modulates sperm long noncoding RNAs expression.

Declaration of originality

This is to certify that:

1. This thesis comprises only my own original work towards the degree of a Doctor of Philosophy, except where indicated in the preface;

2. Due acknowledgment has been made in the text to all other work conducted with assistance or in collaboration;

3. This thesis is fewer than 100,000 words in length, excluding tables, bibliographies, and appendices, as approved by the Higher Degree Research Committee.

_____

Lucas B. Hoffmann

Preface

The work presented in chapter 2 was accepted for publication by BMC Biology in May 2023. This chapter had editorial changes from the peer review process. Contributions from listed authors are as follows:

L. B. Hoffmann, E. A. McVicar, R. V. Harris, C. Collar-Fernández, M. B. Clark, A. J. Hannan, T. Y. Pang*.

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L. B. Hoffmann	planned and conducted the experiments, performed all data analyses, wrote the manuscript and approved its final version (84%)
E. A. McVicar	assisted in the animal studies, read and approved the final manuscript (2%)
R. V. Harris	performed the sperm collection and DNA extractions, read and approved the final manuscript (2%)
C. Collar-Fernández	performed the sperm long-read DNA sequencing study and data analysis, read and approved the final manuscript (5%)
M. B. Clark	planned and supervised the sperm long-read sequencing study, reviewed and approved the final manuscript (1%)
A. J. Hannan	reviewed, edited the manuscript and approved its final version (2%)
T. Y. Pang	conceived and designed the study, supervised data collection and analysis, reviewed, edited the manuscript and approved its final version (4%)

The work presented in chapter 3 was submitted for publication to Molecular Psychiatry in June 2023. Contributions from listed authors are as follows:

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L. B. Hoffmann	contributed to planning experiments, conducted all experiments and analyses, and wrote the manuscript (70%)
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Q. Zhao	performed the CaptureSeq gene expression analysis (2%)
W. Wei	performed the CaptureSeq gene expression analysis (2%)
L. J. Leighton	conducted the capture sequencing (2%)
T. W. Bredy	supervised the CaptureSeq and gave critical feedback to its analysis (1%)
T. Y. Pang	envisioned the study, supervised study design, data collection and statistical analyses, and reviewed the manuscript (5%)
A. J. Hannan	funded the study, contributed to planning experiments, provided critical feedback throughout the experiments, and reviewed the manuscript (3%)

The work presented in chapter 4 is unpublished material not submitted for publication. Submission for publication is planned for July 2023. Contributions from listed authors are as follows:

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T. Y. Pang	envisioned the study, performed the tissue collection, supervised study design and statistical analyses, and reviewed the manuscript (5%)
A. J. Hannan	envisioned and funded the study, contributed to planning experiments, provided critical feedback throughout the experiments, and reviewed the manuscript (3%)

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No other work in this thesis was carried out prior to my enrolment in the degree nor has this work been submitted for any other qualification. No third-party editorial assistance was provided in the preparation of this thesis, except for Chapter 2 that had editorial changes from the peer review process.

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"Apesar do mundo torto, a vida é bela!"

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Chapter 1

Literature review

1.1. Introduction to epigenetic inheritance

A handful of epidemiological studies have reported that preconceptual parental exposure to adverse experiences affects phenotypes and disease predisposition across generations. One of the first studies on this topic investigated post-traumatic stress disorder (PTSD) and depression rates in the adult children of Holocaust survivors, where they reported that having a parent that survived the Holocaust was associated with higher rates of depressive disorder [1]. A follow-up study reported that when both parents were Holocaust survivors who developed PTSD, their adult children had altered glucocorticoid receptor responsiveness [2], suggesting that the mechanism for their increased vulnerability to psychiatric disorders lies in the hypothalamus-pituitary-adrenal (HPA) axis responsivity. Less than 100 subjects per group were recruited in each of these studies, which might restrict their conclusions to that particular population sample; furthermore, those studies did not explore the mechanisms underlying the results they found. For these reasons, despite their trailblazing contribution to the field, it begs for more studies, including preclinical ones. Following epidemiological studies investigated the impact of the preconceptual exposure to other stressful conditions across generations, reporting that daughters of women who were evacuated to Swedish foster families during the World War II present higher chances of being hospitalised for mental health problems [3], and children of Australian men who served in the Vietnam war show higher rates of mental health problems, which was observed in a smaller sample of a few hundred individuals [4], but also in a larger sample of almost 2,000 adults [5]. Lastly, in another study that imaged the brains of neonates whose mothers experienced stressful prenatal life events, the authors reported alterations in brain regions associated with psychiatric disorders, including autism spectrum disorders. The authors speculated this relationship could be due to changes in hormones and neurotransmitters *in utero* or to increased levels of proinflammatory markers during pregnancy, as well as to epigenetic mechanisms [6].

The phenomenon of "parental effects", or the preconceptual parental environment affecting the offspring, has been pointed out as potentially contributing to the "missing heritability" problem [7], which refers to the fact that genome-wide association studies (GWAS) have failed to fully account for all of the genetic underpinnings of complex neuropsychiatric disorders, such as depression and anxiety disorders. Therefore, it is likely that other factors independent of the DNA sequence also influence disease risk [8], determining vulnerability only under certain environmental conditions [9]. As the "parental effects" phenomenon is not believed to involve genetic mutations, it has been proposed that it involves epigenetic mechanisms instead and has been therefore named epigenetic inheritance. Epigenetics refer to the regulation of gene expression that do not involve changes in DNA sequence. Instead, the regulation

happens through epigenetic marks, which can be DNA methylation, histone modifications, regulatory RNAs, or chromatin accessibility [10] (Fig. 1.1).

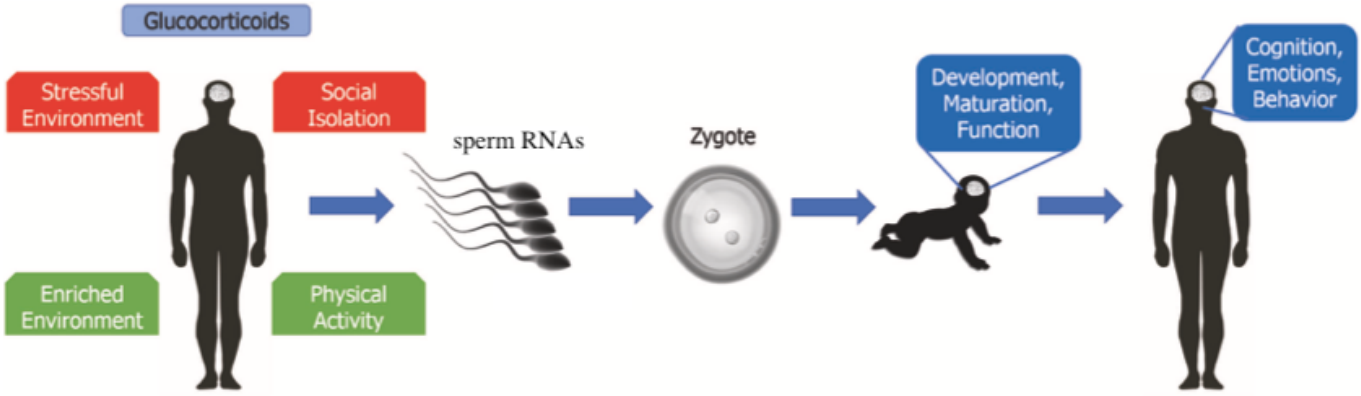


Fig. 1.1. Representation of the epigenetic inheritance in humans. Environmental exposures, both 'positive', such as physical activity, or 'negative', such as social isolation, can have impacts on the sperm epigenome. These epigenetic marks are delivered to the zygote, which will later affect brain development and maturation. Ultimately, they contribute to changes in adult behaviour. Figure adapted from Yeshurun and Hannan, 2018.

The epigenetic inheritance can be expressed intergenerationally or transgenerationally, depending on the number of generations directly affected by the environmental challenge. In other words, an intergenerational effect happens when the germ cells are exposed to the environmental challenge, which is the case for the offspring of males exposed to such challenge, and also to the future offspring of foetuses in the womb of females exposed to the environmental challenge. Any differential phenotype that might be observed in subsequent generations is considered transgenerational [10] (Table 1.1). Following the findings reported in epidemiological studies, several different preclinical paradigms have been adopted to study epigenetic inheritance in rodents. The matrilineal exposure to different environmental conditions has been established to affect the offspring due to potential changes in the *in utero* environment or postnatal care [11], which is proposed to modify the offspring's epigenome and subsequent behaviours in adulthood [12]. The patrilineal exposure, on the other hand, removes such confounding factors and allows us to investigate more conclusively an inherently germline effect, as the fathers are not required for caring for the offspring. Overall, it is not uncommon that the proportion of male and female offspring affected differs, which suggests the inheritance of epigenetic factors occurs through a sexual dimorphic fashion [13].

Table 1.1. Inter and transgenerational inheritance according to different exposures.

Exposure	F_1	F_2	F_3
Paternal	Intergenerational	Transgenerational	Transgenerational
Maternal (preconception)	Intergenerational	Transgenerational	Transgenerational
Maternal (pregnancy)		Intergenerational	Transgenerational

1.2. 'Positive' paternal interventions and their effects

The paradigms used in preclinical studies can be roughly divided into 'positive' or 'negative' paternal environmental interventions. The positive paternal approaches encompass environmental modifications such as environmental enrichment, exercise, and spatial training. Different models of paternal exercise have been used consisting of either voluntary or involuntary physical training, and the studies have focused primarily on their effects on offspring memory. The reported effects usually have been of positive nature, with an improvement in spatial memory [14–16]. However, the prevalence of beneficial effects of paternal exercise in the literature could be reflective of a bias towards publishing positive effects. One of these studies assessed global sperm DNA methylation using a commercial methylated DNA quantification kit and did not find statistically significant changes [15], but changes in sperm miRNA expression have been reported [17]. In our laboratory we have used voluntary paternal running wheel exercise [18] to investigate offspring affective disorders, such as anxiety-like and depression-like behaviours. The paternal exercise is based on voluntary access to running wheels in the home cages of mice for 4 weeks, and this paradigm was developed to allow voluntary exercise, as forced physical training can be stressful, and to cover a full spermatogenic cycle which corresponds to roughly 28 days. We observed that paternal running wheel exercise induces lower anxiety levels in the male offspring and more robust fear extinction memory in the male offspring, and these changes are accompanied by an altered small noncoding RNA profile, with 3 differentially expressed miRNAs (miR-19b, miR-455 and miR-133a) and 2 tRNA-derived RNAs (tRNA-Gly and tRNA-Pro) [18].

Other examples of 'positive' paternal environmental exposures include spatial training and environmental enrichment, which can also be presented with running wheel access. In models of paternal spatial training consisting of exposing them to the Morris water maze, it was shown that the male offspring and grand offspring show increased spatial memory [19], and the same effect in the male offspring was observed by another group, which also reported an enhancement of synaptic plasticity [20]. In models of paternal environmental enrichment alone, it has been reported that their male offspring show higher activity in the open field test [21], and their grand offspring show lower latency to floating in the forced-swim test [22]. In paternal environmental enrichment models that included running wheel access, it has been shown that they confer enhancement of hippocampal long-term potentiation [23,24], although another study reported impairment of spatial memory [25].

1.3. 'Negative' paternal interventions and their effects

The paternal interventions based on negative approaches encompass acute traumatic stress exposures during adulthood [26] or early-life [27], or chronic (daily) mild stress [28]. The models based on stress exposure have been adopted primarily for assessing parameters such as HPA axis reactivity and depressive- and anxiety-like behaviours, across generations [27–29]. The first studies using these models reported behavioural changes in the offspring, such as female offspring of stressed fathers spending longer time floating in the forced-swim test and showing reduced latency to enter the centre of the open field test [27]; reduced HPA axis responsivity [29]; and reduced time spent in the open arms of the elevated-plus maze in the male offspring of stressed fathers [28]. Some of these studies also showed changed in the grand offspring, such as increased time spent floating in the forced-swim test in male grand offspring, and female grand offspring showing reduced latency to enter the centre of the open field test [27]; and increased time spent in the open arms of the elevated-plus maze in male and female grand offspring, and increased time spent floating in the forced-swim test in the male grand offspring of stressed mice [28]. These phenotypical changes in the offspring are related to epigenetic modifications in their brains, but also in the paternal sperm. Sperm epigenetic modifications found after stress exposure include post-translational histone modifications [30], DNA methylation [27] and small RNAs [31], including circular RNAs [32].

In our laboratory, we have also been modelling paternal exposure to daily mild stress through corticosterone supplementation in the drinking water to explore transgenerational epigenetic mechanisms. Our paradigm was adapted from protocols used to model metabolic syndrome stemming from excessive glucocorticoid levels by Gourley and Taylor (2009), and it was adopted to standardise the paternal stress across studies, as most experiments on paternal epigenetic inheritance have relied on maternal separation paradigms to model early-life stress, which is both more time-consuming and also frequently yields inconsistent results [34]. Therefore, even though this model potentially does not encompass all the physiological effects of stress-exposure, it instead focusses on modelling the underlying universal HPA axis activation during stress, doing so in a more predictable way. In our laboratory, the paternal corticosterone has yielded consistent results towards an increased anxiety-like phenotype in the male offspring and depressive-like phenotype in the male grand offspring [28], while female progeny are largely spared. Small RNA sequencing of sperm samples collected from the male breeders showed that corticosterone exposure induces altered expression of 101 miRNAs and 61 tRNAs. In humans, one study that investigated the paternal exposure to systemic corticosteroids a few months prior to conception, using a nationwide data of singletons born from 1997 through 2013, investigated adverse birth outcomes and

reported no effects regarding congenital abnormalities, pre-term birth or small-for-gestational age [35]. However, it would be interesting to follow up on this cohort by investigating potential behavioural changes in the children of the men who were treated with systemic glucocorticoids before conception.

The field of the epigenetic inheritance have been continuously advancing with both the 'negative' and 'positive' paternal models, but there are still many questions left unanswered. Despite different behavioural and epigenetic effects have been reported in the offspring after these different paternal exposures, the adaptive value of such effects has yet to be determined. The investigation of other potential behavioural changes could also inform us about the impact of such inheritance to population adaptiveness, and lastly, other epigenetic mechanisms could also be investigated. The following paragraphs will explore these topics.

1.4. Sex-typical social behaviours and their importance to rodents

Many of the behavioural studies have focused on neuropsychiatric disorders, such as depression and anxiety, due to their complex inheritance. It is known that depression and anxiety are associated with deficits in social behaviours in humans, especially the expression of social isolation or lower quality of social interactions [36–38], making it part of the diagnostic criteria the presence of a clinically significant impairment in social functioning [39]. Accordingly, pre-clinical studies have identified that the projections from the basolateral amygdala to the ventral hippocampus modulate both anxiety-related behaviours and sociability [40]. Additionally, different classes of proteins belonging to the synaptic cell adhesion molecule group have been found to regulate social behaviours in humans, such as neurexins, neuroligins, protocadherins, and SH3 And Multiple Ankyrin Repeat Domains (SHANK) proteins [41], which could have their expression regulated by epigenetic mechanisms. Investigating how different conditions affect sociability across generations is particularly relevant due to the importance of appropriate social behaviour in determining access to resources and fitness in social animals [42].

Sociability is usually assessed in animal studies with the three-chamber interaction test, which was developed as a method to assess tendencies for social avoidance in mouse models of autism [43]. In the context of the epigenetic inheritance, this test was used to show that a model of paternal early-life trauma induces abnormal sociability across generations [44], which is a similar result to that reported by a study on a parental model of prenatal social instability [45]. Another study showed altered sociability in the offspring following *in utero* exposure to stress [46]. In our laboratory, sociability as assessed in the 3-chamber social test was investigated in the paternal corticosterone

paradigm and no differences were found on the offspring [47]. However, measures of sociability in the three-chamber interaction test have limited or confounding effects [48], as they do not rule out the possibility that other social aspects, such as agonistic or mating behaviours, could be involved. This is because sociability in sexually mature animals can have an underlying motivation towards agonistic behaviour or mating behaviour [49,50], and it is unlikely that sociability, as assessed in the three-chamber test, encompasses and distinguishes all these components.

Agonistic and mating behaviours are observed after sexual maturation and are considered sex-typical social behaviours. They depend on sexual hormones and the correct functioning of the hypothalamus-pituitary-gonadal axis (HPG). The HPG axis has been shown to be functionally connected to the HPA axis in an opposing way, thus being suppressed during periods of frequent activation of the HPA axis [51], such as during stressful conditions [52]. We are not aware of any studies that investigated the effects of the epigenetic inheritance on offspring HPG axis function, such as testosterone production. Given that our paternal corticosterone paradigm induces an anxiety-like phenotype and drives heightened responsivity of the HPA axis (to be published) in the offspring, it is important to investigate whether its effects extend to social behaviours that develop during sexual maturation, such as agonistic and mating behaviour, especially when considering their importance to determine fitness. In the sub sections below, we will define each of these behaviours and explain how they can affect health.

1.4.1. Agonistic behaviour

Agonistic behaviour encompasses aggression, threats, fights, or submission [53], and males engage in agonistic behaviour with the purpose of establishing hierarchy [54] and claiming resources and mates [53]. The component that underlies agonistic behaviour is the social dominance [55,56], which can be defined as the consistent capacity of one individual to prevail in conflicts across repeated agonistic interactions against another individual [55]. Importantly, dominance and aggression are not synonymous. It has been suggested that aggression might be required for establishing hierarchies, but once they are set and stable, aggression subsides [56]. Indeed, it has been proposed that one of the benefits of a hierarchy is reduced aggression levels within the group [55].

Social dominance is important because it affects essential aspects of social life, such as survival and reproductive success [57], but also physical and mental health [58]. This is because the position in the hierarchy determines access to resources and fitness, and stress levels, most commonly assessed by cortisol/corticosterone measures, vary depending on the social rank and also the hierarchy stability, which can in turn contribute to general health [59]. Indeed, the rodent model of chronic social defeat stress

is used to induce depressive-like phenotype on the defeated mouse, which attests the importance of the social rank to contribute to the development of psychopathologies [60].

1.4.2. Mating behaviour

Mating behaviour, in its turn, comprises the behaviours that lead to reproduction and encompasses mate-choice, intrasexual competition for mates and parental care [61]. Mate-choice is the stage when mate quality is assessed. Females usually have to be more selective when choosing mates than males are, because eggs are more costly to produce than sperm cells, females often only mate once during reproductive seasons, they bear the pregnancy and usually are responsible for parental care (in most mammalian species), thus suboptimal matings are more detrimental to females than to males [62]. During the mate-choice, the male quality or "attractiveness" predicts the likelihood of first copulation and mating duration [63] and therefore is highly determinant of mating possibilities and subsequent reproduction. Male quality is determined by scent marking [64], vocalisations [65], infection with parasites [66], previous sexual experiences [67] and environmental manipulations [68]. Many studies report that male quality determines the amount of maternal care [69,70], which has important impacts on offspring's phenotype [71], including their social behaviour [72] and social dominance [73].

1.4.3. Sex-typical behaviours and epigenetic inheritance

Social dominance and male quality have not been studied thoroughly in the context of the epigenetic inheritance. Social dominance has been explored briefly. For instance, in a study that investigated whether paternal social competition status, as assessed through the success in claiming territories, influences offspring metabolism, it was shown that the sons of dominant fathers had higher body weight [74]. A seminal study, and its follow up, reported that male quality, assessed in the mate-preference test, can be modified transgenerationally by vinclozolin pollutant exposure, an endocrine-disrupting chemical used as pesticide [75].

The interactions between social dominance and mate quality in epigenetic inheritance have also been explored briefly. A study observed differential inheritance of male attractiveness and volatile pheromone levels from dominant or submissive fathers. The male attractiveness was initially similar between sons of dominant or submissive fathers, but when the offspring faced repeated male-male competition, the sons of dominant fathers became more dominant and more attractive, and this was reflected

on the levels of volatile pheromones [76]. In another study, when wild mice were domesticated by being exposed to 8 generations of captivity, in which monogamous breeding schemes were applied, and then were given free access to a promiscuous breeding scheme for 2 generations, the males that were introduced to the promiscuous breeding scheme were more attractive than the males from the monogamous group [77]. By using a model of paternal environmental enrichment, with access to running wheel exercise, from post-weaning to adulthood, we reported that the male offspring shows reduced social dominance in the tube test and reduced attractiveness in the mate-choice test [25].

1.5. Paternal-driven modifications to the offspring epigenome

Accordingly to what was predicted to underlie the epigenetic inheritance, altered epigenetic marks were found in the somatic tissue of the offspring whose phenotype was changed following paternal environmental exposures, some of them also reporting the same changes in the sperm of the fathers. Many studies have explored DNA methylation, with some reporting changes in the promoter of individual genes that might be linked to the observed offspring behavioural phenotype [27,78], while others reported changes in global DNA methylation in specific brain regions [79], but also altered DNA methylation of glucocorticoid receptor genes in the hippocampus of the offspring of stressed fathers [80–82]. Altered expression of miRNAs in somatic tissue of the offspring, specifically the miRNA-375 in serum and hippocampus, has also been reported, which was shown to be implicated in stress response [31]. It is still unclear how the epigenetic marks found in the paternal sperm can be transmitted to the offspring, although some studies have attempted to establish this causality.

1.6. Epigenetic mechanisms and the importance of microinjection studies to determine causality

Current technologies are still severely limited in enabling direct manipulations of sperm histone modifications or DNA methylation, thereby preventing precise mechanistic studies of their roles in paternal inheritance and their contribution to offspring phenotypes. For this reason, studies that investigated these epigenetic marks in the sperm were not covered in this review. At this time, only sperm RNAs have been systematically investigated for their role in epigenetic inheritance through direct manipulations of their expression levels via microinjection of fertilised oocytes. Sperm RNAs can be divided in two different populations, small and long. Small noncoding RNAs

range from 18 to 30 nucleotide-long and encompass miRNAs, tRNA fragments, piRNAs, snoRNA and others [83]. Long noncoding RNAs, in their turn, are a heterogeneous class of transcripts longer than 200 nucleotides that are not translated into proteins [84]. Different studies have observed biological effects from the microinjection of sperm total RNA [31], small noncoding RNAs [85,86], tRNA fragments [87,88] and miRNAs [26,89,90] into fertilised oocytes, resulting in phenotypical changes in the resulting offspring that encompass developmental, physical, metabolic or behavioural effects. However, none of these studies investigated how the microinjected sperm RNAs promoted the phenotype change in the offspring. Currently, many of these investigators propose that these sperm RNAs conveyed by the sperm and inherited by the offspring would reprogram the epigenome during early embryonic stages [10].

The mechanisms behind this reprogramming are far from being explained, as there is a lack of studies on it and no clear theoretical framework to support them. The main question is: how do noncoding RNAs, known to be short-lived epigenetic marks, induce long-term phenotypic modifications in the developing embryo and postnatal offspring? The amount of RNA delivered by a sperm cell into the oocyte is reported to be around 5 to 10 fg, compared to around 1 ng of maternal RNA [91], and would suffer a two-fold dilution in every mitosis after the first zygote division [92]. Indeed, at least in fruit fly S2 cells (a cell line derived from late stage embryos), it has been reported that the most expressed miRNAs have a median half-life of 11.4 h, which is dependent on their association with argonaute protein to form stable miRNA-induced silencing complexes (miRISC) [93]. This suggests that paternal RNAs would have to bind to their targets during the early embryonic stages, otherwise they would become too diluted or be degraded [94], unless these noncoding RNAs induced their own expression through the embryonic transcriptional machinery [95], which has been recently reported to occur in the mouse embryo, in a non-peer reviewed study [96]. Additionally, a new mechanism of RNA-templated replication (replicons) through RNA-dependent RNA polymerases has been recently reported, although it is not known whether miRNAs and other mammalian RNAs undergo such process [97]. On the other hand, other epigenetic mechanisms, such as DNA methylation and histone modifications, are stable across multiple cell divisions as they are copied into the new synthesised chromatin using the daughter chromatid as a template. This occurs through DNA Methyltransferase 1 (*Dnmt1*) and its cofactor Ubiquitin Like With PHD And Ring Finger Domains 1 (*Uhrf1*) for DNA methylation [98] and a yet not very well defined enzymatic machinery for histone modifications [99], but seemingly involving the inheritance of histone-associated methyltransferases which copy the modifications [100]. Nevertheless, the copy of histone modifications is very precise and reproduced with high accuracy [101]. The stability and reproducibility of both mechanisms make them better mechanistic candidates for explaining the reprogramming effects thought to be involved in the epigenetic inheritance (Fig. 1.2).

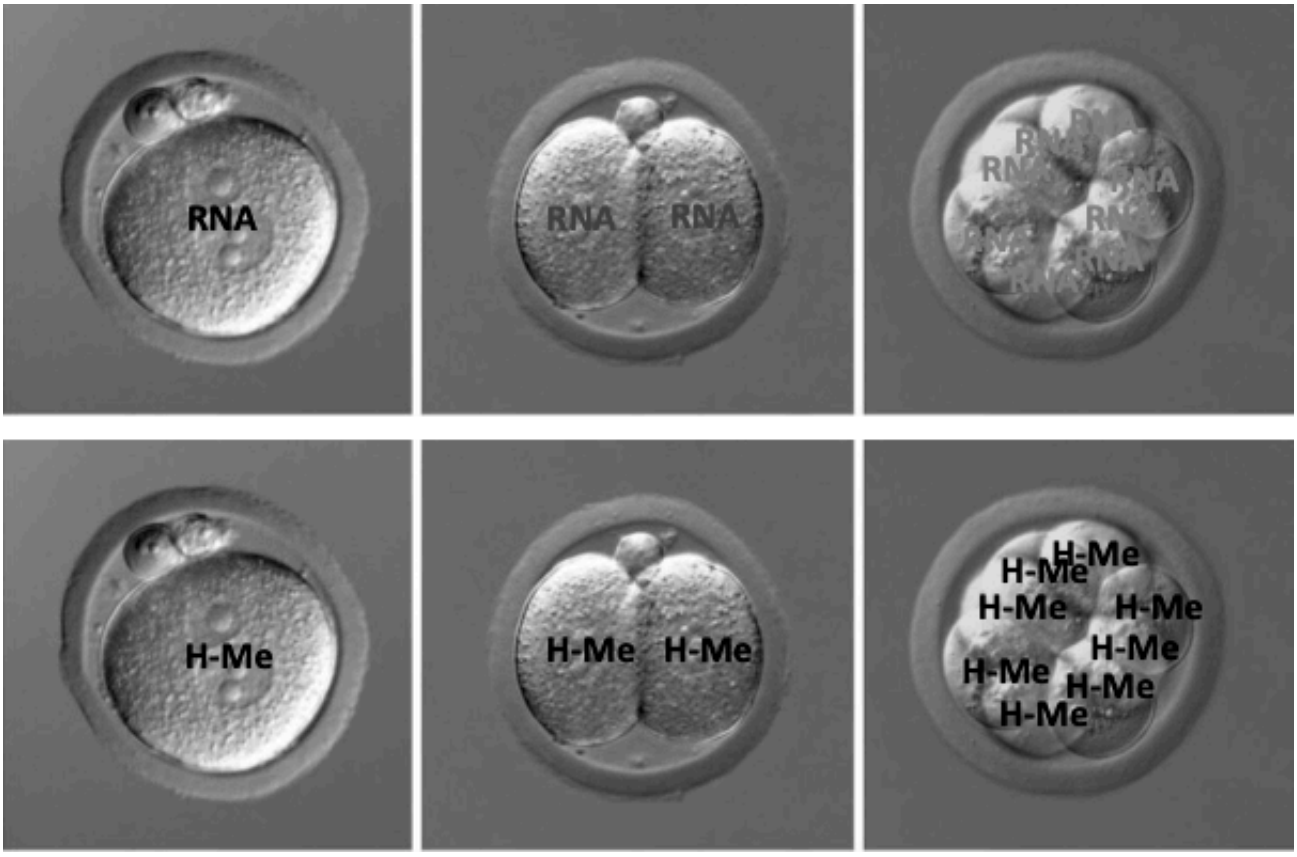


Fig. 1.2. Representation of the stability of different epigenetic marks in the early embryo. (Top) Due do the absence of mechanisms that allow their replication, sperm RNAs are expected to have their concentration levels reduced across early-embryo development. (Bottom) Histone modifications (H-Me), but also DNA methylation, on the other hand, are copied with great reproducibility in every cell division, which allows them to be kept across embryonic development.

Perhaps for this reason many authors suggested that the offspring inherit DNA methylation and/or histone/protamine modification patterns from the paternal chromosomes [17,27,81,102–104]. Although, as explained before, such mechanisms have not had their causality explored, this hypothesis is weakened by the fact that the embryo undergoes extensive reprogramming and erasure of epigenetic marks (except for imprinted regions). This is a complex process that involves replacement of protamines from the paternal DNA by maternally inherited histones, facilitation of chromatin accessibility through histone modifications and global DNA demethylation, and epigenetic remodelling to determine cell lineage fate through recruitment of Polycomb Repressive Complex 2 (PRC2) [105]. These processes happen so as the zygote can restore totipotency [105,106] and also undergo the Maternal to Zygotic Transition, which refers to the initiation of the zygotic transcriptional machinery and translation of zygotic genes to replace the maternal supply inherited in the oocyte [106,107].

Global methylation is almost universal, except for some repeat-poor regions that comprise imprinted regions [98] that have their methylation pattern preserved throughout

development and are demethylated only during gametogenesis [98,108]. Some non-imprinted genes can also escape demethylation and preserve their methylation pattern inherited from the parental DNA, although this was reported for oocyte-derived DNA only [109]. When it comes to histones and their replacement by protamines during spermatogenesis, only 1% of the nucleosomes in sperm DNA maintain H3.3 histones [110] and are therefore inherited. These histones are enriched for genes that are necessary for embryo development [111] and, indeed, when methylation of these histones is compromised, developmental problems and reduced survivability are observed [112]. Given that global epigenetic reprogramming occurs, except for differential methylation inherited in imprinted regions, it is unlikely that the inheritance of paternal DNA methylation or histone modifications would explain the plethora of effects observed in epigenetic inheritance.

Therefore, the microinjection of sperm RNAs into fertilised oocytes remains the best tool for investigating the causal mechanisms behind the epigenetic inheritance, especially because it promotes long-term effects in the offspring, seemingly without affecting embryonic survival and development.

1.7. The relevance of long noncoding RNAs

Considering that sperm noncoding RNAs should bind to their targets during a short time window during early-embryo stages to induce long-term effects, and that DNA methylation or histone modifications are the only known epigenetic mechanisms that are stable throughout lifetime, we hypothesise that the transcriptional regulatory information encoded by short-lived sperm noncoding RNAs are translated into more stable marks, such as histone modifications, during the early-embryo stages.

However, with the exception of piwi-interacting RNAs (piRNAs) being capable of inducing methylation of transposable DNA, there are yet no reports on other small noncoding RNA-induced chromatin modification in mammals [113]. On the other hand, long noncoding RNAs (lncRNAs), which are noncoding RNA molecules with at least 200 nt, interact with DNA to form triplex structures through non-canonical base pairing known as Hoogsteen bonds [114–116], which recruit PRC2 for chromatin repression [117–120] and help determine cell fate [119,121,122]. PRC2 is important for reorganisation of histones during programming in foetal germ cells [123] and is involved in paternal epigenetic inheritance [124]. Therefore, lncRNAs and their recruitment of PRC2 is a potential mechanistic candidate that could transform the information encoded by unstable carriers in the sperm into stable zygotic epigenetic marks (Fig. 1.3). Small noncoding RNAs could have effects on lncRNAs stability, as they can act as decoy targets or miRNAs

"sponges" [125]. There is only one study that microinjected lncRNAs into fertilised oocytes and compared the effects to the microinjection of miRNAs, which reported that lncRNAs extracted from the sperm of stressed mice induced increased food intake and glucose response to insulin in the male offspring, whereas miRNAs extracted from the same group of mice induced increased body weight and time spent floating in the forced-swim test in the male offspring [126].

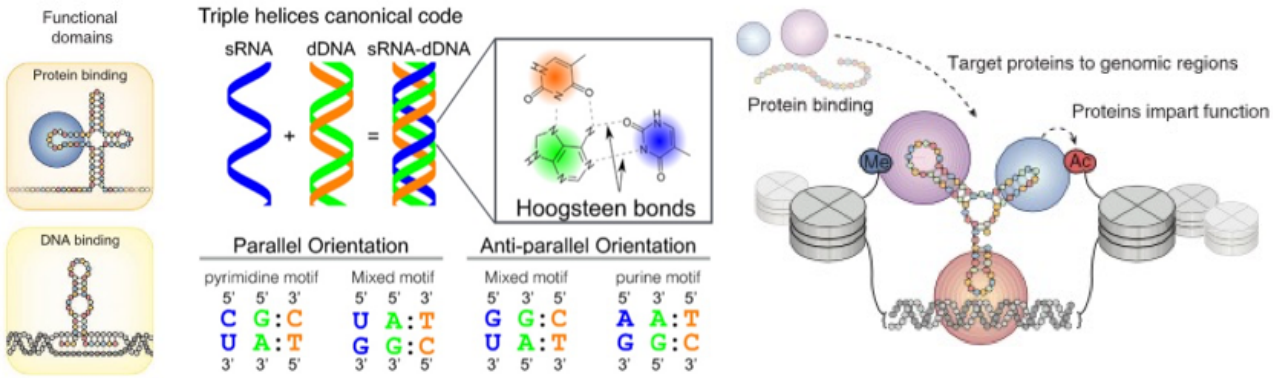


Fig. 1.3. Long noncoding RNAs and their function in epigenetic modulation. (Left) Long noncoding RNAs can have functional domains, such as protein and DNA binding, that confer their biological function. (Middle) Through Hoogsteen bonds, they bind to specific sequences of double-stranded DNA according to either a parallel or anti-parallel orientation, and then form triple helices that attract proteins. (Right) Chromatin modifiers are recruited, which induce histone modifications on the double-stranded DNA sequence with which the long noncoding RNA formed a triple helix. Figure adapted from Kuo et al., 2019; and Mercer and Mattick, 2013.

1.8. The relevance of transposable elements

A special class of functional elements within the long noncoding RNA realm that deserves attention, particularly due to their biological action during embryogenesis, is the transposable elements, the "jumping genes" (Fig. 1.4). It has been shown that retrotransposons long interspersed nuclear elements (LINE-1) regulate chromatin accessibility in the embryo [127], are required for rRNA synthesis and 2-cell embryo developmental progression [128], orchestrate the genetic function [129] and help determine cell fate in early embryos [130]. Transposable elements are especially expressed in early embryos, foetal germline [128] and neural progenitor cells [131], as well as in adult neurons, whose expression is modulated by Methyl-CpG-binding Protein 2 (MeCP2) [132]. Coincidently, these cell types are the most relevant for the transgenerational epigenetic inheritance of affective behaviours. Some studies show that LINE-1 activation can be modulated by the environment [133], including by the level of maternal care [134], which is surprisingly similar to how epigenetic mechanisms operate. Indeed, the activity of

transposable elements is controlled by epigenetic mechanisms that could be inherited [135]. Curiously, one of the first reports on transgenerational epigenetic inheritance in mammals involved the retrotransposon IAP LTR (intracisternal-A Particle Long Terminal Repeat) within the Axin-fused (*Axin^{Fu}*) gene, affecting its expression and promoting inheritable phenotypic variation [136]. There are other studies reporting small noncoding RNAs regulating retrotransposon RNAs [137] and sperm tRNA fragments controlling murine endogenous retrovirus-L (MERVL) activity in the embryo [138]. Therefore, transposable elements are interesting targets to be studied in the context of epigenetic inheritance, as they can be modulated by sperm RNAs and can themselves initiate embryonic transcription, which could be a mechanism that should be involved in the reprogramming induced by the epigenetic inheritance.

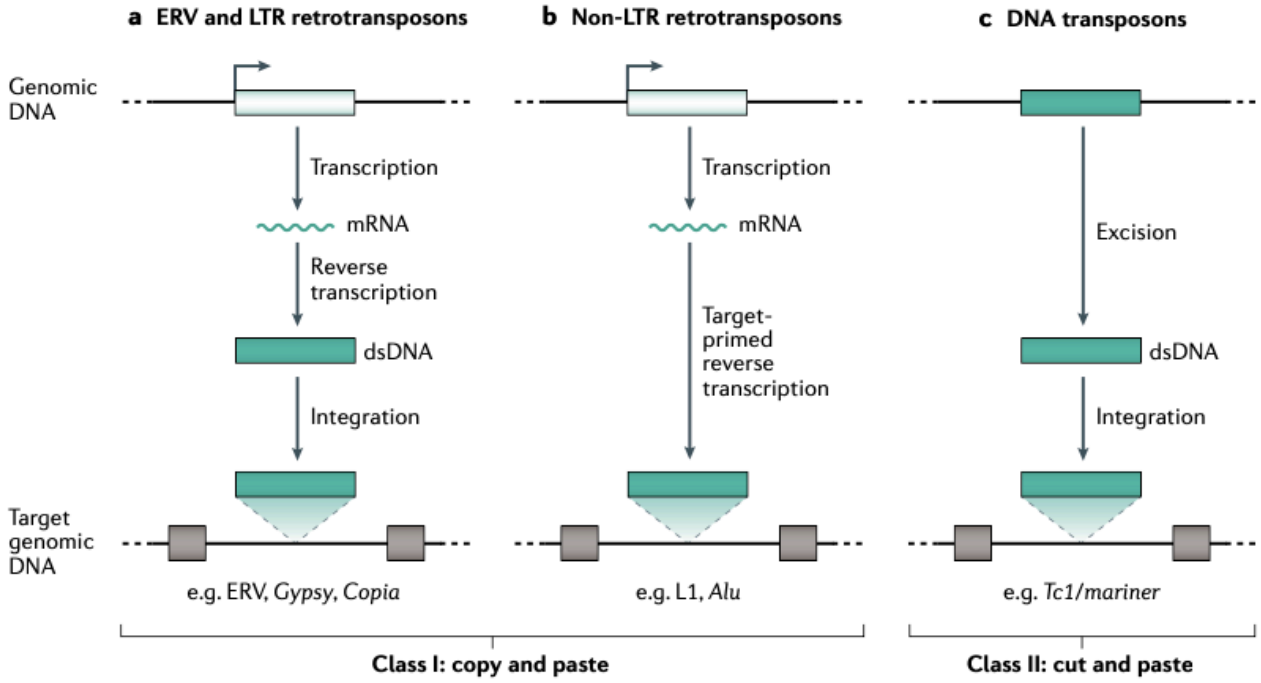


Fig. 1.4. Mechanisms of transposon mobility. Retrotransposons mobilise through a "copy and paste" mechanism, by first expressing an mRNA that can be reverse transcribed multiple times into double-stranded DNA, which in turn can insert themselves into different genomic regions. While elements of the ERV and LTR families do not express an endogenous reverse transcriptase, elements of non-LTR families (such as L1 and Alu elements) are autonomous. DNA transposons, on the other hand, mobilise through a "cut and paste" mechanisms, therefore literally "jumping" to different genomic regions. Figure taken from Lanciano and Cristofari, 2020.

1.9. Epigenetic inheritance as a "neo-Lamarckian" mechanism

The idea that epigenetic inheritance is at the forefront of a "neo-Lamarckian" resurrection has been suggested by some authors, due to its potential to facilitate phenotypic modification and its inheritance caused by environmental influences [140]. Importantly though, these processes happen because of epigenetic mechanisms, which operate under the regulation of Darwinian mechanisms. Hence, this type of inheritance was subjected to evolutionary pressures and, as such, was selected due to its benefits on fitness (i.e. via natural selection). Therefore, the study on transgenerational epigenetic inheritance raises the question about its relevance to evolution and adaptiveness [141,142].

These concepts become clearer when we investigate environment-induced inheritable phenotypic changes that are particularly important for determining fitness. Examples of parental environmental changes modifying offspring phenotype towards adaptation have been reported in ecological studies many years ago; for instance, the water flea *Daphnia sp.* develops a protective helmet-like structure when their parents are exposed to predator cues [143], and the desert locust assumes a gregarious behaviour when their parents lived in a high-density population [144]. Adaptation has been reported in experimental studies as well. In *C. elegans*, the expression of a type of virus in the soma results in the production of an interference RNA that is transmitted to the next generations in a non-Mendelian way, conferring them adaptive benefits [145]. In rats, the chronic history of hepatic wound healing through exposure to hepatotoxin in the parental generation reduced fibrotic healing across generations, an effect that was associated with epigenetic modifications in fibrogenic factors and transmission of the epigenetic marks to sperm cells through the serum [146].

It is important to consider that evolution happens through selection and fitness, which comprises survival in the environment (natural selection) but also advantage over competition for resources and for mates (sexual selection), as described by Darwin [147]. Therefore, if the epigenetic inheritance had an effect on sexual selection through modifying sex-typical social behaviours, such as social dominance and male attractiveness, it could possibly have a higher impact for evolution than if it operated through modifying natural selection alone, as the consequences for such changes would become more dramatic over multiple generations, as small changes amplify over time [42]. This adds the exciting possibility that not only the epigenetic inheritance would alter epigenetic marks, which are not expected to be permanent across generations once the stimulus ceases [148], but by modifying reproduction rates, it would favour the spread of its specific environment-induced epimutations into the population. In fact, epigenetic inheritance through the paternal lineage has been theorised to contribute to sexual selection [149], and a study suggests that in species that males do not provide direct benefits for the females – as we observe in mice – female mate choice would be more likely to have evolved if fitness depended on non-genetic inheritance of environmentally induced factors, and that such mechanism would be advantageous to guarantee male

investment when females can mate multiple times [148]. Additionally, it has been suggested that male attractiveness could have evolved with the aid of epigenetic mechanisms and the female mate choice [76]. Therefore, the epigenetic inheritance could be adaptive by inducing targeted behavioural changes that affect sexual selection through modulating specific genomic networks. As a side note, it is interesting to consider that the environmental influence across generations should increase fitness more effectively through increasing reproduction rates rather than enhancing survivability in such environment, especially because if the environmental conditions changed between generations, the reprogramming could instead become disadvantageous due to a mismatch [150,151].

1.10. Genes involved in sex-typical social behaviours

Considering that sex-typical social behaviours are particularly important for fitness, it is then important to investigate whether the genes involved with such behaviours can have their expression modulated by epigenetic mechanisms and, most importantly, if their expression can be modified by the epigenetic inheritance. Regarding male quality, as assessed in the female mate-choice test, olfactory cues, or male pheromones, contribute greatly to attractiveness [64], which are primarily determined by the Major Urinary Proteins (MUPs) in the urine [152]. These small glove-shaped proteins are synthesised in the liver and secreted to the urine. They encase volatile pheromones [153,154], and can act themselves as non-volatile pheromones [152]. The levels of these proteins in urine can also predict and be modified by social status [155], as well as convey information about the male quality, social status and individuality [76,153,155–158]. The expression of these proteins is controlled by androgens [159] and by *Zhx2* in the liver (Zinc fingers and homeoboxes 2) [160]. Of particular importance, MUP20, also called Darcin, is male-specific and associated with male attractiveness [152], although recent studies have shown that its attraction potential depends on the expression of other MUP proteins [161].

Importantly, male quality has been reported to be modified by environmental conditions and some of the genes reported to affect this behaviour can have their expression controlled by epigenetic mechanisms. For instance, male attractiveness increases after exposure to environmental enrichment [68]. Interestingly, one of the first studies to propose the occurrence of epigenetic inheritance in mice showed that the transference of zygote pronuclei to eggs of a different genotype resulted in major repression of *Mup* genes in the liver, which was associated with increased levels of DNA methylation [162] that could be inherited by the offspring of these males [163]. On the other hand, that are reports on upregulation of *Mup* genes in the liver of male offspring from obese mothers [164] and when males ascend to higher social ranks [156]. *Mup* expression can

be regulated by DNA methylation of CpGs in their promoter [77]. Additionally, ZHX2 can have its expression silenced through hypermethylation, at least in humans [165].

Interestingly, it has been shown that the *Mup* gene evolution is recent, with 22 *Mup* genes and 29 pseudogenes all located in a cluster, a result of gene duplication and variation of expression, which are characteristic of a recent "evolutionary bloom" [166–168]. The heavy presence of repeated elements in the same chromosomal cluster and the lack of similarity with the rat genome are also indicative of its recent evolution. The *Mup* genes are an example of genes that show a fast evolution due to pressures originating from their importance in contributing to behaviours that determine fitness. The association between fast-evolving genes that regulate fitness and the epigenetic inheritance is an interesting phenomenon that deserves more attention.

1.11. Transposable elements as the agents of evolution in the epigenetic inheritance

If the expression of transposable elements can be regulated by epigenetic mechanisms, then the epigenetic inheritance could contribute to gene duplication events, such as those that happened in the *Mup* cluster, a process that could be adaptive. Transposable elements are "generators of evolutionary novelty" [169]: they can create phenotypic variability [135], increase genomic diversity by duplicating genes, creating pseudogenes and alternative splicing [170] and aid in genomic network development, as they can duplicate hubs [171] which allows mutations to happen with lower risk of inducing mortality. Transposition can also create new lncRNAs [172], which are then subject to natural selection. Accordingly, more than two thirds of the lncRNAs in humans and mice have retrotransposon insertions [173]. Transposable elements and their functional RNAs are important for the embryonic transcriptional program activation, which has been suggested as highly robust due to the repeated nature of transposable elements, but also as highly adaptable due to the rapid evolution of their RNAs, as well as their ability to quickly respond to conditions of stress with an increase in transposition [128]. For this reason, transposable elements and lncRNAs act symbiotically to drive evolution [172] and such intertwined relationship seems reflected in the fact that transposable elements comprise more than half of the genome [174] and, at least in humans, the vast majority of all the transcripts are noncoding [172]. Transposition does not seem to favour specific genomic regions or networks, instead affecting any genomic region indiscriminately.

Overall, the occurrence of the epigenetic inheritance denounces the permeability of the theoretical Weismann barrier, a principle that determined a barrier separating

somatic cells and germ cells, implying that the environment could not modulate germ cells [175]. Even though the mechanisms for such permeability are not clear, there is evidence to suggest that the environment can in fact affect not only germ cells, but also the expression of transposable elements in the embryo [176]. Therefore, if the epigenetic inheritance recruits transposition, its action might be responsible for global effects towards increasing genomic variability, which would be highly beneficial for natural selection within a long-term evolutionary framework, rather than just conferring immediate adaptation to the next generation through modifying specific behavioural phenotypes.

Moreover, if the epigenetic inheritance could be modifying sex-typical social behaviours towards affecting sexual selection, but also recruiting transposition towards increasing whole genomic variability, both mechanisms could work in synchrony in favour of enhancing the spread of new transposition insertions to subsequent generations, as long as the immediate generation was sufficiently adapted to the environment that induced these changes. The epigenetic inheritance could be contributing to evolution through specific short-term effects that are expressed in a few generations and stop once the stimulus ceases, but also through global long-term effects with the mobilisation of transposable elements. As epigenetic mechanisms evolved to control retrotransposition [177], both mechanisms could have been co-opted together through facilitating adaptation when environmentally challenging conditions surge. Therefore, when studying the molecular mechanisms underlying the epigenetic inheritance, it would be important to consider the possibility of multiple epigenetic mechanisms coexisting, some favouring specific processes that modify targeted genomic networks, and some operating global modifications. For instance, the recruitment of PRC2 by lncRNAs might be involved in modifying targeted genomic networks, whereas transposon methylation status by MeCP2 might be involved in increasing whole genomic variability.

1.12. Aims of this study

The specific aims for each chapter are as follows:

Chapter 2: given that the paternal corticosterone exposure paradigm induces anxiety-like behaviour in the male offspring, and depressive-like phenotype in the male grand offspring, this project aims to investigate sex-typical social behaviours, specifically social dominance and male attractiveness, due to their importance for determining sexual selection. This will be followed up by investigating MUP protein levels and *Mup*

gene expression in the male progeny of these animals. The results from this study are present in chapter 2.

Chapter 3: the modulation of social behaviours by paternal corticosterone administration through the epigenetic inheritance should recruit mechanisms that are pivotal for early-embryo reprogramming, such as lncRNAs and transposons. Therefore, the aims for this chapter were to follow up on the behavioural studies conducted in chapter 2 by sequencing long noncoding RNAs isolated from the sperm of males exposed to corticosterone, and also to investigate transposable element transcripts and circular RNAs. We then performed the microinjection of these long noncoding RNAs into fertilised oocytes to assess their effect on the adult phenotype of the resulting offspring. The results from this study are present in chapter 3.

Chapter 4: lastly, to determine how different environmental interventions can affect the sperm long noncoding RNA profile, we aimed to assess the effects of a positive environment by exposing males to voluntary running wheel exercise and investigating the sperm long noncoding RNA profile. We then explored how the modulation of lncRNA and retrotransposon expression could have impacts on embryonic health. The results from this study are present in chapter 4.

Chapter 2

Increased paternal corticosterone exposure influences offspring social behaviour and expression of urinary pheromones

2.1. Abstract

Background: Studies have shown that paternal stress prior to conception can influence the innate behaviours of their offspring. The evolutionary impacts of such intergenerational effects are therefore of considerable interest. Our group previously showed in a model of daily stress that glucocorticoid treatment of adult male mouse breeders prior to conception leads to increased anxiety-related behaviours in male offspring. Here, we aimed to understand the transgenerational effects of paternal stress exposure on the social behaviour of progeny and its potential influence on reproductive success.

Results: We assessed social parameters including social reward, male attractiveness and social dominance, in the offspring (F_1) and grand-offspring (F_2). We report that paternal corticosterone-treatment was associated with increased display of subordination towards other male mice. Those mice were unexpectedly more attractive to female mice while expressing reduced levels of the key rodent pheromone Darcin, contrary to its conventional role in driving female attraction. We investigated the epigenetic regulation of major urinary protein (*Mup*) expression by performing the first Oxford Nanopore direct methylation of sperm deoxyribonucleic acid (DNA) in a mouse model of stress, but found no differences in *Mup* genes that could be attributed to corticosterone-treatment. Furthermore, no overt differences of the prefrontal cortex transcriptome were found in F_1 offspring, implying that peripheral mechanisms are likely contributing to the phenotypic differences. Interestingly, no phenotypic differences were observed in the F_2 grand-offspring.

Conclusions: Overall, our findings highlight the potential of moderate paternal stress to affect intergenerational (mal)adaptive responses, informing future studies of adaptiveness in rodents, humans and other species.

Keywords: paternal stress, epigenetic inheritance, social dominance, mate choice, major urinary protein, reproductive success

2.2. Background

Recent studies have demonstrated that the accumulation of paternal experiences before conception indirectly influence offspring behavioural phenotypes, and are largely attributed to epigenetic inheritance [25,75,81,178,179]. This phenomenon has been studied in various animal models, with altered offspring phenotypes linked to paternal stress exposures persisting until the third generation of offspring [180]. One common finding of studies to-date is a selective impact on offspring stress-relevant behaviours e.g. anxiety-like behaviour and social withdrawal. Studies of the potential mechanisms underlying

these intergenerational effects have identified contributions of distinct subpopulations of non-coding ribonucleic acids (RNAs). For example, through microinjection studies of fertilised oocytes and zygotes, paternal preconception stress-associated changes to sperm small non-coding RNAs in mice were found to influence anxiety-related behaviours and the stress-induced corticosterone response of adult F_1 offspring [26,31]. There is also emerging evidence of a specific contribution of sperm long non-coding RNAs to influence adult F_1 offspring metabolic phenotypes [126]. Thus, paternal stress-driven intergenerational adaptations could be a contributor to the 'missing heritability' problem associated with anxiety, depression and other psychiatric disorders in humans [7]. Additionally, some speculate about the evolutionary advantages that such heritability could confer, such as adding phenotypic variation [181,182].

Our previous work on the paternal corticosterone-supplementation model of generalised daily stress had reported elevated anxiety-like behaviours of male F_1 offspring (paternal corticosterone – PatCort) and the emergence of depressive-like behaviours in male F_2 grand-offspring (grand-paternal corticosterone – GPCort) [28]. We subsequently found that PatCort mice were resistant to the anxiolytic effects of environmental enrichment (routinely reported in the wider literature) and had reduced sensitivity to the selective serotonin reuptake inhibitor sertraline [183]. Other independent preclinical studies of distinct mouse models of stress have also found defects in sociality and social recognition accompanying impaired serotonergic signalling [44], as well as dysregulation of the physiological stress response [26]. In rodents, appropriate social behaviour is particularly important for reproduction and survival, and thus influences individual fitness. Given the increasing evidence that epigenetic inheritance influences behavioural endophenotypes, it is possible that epigenetic inheritance also underlies social behaviours relevant to successful reproduction, with consequences for adaptivity and species evolution [140,142].

Here, we embarked on a transgenerational study of rodent social behaviours highly relevant to their reproductive success. We investigated social dominance and male attractiveness across two generations of progeny in the paternal corticosterone-supplementation model of paternal stress. We hypothesised that a paternal history of stress would result in offspring displaying lower preference for social reward, increased subordinate behaviour during social interaction, and reduced preference from potential female mates. We followed up on these findings by investigating the expression of major urinary proteins (MUPs), which are non-volatile pheromones that have been associated with male attractiveness and sexual signalling [152,161]. Here, we present evidence that paternal stress exposure preconception can exert an intergenerational influence over behavioural endophenotypes that determine reproductive success in offspring. Our initial molecular and epigenetic studies also highlight the complex involvement and regulation of MUPs in rodent social behaviour, and the regulation of MUP expression by the paternal exposure to corticosterone.

2.3. Results

2.3.1. Corticosterone (Cort)-treatment diminishes adult male social dominance but does not adversely affect mate attraction

We first evaluated the effect of Cort-treatment on the social behaviours of adult male mice. 4 weeks of Cort-treatment significantly reduced the percentage of wins in the tube test (Fig. 2.5A, χ^2=30.25, p<0.001), which did not correlate to their body weight (Sup. Fig. S2.12). Based on this, we expected that Cort-treated males would be less preferred by potential female mates. However, there were no differences across all three variations of the test we conducted ('Standard' set up: Sum of Signed Ranks W=8.000, p=0.8603, Fig. 2.5B; 'Mouse only' set up: Sum of Signed Ranks W=32.00, p=0.4332, Fig. 2.5C; 'Bedding only' set up: Sum of Signed Ranks W=36.00, p=0.3755, Fig. 2.5D). Based on interaction times, females did not show preference for one group of males over the other (Fig. 2.5E, 'Standard' set up: χ^2=0.2500, p=0.6171; Fig. 2.5F, 'Mouse only' set up: χ^2=1.000, p=0.3173; Fig. 2.5G, 'Bedding only' set up: χ^2=0.2500, p=0.6171). Therefore, we found that Cort-treatment reduces social dominance and does not affect male attractiveness.

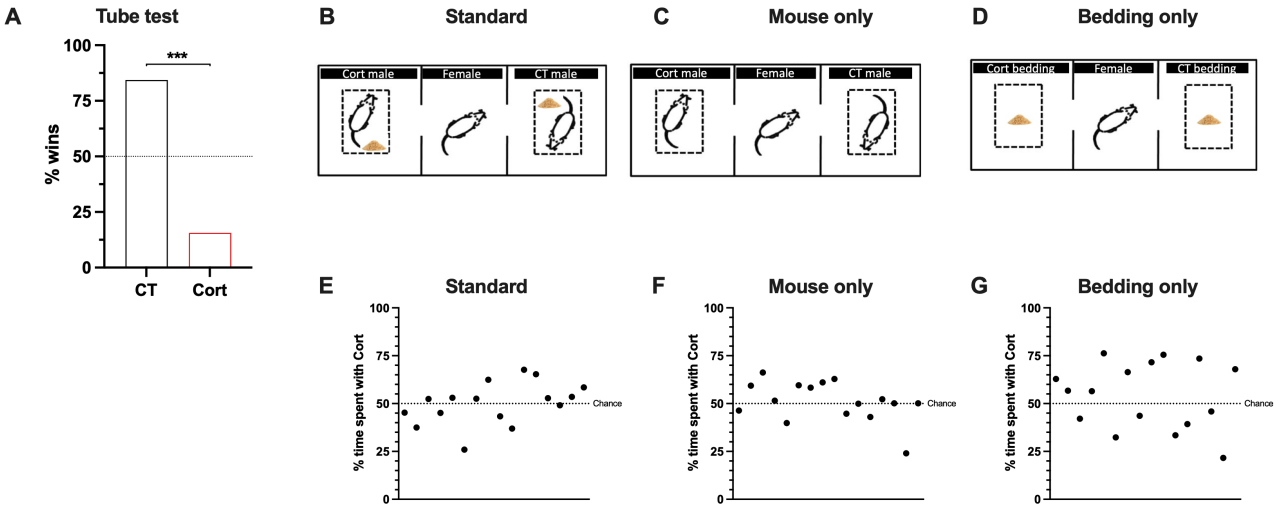


Fig. 2.5. Assessing effects of Cort-treatment on social dominance and mate-choice attractiveness. (A), corticosterone treatment reduces male dominance as assessed by the percentage of wins in the tube test. % wins calculated as the number of wins per group in the total number of interactions. n=16 per group, with a total of 64 unique interactions. (B – D), figures adapted from Toth and Neumann, 2013. In all of them, a female mouse is represented in the centre of a 2-chamber apparatus. In figure B, male mice from CT and Cort group are located at each end of the apparatus, alongside soiled bedding from their home cage. In figure C, male mice from CT and Cort group are

presented without bedding. In figure D, only soiled bedding from CT and Cort cages is presented. (E – G), male attractiveness was not affected by corticosterone-treatment. Each point represents the results from one female mouse, n=16 per group. A, E – G: one-sample chi-squared test. *** p<0.001.

2.3.2. Paternal Cort-treatment affects social dominance and mate attraction of adult male offspring

Juvenile (post-natal day 35 – PND 35) F_1 male and female PatCort offspring displayed a clear preference for the social-conditioned bedding (Fig. 2.6A, Males: $F_{(1,72)}$=12.76, p<0.001; Fig. 2.6C, Females: $F_{(1,74)}$=8.227, p=0.0054). No effects of paternal treatment were found for males (Fig. 2.6B, U=601, p=0.3811) nor for females (Fig. 2.6D, U=640.5, p=0.4035). Therefore, F_1 PatCort offspring mice displayed normal preference for social reward, and it was independent of the paternal corticosterone exposure.

In the tube test, adult F_1 male PatCort offspring recorded significantly fewer winning interactions, indicative of a lower order of social dominance (Fig. 2.6E, χ^2=15.75, p<0.001). In the mate-choice test, *naïve* females investigated F_1 male PatCort offspring for significantly longer periods than their paternal control (PatCT) counterparts (Sum of Signed Ranks W=241.0, p=0.0080), and a significantly higher percentage of time (Fig. 2.6F, χ^2=7.759, p=0.0053). No differences were observed between the groups for the 'mouse only' nor the 'bedding only' set ups ('Mouse only': Sum of Signed Ranks W=-40.00, p=0.3225; 'Bedding only': Sum of Signed Ranks W=-18.00, p=0.5186), and the total percentage of time was comparatively similar between the groups (Fig. 2.6G, 'mouse only' set up, χ^2=1.000, p=0.3173; Fig. 2.6H, 'bedding only' set up, χ^2=1.333, p=0.2482). Additionally, analysis of the total time each male directly interacted with the female when she approached them revealed no differences (Fig. 2.6I, $t_{(26)}$=0.8106, p=0.4249). Thus, paternal Cort-treatment was associated with intergenerational shifts in social-relevant behaviours of adult F_1 male PatCort offspring.

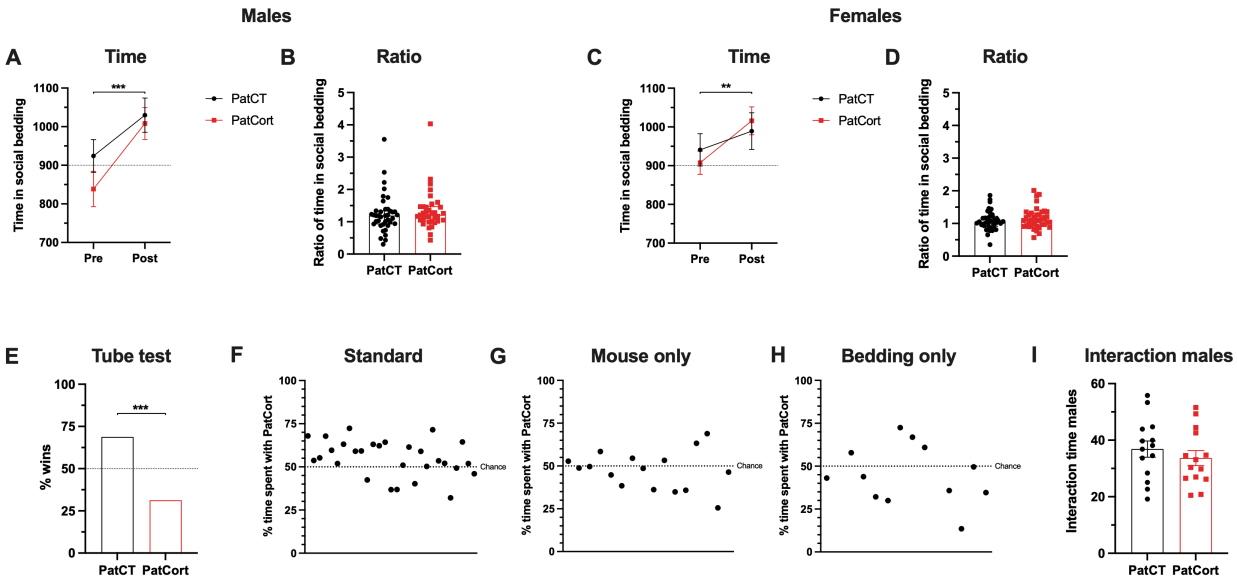


Fig. 2.6. Assessing effects of paternal Cort-treatment on offspring social behaviour. (A – D), the degree of social reward in male or female offspring was not affected by paternal Cort-treatment. Time and ratio of the time spent in the social-conditioned bedding. Males: *n*=39/35; females: *n*=37/39. (E), paternal Cort-treatment reduces male dominance in the tube test. % wins calculated as the number of wins per group in the total number of interactions. *n*=28 per group, with a total of 112 unique interactions. (F – H), paternal corticosterone treatment increases male attractiveness in the 'standard' set up only. Each point represents the results from one female mouse. Standard: *n*=29 per group; Mouse only: *n*=16 per group; Bedding only: *n*=12 per group. (I), PatCort mice do not interact more with female mice, compared to PatCT. *n*=14 per group. A and C: 2-way ANOVA, data represented as mean ± SEM. B and D: Mann-Whitney test, data represented as median ± interquartile range. E – H: one-sample chi-square test. I: unpaired t-test, data represented as mean ± SEM. ** p<0.01, *** p<0.001.

2.3.3. Paternal Cort-treatment effects do not transgenerationally alter grand-offspring social behaviours

Juvenile F_2 male and female grand-offspring showed a preference for the social-conditioned bedding (Fig. 2.7A, Males: $F_{(1,76)}$=35.14, p<0.001; Fig. 2.7C, Females: $F_{(1,80)}$=12.09, p<0.001). No differences in social reward were found between the groups for males (Fig. 2.7B, U=714.5, p=0.6527) nor for females (Fig. 2.7D, U=755, p=0.4547).

In the tube test, GPatCort and GpatCT groups recorded similar numbers of wins (Fig. 2.7E, χ^2=2.667, p=0.1025). In the mate-choice test, females spent similar amounts of time interacting with both groups of mice across all set ups (Sum of Signed Ranks W=-10.00, p=0.8209; 'Mouse only' set up: Sum of Signed Ranks W=40.00, p=0.3160; 'Bedding only' set up: Sum of Signed Ranks W=-40.00, p=0.3225). The percentage of time spent by the female interacting with both groups was similar (Fig. 2.7F, 'Standard' set up: χ^2=1.000, p=0.3173; Fig. 2.7G, 'Mouse only' set up: χ^2=1.000, p=0.3173; Fig. 2.7H, 'Bedding only' set up: χ^2=0.2500, p=0.6171). Therefore, no transgenerational effects on

social-relevant behaviours of F_2 GPCort grand-offspring were observed in association with grand-paternal Cort-treatment.

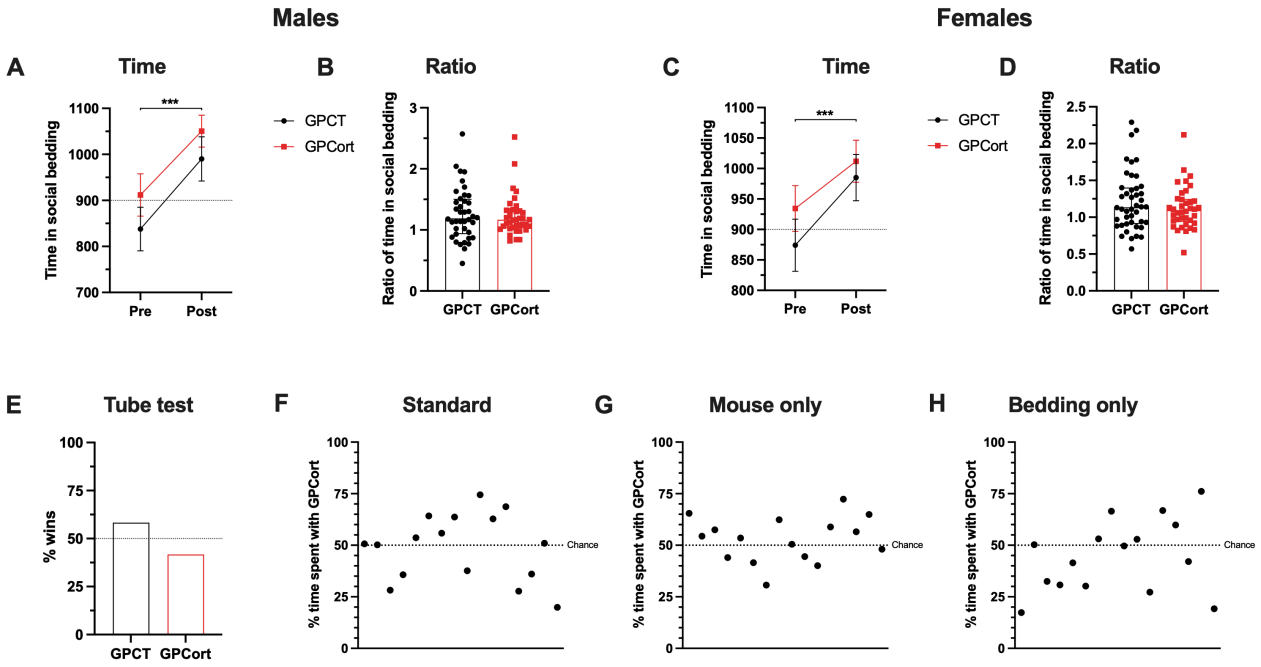


Fig. 2.7. Assessing effects of grand-paternal Cort-treatment on grand-offspring social behaviour. (A – D), the degree of social reward in male or female grand-offspring was not affected by grand-paternal Cort-treatment. Time and ratio of the time spent in the social-conditioned bedding. Males: *n*=40/38; females: *n*=44/38. (E), social dominance in male grand-offspring was not affected by grand-paternal Cort-treatment. % wins calculated as the number of wins per group in the total number of interactions. *n*=24 per group, with a total of 96 unique interactions. (F – H), male attractiveness in male grand-offspring was not affected by grand-paternal Cort-treatment. Standard: *n*=16 per group; Mouse only: *n*=16 per group; Bedding only: *n*=16 per group. A and C: 2-way ANOVA, data represented as mean ± SEM. B and D: Mann-Whitney test, data represented as median ± interquartile range. E – H: one-sample chi-squared test. *** $p<0.001$.

2.3.4. Paternal Cort-treatment alters the expression of a subpopulation of MUPs in F_1 offspring

Since pheromones play an important role in rodent male social hierarchy and mate attraction, we quantified the expression profiles of MUPs in the urine of F_1 male offspring.

We first assessed urinary creatinine content to account for urinary dilution, and determined that it was not different between PatCT and PatCort male offspring (Fig. 2.8A, $t_{(12.74)}$=1.704, p=0.1126), which indicates that their urination volume did not differ [156]. The relative levels of total MUPs also did not differ between the groups (Fig. 2.8B, $t_{(15.24)}$=1.792, p=0.0930). Next, we differentiated between the three major MUP bands

(Fig. 2.8C), in agreement with previous publications [77,152]. Semi-quantification of the protein bands revealed that PatCort males had reduced concentrations of the small MUP band (Fig. 2.8D, $t_{(15.41)}$=2.252, p=0.0393) and of Darcin (Fig. 2.8F, U=53, p=0.0243). No difference in the big MUP band was observed (Fig. 2.8E, $t_{(14.09)}$=1.904, p=0.0775). Additionally, the variability of all three bands of MUP proteins was higher in the PatCort mice (Small MUP band: $F_{(12,15)}$=5.693, p=0.0022; Big MUP band: $F_{(12,15)}$=9.406, p=0.0001; Darcin: $F_{(12,15)}$=5.614, p=0.0024; F test to compare variances). Therefore, there appears to be some selectivity in terms of the intergenerational impacts of paternal Cort-treatment on offspring production of MUPs.

Darcin was proposed to be the key MUP subtype to act as a female attractant pheromone [152]. However, urinary Darcin concentrations of male mice of both PatCT and PatCort groups were not significantly correlated with total time being investigated by the female in the mate-choice test (Fig. 2.8G, PatCT: Pearson's correlation R^2=0.1112, p=0.2068; PatCort: Pearson's correlation R^2=0.0921, p=0.3134).

Since MUP proteins are produced and secreted by the liver [185], we further investigated hepatic gene expression of the highly expressed *Mup3* [77], *Mup20* (Darcin), and the general *MupB* population by using non-specific primers, and *Zhx2* (a transcript factor for *Mup* genes) [160]. However, *Mup* gene expression in PatCort mice did not differ from PatCT mice (Fig. 2.8H, *Mup3*: $t_{(21.88)}$=1.666, p=0.1100; Fig. 2.8I, *Mup20*: U=107, p=0.4393; Fig. 2.8J, *MupB*: $t_{(30)}$=1.235, p=0.2264), despite the increased variability in the gene expression of *Mup3* (*Mup3*: $F_{(15,15)}$=4.117, p=0.0094; *Mup20*: $F_{(15,15)}$=1.555, p=0.4020; *MupB*: $F_{(15,15)}$=2.444, p=0.0939; F test to compare variances) in PatCort mice. There was also no difference in hepatic *Zhx2* gene expression (Fig. 2.8K, *Zhx2*: $t_{(30)}$=0.5436, p=0.5907).

Mup gene expression levels displayed a strong positive correlation with MUP protein populations in the PatCort offspring. *Mup3* correlates very strongly with total MUP urinary concentration in the urine of PatCort mice (Fig. 2.8L, Pearson's correlation R^2=0.6988, p<0.001). Interestingly, this correlation was not observed for PatCT mice (Fig. 2.8L, Pearson's correlation R^2=0.0003, p=0.9498). *Mup20*, in its turn, correlates very strongly with the Darcin band population of MUPs in the urine of PatCort mice (Fig. 2.8M, Pearson's correlation R^2=0.6823, p=0.0005), but did not correlate in PatCT mice (Fig. 2.8M, Pearson's correlation R^2=0.041, p=0.4521). The Darcin band has been attributed to the *Mup20* gene in previous publications [152,156]. These findings suggest that in PatCort mice, the high variability in the gene expression of *Mup3* is likely causing the high variability in the protein level.

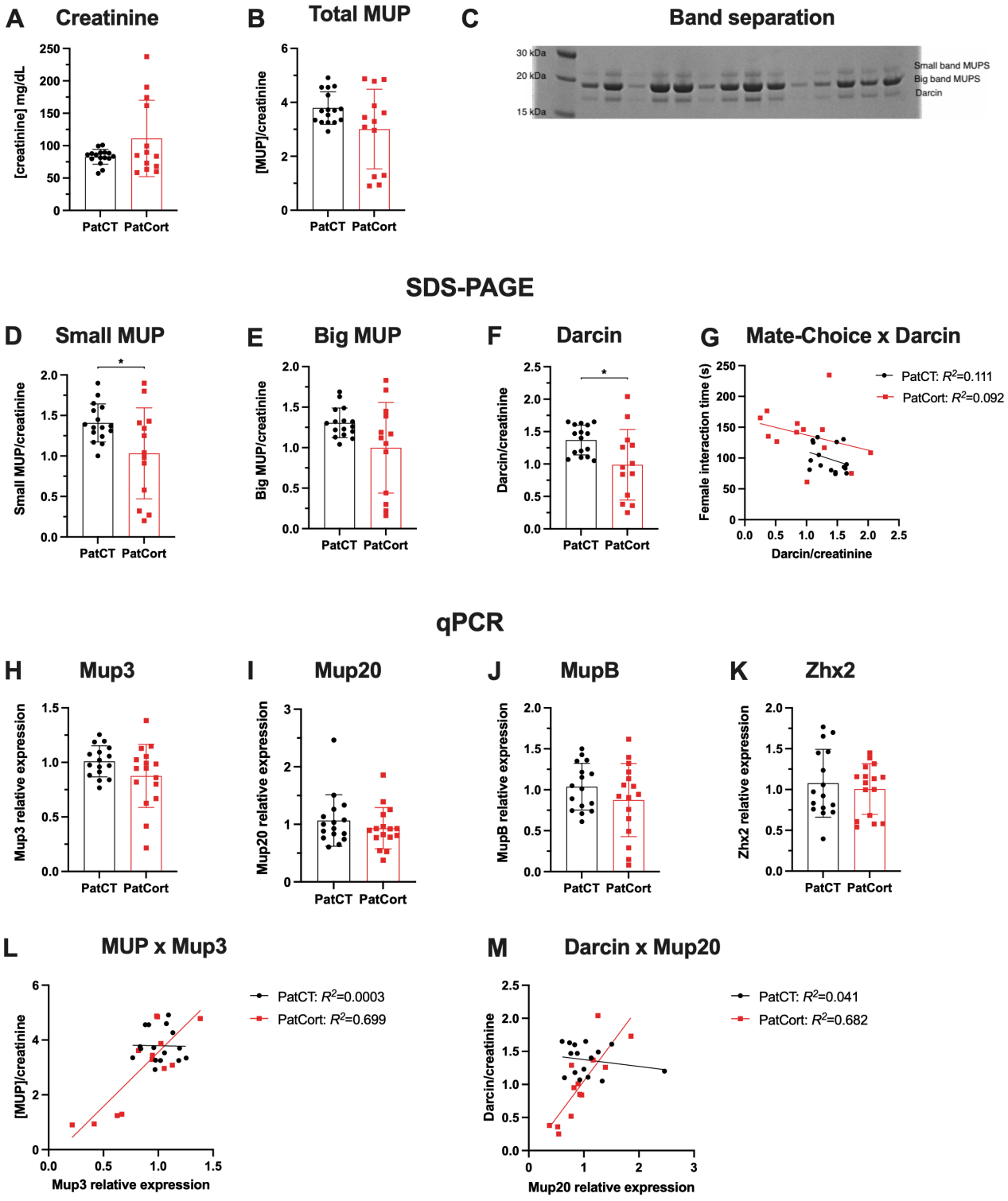


Fig. 2.8. Assessing effects of paternal Cort-treatment on offspring urinary MUP levels and liver gene expression. (A – B), urinary creatinine or total MUP were not affected by paternal Cort-treatment. *n*=16/13. (C), after SDS-PAGE of mouse urine, three different MUP bands can be seen, whose molecular weight correspond to previously published literature. Heavier band = 'Small MUP', middle band = 'Big MUP', lighter band = 'Darcin'. (D – F), paternal Cort-treatment induces reduced specific MUP populations in the urine. n=16/13. (G), time being investigated by the female does not correlate with urinary Darcin concentration. *n*=16/13. (H – K), Mup gene expression in the liver was not affected by paternal Cort-treatment. *n*=16 per group. (L – M), Mup genes correlate with specific MUP

populations in PatCort, but not in PatCT. *n*=16/13. A – B: unpaired t-test with Welch correction. D and E: unpaired t-test with Welch correction. F: Mann-Whitney test. G: correlation of Pearson and simple linear regression. H: unpaired t-test with Welch correction. I: Mann-Whitney test. J and K: unpaired t-test. L – M: correlation of Pearson and simple linear regression. Data represented as mean ± standard deviation. * $p<0.05$.

2.3.5. MUP profile is not transgenerationally influenced by paternal Cort-treatment

We also assessed urinary creatinine and MUP protein concentrations of F_2 male grand-offspring mice. Both urinary creatinine (Fig. 2.9A, $U=101.5$, $p=0.8897$) and total MUP concentrations (Fig. 2.9B, $t_{(27)}=0.9832$, $p=0.3343$) did not significantly differ between the groups. No between-group differences were also detected for any of the three major MUP bands, namely small MUPs (Fig. 2.9C, $t_{(27)}=0.4547$, $p=0.6529$), big MUPs (Fig. 2.9D, $t_{(27)}=0.7838$, $p=0.4400$) and Darcin (Fig. 2.9E, $t_{(27)}=0.5951$, $p=0.5568$). Additionally, there were no differences in variability between the groups.

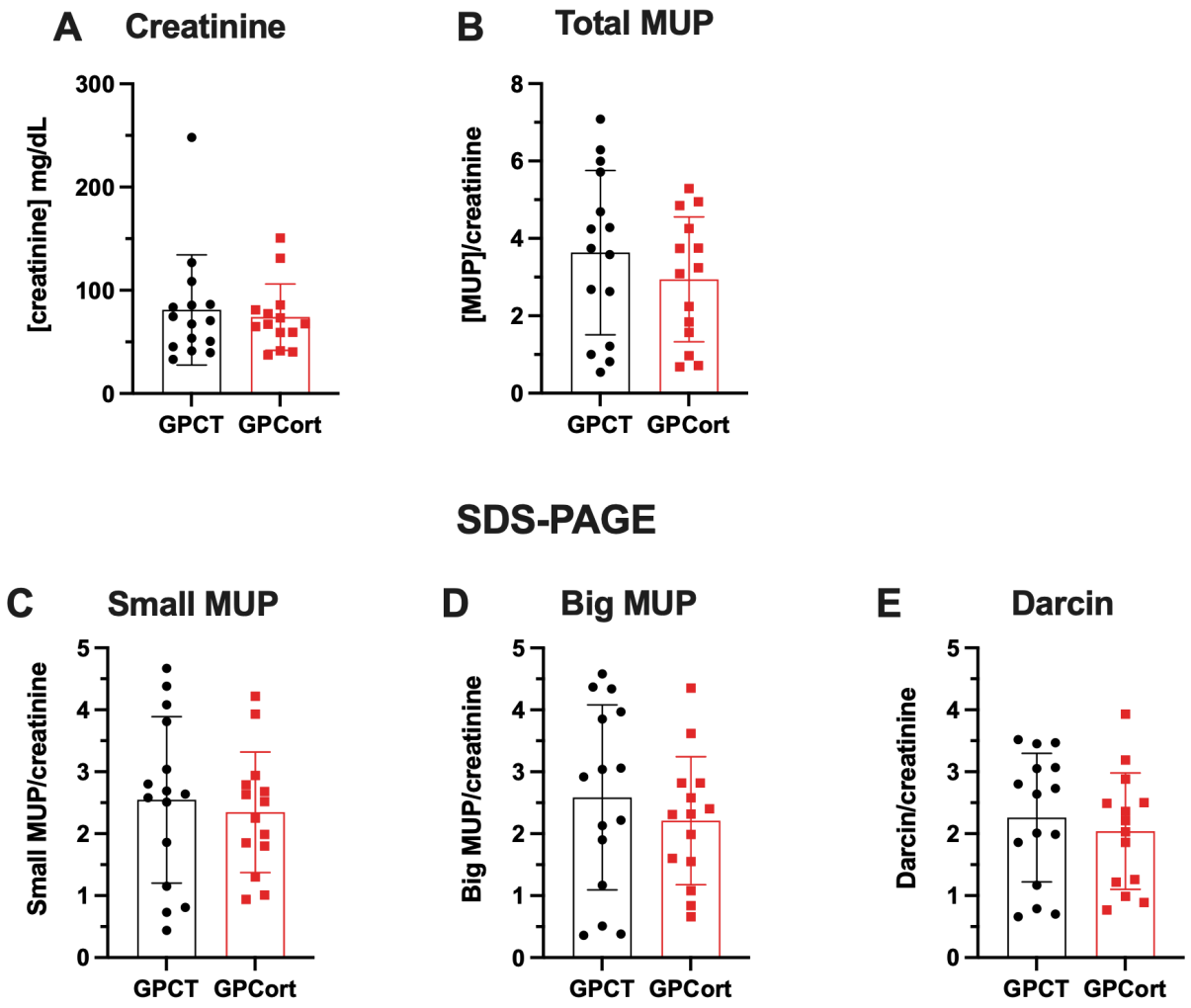


Fig. 2.9. Assessing effects of grand-paternal Cort-treatment on grand-offspring urinary MUP levels. (A – B), urinary creatinine or total MUP were not affected by grand-paternal Cort-treatment. *n*=15/14. (C – E), specific MUP populations in the urine were not affected by grand-paternal Cort-treatment. *n*=15/14. A: Mann-Whitney test. B – E: unpaired t-test. Data represented as mean ± standard deviation.

2.3.6. Cort-treatment does not alter *Mup* gene DNA methylation

Differential methylation of *Mup* genes has been reported, but the epigenetic regulation of *Mup* gene expression remains unclear [162,163,186]. To address this, we developed an optimised sperm DNA extraction protocol that enabled us to conduct the first long-read nanopore sequencing study of *Mup* gene methylation in sperm DNA harvested from Cort-treated and control F_0 males [187,188]. Overall, CpG methylation indicated a very high methylation frequency across the genome (not shown), which is expected for sperm since it is a transcriptionally quiescent cell population. CpG methylation located within the *Mup* gene cluster was also found to be highly methylated (Fig. 2.10H), with no obvious differences between both groups (Fig. 2.10A). We noted a potential pattern of increased methylation associated with Cort-treatment at the promoter region of *Mup20* and decreased methylation in the 3' downstream region of the gene (Fig. 2.10B); these would be consistent with decreased gene expression of *Mup20* and decreased expression of Darcin protein we had found [189]. Therefore, we assessed CpG methylation at the promoters of the *Mup20* gene, whose locations were obtained from the University of California Santa Cruz (UCSC) genome browser promoter track. However, no differences in methylation were found (p=0.96 – Fig. 2.10J). We further inspected methylation of the transcription factor *Zhx2* and additional *Mup* genes of interest (*Mup3*, *Mup2*, *Mup15*, *Mup18* – Fig. 2.10C – 2.10G), identifying no major differences. Consistent with this, an analysis of differentially methylated regions (DMRs) using dispersion shrinkage for sequencing data (DSS) revealed no significant differences between the groups at the *Mup* locus. Thus, it appears that DNA methylation is not a major epigenetic regulator of *Mup* expression in this rodent model.

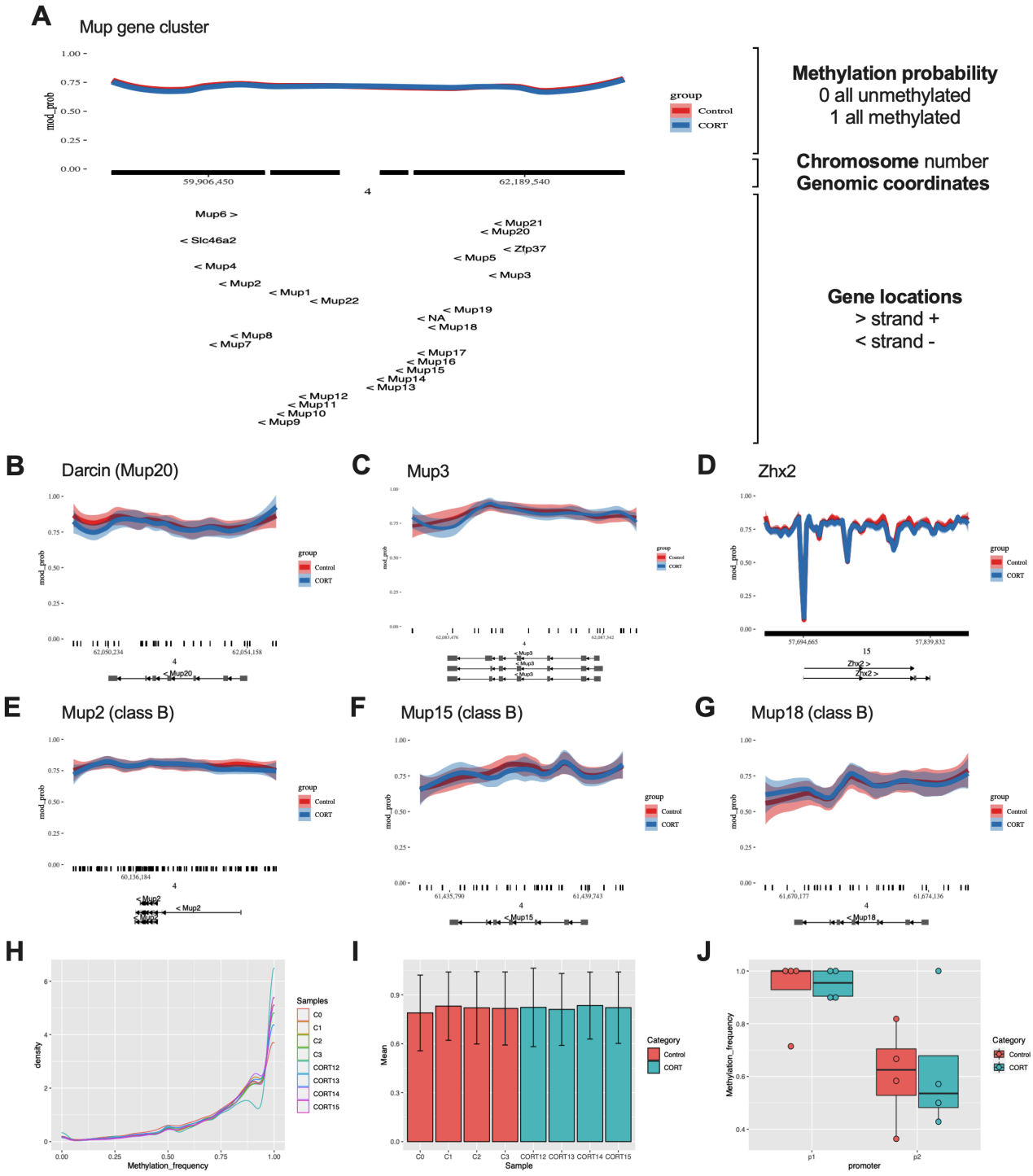
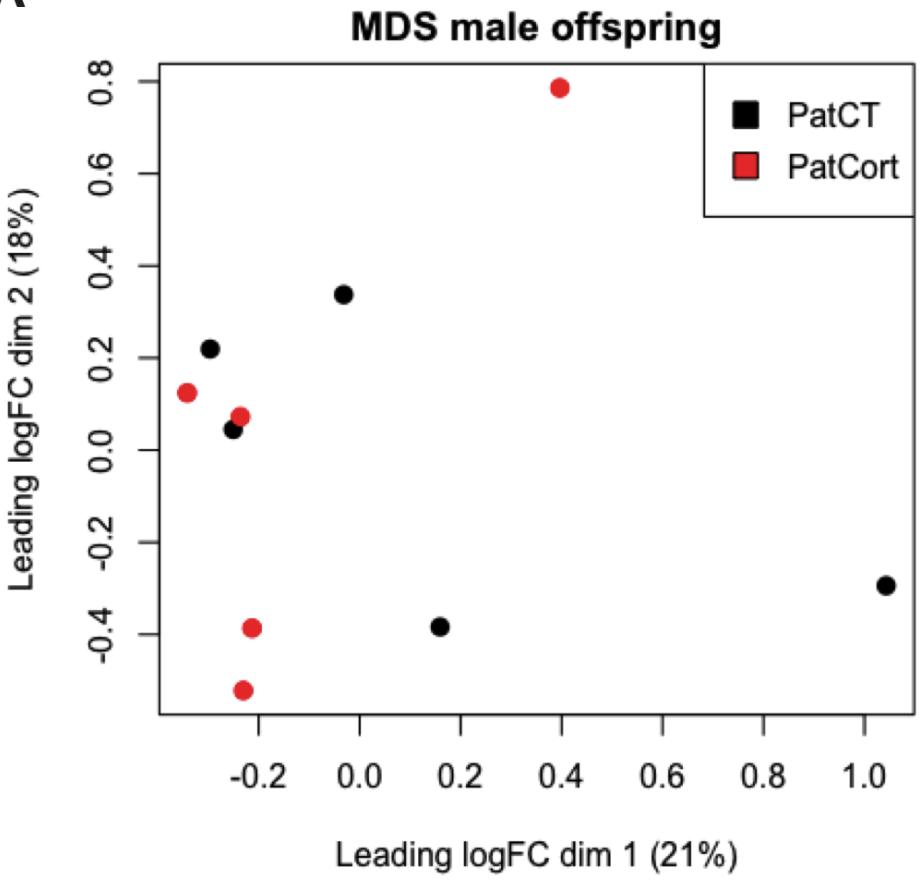


Fig. 2.10. DNA methylation profile of *Mup* genes. (A), whole *Mup* cluster genomic region. (B), *Mup20*. (C), *Mup3*. (D), *Zhx2*. (E), *Mup2*. (F), *Mup15*. (G), *Mup18*. (H), *Mup* cluster methylation density plot. (I), *Mup20* gene methylation frequency per sample. (J), *Mup20* promoters 1 and 2 methylation frequency. Methylation frequency=1: Methylated CpG. Methylation frequency=0: Unmethylated CpG [187,188].

2.3.7. Adult male offspring prefrontal cortex gene expression is relatively unchanged by paternal Cort-treatment

The rodent prefrontal cortex is heavily implicated in displays of social dominance [190], as well as anxiety-relevant behaviours that we have reported in this model [28,191]. We therefore conducted transcriptome profiling of this brain region to determine whether gene expression differences underlie the F_1 offspring phenotypes we observed [192]. Overall, we found that samples were very similar, independent of their group (Fig. 2.11A). Only 32 genes were found to have $p<0.05$ and log-fold change (LFC) threshold = 1, with 3 upregulated and 29 downregulated in the PatCort male mice (Fig. 2.11B, Table 2.2), although they were not statistically significant after false discovery rate (FDR) correction. Similarly, Gene Set Enrichment Analysis (GSEA) was performed, but no significant gene sets were found (Table 2.3).

A


MDS male offspring
PatCT
PatCort
Leading logFC dim 2 (18%)
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
Leading logFC dim 1 (21%)

B

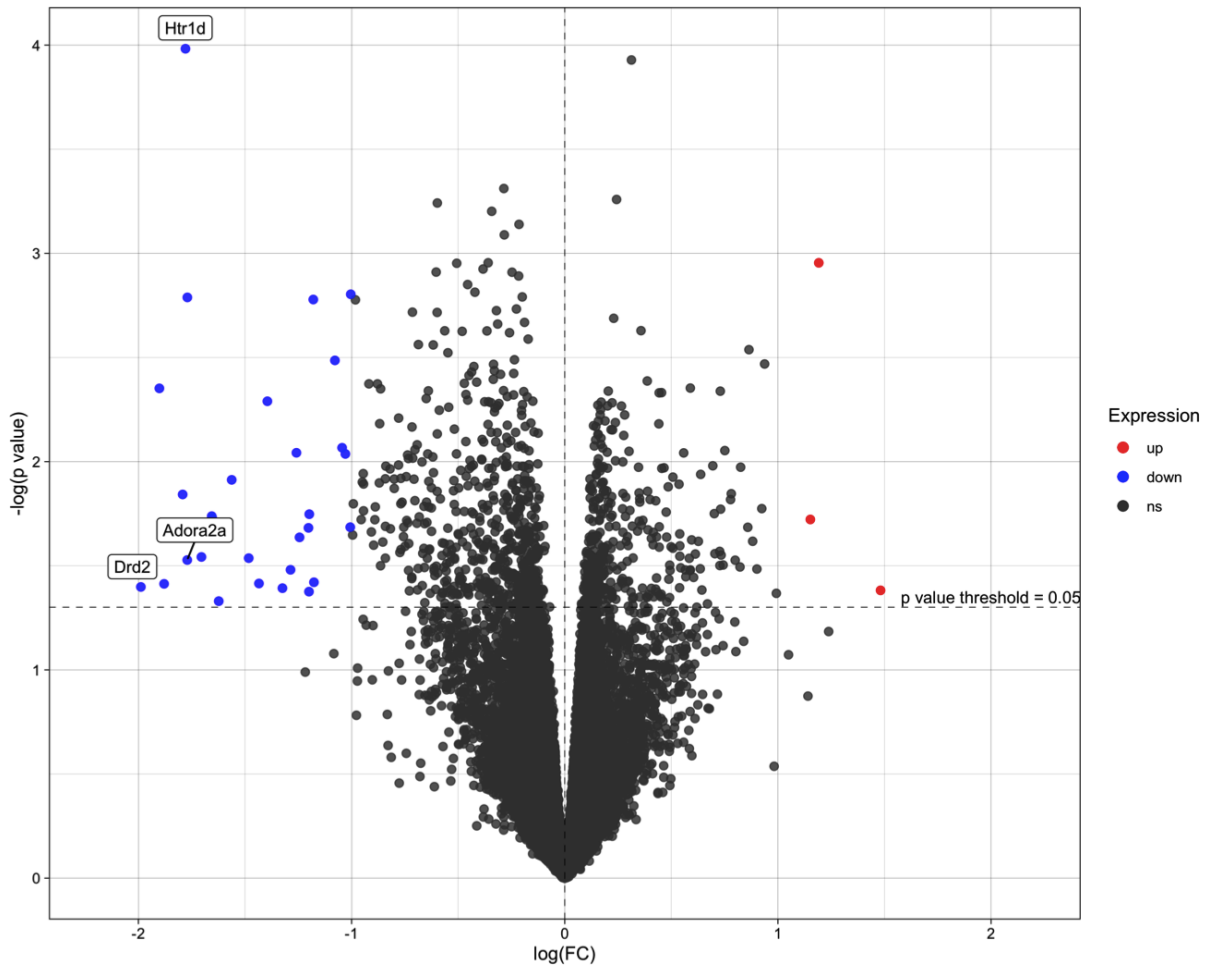

Htr1d
Adora2a
Drd2
p value threshold = 0.05
-log(p value)
log(FC)
Expression
up
down
ns

Fig. 2.11. Assessing effects of paternal Cort-treatment on offspring prefrontal cortex gene expression. (A), multidimensional scaling plot of distances between the male F_1 offspring prefrontal cortex gene expression profiles. (B), volcano plot showing genes with $p<0.05$ and log-fold change threshold of 1 prior to FDR correction. Red: genes upregulated in PatCort. Blue: genes downregulated in PatCort, compared to PatCT [192].

Table 2.2. List of genes with $p<0.05$ in the PFC of PatCort male mice prior to FDR correction. Legend: genes included with $p<0.05$ and LFC threshold = 1.

gene name	logFC	logCPM	F	PValue	description
Htr1d	-1.7794088	0.1134831	26.9624767	0.00010408	5-hydroxytryptamine (serotonin) receptor 1D
Gm16253	1.19174884	-0.6984645	16.0603361	0.00111027	predicted gene 16253
6530403H02Rik	-1.0039713	-0.4233215	14.7331383	0.00157162	RIKEN cDNA 6530403H02 gene
Ndst4	-1.7705306	3.04068684	14.6040417	0.0016271	N-deacetylase/N-sulfotransferase (heparin glucosaminyl) 4
Gm13680	-1.1797611	-0.839943	14.5172903	0.00166564	predicted gene 13680
Gm43823	-1.0783109	-0.9661731	12.1374059	0.00326629	predicted gene 43823
Igfbpl1	-1.9017291	0.15683826	11.1175445	0.00444885	insulin-like growth factor binding protein-like 1
Nmbr	-1.395177	1.13344848	10.661352	0.00513102	neuromedin B receptor
Gm16299	-1.0445865	-0.3589326	9.09198402	0.00857875	predicted gene 16299
Gm6473	-1.2588983	1.69703496	8.92938195	0.00906811	predicted gene 6473
Map3k7cl	-1.0292819	-0.8525231	8.89272178	0.0091828	Map3k7 C-terminal like
Ano2	-1.5624926	-0.210955	8.07490909	0.01222935	anoctamin 2
Sh3rf2	-1.7925705	0.40555043	7.62873312	0.01437308	SH3 domain containing ring finger 2
Dlk1	-1.1985842	0.55636387	7.04088197	0.01788999	delta like non-canonical Notch ligand 1
Gm17794	-1.6562058	0.20614033	6.98415481	0.0182789	predicted gene, 17794
Ptprv	-1.6538717	0.15827073	6.93394202	0.01863129	protein tyrosine phosphatase, receptor type, V
Tspan8	1.15212195	-0.8936078	6.88796378	0.01896083	tetraspanin 8
Gm5829	-1.0064098	-0.6207619	6.66503891	0.02065689	predicted gene 5829
Rps15a-ps6	-1.202439	-0.9658333	6.64615544	0.02080842	ribosomal protein S15A, pseudogene 6
Draxin	-1.2443218	-0.5397824	6.37744503	0.0231097	dorsal inhibitory axon guidance protein
Syndig1l	-1.7044298	2.80947959	5.8359385	0.02870111	synapse differentiation inducing 1 like
Glp1r	-1.4826886	0.49739511	5.80394371	0.02907767	glucagon-like peptide 1 receptor
Adora2a	-1.7710575	2.99674199	5.75323668	0.02968623	adenosine A2a receptor
Lrrc10b	-1.2865551	2.85216404	5.49122498	0.03307592	leucine rich repeat containing 10B
Slc35d3	-1.176682	0.80899848	5.16393751	0.03796042	solute carrier family 35, member D3
Gpr6	-1.434612	1.18472521	5.13221195	0.03847698	G protein-coupled receptor 6
Gm47283	-1.8795921	1.28645542	5.12284307	0.03863109	predicted gene, 47283
Drd2	-1.9885276	2.27041218	5.04282907	0.03997688	dopamine receptor D2
Cd4	-1.324498	1.13303266	5.01099357	0.04052746	CD4 antigen
Gm24245	1.4814654	-0.8349585	4.95531498	0.04151175	predicted gene, 24245
Gm7908	-1.199978	-0.2670917	4.92233475	0.0421079	predicted gene 7908
Dnah11	-1.6232851	-0.4577555	4.68139466	0.04677925	dynein, axonemal, heavy chain 11

Table 2.3. Gene sets detected through GSEA analysis of genes detected in the PFC.

NAME	SIZE	ES	NES	NOM p-val	FWER p-val	RANK AT MAX	LEADING EDGE
WP_PEPTIDE_GPCRS	34	-0.7602396	-1.7671843	0	0.1296	1059	tags=32%, list=6%, signal=34%
KEGG_OXIDATIVE_PHOSPHORYLATION	101	-0.5982546	-1.6257622	4.26E-04	0.958	1282	tags=7%, list=7%, signal=7%
WP_OXIDATIVE_PHOSPHORYLATION	45	-0.655493	-1.5960314	0.005150617	0.9946	352	tags=7%, list=2%, signal=7%

2.4. Discussion

This study has uncovered novel evidence of the paternal influence over offspring social behaviours. Our interest on paternal, rather than maternal, epigenetic inheritance was because the latter involves confounding factors derived from differential maternal care, which has been shown to affect long-term epigenetic marks [12]. The study of paternal epigenetic inheritance, therefore, allows us to investigate the effects of epigenetic marks transmitted through the sperm alone. Paternal Cort-treatment was associated with reduced social dominance and increased attractiveness of their male F_1 offspring, in addition to the elevated anxiety phenotype we previously reported [28]. Interestingly, these effects on social behaviours were restricted to the immediate generation (F_1 offspring), with no significant transgenerational effects on the F_2 grand-offspring. Additionally, the male F_1 PatCort offspring also showed reduced and more variable MUP protein output in their urine, in particular the band that corresponds to the male pheromone Darcin. By performing the first sperm DNA methylome sequencing study in mice treated with corticosterone, we established that the abnormal MUP expression is not a result of dysregulated DNA methylation of the various *Mup* genes. Additionally, the absence of differences in the prefrontal cortex (PFC) transcriptome strongly suggests that the altered social responses of F_1 PatCort offspring likely originate in other brain regions key to social interaction, such as the anterior cingulate cortex or the ventromedial hypothalamus nucleus [193,194].

2.4.1. F_0 male mice showed reduced dominance and no difference in attractiveness

Male mice treated with corticosterone in their drinking water for 4 weeks showed a large reduction in their social dominance, with no effects on their attractiveness to

female mice. There is extensive literature on dominance and social hierarchy priming response to stress [59], as it is known that hierarchical rank modulates the hypothalamic-pituitary-adrenal (HPA) system reactivity [195], possibly due to higher-order impacts of the prefrontal cortex on hypothalamic function. Studies on stressful exposures modulating social dominance and hierarchical rank are less common, but it has been reported that a cortico-hypothalamic circuit modulates social dominance [196] and maternal separation reduces adult dominance in a competition for water in water-restricted mice [197]. Furthermore, chronic restraint decreases social dominance in the tube test [198] and severe immobilisation stress heavily reduces social dominance in anxious mice [199]. Another study reported the opposite effect of stress on dominance, with maternal separation resulting in increased social dominance in the tube test in rats [200]. Therefore, dominance can both regulate the response to stress but also be modulated by it, which indicates the complexity of the neural circuitry regulating this social behaviour, as well as its reactivity to corticosterone.

2.4.2. F_1 male and female offspring do not show changes in social conditioned-place preference

Male and female offspring were tested as juveniles in the social conditioned-place preference, and they displayed expression of social reward, with no differential effects of the paternal corticosterone exposure. Social reward has been proposed to drive the approach towards socially relevant environments, as well as the avoidance from predicted social isolation, and is expressed during youth in social animals [50]. Through the acquired social experiences resulting from this behaviour, the social reward would then influence the development of adult sociality [201]. Stress can modulate social reward, as it has been shown that foot-shock stress exposure reduces social reward expression [202], and chronic social defeat stress leads to reduced social reward as measured in the social conditioned-place preference test [203,204]. Stress exposure has also been found to impair sociability [205], with increased aggression in males and social withdrawal in females [206]. The effects of stress on adult sociability could be due to impaired acquisition or expression of social reward, although further studies are needed to investigate this hypothesis. Nevertheless, differences in social reward were not observed in our model, which indicates that the changes in adult social behaviour observed in these mice are not due to altered social developmental trajectories.

2.4.3. Male F_1 offspring show lower social dominance and increased attractiveness

Male offspring were tested as adults in the social dominance tube test and mate-choice test. PatCort F_1 offspring showed reduced social dominance in the tube test, like their fathers. This effect was expected, due to their increased anxiety-like behaviour, as studies have showed that anxiety affects social behaviours [40,207], although other studies did not show association between anxiety-like behaviour and social rank in the tube test [208]. These mice also showed increased attractiveness, which was not expected. It is important to consider that the setup we used for the mate-choice test intentionally did not let the males display territoriality, compete over mating opportunities, or initiate an interaction with the female, rendering them passive to female mouse discretion. We chose this approach because we wanted to focus on the female choice towards the male, therefore focussing on her assessment of male quality, without male coercion. Therefore, the results in attractiveness could have been different if a competitive setup had been used where the males could display their dominance.

Both MUPs and ultrasonic vocalisations (USVs) have been shown to attract females [209,210]. When males are exposed to females, they emit USVs as part of a 'male song' [211]. Additionally, males excrete MUPs in their urine. These proteins have pockets that bind volatile pheromones, but they also act as non-volatile pheromones. MUP expression is complex and conveys a plethora of information, ranging from sex, health status, individuality, and attractiveness [212]. By using different setups in the mate-choice test, we tried to determine the key features underpinning, and potentially driving, the increased attractiveness in the PatCort mice. This may include the USVs being derived from the physical presence of the mice in the apparatus, and the MUPs being derived from the presence of soiled bedding. For instance, in our 'standard' setup, where we added both mice and their soiled bedding, we accounted for both USV and MUPs.

USVs have been shown to travel short distances and therefore are more relevant for short-distance communication [213]. MUPs and other olfactory-based signals, on the other hand, can travel long distances and have therefore been associated with long-distance communication [209]. In a natural setting, pheromone marks scattered around a certain area would indicate to a female mouse an attractive socially relevant stimulus. If there was a male mouse nearby, he would then vocalise once the female mouse approached him. Therefore, it is possible that in the mate-choice test setups we used, olfactory marks devoid of pairing with USVs, or vice-versa, could have not been enough to signal attractiveness, hence no differences were observed in the 'bedding-only' and the 'mouse-only' setups. Additionally, we assessed the male interaction response towards the approaching female in the 'standard' set up, to determine whether a differential interaction could have accounted for the changes in attractiveness. However, no differences were observed.

2.4.4. F_2 male and female grand-offspring do not show changes in social conditioned-place preference

Following the same experimental design, male and female grand-offspring were tested as juveniles in social conditioned-place preference, and male grand-offspring were assessed as adults in the social-dominance tube test and mate-choice test. However, despite the previously observed increase in depressive-like behaviour in the adult male mice, no differences were observed for any of the social behaviours tested. This shows the limited heritability of the effects that the paternal corticosterone exposure has on social behaviours, which is also observed in other studies, with phenotypes spanning across only one generation following the environmental exposure [7].

2.4.5. F_1 offspring mRNA sequencing does not show overt changes in gene expression

Following the reduced social dominance in the PatCort male offspring, we assessed gene expression in the prefrontal cortex of these mice, to investigate potential transcriptomic regulation underpinning this behaviour. We chose the prefrontal cortex because of the association between this brain region and social dominance. For instance, studies have shown that the synaptic efficacy regulated by AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors in this region controls the expression of social dominance [214], that the increase in social dominance as assessed in the tube test accompanies modifications of the stable actin fraction in synaptic spikes in this region [200], and that social dominance is followed by differential gene expression in this region [208]. Lastly, neuronal population activity in the medial prefrontal cortex (mPFC) predicts social rank and success in competitive settings [196].

However, no differentially expressed mRNAs were detected after a rigorous False Discovery Rate correction for multiple comparisons. This effect might be due to four possibilities: 1) Animals were culled at baseline (without acute stress). Unpublished data from our group showed that PatCort males do not differ from controls in baseline plasma corticosterone, but only after a restraint stress. 2) Sequencing was done from bulk-tissue RNA. When considering the heterogeneity of the cellular populations in the prefrontal cortex, it is possible that cell-specific differences in gene expression are not detected (i.e. 'diluted out') by bulk-mRNA sequencing. 3) Differential gene expression occurs during development, and we only assessed adult PFC. 4) We only assessed adult PFC, and transcriptomic changes may have been present in one or more other relevant brain areas. We hypothesise that these mice exhibit behavioural changes in adulthood due to differential developmental trajectories that result in subtle neuronal changes, such as modifications in synaptic architecture, spine density or dendritic arborisation.

Therefore, differences in gene expression may have been present during critical periods of development, such as during embryonic, early postnatal and/or adolescent stages.

2.4.6. F_1 male offspring MUP protein analyses

Urine was collected from adult male F_1 offspring for quantification of MUP proteins. PatCort mice showed reduced levels of specific MUP bands observed after separation in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Three different bands with molecular weight of around ~17 kDa to ~23 kDa were identified, which is similar result to what have been described before [77,152]. The band with the lowest molecular weight has been shown to be present in males only and to be the most relevant to signal male attractiveness, and it has been named 'Darcin' [152]. In our dataset we have observed a reduction in the Darcin and the small band MUP populations (the band with higher molecular weight).

Importantly, contrary to the current literature [152,215], in the mate-choice test we did not observe a correlation between urinary Darcin intensity and male attractiveness. Mate choice is a complex decision that depends on the integration of multiple sensory, affective and cognitive systems [209]. Therefore, we hypothesise that although PatCort males exhibit lower levels of Darcin in their urine, other factors might be modulating their attractiveness. Indeed, the different setups we used for the mate-choice test indicate that a combination of both olfactory and auditory stimuli seems to be necessary to signal the differential attractiveness in these mice. Additionally, the highly individual and complex urinary MUP pattern, which is pronounced in wild mice, indicates that female mice use this information for additional profiling of the male quality [216], and therefore could modulate the attractiveness signalled by Darcin alone. It is also important to consider the role of volatile pheromones that bind to and are slowly-released from MUPs, which can have their concentration proportionally altered by social status [217]. Due to the reduced social dominance in the PatCort mice, it would be interesting to profile the volatile pheromone content in their urine.

2.4.7. F_1 male offspring *Mup* mRNA expression analysis and correlation with MUP bands in SDS-PAGE

Due to the changes in MUP protein expression, we collected the liver from the adult male offspring for *Mup* mRNA expression assessment. No differences were observed between the groups for *Mup3*, *Mup20*, class-B *Mups* and *Zhx2*. However, there was a very strong correlation between *Mup3* gene expression and urinary MUP output in the PatCort only, with no correlation in the PatCT.

The MUP protein concentration normalised by creatinine output, as analysed in this study, is determined by a range of factors. To begin with, the expression of *Mup* genes is induced by many different factors, such as testosterone [218], pulsatile growth hormone [185], and circadian glucocorticoid [219]. Social factors also modify the expression of MUPs, with social dominance proportionally affecting MUP production [220], possibly due to changes in testosterone levels [218]. The differential transcription of *Mup* gene paralogs results in the large diversity of MUP proteins observed in the urine [221], and one of their known transcription factors is *Zhx2* [160]. There is no evidence of post-transcriptional processes regulating MUP protein concentration, as the mRNA expression predicts the urinary protein output [221], and MUPs are not reabsorbed in the kidney [222]. Lastly, creatinine levels relate to the muscle mass and are a marker of glomerular filtration [218], and were used in this study to normalise the absolute MUP protein levels in the urine. This normalisation accounts for urine dilution, which can also be changed by social status, with submissive mice reducing their urine production and subsequently increasing their urinary creatinine concentration [220]. Therefore, the urinary MUP concentration normalised by creatinine output represents the instantaneous MUP expression relative to the protein levels in the body.

The very strong correlation between *Mup* gene expression and protein output in the PatCort indicates that the overall effect of the factors described above should be homogeneous across this group, resulting in a deterministic association between *Mup* gene expression and its protein output. However, despite no differences in gene expression between the groups, MUP protein is lower in the PatCort. More studies would be necessary to determine the regulatory mechanisms underpinning this result. It is interesting as well that most measures of MUP band levels and *Mup* gene expression have higher variability in the PatCort group only, compared to the PatCT counterpart, which suggests that paternal-Cort exposure affects the expression of these genes, but not homogenously.

Additionally, the primers we used for detecting class-B *Mups* aligned with a range of different *Mup* genes (*Mups 1*, *2*, *7*, *8*, *9*, *10*, *11*, *12*, *13*, *14*, *15*, *17*, *18*, *19* and *22*) to cover some of the *Mup* genes that are evolutionary related [168]. Therefore, we may have missed resolving individual *Mups* that are also implicated as female attractants. However, it is important to notice that Darcin (*Mup20*), *Mup2* and *Mup3* are highly expressed compared to the other *Mup* genes [77].

2.4.8. No differences in MUPs in male F_2 grand-offspring

Despite no differences in attractiveness observed in the male grand-offspring, urine was collected from these mice for quantification of MUP proteins. As expected, no differences were observed in MUP protein concentration, and neither for the bands

separated in the SDS-PAGE. Whilst we know that F_0 paternal-corticosterone treatment can have effects that transmit to F_2 grand-offspring, particularly with respect to depression-like behaviour [28], it appears that this transgenerational epigenetic inheritance is specific and does not generalise to the social behaviours and MUP expression that we now report as changed in the F_1 offspring.

2.4.9. No differences in methylation of *Mup* genes in male F_0 sperm

Sperm DNA methylation was assessed through DNA long-read sequencing to determine whether the increased male offspring attractiveness and reduced urinary MUP protein levels could be due to the inheritance of Cort-treatment-induced altered DNA methylation. Although most of the parental DNA marks get erased during early development due to the embryonic reprogramming [105], it has been suggested that certain DNA marks can escape this process [223], as it has been shown to occur in regulatory regions of glucocorticoid and estrogen receptors [80]. Additionally, a previous study has showed increases in the DNA methylation of *Mup* genes in adult mice, induced by early-embryonic manipulations, which also resulted in repression of *Mup* transcription [162]. However, no overall differences in CpG methylation spanning the *Mup* gene cluster were found in our study, which suggests that other regulatory mechanisms underlie the decreased MUP protein levels in the PatCort male mice.

2.5. Conclusions

Overall, these new results have implications for our understanding of adaptiveness in the context of the epigenetic inheritance, as social interactions are known to contribute to fitness in mice [224]. More specifically, social hierarchy can affect survival due to differential access to resources [42] and modulation of the HPA axis responsiveness [58] and, together with male attractiveness, they can impact access to mating opportunities [57,63]. The sons (F_1 offspring) of corticosterone-treated mice showed reduced dominance and, although they showed increased attractiveness, it was only when they were in proximity to their urinary marks. Therefore, although it is not possible to establish the causality between low urinary output and dominance, we hypothesise that in a natural environment, due to their lower dominance, these mice would have lower total urine production and success in marking territory, which has been shown to affect reproductive success in wild mice [225]. These male offspring (whose fathers had elevated stress hormone levels) would also have a decline in their survival rates.

Aspects of epigenetic inheritance in mammals are still met with some scepticism, with one of the questions being why such inheritance evolved if its impact is rarely

observed across many generations [226]. However, the hypothesised decrease in survival proposed above due to the reduced social dominance observed in the PatCort mice suggests that by modulating endophenotypes that determine fitness, epigenetic inheritance could impair reproduction and survival, which could then heavily impact the generations to come, even though the changes in behaviour are observed in only one generation. This 'trans-populational impact' has been suggested in mice before [75], and has been shown to occur in *C. elegans* [227].

Another factor that can impact adaptiveness and needs to be considered is the mismatch between the environments experienced by the fathers and the offspring/ grand-offspring. Some studies indicate that the epigenetic inheritance could fit into the mismatch hypothesis of disease [151], which posits that changes in the environment during development induce adaptive changes that can prime the individual for that environment (within genetic constraints). For instance, adult-generated neurons born during chronic social stress are uniquely adapted to respond to subsequent chronic social stress [228]. However, when an environmental mismatch happens between the timepoints, it can result in maladaptation and disease. Similar mechanisms might be at play behind how the epigenetic inheritance functions. For instance, it has been suggested that when environmental cues are not reliable predictors of offspring environment, in the case of environmental conditions changing between generations, the epigenetic inheritance could instead reduce fitness [229,230].

Lastly, it has been suggested that male attractiveness could have evolved with the aid of epigenetic mechanisms and female mate choice [76]. The evolutionary expansion of mouse *Mup* genes is recent [167], occurring due to multiple duplication events [168] that have led to the emergence of many pseudo-genes [154]. This indicates selective pressures shaping scent signals relevant for social communication [166], and this is in accordance with olfactory signals that mediate territorial behaviour and sexual selection being under evolutionary pressure [166]. Interestingly, one of the first reports on epigenetic inheritance in mice showed differential methylation of *Mup* genes [163], and it has been shown that different sociality levels can have transgenerational effects on MUP expression [77]. These studies indicate that one of the avenues to understand the evolutionary relevance of the epigenetic inheritance could be through investigating its regulation of genes that are relevant for sexual selection. For instance, epigenetic reprogramming of reproductive function, driven by early-environment, has been shown in humans [231].

In conclusion, in this study we showed that epigenetic inheritance can modulate social behaviours that are important for determining reproductive success, thus potentially impacting many generations. The present findings, along with other studies investigating epigenetic inheritance, help shed light on the evolutionary impact that this type of inheritance confers. The observed phenotype does not necessarily accompany altered gene transcription in adult offspring (in the limited tissues examined at a single

adult stage), which suggests developmental processes are at play. Therefore, it is essential that studies on gene expression focus on the critical developmental timepoints and explore additional relevant tissues and cell population in offspring. Lastly, epigenetic mechanisms underpinning this type of inheritance need to be investigated further, including sperm epigenetics and post-conceptual transfer of epigenetic information via developmental algorithms.

2.6. Methods

Mice and husbandry:

Unless indicated, all mice were housed in groups of 3 to 5 in open-top cages with Aspen shaving bedding (Romania) and 2 sheets of paper tissue for nesting. Cages were changed weekly, and food and water were provided *ad libitum*. 7-week-old *naïve* male and female C57BL/6 breeders were obtained from the Animal Resources Centre (Murdoch, WA, Australia). One week later, male breeders were single-housed and randomly assigned to the control (CT) or corticosterone (Cort) group (total liquid consumption can be found on Sup. Fig. S2.13). One week before the end of the corticosterone treatment described below, male breeders were tested for the mate-choice and social-dominance tube tests at PND 77. After the designated corticosterone treatment period, CT and Cort male breeders were individually and randomly assigned to *naïve* female breeders and paired for 5 days, after which the males were culled. The females were left single-housed and undisturbed for 19 days, after which they were checked daily for newborn litters. Pups from CT or Cort fathers (Paternal CT – PatCT or Paternal Cort – PatCort groups) were weighed on post-natal day (PND) 7, 14 and 21, during which the boxes were changed. Pups were weaned at PND 28 and were group-housed according to sex and to paternal treatment, with pups from multiple different litters being housed together to avoid litter effects. Male and female offspring were tested for the social conditioned-place preference test at PND 35, and the male offspring were tested for the mate-choice and social-dominance tube tests at PND 77. When the behavioural testing was complete, the male offspring were single-housed and paired with *naïve* females to generate the grand-offspring: grand-paternal control (GPCT) and grand-paternal corticosterone (GPCort); the procedure was the same as described above (Sup. Fig. S2.14). All male mice tested were also paired with a *naïve* female for mating. All procedures were approved by the Florey Institute of Neuroscience and Mental Health Animal Ethics Committees (AEC), complying with the Australian Code for the Responsible Conduct of Research and the Australian Code for the Care and Use of Animals for Scientific Purposes. For each generation at least 16 mating pairs were used per group, generating at least 9 litters per group. The *n* size per group for the behavioural experiments was 16-29, and the individual numbers can be found in the figure legends.

The numbers of pups and litters used per group can be found in Table 2.4. Maternal behaviour was scored during PND 2, 7 and 14, when the dams were given a score from 0 to 3 according to their presence in the nest and grooming of the pups or arched-back nursing, every 5 minutes until 7 measures were taken. No differences in maternal behaviour were found. Culling of mice, urine and liver collection were performed from around 10 am to 1 pm.

Table 2.4. Number of litters and pups per group.

Group	Number of litters	Number of pups
PatCT	21	162
PatCort	17	125
GPCT	9	66
GPCort	14	92

Corticosterone treatment:

Corticosterone treatment was as per our published protocols [28,183]. Briefly, the Cort group of male mice was given 25 µg/mL corticosterone hemisuccinate (Steraloids Inc., Newport, RI, USA) in their drinking water, changed twice a week, for 4 weeks. Control (CT) male mice received the same drinking water, without corticosterone added.

Behavioural experiments:

Social conditioned-place preference: the protocol was adapted from Dölen et al., 2013; Nardou et al., 2019. This test was used to assess social reward, which is the result of the interaction between the approach towards socially salient stimuli, and the avoidance of cues that predict social isolation, which is more easily observed in juvenile mice when social interactions are not affected by sex-specific interests [50]. Male and female offspring and grand-offspring, from paternal and grand-paternal CT and Cort treatment respectively, were tested on PND 35. In a locomotor chamber (ENV-510, Med Associates, Fairfax, VT, USA) divided in two similar halves with a door connecting the two halves, two different types of bedding were used to cover the floor in each half (CornCob or Alpha Dri), around 1 cm height. Mice were first assessed in the locomotor chamber and had their activity recorded for 30 min, and the time spent on each side was measured (pre-conditioning). Soon after the exploration, all mice from the same cage were housed together in a new cage with one of the bedding types for 24h, after which they were single housed with the other bedding type for 24h. Mice were then assessed in the locomotor chamber once again (post-conditioning). The time and ratio of the time spent in the social-conditioned bedding during post- and pre-conditioning were evaluated.

Mate-choice test: the protocol was adapted from Hoffmann et al., 2020; Mitra and Sapolsky, 2012. This test was used to assess male quality or "attractiveness" as perceived by a fertile female, which predicts the likelihood of first copulation and mating duration [63]. Male quality is determined by scent marking [64] and vocalisations [65]. Using a 3-chamber interaction test, a fertile female (assessed daily before the test by vaginal smear) explored the apparatus for 10 min. Then, one male from each experimental group was put inside a small cage on each side of the apparatus, and the female explored the apparatus again for 10 min, during which the time she spent interacting with each male mouse was measured. Different set ups were used for this test in order to investigate the underlying factor for an eventual change in attractiveness. The 'standard' set up consisted of presenting mice and the respective soiled bedding from their home cage (Fig. 2.5B). The 'bedding only' set up consisted of presenting the soiled bedding from their home cage, to assess if pheromones alone determine attractiveness (Fig. 2.5C), whereas the 'mouse only' set up consisted of presenting the mice alone, to assess if ultrasound vocalisations or the male interaction *per se* determine attractiveness (Fig. 2.5D). A different female mouse was used for every round of assessment, including for mouse only and bedding only sessions. Females were not tested because this test relies on behavioural responses linked to the development of male secondary sexual characteristics. As follow up on the results found for the F_1 male offspring, we manually analysed their Mate-Choice trial video recordings to quantify each male's responsive interaction to the approaching female, to determine if there were differences between the groups for this measure. For this analysis, we quantified the time that each male spent with its snout directed towards the female when she approached the interaction zone.

Social-dominance tube test: the protocol was adapted from Tada et al., 2016; Zhou et al., 2016. This test was used to assess social dominance, which underlies agonistic behaviours [56] and can be defined as the capacity to prevail in conflicts encompassing aggression, threats, fights or submission [53,55]. The apparatus consisted of a 30-cm long clear plastic tube. During habituation each mouse was trained to go through the tube for 10 times. On the following day, during testing, each mouse from a CT cage faced every mouse from a Cort cage, in a total of 4 interactions per mouse per cage. Each mouse was tested once every after 7 to 9 interactions, so as to allow resting time between the face offs. The number of wins by the Cort group was compared to the null hypothesis on a chi-square test to determine statistical significance. Females were not tested because this test relies on behavioural responses linked to the development of male secondary sexual characteristics.

Other procedures:

Urinary component assessments

Immediately prior to being culled, urine was collected from each male by scruffing, and frozen at -80 °C immediately. Mouse urine was thawed and diluted 1/4 in MilliQ water. Previous studies have determined that most of the mouse urinary protein content corresponds to MUP proteins [235]. Therefore, MUP concentration was determined using the Quick Start™ Bradford Protein Assay, according to the manufacturer's instructions. Briefly, diluted urine was incubated with Bradford reagent at room temperature for 5 min, after which it was read at 595 nm in Epoch 2 Microplate Spectrophotometer (Biotek/Agilent). The standard curve was constructed using BSA dilutions ranging from 125 to 1,000 µg/mL (Quick Start Bovine Serum Albumin Standard, Cat. #5000206). Creatine concentration was determined using Creatine (urinary) Colorimetric Assay Kit Cayman Chemical Item No 500701 to account for urine dilution [212], according to the manufacturer's instructions.

SDS-PAGE for MUP protein analysis

The protocol was adapted from Lee et al., 2017; Nelson et al., 2015. Mouse urine was thawed and diluted 1/50 in MilliQ water. Beta-mercaptoethanol and SDS loading buffer were added to each sample and heated for 5 min at 95 °C. 10 µL of each sample was loaded into 4-15% gel and run at 200 V for around 20 min. The bands were stained with 0.1% colloidal blue in ethanol using the Coomassie R-250 staining protocol. A reference comprised of pooled urine from 8 mice was used for semi-quantification, which was run in every gel and used as a normaliser across all gels, after measuring its band intensity with ImageJ (v2.1.0/1.53c).

Real-time polymerase chain reaction (RT-qPCR) of *Mup* genes

Mice were killed by cervical dislocation and the right lobe of the liver was dissected, then frozen at -80 °C. Liver RNA was extracted using QIAzol according to the manufacturer's instructions and quantified in Nanodrop (2000c Thermo Scientific). 1000 ng of RNA was reverse transcribed with SuperScript™ VILO™ cDNA Syntesis Kit (Invitrogen, Cat. #11754050). cDNA was diluted 1/10 for qPCR gene expression studies. Relative expression was determined using the comparative ΔΔCt method, with ß-actin as the endogenous control gene. The primers used in this study can be found in Table 2.5.

Table 2.5. Primers used for RT-qPCR.

Gene	Forward	Reverse
Mup3	5'-GCTTCTGCTCCTGTGTTTGGA-3'	5'-CATCAGAGGCTTCAGCAATAGAA-3'

Mup20 (Darcin)	5'-GTGCTGCTGCTGTGTTTGGG-3'	5'-TGTCAGTGGCCAGCATAATAGTA-3'
Class B *Mups*	5'-CAGAAGAAGCTAGTTCTACGGG-3'	5'-GAGGCCAGGATAATAGTATGCC-3'
Zhx2	5'-AGGCCGGCCAAGCCTAGACA-3'	5'-TGAGGTGGCCCACAGCCACT-3'
ß-actin	5'-TATAAAACCCGGCGGCGCA	5'-ATGGCTACGTACATGGCTGG-3'

Offspring prefrontal cortex mRNA sequencing

Mouse prefrontal cortex (bregma +1.42 mm, interaural 5.22 mm) was dissected and snap frozen in dry ice. The RNA was extracted using a standard QIAzol Lysis Reagent (QIAGEN, Cat # 79306) procedure, according to the manufacturer's instructions. RNA was purified from potential DNA contamination with DNA-*free*™ Kit (Ambion, Cat # AM1906), according to the manufacturer's instructions. RNA quality was assessed using the Agilent 4200 TapeStation system. Samples with RIN value higher or equal to 7.5 were sent for sequencing at the Australian Genome Research Facility (AGRF) in Parkville, VIC, Australia. Library preparation was performed using Illumina Stranded mRNA protocol, and sequencing was done in the Illumina Novaseq platform on a SP flowcell. 100 bp long reads were single-end sequenced at a depth of 20M to 49M. Adapters were trimmed by the Casava software used by the Illumina platform.

mRNA Sequencing data analysis

The Galaxy Australia (v1.0) platform was used for quality control, read alignment and generation of count matrix. Read quality control was done with FastQC (v0.72). Alignment was done with HISAT2 (v2.1.0) [236] using mm10 as the reference. Gene count matrix was generated with HTSeq-count (v0.9.1) [237] with the comprehensive gene annotation of the GENCODE M25 release (GRCm38.p6) as reference. Lowly expressed genes were filtered out using the default filtering conditions from the edgeR package (v3.34.1) [238,239]. Differential analysis expression was done using edgeR, and the volcano plot was made with ggplot2 (v3.3.5) [240]. Log-fold change of 1 was set for an exploratory analysis of the non-differentially significant data. Gene Set Enrichment Analysis was performed using GSEA (v4.2.3) [241,242], with the c2.cp.v7.5.1 gene set database.

Sperm collection

For sperm collection, mice were culled by cervical dislocation for immediate dissection of both epididymides. The caudal epididymis was bisected with a clean surgical blade, then immersed into 1.0 mL mt-PBS that was pre-warmed to 37°C and incubated at that temperature for at least 30 mins. Sperm counts were determined, and samples were examined for the absence of tissue debris under a light microscope. Samples were then centrifuged at 400g for 15 mins and excess mt-PBS was carefully

removed leaving approximately 100 µL as the final volume. Samples were immediately frozen down and stored at -80°C until subsequent use.

Long-read sperm DNA sequencing

High molecular-weight sperm DNA collected from F_0 males was obtained using MagAttract HMW DNA Kit (QIAGEN, Cat # 67563), following a modified version of the manufacturer's instructions which consisted of replacing the digestion step with Proteinase K with 40 µL of the reducing agent TCEP (tris(2-carboxyethyl)phosphine hydrochloride, Sigma-Aldrich, St. Louis, Missouri, United States). 5 µg of sperm DNA from 4 CT and 4 Cort F_0 mice were fragmented by centrifugation for 60 s at 7200 rpm using g-TUBES (Covaris, Woburn, MA, USA) to obtain 8 kb-long fragments. These fragments were prepared for sequencing with the Ligation Sequencing Kit (Cat # SQK-LSK 109) and sequenced on the PromethION and GridION platforms (Oxford Nanopore Technologies, Oxford, UK) with a mean genome coverage of ~20X, to ensure methylation calling power.

Sperm DNA methylation analysis

Samples were base called using guppy (v4.2.2) and megalodon (v2.2.9) (Oxford Nanopore Technologies Ltd.) against the configuration file "res_dna_r941_prom_modbases_5mC_v001.cfg". NanoStat [243] was used for data inspection and quality control. Fastq files obtained from megalodon were aligned to the mm10 genome using minimap2 (v2.17-r941) [244], and sam files were sorted and transformed into bam files using samtools (v1.10) [245]. The tool f5c (v0.5) [246] was used to call CpG methylation per read, as well as to calculate frequencies of methylation per CpG. A methylation matrix corresponding to the location of the *Mup* gene cluster and its flanking genes Slc46a2 and Zfp37 was generated using the coordinates Chr4: 59,904,830-62,212,385, totalling 11,026 CpGs. Differentially methylated regions (DMRs) between CT and Cort were determined with the program DSS (Dispersion shrinkage for sequencing data) (v2.43.2) [247], as previously described for Oxford Nanopore [248,249]. Specific methylation at the *Mup20* promoters was determined using genomic coordinates (Table 2.6) obtained from the UCSC genome browser promoter track [250]. Methylation plots were generated using Nanomethviz [251], and density plots, dotplots and boxplots were generated using ggplot2 (v3.3.5) [240]. Statistical analysis of methylation frequencies and data visualisation were performed using RStudio (v4.0.5). The code used for this analysis can be found in the zenodo page (DOI: 10.5281/zenodo.7956197).

Table 2.6. Genomic coordinates for the *Mup20* promoters, obtained from the UCSC genome browser promoter track.

chrom	chromStart	chromEnd	name	score	strand	thickStart	thickEnd
chr4	61959968	61960028	Mup20_2	900	-	61959968	61959979
chr4	62054106	62054166	Mup20_1	900	-	62054106	62054117

Statistical analysis

Data was tested for normality with D'Agostino-Pearson tests. If data distribution was normal, it was tested with unpaired t-tests and, if variance differed between the groups, t-tests with Welch Correction were used. If data was not normally distributed, Mann-Whitney tests were used. The mate-choice test was analysed by quantifying the total time spent interacting with each male, which was analysed with the Wilcoxon Matched-Pairs Signed-Ranks test, in accordance with Mitra and Sapolsky, 2012. Additionally, the data was also analysed with the chi-square test for relative preference for each male. The social-dominance tube test was analysed using a Chi-square to assess difference from an expected chance of 50:50, in accordance with Zhou et al., 2016. Comparisons of variance for MUP protein and gene expression data were performed with F test. Statistical analysis was performed using GraphPad Prism 9 for MacOS (v9.3.1). Statistical significance was reached when $p<0.05$. Graphs are represented as mean ± standard error of the mean (SEM) for normally distributed data, or median ± interquartile range for non-normally distributed data. Data of MUP protein levels and *Mup* gene expression (Figs. 4 and 5) is presented as mean ± standard deviation to showcase differences in variance between groups.

List of abbreviations

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid. Cort: corticosterone. CT: control. DMR: differentially methylation region. DNA: deoxyribonucleic acid. DSS: dispersion shrinkage for sequencing data. FDR: false discovery rate. GPCort: grand-paternal corticosterone. GPCT: grand-paternal control. GSEA: gene set enrichment analysis. HPA: hypothalamic pituitary adrenal. LFC: log-fold change. mPFC: medial prefrontal cortex. MUP: major urinary protein. PatCort: paternal corticosterone. PatCT: paternal control. PFC: prefrontal cortex. PND: post-natal day. RNA: ribonucleic acid. RT-qPCR: real-time polymerase chain reaction. SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis. SEM: standard error of the mean. TCEP: tris(2-carboxyethyl)phosphine hydrochloride. UCSC: University of California Santa Cruz. USV: ultrasonic vocalisation.

2.7. Declarations

Ethics approval and consent to participate

All procedures were approved by the Florey Institute of Neuroscience and Mental Health Animal Ethics Committees (AEC) #19-064, complying with the Australian Code for the Responsible Conduct of Research and the Australian Code for the Care and Use of Animals for Scientific Purposes.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article, its supplementary information files, and publicly available repositories. The sequencing datasets generated and/or analysed during the current study have been deposited in the European Nucleotide Archive (ENA) repository at EMBL-EBI under accession numbers PRJEB60786 (https://www.ebi.ac.uk/ena/browser/view/PRJEB60786) [192] and PRJEB60812 (https://www.ebi.ac.uk/ena/browser/view/PRJEB60812) [187]. The code used for analysing the methylation data has been deposited at Zenodo (https://zenodo.org/record/7956197) [188].

Competing interests

M.B.C has received support from Oxford Nanopore Technologies (ONT) to present their findings at scientific conferences. However, ONT played no role in study design, execution, analysis or publication. The other authors declare that they have no competing interests.

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Authors' contributions

L.B.H. planned and conducted the experiments, performed the data analyses and wrote the manuscript. E.A.M. assisted in the animal studies. R.V.H performed the sperm collection and DNA extractions. C.C.F. performed the sperm long-read DNA sequencing study and data analysis. M.B.C. planned and supervised the sperm long-read sequencing study. T.Y.P. conceived the study, designed the study, supervised data collection and analysis. A.J.H. and T.Y.P. reviewed and edited the manuscript. All authors read and approved the final manuscript.

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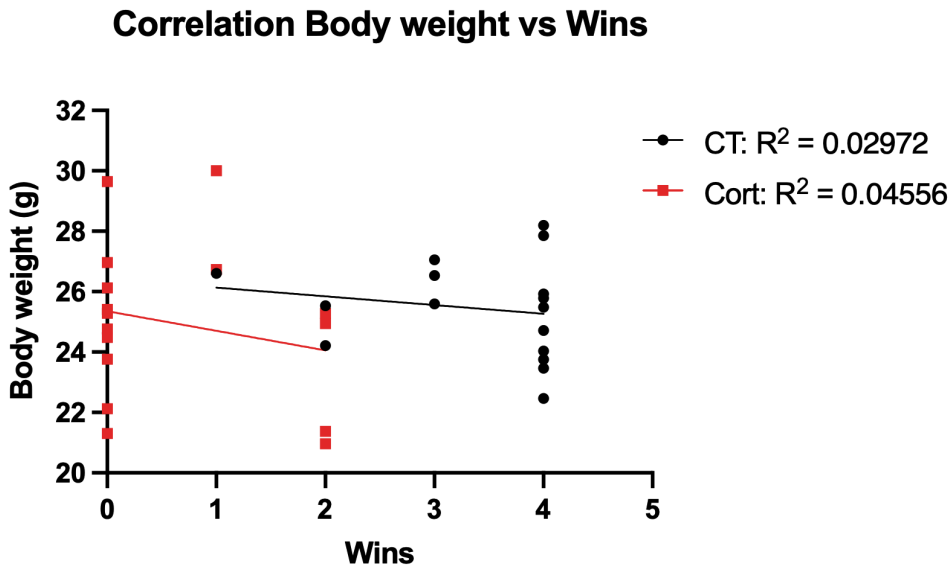
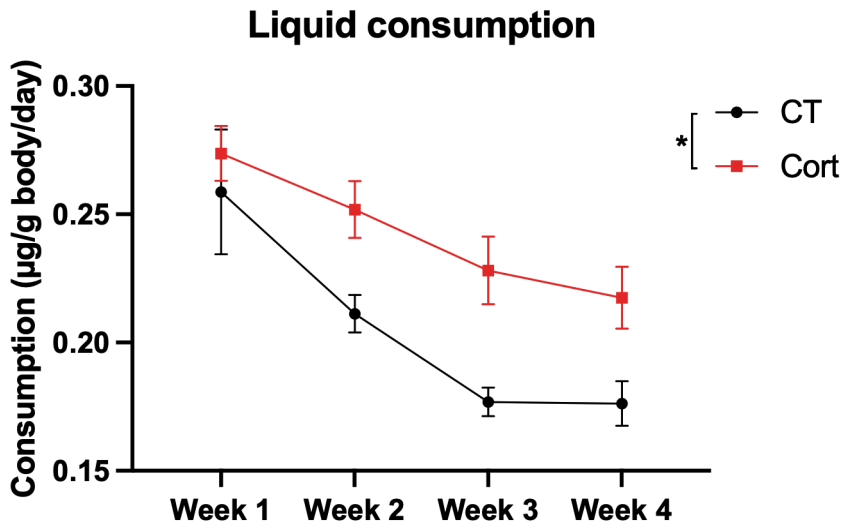
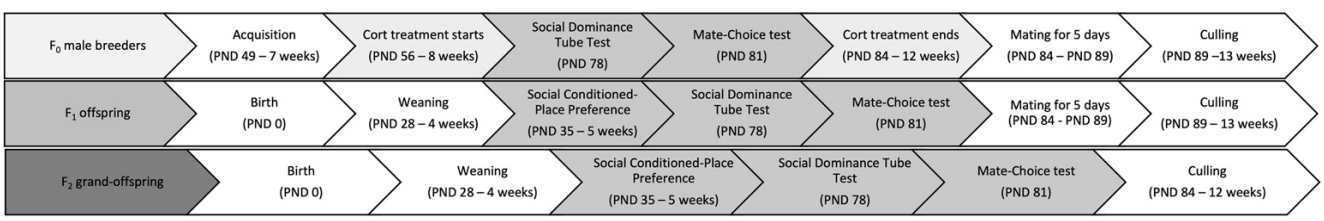
2.8. Supplementary material



Sup. Fig. S2.12. Correlation between body weight and performance in the social dominance tube test. No statistically significant correlation was found for CT or Cort groups. Simple linear regression. CT p=0.5232, n=16; Cort p=0.4273, n=16.



Sup. Fig. S2.13. Assessing liquid consumption during Corticosterone treatment. As expected, Cort-treated mice drink more liquid compared to CT. Repeated-measures ANOVA. CT n=16, Cort n=16. Group: $F_{(1,30)}$=5.835, p=0.04388. * p<0.05.



Sup. Fig. S2.14. Experimental design.

Chapter 3

Chronically high stress hormone levels dysregulate sperm long noncoding RNAs and their embryonic microinjection alters development and affective behaviours

3.1. Abstract

Previous studies on paternal epigenetic inheritance have shown that sperm RNAs play a role in this type of inheritance. The microinjection of sperm small noncoding RNAs into fertilised mouse oocytes induces reprogramming of the early embryo, which is thought to be responsible for the differences observed in adult phenotype. While sperm long noncoding RNAs (lncRNAs) have also been investigated in a previous study, their microinjection into fertilised oocytes did not yield conclusive results regarding their role in modulating brain development and adult behavioural phenotypes. Therefore, in the current study we sought to investigate this further. We used our previously established paternal corticosterone (stress hormone) model to assess sperm lncRNA expression using CaptureSeq, a sequencing technique that is more sensitive than the ones used in other studies in the field. Paternal corticosterone exposure led to dysregulation of sperm long noncoding RNA expression, which encompassed lncRNAs, circular RNAs and transposable element transcripts. Although they have limited functional annotation, bioinformatic approaches indicated the potential of these lncRNAs in regulating brain development and function. We then separated and isolated the sperm lncRNAs and performed microinjections into fertilised oocytes, to generate embryos with modulated lncRNA populations. We observed that the resulting adult offspring had lower body weight and altered anxiety and affective behavioural responses, demonstrating roles for lncRNAs in modulating development and brain function. This study provides novel insights into the roles of lncRNAs in epigenetic inheritance, including impacts on brain development and behaviours of relevance to affective disorders.

Keywords: long noncoding RNAs, microinjection, paternal epigenetic inheritance.

3.2. Introduction

There is a growing body of work showing that parental environmental exposures can affect the phenotype of future generations, including paternal stress [28,29,81]. This type of non-Mendelian inheritance involves epigenetic mechanisms and has been reported to affect the behaviours of the F_1 offspring, F_2 grand-offspring, and beyond [180]. We previously reported that paternal corticosterone exposure preconception (modelling moderate chronic stress) induced sex-specific increases in anxiety-like behaviours of male F_1 offspring [28] while apparently sparing female F_1 offspring. Importantly, we and others have found that the intergenerational effects of paternal stress were mediated by sperm small noncoding RNAs, especially miRNAs which we found to be downregulated in corticosterone-treated animals [28].

In the context of epigenetic inheritance, sperm small noncoding RNAs (sncRNAs) have been the most intensively studied molecular mediators. The ability to isolate sncRNAs from the sperm and microinject them into fertilised oocytes [26,31] have enabled mechanistic studies of their contributions to intergenerational effects. Although the microinjection of sperm sncRNAs has been claimed in some studies to partially replicate the intergenerational effects on offspring phenotypes observed through natural mating, the contribution of other forms of noncoding RNAs remains unclear. Due to the short half-life that sperm sncRNAs are presumed to have in the early embryo, and the intense cell division and differentiation that occurs during embryonic development, it is unclear how sperm sncRNAs affect reprogramming to bring about phenotypic changes that persist until adulthood.

Long noncoding RNAs (lncRNAs) are another subpopulation of noncoding RNAs that are likely to play a role in the observed intergenerational effects of paternal stress exposures. There are thousands of lncRNAs reported to be present in the sperm [252] and they are poorly understood, with mostly unknown functions. However, it is known that lncRNAs can recruit and act as scaffolding for the Polycomb Repressive Complex (PRC), which induces histone modifications resulting in chromatin repression [253,254]. Various lncRNAs could therefore be modulators of the intergenerational effects by regulating the extent of histone modifications, which are replicated with high confidence during cell divisions and persist across development until adulthood. There has only been one other study attempting to elucidate the contribution of sperm lncRNAs in the transmission of paternal stress effects. Using their early-life stress model, Gapp and colleagues microinjected sperm lncRNAs into fertilised oocytes, and found no evidence that they modified the F_1 offspring behavioural phenotype, but instead exerted some metabolic health effects [126]. More studies are needed further to elucidate how stress, and associated stress hormone elevation, affects the lncRNA content of sperm and its impact on offspring behavioural phenotypes, including cognitive and affective function.

In this study, we employed CaptureSeq [255] to reveal the impact of corticosterone-exposure on the mouse sperm lncRNA profile. We observed thousands of differentially expressed lncRNAs, and significant changes were also found for transposable element transcripts and circRNAs. We carried out exploratory bioinformatic analyses to identify potential mechanisms in reprogramming the embryonic epigenetic landscape that would ultimately alter adult behaviour. We hypothesised that the microinjection of fertilised oocytes with lncRNAs isolated from the sperm of corticosterone-treated male mice would lead to discernible alterations in affective behavioural responses. Indeed, we observed that the microinjection of lncRNAs leads to alterations in offspring behavioural responses in the light-dark box and Porsolt swim test, although the effects were mostly indistinguishable between mice born from microinjection with lncRNAs extracted from control or corticosterone-treated mice. Therefore, our findings in the

present study implicate sperm lncRNAs in the intergenerational modification of affective phenotypes.

3.3. Materials and Methods

Mice and husbandry:

C57BL/6 mice were sourced from two different facilities: C57BL/6 mice from ARC (Perth, Australia) were used for generating the sperm lncRNAs for CaptureSeq and for the microinjections. C57BL/6 mice from ARC were used in previous studies for behavioural phenotyping. Mice and fertilised oocytes from WEHI (Kew, Melbourne, Australia) were used for the behavioural studies and microinjections.

Mice were weaned and weighted weekly from PND 42 (Week 6) to PND 63 (Week 9). Behavioural testing started at around PND 75. Mice were culled when they were around PND 84.

Mice were housed in open-top cages, with lights on at 7 am and off at 7 pm. Male mice were housed with maximum 4 mice in a box, and females were housed with maximum 6 mice per box. Food and water were provided *ad libitum*. All mice were culled by cervical dislocation. Information about each group can be found in the Table 3.7. A timeline can be found in Fig. 3.18A and Fig. 3.18B.

Table 3.7. Groups used, their source and whether they were generated via microinjection.

Group	Mouse source	Source microinjected RNA
PatCT	WEHI, Kew	Not applicable
PatCort	WEHI, Kew	Not applicable
InjCT	WEHI, Kew	ARC, Perth
InjCort	WEHI, Kew	ARC, Perth

Sample sizes were based on prior calculations and experience with experimental variance, effect sizes and reproducibility associated with each experimental technique and measure. All procedures were approved by the Florey Institute of Neuroscience and Mental Health Animal Ethics Committees (AEC), complying with the Australian Code for the Responsible Conduct of Research and the Australian Code for the Care and Use of Animals for Scientific Purposes.

Corticosterone treatment:

When male breeders were 8 weeks old (PND 56) days old, those randomised to corticosterone treatment (versus drinking-water controls) were caged individually and given 25 mg/L of corticosterone hemisuccinate (Steraloids Inc., Newport, RI, USA) in their drinking water for 4 weeks. The corticosterone solution was replaced with a new solution twice weekly, and their consumption was measured weekly. At the end of the exposure, mice sourced from ARC were culled for sperm extraction (for sequencing and microinjection), whereas mice sourced from WEHI were put to mate with age-matched females.

Behavioural experiments:

All behavioural phenotyping was done in mice sourced from WEHI at Kew. Mice were left in the room to habituate for at least 30 min before the behavioural testing commenced. The experimenter was blinded to experimental condition.

Elevated-plus maze: it was used to assess anxiety-like behaviour. The apparatus consisted of a plus-shaped maze, with 2 open and 2 closed arms, in each axis, with a central area connecting them. The luminosity in the open arms was of 20 lux. Mice were put in the centre of the maze facing the open arm, and were left to explore it for 5 min, where a camera placed above the maze recorded its movement. We used TopScan software (CleverSys Inc, Reston, VA), to track movement in the plus maze. The percentage of time in the open arms was assessed, the number of entries into the open arms and the latency to first entry into an open arm were recorded.

Light-dark box: it was used to assess anxiety-like behaviour. The apparatus consisted of locomotor chambers with automatic tracking system (ENV-510, Med Associates, Fairfax, VT, USA), with a dark chamber attached to it, dividing it in half. In the light zone the luminosity was of at least 550 lux (around 750 lux). Mice were placed inside the dark zone and were left to explore the apparatus for 10 min. Latency to enter the light zone, total time spent in the light zone, and the total number of entries into the light zone were recorded.

Social dominance tube test: This test was used to assess social dominance in male mice. The apparatus consisted of a 30-cm long clear plastic tube, with a camera located above it. Mice were first habituated to the apparatus by going through the tube for 10 times, either from the right or the left end at each time. One day after habituation, each mouse from a PatCT cage of 4 mice faced each mouse from a PatCort cage of 4 mice. The number of wins by the Cort was compared to the null hypothesis on a chi-square test to determine statistical significance. The protocol was adapted from Tada et al., 2016; Zhou et al., 2016.

Mate-choice test: We used this test to assess attractiveness in male mice. The apparatus consisted of a 3-chamber social interaction test, with a camera located above

it. First a fertile female on oestrous (assessed by vaginal smear) was put in the apparatus to explore it for 10 min as a baseline. Then, one male from each group was put inside a small cage on each side of the apparatus, and the female mouse explored it again for 10 min. Time spent by the female mouse interacting with each male mouse was recorded by the TopScan software (CleverSys Inc, Reston, VA). One different female was used for each trial. The protocol was adapted from Hoffmann et al., 2020; Mitra and Sapolsky, 2012.

Porsolt swim test: This test was used to assess depressive-like behaviour. The apparatus consisted of a 2L beaker filled with approximately 1.8 L of water at room temperature (22-24 °C), and a camera placed in front of it to record movement. Each mouse was placed inside the beaker for 5 minutes, and the water was replaced after each trial. Videos were analysed using the TopScan Forced Swim software (CleverSys Inc, Reston, VA), excluding the first minute of it and only assessing the last 4 minutes of the video. Total time floating, swimming and 'struggling' was recorded.

Other procedures:

Sperm collection

The cauda epididymis was dissected with fine scissors and cut with a blade to allow sperm cells to flow into warm PBS (37 °C) for at least 30 min. The sperm solution was centrifuged for 15 minutes at 400 g, the supernatant was discarded, and the sperm was frozen at -80 °C until used.

RNA extraction and quality control for lncRNA Capture-Seq

Sperm pellets were disrupted in 500 µL of NucleoZOL (Cat #740404, Macherey-Nagel) using a glass dounce homogeniser. Water was added and samples centrifuged to remove contaminants according to the manufacturer's instructions. Supernatant was mixed 1:1 with ethanol and RNA captured on a Zymo-Spin IC column (Zymo Research). DNAse treatment was performed on-column and RNA was washed and eluted according to the manufacturer's directions. RNA concentration was measured by Qubit assay (Invitrogen).

RNA extraction and quality control for microinjection

RNA was extracted from sperm using QIAzol Lysis reagent (Cat #79306, QIAGEN) and the miRNeasy Mini kit (Cat #217004 , QIAGEN), to separate long and small RNAs,

according to the manufacturer's instructions. Each sample was pooled from the sperm of 5 males. RNA quality was assessed on Nanodrop and TapeStation (Agilent Technologies, Santa Clara, CA) (Sup. Fig. S3.23). RNA solutions were diluted to 5 ng/µL, so that the total amount of RNA microinjected into each oocyte corresponds to roughly 5 to 10 fg of RNA, equivalent to roughly the amount that is delivered by each sperm cell [91].

Oocyte microinjections

Microinjections into fertilised oocytes were performed at the WEHI facility at Kew by experienced personnel. The RNA solution was microinjected until the distension of the male pronucleus was observed (which corresponded to around 1 to 2 pL). The concentration of the solution containing the lncRNAs was such that 5 to 10 fg of lncRNA would be injected, which is what is believed to be delivered by a single sperm cell during fertilisation [91]. A total of 123 embryos were microinjected with lncRNA from CT mice, and 124 embryos were microinjected with lncRNA from Cort-treated mice. Of these, 93 CT 2-cell embryos were transferred into 7 recipient females, whereas 98 Cort 2-cell embryos were transferred into 7 recipient females. Subsequently, 42 Inj-CT and 43 Inj-Cort pups were littered. Chi-squared statistical testing revealed no differences in survival between the pups born from microinjection ($\chi^2_{(3)}$=1.708, p=0.6351, Table 3.8).

Table 3.8. Number of embryos and resulting mice from the microinjection procedures.

Group	Embryos	2-cell	Pups born	Survival	Males	Females
InjCT	123	93	42	39	21	18
InjCort	124	98	43	36	18	18

Sequencing:

Sperm lncRNA capture-Seq

2 µg of total RNA was used to generate RNA library. First, total RNA was treated with NEBNext® rRNA Depletion Kit (NEB) to remove rRNA and RNA libraries were generated using the NEBNext® Ultra™ II RNA Library Prep Kit for Illumina® (NEB). Around 28000 custom-designed probes (Roche), which targets all known lncRNA from the GENECODE, were used to capture lncRNAs followed the SeqCap EZ Hybridization manual and Wash Kit and SeqCap EZ Accessory Kit (Roche) [256]. Captured lncRNA libraries were sequenced on the Illumina HiSeq 4000 platform. 200 bp long reads were double-end sequenced at an average depth of 100 M, and the average mapping success was 56.63%.

Sequencing data analysis

Using cutadapt (v1.17) [257] we trimmed low-quality nucleotides with Phred quality score below 20 and removed Illumina adaptor sequences from the 3' end of each read. Processed reads were aligned to the reference genome of mouse (mm10) using HISAT2 (v2.1.0) [236]. SAMtools (v1.8) [245] was then used to convert "SAM" files to "BAM" files, remove duplicate reads, sort and index the "BAM" files. Only properly aligned reads with mapping quality at least 20 were kept for downstream analyses. StringTie (v2.0.3) [258] was used to quantitate both the gene and the transcript expression level for each sample, with the option of "-e -G gencode.vM24.long_noncoding_RNAs.gtf" to limit the processing of read alignments to estimate transcripts matching the GENCODE long noncoding RNAs. Genes with low expression were filtered out using the 'filterByExpr' function of the edgeR package (v3.34.1) [238,239]. We then normalised the RNA composition using the 'calcNormFactors' function of edgeR. Dispersion estimates were obtained through the Cox-Reid profile-adjusted likelihood method. Lastly, differential expression analysis was determined using the quasi-likelihood F-test. Volcano plots were generated using ggplot2 (v3.3.5) [240].

Gene Ontology analysis was done with GO Enrichment Analysis, accessed at http://geneontology.org. A list of every protein coding gene overlapping every read detected in our sequencing was used as the reference list, which was obtained using bedtools. The analysis type was the PANTHER Overrepresentation Test (Released 20221013), with the GO ontology database present in (DOI: 10.5281/zenodo.6799722) and release date (2022-07-01). Due to the poor functional annotation for lncRNAs, bedtools was used to obtain a list of protein-coding genes that overlap with the detected lncRNAs, which was then used as the reference list for the test. Statistical testing was done with a binomial test type using the False Discovery Rate for correction [259–261]. Transposable-element derived lncRNA analysis was performed using TEtranscripts (v2.2.1), using the default parameters [262]. circRNAs were analysed using CIRI2 (v2.0.6) [263,264], with the following filtering parameters: min.count=3, min.total.count=18.

To assess the abundance of transposable elements located in the promoter region of sperm lncRNA, we used bedtools to intersect the coordinates of transposable elements obtained from the mm10 RepeatMasker reference (Dec 2011, RepeatMasker open-4.0.6 - Dfam 2.0) and the coordinates of the 1kb upstream region from the Transcription Start Site (TSS) of the first exon of each lncRNA. The statistical test was done with the function chisq.test of the package stats (v4.1.1), and visualisation of the correlation was performed with corrplot (v0.92).

Statistical analysis

Data collected from the elevated-plus maze, light-dark box and Porsolt swim test were analysed with a 2-way ANOVA, followed by Tukey *post-hoc* test. Latency data from the light-dark box and Porsolt swim test were analysed by Kaplan-Meier survival analysis. Body-weight measures were analysed with repeated-measures ANOVA. The social dominance tube test data was analysed using binomial test, and the mate-choice test data was analysed using Wilcoxon matched-pairs signed rank test. Statistical testing and graphs were done in GraphPad Prism 9 for MacOS (v9.4.1). Statistical significance was reached when $p<0.05$, data is represented as mean ± standard error of the mean (SEM).

3.4. Results

3.4.1. Corticosterone treatment induces many changes in sperm lncRNA expression

We performed CaptureSeq to establish whether, and to what extent, corticosterone (Cort) treatment impacted the lncRNA content of sperm. The MDS plot showed that CT and Cort groups separate very distinctly (Fig. 3.15A), which is reflected in the number of differentially expressed lncRNAs. A total of 10816 transcripts were detected, and after the exclusion of lowly expressed reads, the volcano plot (Fig. 3.15B) shows out of 7552 lncRNAs, 2382 of them were found to be differentially expressed, with 772 upregulated and 1610 downregulated. The heatmap also shows stark differences (Fig. 3.15C). Specific lncRNAs, such as Meg3, Rian and Mirg, were found to be downregulated in the Cort sample and, although most of the lncRNAs have unknown functions, these 3 belong to the *Dlk1-Dio3* cluster, which is maternally imprinted and has been somewhat studied for their function on stem cell differentiation. For instance, MEG3 has been found to be needed for the expression of neural lineage genes, as well as neurite formation, during the differentiation of human embryonic stem cells into neurons [265]. To determine whether variations in stress severities differentially impact sperm lncRNAs, we compared our differentially expressed lncRNAs to those identified in a mouse model of early life adversity (MSUS) [126]. Although we did not have access to their full dataset, out of the 74 DE lncRNAs they detected with edgeR and reported in their study [126], we detected 21 of them in our sequencing, with 9 of these lnRNAs also being differentially expressed.

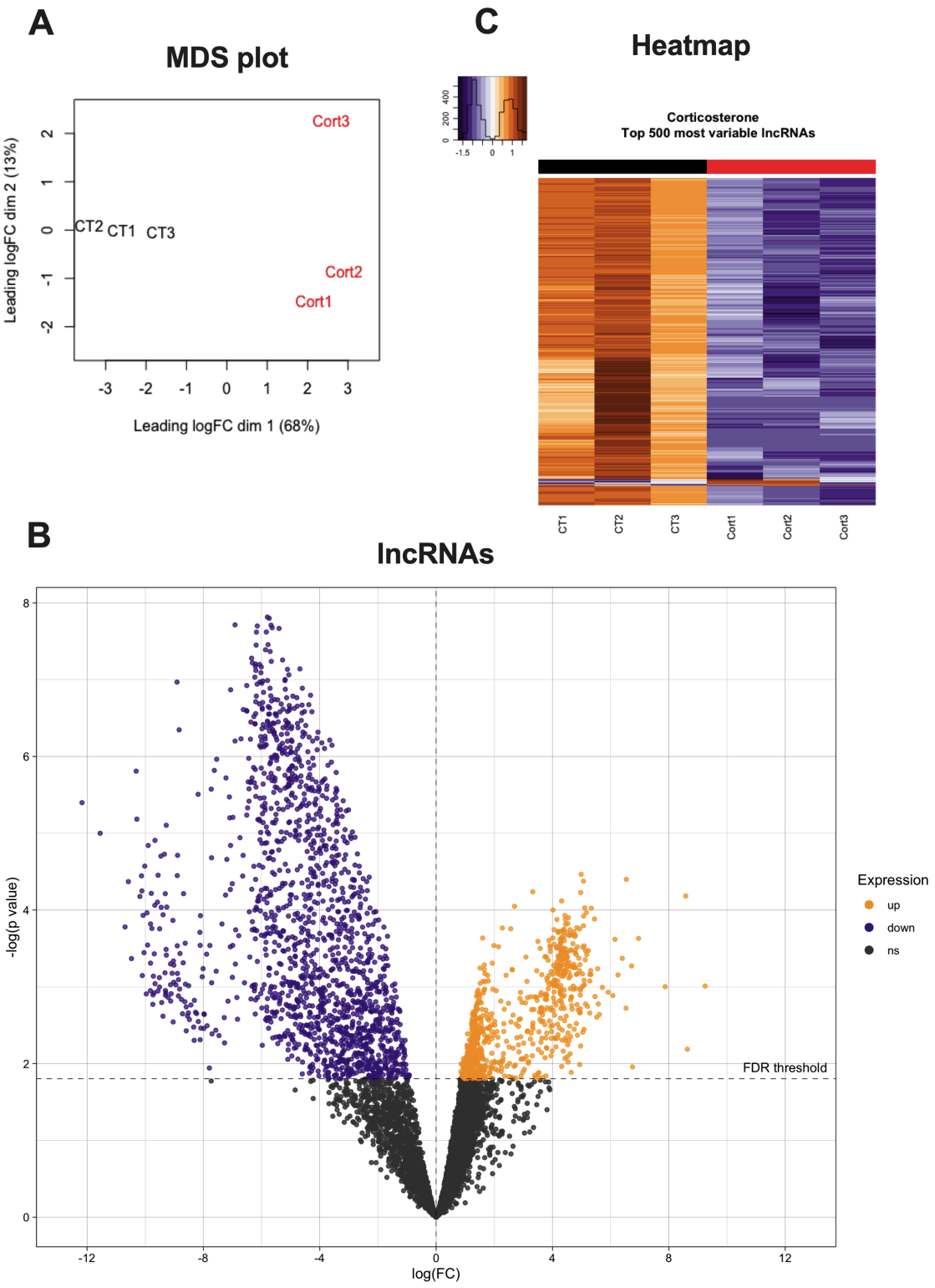


Fig. 3.15. Long noncoding RNA capture sequencing data analysis. (A) Multidimension scaling analysis showing clear separation between CT and Cort sperm samples, indicating that CT and Cort samples show distinct lncRNA expression pattern. (B) Volcano plot of the lncRNA analysis. Up: upregulated lncRNAs. Down: downregulated lncRNAs. ns: non-significant. Significance was determined based on False-Discovery Rate threshold. (C) Heatmap of the top 500 most variable lncRNAs, showing a clear distinction between CT and Cort samples, which is reflected on the Multidimension scaling plot.

We also investigated other types of lncRNAs present in the sperm, starting with Transposable Element (TE) transcripts, using the tool TEtranscripts. We detected 1114 different TEs, with 167 differentially expressed, of which 13 were upregulated and 154 were downregulated (Fig. 3.16A). The ERVK element IAPEz-int, which is a member of the LTR family, was found to be differentially expressed, similar to what has been described by Gapp et al., 2018.

Lastly, we used CIRI2 to analyse the differential expression of cicRNAs. We found 705 differentially expressed circRNAs in our dataset, with 665 upregulated and 40 downregulated (Fig. 3.16B).

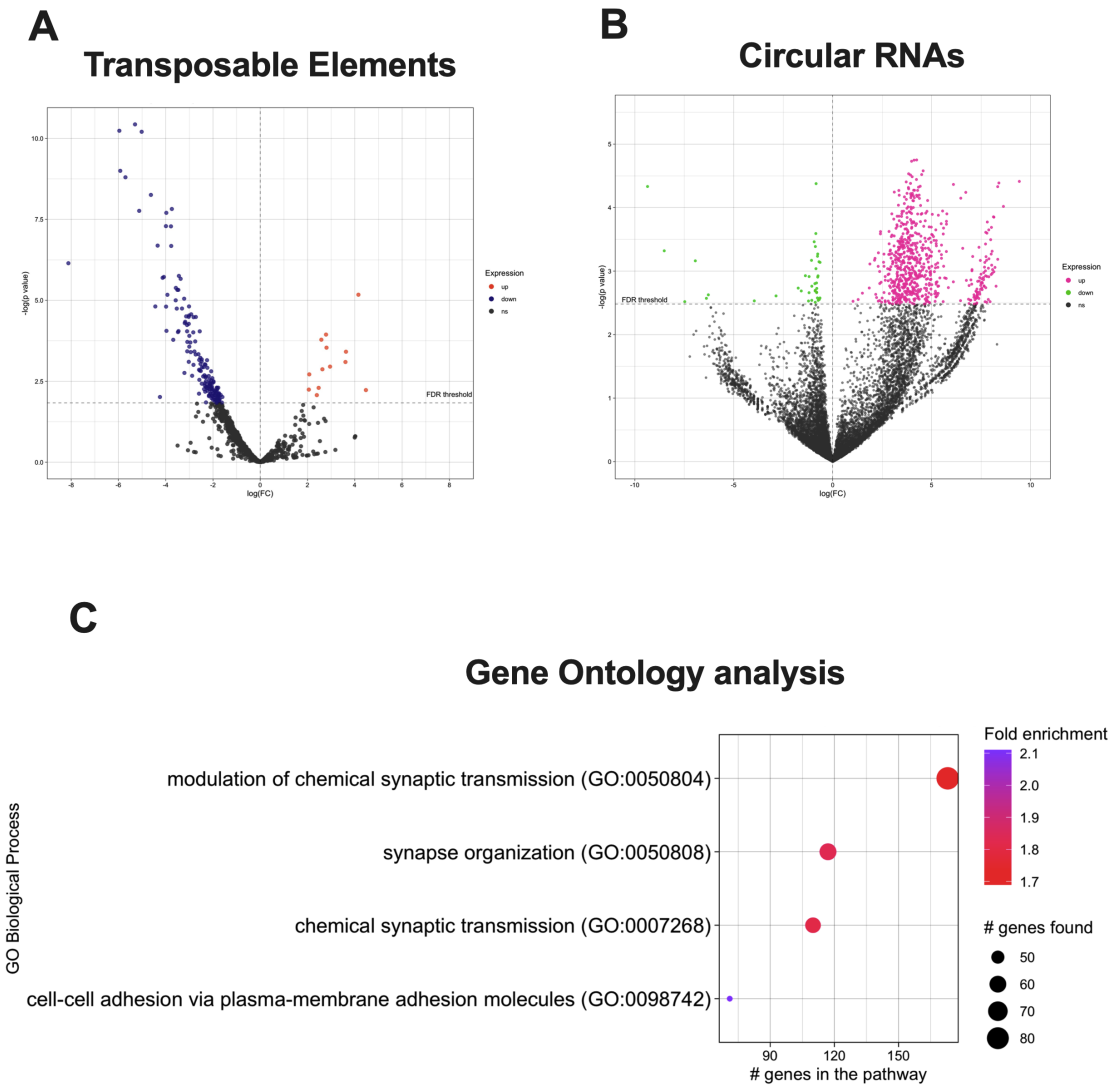


Fig. 3.16. Investigation of other long RNA types, and cis-target prediction. (A) Volcano plot of the Transposable Element transcript analysis. Up: upregulated TE transcripts. Down: downregulated TE transcripts. Ns: non-significant transcripts. Significance was determined based on False-Discovery Rate threshold. (B) Volcano plot of the circRNA analysis. Up: upregulated circRNAs. Down:

downregulated circRNAs. ns: non-significant circRNAs. Significance was determined based on False-Discovery Rate threshold. (C) Gene Ontology analysis, showing the main pathways found to be statistically significant. The fold enrichment for each pathway is colour-coded, and the number of genes detected that belong to the pathway are proportional to the size of the circle.

3.4.2. Cort-induced sperm lncRNAs might modulate synaptic function

An important question regarding the function of lncRNAs is how they drive embryonic reprogramming that result in behavioural changes in adult life. It is known that lncRNAs have their expression correlated with genes that are in proximity to their chromosomal location, which is known as cis-action [84]. By using bedtools, we compiled all the genes that overlap with the lncRNAs we detected in our sequencing, and those that overlap with the DE lncRNAs. We found 4240 overlapping protein-coding genes in total, and 1180 of them overlap with the DE lncRNAs. When running the Gene Ontology analysis, we found that the main terms are related to synaptic function, more specifically cell-cell adhesion via plasma-membrane adhesion molecules (GO:0098742), synapse organization (GO:0050808), chemical synaptic transmission (GO:0007268) and modulation of chemical synaptic transmission (GO:0050804) (Fig. 3.16C). Sperm lncRNAs affecting these processes during brain development could, therefore, underlie the altered behaviour that was observed in this study.

To clarify the relevance of sperm lncRNAs to brain function, we compared this current data to our previous CaptureSeq study of lncRNAs in the mouse infralimbic prefrontal cortex, both of which used the same custom-designed probe set [256]. 6677 unique lncRNAs were detected in the brain, compared to 7544 in sperm. Of these, 3982 unique lncRNAs were found to be present in both datasets, which corresponds to 52.78% of the sperm lncRNAs that are also expressed in the Infralimbic Prefrontal Cortex (ILPFC) of adult mice. Among the 2382 differentially expressed sperm lncRNAs, 1402 were found to be expressed in the ILPFC of adult mice, corresponding to 58.86% of the differentially expressed lncRNAs (Sup. Fig. S3.21), of which Malat1, Meg3, Tug1, Rian and Miat are present, to name a few. The majority of these 1402 DE lncRNAs that are also expressed in the ILPFC are downregulated in the sperm of corticosterone-treated males (1114 downregulated and 288 upregulated lncRNAs). These findings corroborate the possibility that sperm lncRNAs are also relevant to adult brain function.

Given that lncRNAs are a product of transcription during spermatogenesis [252], and that the germline transcriptome is driven by endogenous retroviruses that function as active enhancers [266], we investigated the abundance of transposable elements in the promoter of differentially expressed sperm lncRNAs. By using the mm10 RepeatMasker as reference for repeat location and bedtools to identify every transposable element located within 1kb upstream of the transcription start site (TSS) of each lncRNA, we identified 1194 transposable elements in the promoter region of upregulated lncRNAs,

with the 3 most abundant families being Alu (309 elements), B2 (163 elements) and B4 (150 elements), Fig. 3.17A. For the downregulated lncRNAs 2344 elements were found, with the 3 most abundant families being L1 (535 elements), ERVL-MaLR (339 elements) and Alu (273 elements), Fig. 3.17B. The patterns of family abundance were statistically different between up and downregulated lncRNAs ($\chi^2_{(78)}$=380.79, p<2.2e-16, Fig. 3.17C), with Alu and B2 being overrepresented and ERVL-MaLR underrepresented in upregulated lncRNAs, whereas in downregulated lncRNAs L1 is overrepresented and Alu and B2 are underrepresented (contribution of each residual to the χ^2 value is depicted in Fig. 3.17D). These results suggest that the differential expression we found between up and downregulated lncRNAs might be driven by different transcription regulators.

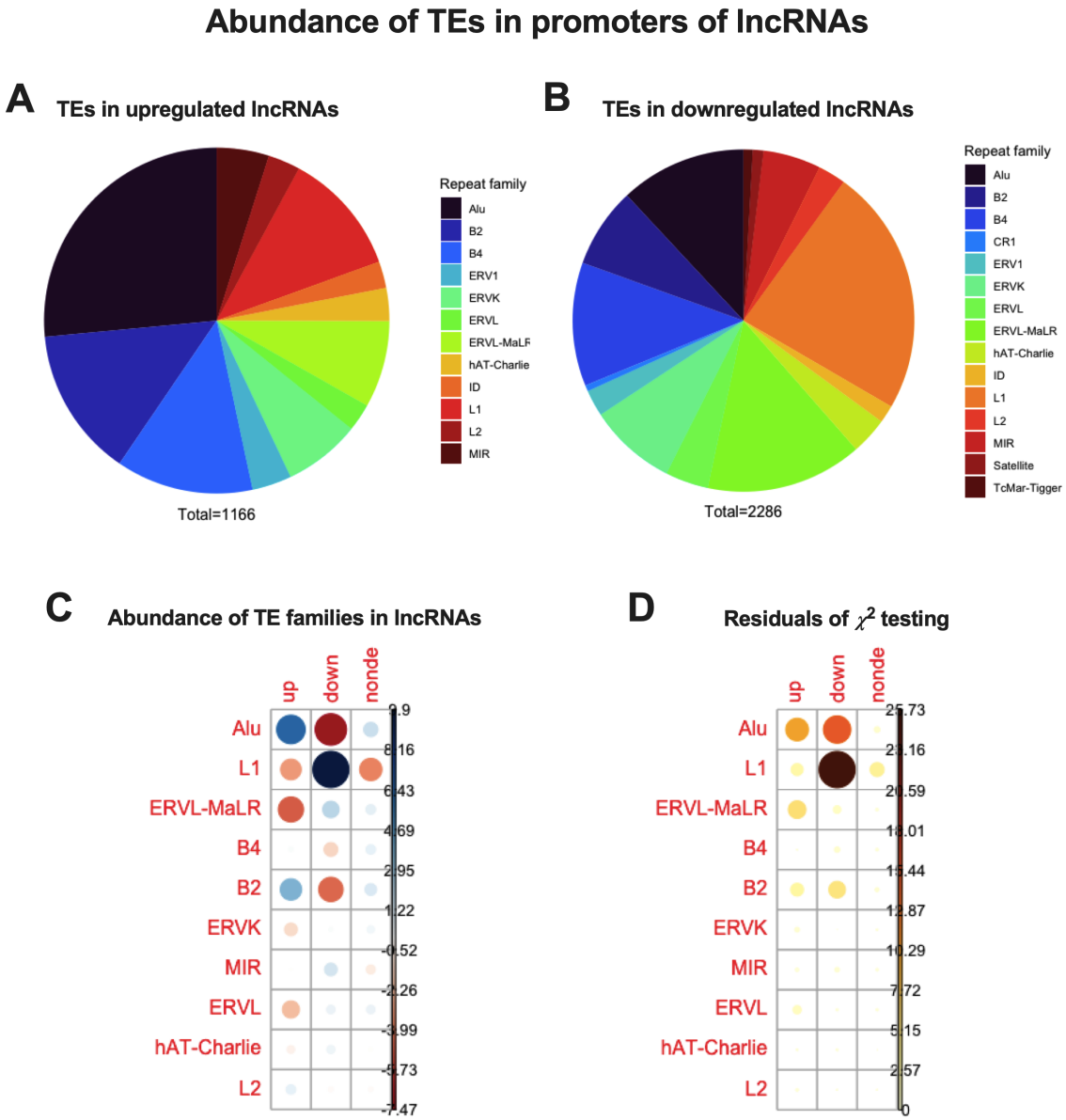


Fig. 3.17. Analysis of transposable elements in the promoter regions of up and downregulated lncRNAs. (A) Pie chart showing abundance of different families of transposable elements in the

promoter region of upregulated sperm lncRNAs. Only families with more than 10 elements are shown. (B) Pie chart showing abundance of different families of transposable elements in the promoter region of downregulated sperm lncRNAs. Only families with more than 10 elements are shown. (C) Representation of statistical results of abundance of each family of transposable elements. Blue: overrepresented. Red: underrepresented. (D) Representation of contribution to each residual to the χ^2 value.

3.4.3. Microinjection of sperm lncRNAs into fertilised oocytes affects physical growth during early adulthood

To determine whether sperm lncRNAs play a role in the intergenerational effects of corticosterone treatment on offspring phenotypes, we microinjected fertilised mouse oocytes with the lncRNA fraction isolated from control or corticosterone-treated male breeders. First, we exposed adult male mice to the 4-week corticosterone treatment or to control conditions, after which we extracted sperm from the caudal epididymis. Total RNA was extracted, which was further separated into long and small RNA populations. Long RNA populations were microinjected into fertilised oocytes in two rounds of microinjections.

Concomitantly, we generated cohorts of offspring born from 'natural mating', after their fathers were exposed to the corticosterone treatment or to control conditions, as per our previously published protocol [28]. These mice were reared and tested together with the mice born from the microinjection procedure.

The offspring born from natural mating and microinjection were weighed weekly, from week 6 (PND 42) to week 9 (PND 63). We observed that for the male offspring (Fig. 3.18C) there was a significant effect of age ($F_{(1.844,164.1)}$=467.8, p<0.0001), group ($F_{(3,89)}$=47.62, p<0.0001), and an age x group interaction ($F_{(9,267)}$=2.344, p=0.0147). PatCT was heavier than PatCort (p=0.0280), and so were the InjCT compared to InjCort (p=0.0168) in week 6, but these within-group differences were not significant thereafter. In every timepoint measured, mice born from microinjection were heavier than mice born from natural mating (p<0.0001).

In the females (Fig. 3.18D), we observed a significant effect of age ($F_{(2.538,231.0)}$=53.01, p<0.0001) and group ($F_{(3,91)}$=25.44, p<0.0001), but no significant interaction ($F_{(9,273)}$=0.6541, p=0.7501). PatCT offspring were heavier than PatCort offspring at 6-weeks of age (p=0.0421). By 7 weeks of age, the InjCort group was heavier than PatCort (p=0.0068). In the following weeks, mice born from microinjection were heavier compared to mice born from natural mating.

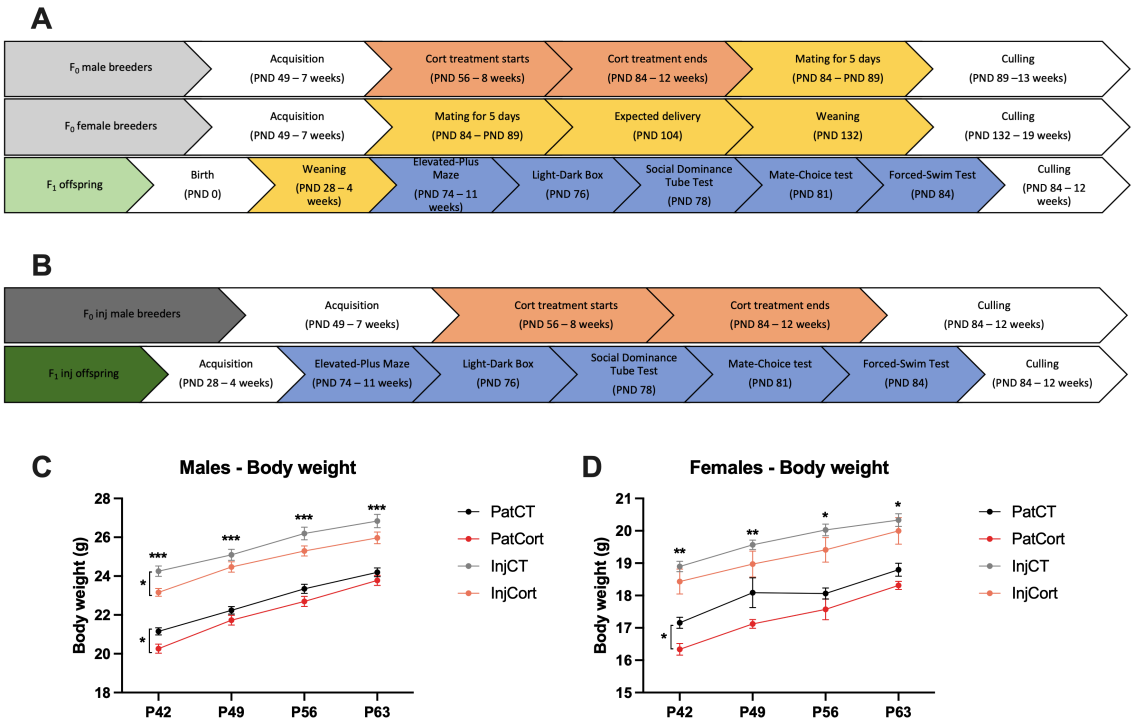


Fig. 3.18. Experimental design and offspring body weight. (A) Timeline of the mice born from natural mating. (B) Timeline of the mice born from microinjection procedure. (C) Male mice born from microinjection procedure are consistently heavier than mice born from natural mating across all the timepoints. PatCT *n*=28, PatCort *n*=26, InjCT *n*=21, InjCort *n*=18. (D) Female mice born from microinjection procedure are consistently heavier than mice born from natural mating across all the timepoints. PatCT *n*=26, PatCort *n*=33, InjCT *n*=18, InjCort *n*=18. Data analysed by repeated Measures ANOVA and represented as mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.4.4. lncRNA microinjection induced changes in affective behaviours of adult offspring

In the elevated-plus maze test for anxiety, male mice did not show differences in percentage of time in the open arms (Fig. 3.19A, Paternal Treatment: $F_{(1,88)}$=0.04806, p=0.8270, Conception Method: $F_{(1,88)}$=1.046, p=0.3093, Interaction: $F_{(1,88)}$=0.03169, p=0.8591) or number of entries into the open arms (Sup. Fig. S3.22A, Paternal Treatment: $F_{(1,88)}$=0.6818, p=0.4112, Conception Method: $F_{(1,88)}$=0.2045, p=0.6522, Interaction: $F_{(1,88)}$=1.588, p=0.2110). In the females, differences were found in the percentage of time spent in the open arms (Fig. 3.19D, Paternal Treatment: $F_{(1,84)}$=0.001474, p=0.9695, Conception Method: $F_{(1,84)}$=5.992, p=0.0165, Interaction: $F_{(1,84)}$=3.636, p=0.0599), with mice born from microinjection spending less time in the open arms compared to mice born from natural mating, independently of paternal treatment. No differences were found in number of entries into the open arms (Sup. Fig. S3.22E, Paternal Treatment: $F_{(1,84)}$=0.1694, p=0.6817, Conception Method:

$F_{(1,84)}$=0.008004, p=0.9289, Interaction: $F_{(1,84)}$=7.767, p=0.0066, with no statistically significant results *post-hoc*).

In the males, there were differences in the percentage of time spent in the light zone of the light-dark box (Fig. 3.19B, Paternal Treatment: $F_{(1,89)}$=11.00, p=0.0013, Conception Method: $F_{(1,89)}$=18.14, p<0.0001, Interaction: $F_{(1,89)}$=1.580, p=0.2120), with mice born from microinjection spending more time in the light zone compared to mice born from natural mating, and Cort mice spending more time in the light zone compared to CT mice. No differences were found in latency to light zone (Sup. Fig. S3.22B, χ^2=2.008, p=0.5708). There were differences in the ambulatory distance (Sup. Fig. S3.22C, Paternal Treatment: $F_{(1,89)}$=1.476, p=0.2277, Conception Method: $F_{(1,89)}$=19.88, p<0.0001, Interaction: $F_{(1,89)}$=0.04561, p=0.8314), with mice born from microinjection moving longer distances compared to mice born from natural mating. In the females, similar differences were found. There were differences in the percentage of time spent in the light zone (Fig. 3.19E, Paternal Treatment: $F_{(1,86)}$=0.4776, p=0.4914, Conception Method: $F_{(1,86)}$=11.53, p=0.0010, Interaction: $F_{(1,86)}$=0.5541, p=0.4587), with mice born from microinjection spending longer time in the light zone compared to mice born from natural mating, independently of paternal treatment. No differences were found in latency to light zone (Sup. Fig. S3.22F, χ^2=1.434, p=0.6975). There were differences in the ambulatory distance (Sup. Fig. S3.22G, Paternal Treatment: $F_{(1,86)}$=3.043, p=0.0847, Conception Method: $F_{(1,86)}$=27.26, p<0.0001, Interaction: $F_{(1,86)}$=0.2841, p=0.5954), with mice born from microinjection travelling longer distances compared to mice born from natural mating. As the hyperactivity displayed in the light-dark box apparatus is a confounding factor to the anxiety-like behaviour assessed at such test, these results show us that female mice born from microinjection were more anxious than mice born from natural mating, and mice showed hyperactivity in the light-dark box apparatus, irrespective of sex.

We also tested mice in the Porsolt swim test, to measure depressive-like behaviour. In the males, there were differences in the total immobility time (Fig. 3.19C, Paternal Treatment: $F_{(1,89)}$=0.8551, p=0.3576, Conception Method: $F_{(1,89)}$=18.57, p<0.0001, Interaction: $F_{(1,89)}$=0.7076, p=0.4025), with mice born from microinjection spending more time immobile compared to mice born from natural mating, irrespective of paternal treatment. There were also differences in the latency to floating (Sup. Fig. S3.22D, χ^2=22.73, p<0.0001), with InjCT showing an overall lower latency to float compared to PatCT (p<0.0001). Similar results were found in the females: there were differences in the immobility time (Fig. 3.19F, Paternal Treatment: $F_{(1,87)}$=0.04308, p=0.84, Conception Method: $F_{(1,87)}$=40.33, p<0.001, Interaction: $F_{(1,87)}$=0.8240, p=0.37), with mice born from microinjection spending more time floating compared to mice born from natural mating, independently from paternal treatment. There were also differences in the latency to float (Sup. Fig. S3.22H, χ^2=17.6, p=0.0005), with InjCort being quicker to float compared to PatCort (p=0.0004). These results show that mice

born from microinjection present more depressive-like behaviour compared to their counterparts born from natural mating, irrespective of sex.

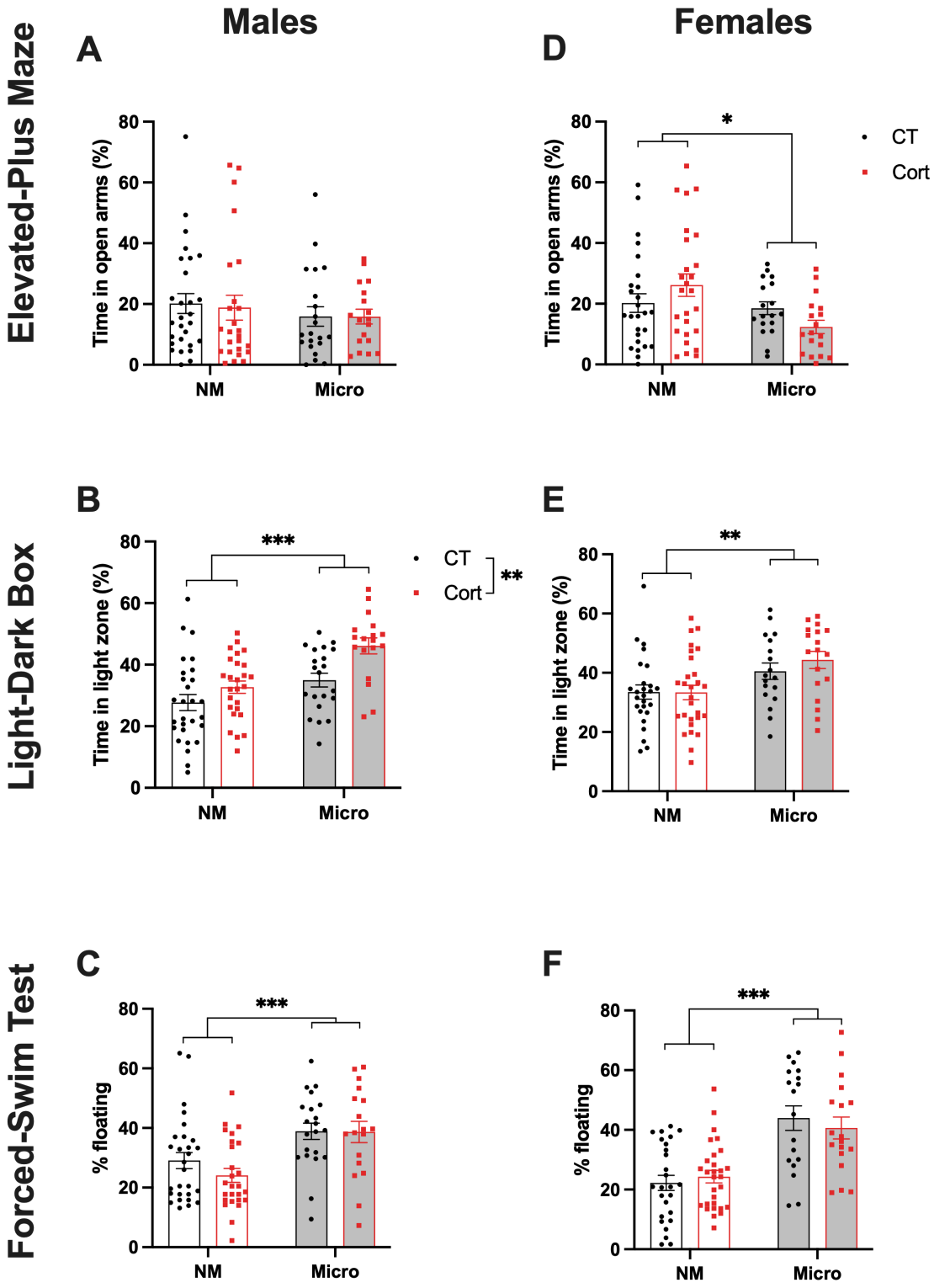


Fig. 3.19. Assessment of anxiety- and depressive-like behaviours. (A) No evidence of differences between the male groups in the percentage of time spent in the open arm in the elevated-plus maze. (B) Male mice born from microinjection spent more time in the light zone of the light-dark box

compared to male mice born from natural mating, and male Cort mice spent more time in the light zone of the light-dark box compared to their CT counterparts. (C) Male mice born from microinjection spent longer times immobile compared to mice born from natural mating. (D) Female mice born from microinjection spent less time in the open arms in the elevated-plus maze compared to mice born from natural mating. (E) Female mice born from microinjection spent more time in the light zone of the light-dark box compared to mice born from natural mating. (F) Female mice born from microinjection spent longer times immobile compared to mice born from natural mating. Males: PatCT *n*=28, PatCort *n*=25-26, InjCT *n*=21, InjCort *n*=18. Females: PatCT *n*=26, PatCort *n*=26-29, InjCT *n*=18, InjCort *n*=18. Data analysed by 2-way ANOVAs and represented as mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.4.5. Microinjection of sperm lncRNAs does not have effects on social behaviours

We recently published in the paternal corticosterone-exposure and in a separate paternal stress model that male offspring social behaviours are subject to intergenerational modification [267,268]. In assessing social dominance, we found that PatCort showed an increased percentage of wins against their PatCT counterpart (Fig. 3.20A, $p=0.0037$). However, we found no difference in social dominance between both groups of microinjected mice (Fig. 3.20A, $p=0.3817$). These results show that PatCort mice were more dominant than PatCT (Fig. 3.20B), but the microinjections did not replicate this dynamic of dominance between the groups.

In the mate-choice test, which was used to assess male attractiveness, there were no differences in the time being investigated by female mice, within the mice born from natural mating ($W=-56$, $p=0.1591$), nor within the ones born from microinjection procedure ($W=-2$, $p=0.9799$) (Fig. 3.20C). These results show that male mice were not found to have different attractiveness.

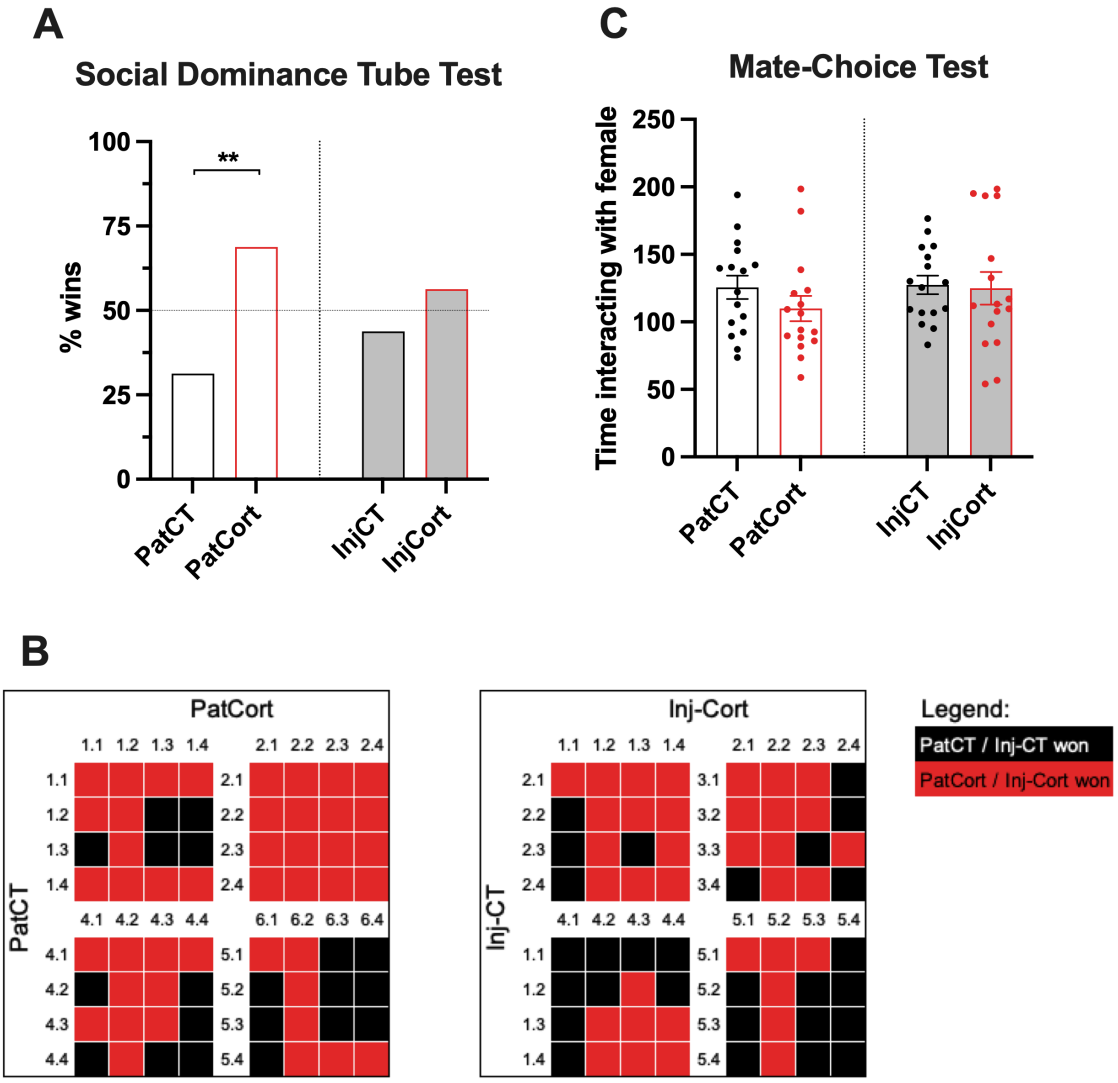


Fig. 3.20. Assessment of social behaviours. (A) PatCort won more often compared to PatCT in the social dominance tube test. No differences were observed within the mice born from microinjection. PatCort. PatCT *n*=16, PatCort *n*=16, InjCT *n*=16, InjCort *n*=16, in a total of 64 unique interactions between PatCT and PatCort, and 64 unique interactions between InjCT and InjCort. Data analysed by Binomial test. (B) Schematic representing the outcomes for each face-off in the social dominance tube test. (C) No differences were found in the mate-choice test for mice born from natural mating or from microinjection. PatCT *n*=16, PatCort *n*=16, InjCT *n*=16, InjCort *n*=16. Wilcoxon matched-pair signed rank test. Data represented as mean ± SEM. ** $p<0.01$.

3.5. Discussion

In this study we showed that the Cort-treatment induced many altered lncRNAs, transposable element transcripts and circRNAs, which might be responsible for some of the phenotypic effects we observed after microinjection of lncRNAs into fertilised oocytes. These effects included increased body weight, adult behavioural changes towards a reduction of time spent in the open arms of the elevated plus-maze, increased

time spent in the light zone of the light-dark box, as well as hyperactivity in the light-dark box, and increase in immobility time in the Porsolt swim test. In addition to these effects being mostly independent of the origin of sperm lncRNA that was microinjected, they were also found to be independent of sex, which is unusual as sex-related phenotypes are usually reported in studies of the paternal epigenetic inheritance.

These results greatly extend upon the previous study by Gapp et al., 2018, where they saw an effect of lncRNA microinjection, particularly on metabolic measures. Compared to our current study, where we observed ample effects on behavioural measures, these differences could have been due to the amount of lncRNA we microinjected, which was around 10 times higher than that of Gapp et al., 2018. The number of differentially expressed lncRNAs we observed was also higher than what they reported, which could indicate that our paternal Cort-treatment might be particularly impactful on the sperm lncRNA profile, although this difference could be explained by the more sensitive capture-sequencing technique we used, which allowed us to study a far greater number of sperm lncRNAs.

The profile of lncRNAs between CT and Cort we found is fundamentally different, encompassing many different lncRNA types, including transposable element transcripts and circRNAs, and this difference was hypothesised to be reflected in the phenotype observed in the mice born from microinjection procedure. However, InjCT and InjCort were not statistically different in most of the parameters we measured. We believe this could have been because the microinjection technique is based on the addition of lncRNAs into the fertilised oocyte, therefore surpassing the levels of lncRNAs normally delivered by the sperm cell during fertilisation. The nature of this technique also precludes the investigation of the effects that reduced levels of sperm lncRNAs could cause to the embryonic landscape and development. As a result, we could not account for the effects that downregulated sperm lncRNAs could have on the embryonic development, which might have been particularly relevant in our model, as the downregulation of sperm lncRNAs comprised most of the differences that were observed between control and Cort-treated fathers, and therefore this change cannot be replicated by the microinjection procedure. Other techniques, such as the use of antisense oligonucleotides, could be used in future experiments to address this issue.

Because we injected the entire lncRNA fraction isolated from CT or Cort sperm, our results represent the holistic effect of the stress-associated sperm lncRNA payload on development. To investigate the effect of specific lncRNAs or combinations of lncRNAs on development, future studies could be performed involving injection of a more restricted RNA population. Specific lncRNAs could be isolated from sperm RNA using capture probes and then injected into zygotes, with the advantage that any unusual features of these lncRNAs (including the 5' cap or any nucleotide modifications) would be retained in their native state. Alternatively, the impact of the RNA sequence only could be assessed by microinjection of synthetic lncRNAs created by *in vitro*

transcription. Even though these experiments could undoubtedly help uncover the role of lncRNAs in the epigenetic inheritance, it is expected that multiple noncoding RNAs, encompassing both small and long ones, are necessary to transmit the entirety of the phenotypes observed in the epigenetic inheritance.

Nevertheless, our results show that the microinjection procedure induced effects in the adult offspring. The potential stress due to the microinjection procedure, which could impact the embryo, cannot be ruled out, but it is likely that the extra payload of lncRNAs was responsible for inducing the phenotypic differences we observed, compared to the mice born from natural mating. lncRNAs are known to interact with the DNA and recruit chromatin-modifying factors, therefore inducing histone modifications. Additionally, it has also been reported that RNA m6A can drive DNA and determine chromatin accessibility [269]. Thus, it is possible that the informational layer coded by the lncRNA profile could be translated into a more stable algorithm in the form of correlated histone modifications or modified chromatin accessibility. These modifications could persist across embryonic development and, ultimately, affect gene expression in adult life. This is an important hypothesis that, albeit technically challenging to be investigated, should receive more attention in the future. Although the RNA-induced embryonic reprogramming has been hypothesised to be the basis of the epigenetic inheritance, the mechanisms underlying this process have not been pinpointed. We believe this can only be addressed when the functional annotation of lncRNAs advances, so that individual lncRNAs can be linked to neural development and affective behaviour. Studies like the one we performed, which show that lncRNAs are not just 'junk RNA', but instead potentially have biological functions, could leverage this field and justify more targeted, biomolecular approaches to explore the functional annotation of individual lncRNAs.

Importantly, the role of other long RNA types in this paternally mediated epigenetic inheritance should be investigated. Most of the circRNAs have unknown function, but some have been shown to act as miRNA sponges and are highly stable [270]. Recent studies have reported, however, that only a small number of circRNAs is functional, and most are products of splicing errors [271]. RNA derived from transposable elements, on the other hand, have been appointed as initiating transcriptional processes in the early-embryonic development [266], and for conferring functional structures to long RNAs [272]. Additionally, our present study suggests that paternal experiences modify the expression of transposable element transcripts in the sperm. This effect has not been sufficiently addressed in the epigenetic inheritance field and deserves more attention, because these elements can result in mutagenesis but also play important evolutionary roles by duplicating genes [273]. This raises important questions related to the evolutionary importance of the epigenetic inheritance.

Lastly, the affective behavioural phenotype we observed in our study resembles those reported by Gapp et al., 2014a, in which they performed the microinjection of

sperm small noncoding RNAs extracted from male mice exposed to traumatic early life stress into fertilised oocytes, and observed increased locomotion in the light-dark box and floating time in the Porsolt swim test. This points towards small and long noncoding RNAs having effects in the embryonic reprogramming, which shows that lncRNAs might be as important for the epigenetic inheritance as small noncoding RNAs, despite most of the research in the field having studied sncRNAs. Taken together, these experiments also inform on the involvement of sperm RNAs in the inheritance of anxiety and depressive phenotypes induced by paternal experiences to stressful experiences preconception.

Overall, the present study highlights that, on top of the known importance of small noncoding RNAs, long noncoding RNAs also seem to play a role in paternally mediated epigenetic inheritance. Our study also demonstrates the importance of sensitive sequencing techniques to detect lncRNAs and other long RNA types. There is a paucity of mechanistic studies addressing the function of lncRNAs and their potential importance in mediating epigenetic mechanisms. The present findings suggest that sperm lncRNAs are key mediators of epigenetic inheritance and can modulate offspring metabolism, brain development and behaviour.

3.6. Declarations

Conflict of interest

The authors declare no conflict of interest.

Author contributions

L.B.H. contributed to planning experiments, conducted all experiments and analyses, and wrote the manuscript. B.L. assisted in conducting animal behavioural studies. Q.Z. and W.W. performed the CaptureSeq gene expression analysis. L.J.L. conducted the capture sequencing. T.W.B. supervised the CaptureSeq and gave critical feedback to its analysis. T.Y.P. envisioned the study, supervised study design, data collection and statistical analyses, and reviewed the manuscript. A.J.H. envisioned and funded the study, contributed to planning experiments, provided critical feedback throughout the experiments, and reviewed and edited the manuscript.

Data availability

The sequencing datasets generated and/or analysed during the current study have been deposited in the European Nucleotide Archive (ENA) repository at EMBL-EBI under

accession number PRJEB66376 (https://www.ebi.ac.uk/ena/browser/view/PRJEB66376).

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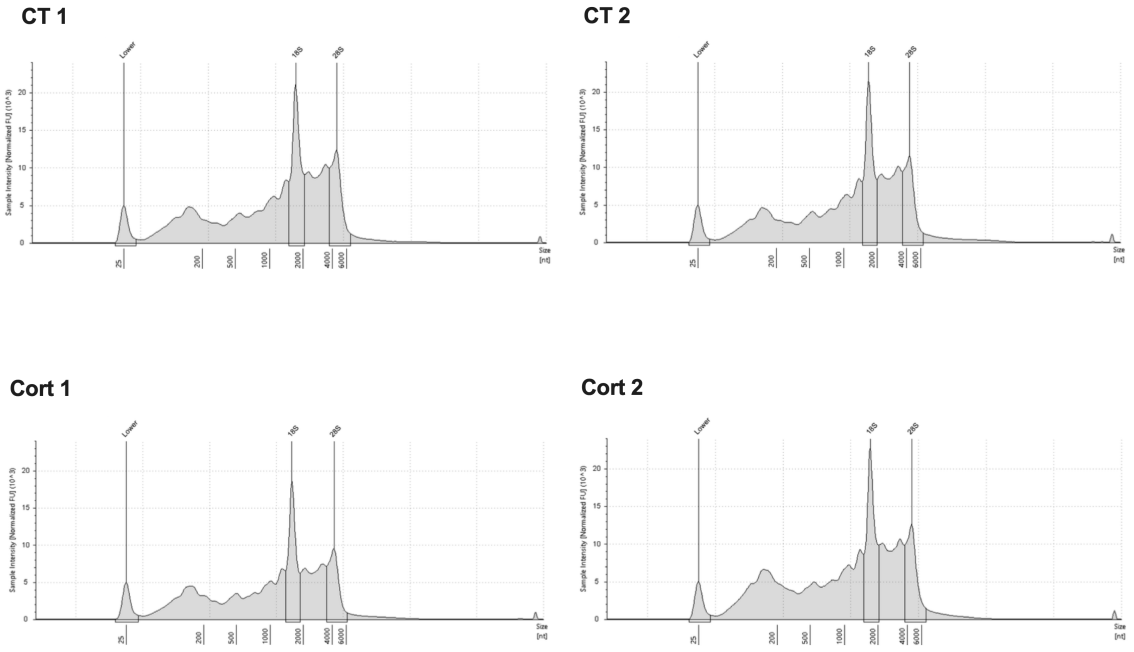
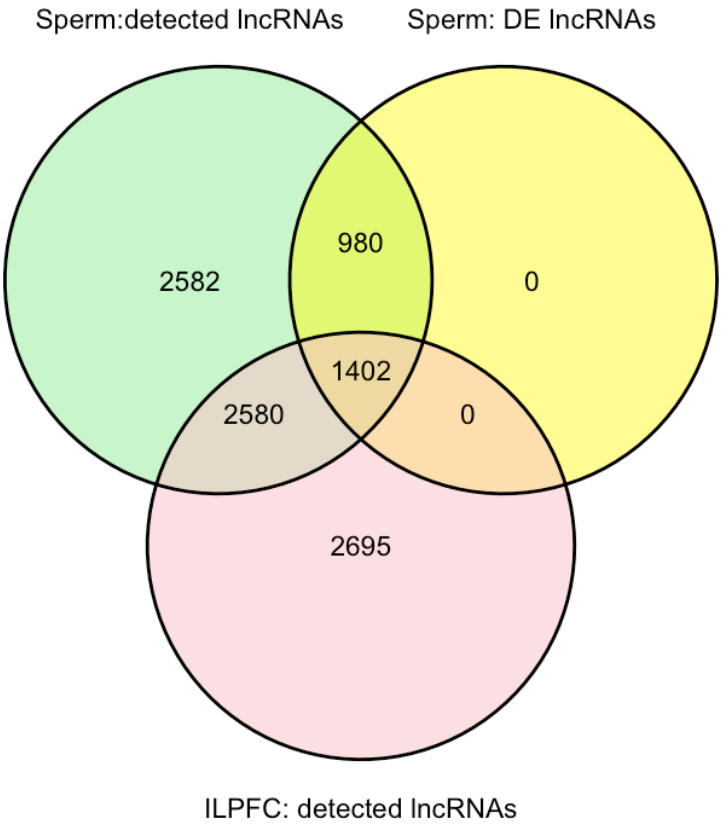
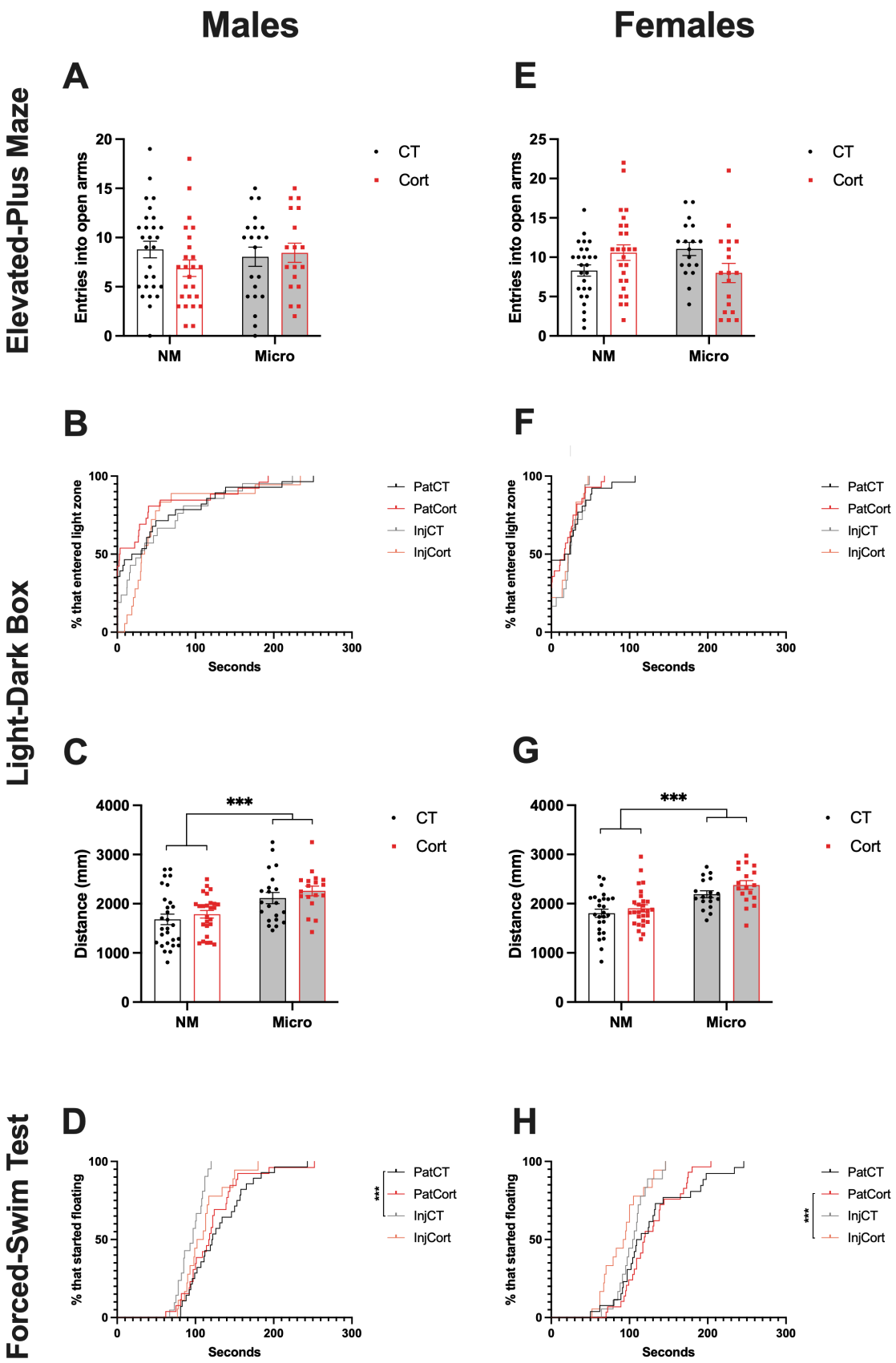
3.7. Supplementary material



Sup. Fig. S3.21. Size distribution of long RNA populations isolated from sperm. Two different samples were extracted from CT and corticosterone-exposed males, each from a pool of 5 mice.



Sup. Fig. S3.22. Venn diagram of comparison to ILPFC CaptureSeq.



Sup. Fig. S3.23. Additional parameters measured for the assessment of anxiety- and depressive-like behaviours. (A) No evidence of differences between the male groups in the number of entries into the open arms in the elevated-plus maze. (B) No evidence of differences between the male

groups in the latency into the light zone in the light-dark box. (C) Male mice born from microinjection moved longer distances in the light-dark box compared to male mice born from natural mating. (D) Male InjCT mice showed lower latency to float in the Porsolt swim test compared to PatCT. (E) No evidence of differences between the female groups in the number of entries into the open arms in the elevated-plus maze. (F) No evidence of differences between the female groups in the latency into the light zone in the light-dark box. (G) Female mice born from microinjection moved longer distances in the light-dark box compared to female mice born from natural mating. (H) Female InjCort mice showed lower latency to float in the Porsolt swim test compared to PatCort. Males: PatCT *n*=28, PatCort *n*=25-26, InjCT *n*=21, InjCort *n*=18. Females: PatCT *n*=26, PatCort *n*=26-29, InjCT *n*=18, InjCort *n*=18. Data analysed by 2-way ANOVAs and represented as mean ± SEM. Latency data was analysed by Kaplan-Meier survival analysis. Data represented as mean ± SEM. *** $p<0.001$.

Chapter 4

Voluntary physical exercise modifies the sperm long noncoding RNA profile

4.1. Abstract

We have previously observed that 4 weeks of paternal running wheel voluntary exercise in mice affects male offspring behaviour, leading to lower anxiety levels and a more robust fear extinction memory, which is accompanied by changes in sperm small noncoding RNA expression, particularly tRNA-derived fragments. In the present study, we sought to follow up on our previous results by determining the sperm expression profile of long noncoding RNAs (lncRNAs) following voluntary exercise in laboratory mice (C57Bl/6 strain). Using CaptureSeq, a technique that improves sequencing sensitivity by capturing long RNAs, we detected hundreds of downregulated lncRNAs. We also aligned the sequencing data to transposable element transcripts using TEtranscripts, where we observed preferential downregulation. Due to the poor functional annotation for lncRNAs, we then used bioinformatic strategies to predict their biological function. We found that almost half of the downregulated lncRNAs are also expressed in the brain, suggesting their activity in this tissue. After exploring their potential *cis* targets, we found that lncRNAs could be associated with selective biological processes, including cell adhesion. Lastly, we explored the transcriptional regulation of the differentially expressed lncRNAs. This is the first report showing that sperm lncRNAs are affected by exercise, which has implications for paternal epigenetic inheritance.

Keywords: voluntary exercise, long noncoding RNAs, sperm, epigenetic inheritance, transposable elements.

4.2. Introduction

There is a growing number of studies reporting that varied paternal exposures to environmental factors can influence offspring phenotypes. This intergenerational transfer of information is generally referred to as epigenetic inheritance, due to the mechanisms that were found to underlie this phenomenon, which includes epigenetic marks found in the sperm [274]. The first reports in humans that point to the involvement of the epigenetic inheritance encompass paternal exposures to traumatic conditions, such as the Holocaust [1], ex-prisoners of war [275] and famine [276]. Accordingly, many of the following animal studies continued to investigate the effects of stressful paternal conditions on epigenetic inheritance in the offspring. However, there is also the interest to investigate whether other, more positive, interventions could instead confer beneficial effects to the offspring.

An example of such positive intervention is physical exercise. In humans, in addition to the commonly reported beneficial effects of physical exercise on glycemia

[277], as well as on anxiety and depression levels [278], recreational physical exercise has also been shown to potentially have a positive effect on semen parameters, such as increasing semen concentration [279] and progressive motility [280], as well as plasmatic levels of testosterone [281]. Very importantly, endurance training promoted alterations in sperm piRNA expression, as well as DNA methylation changes in genes related to neurogenesis, neuron differentiation and axon guidance [282].

In preclinical studies, by exposing male mice to different paradigms that incorporate physical exercise preconception, groups have reported that the effects on the offspring range from improvement in spatial learning, together with decreased hippocampal DNA methylation [15], to cognition improvement and increased adult neurogenesis [16], but also susceptibility to the adverse effects of a high-fat diet [17]. In our laboratory, we gave male breeders access to running wheel voluntary exercise for 4 weeks pre-conception and observed that their male offspring display lower anxiety levels and a more robust fear extinction memory [18].

When we investigated the sperm small noncoding RNAs of the runner fathers, we observed changes in the expression of sperm miRNA and tRNA-derived RNAs [18]. Others have also reported changes in the sperm small RNA profile after paternal exercise [17]. These changes in the sperm transcriptome are believed to reprogram embryonic development, ultimately modifying offspring adult phenotype. Despite the known causal effect that sperm small noncoding RNAs can have in epigenetic inheritance [31], it is known that the sperm has a rich expression of long noncoding RNAs [252], which might also underlie the epigenetic inheritance. These RNA types are defined as longer than 200 nucleotides (nt), which encompass a wide range of heterogeneous non-protein coding RNA types [254], the vast majority of which do not have a well-defined functional annotation. However, it is known that lncRNAs can interact with chromatin-modifying factors and drive histone modifications [283], a mechanism that could explain the embryonic reprogramming that occurs in epigenetic inheritance.

Therefore, in order to understand the impact of physical exercise on sperm lncRNAs, we carried out CaptureSeq, a very sensitive RNA sequencing technique. We observed robust changes in the expression of lncRNAs, with most of them being downregulated. We also assessed the expression of other RNA types: transposable elements, whose expression profile was changed, with most of them being downregulated; and circular RNAs, which did not show any differential expression. Importantly, one of the transposable elements that was found to be downregulated is a mobile and autonomous retrotransposon. We then proceeded to investigate the lncRNA targets using bioinformatic approaches. We found that protein-coding genes associated with differentially expressed lncRNAs belong to a specific gene ontology involving cell-adhesion pathways, and that almost half of the differentially expressed lncRNAs are also expressed in the prefrontal cortex, suggesting roles in brain function. Additionally, we investigated the abundance of different transposable element families in the promoters

of lncRNAs and found substantial changes between upregulated and downregulated lncRNAs. Together, these results show that voluntary exercise modifies the sperm long noncoding RNA profile, which could have biological effects, thus playing a role in epigenetic inheritance by determining offspring phenotypic changes.

4.3. Materials and methods

Mice and husbandry:

C57Bl/6 mice were sourced from the Animal Resources Centre (ARC, Perth, Australia). Mice were housed in open top cages, with lights on from 7 am to 7 pm, and had food and water *ad libitum*. Soiled bedding was changed once a week. Mice were habituated for 2 weeks before being single housed randomly, having access to voluntary running wheel exercise for 4 weeks, in a wheel of 12 cm of diameter (running wheel group – RW). Controls were single housed into regular open top cages (control group – CT). Our previous studies showed that mice run around 50-60 km per week over the 4-week period [18]. At the end of the exercise training, male mice were culled by cervical dislocation for sperm extraction. All experiments were approved by the Florey Animal Ethics Committee and performed in accordance with the animal research guidelines of the National Health and Medical Research Council.

Other procedures:

Sperm collection

Sperm was collected by dissecting the cauda epididymis and slitting it with a blade to let the sperm cells flow into warm PBS (37 °C) for at least 30 min. The solution containing sperm cells was then centrifuged for 15 minutes at 400g. The supernatant was discarded, and the sperm was frozen at -80 °C.

RNA extraction and quality control for lncRNA Capture-Seq

RNA extraction from sperm was performed using Trizol reagent (Invitrogen), according to the manufacturer's instructions. Qubit assay (Invitrogen) was used to measure RNA concentration.

Sequencing:

Sperm lncRNA capture-Seq

To generate the RNA library, 2 µg of extracted total RNA was first treated with NEBNext® rRNA Depletion Kit (NEB) to remove rRNA. Then, NEBNext® Ultra™ II RNA Library Prep Kit for Illumina® (NEB) was used to generate the library. Long noncoding RNAs were captured by using around 28000 custom-designed probes (Roche) to target all known lncRNAs from GENCODE. Then, SeqCap EZ Hybridization manual and Wash Kit and SeqCap EZ Accessory Kit (Roche) were used to generate captured lncRNA libraries. 200 bp-long reads were then double-end sequenced on the Illumina HiSeq 4000 platform, with an average depth of 100 M. The average mapping success was 56.63%.

Sequencing data analysis

Low-quality nucleotides were trimmed off from the reads with cutadapt (v1.17) [257], to remove reads with Phred quality score lower than 20, and to remove Illumina adaptor sequences at the 3' end of each read. Processed reads were aligned to the mouse reference genome (mm10) using HISAT2 (v2.1.0) [236]. SAMtools (v1.8) [245] was used to convert "SAM" files to "BAM" files and to remove duplicate reads, and to sort and index reads. Only the properly aligned reads with mapping quality of at least 20 were kept for downstream analyses. Gene and transcript expression matrices were generated for each sample with StringTie (v2.0.3) [258], with the option "-e -G gencode.vM24.long_noncoding_RNAs.gtf" to limit the processing of read alignments to estimate transcripts matching the GENCODE long noncoding RNAs. Low-expression reads were filtered out using default parameters of the edgeR package (v3.34.1) [238,239]. edgeR was used for differential analysis expression, which used the calculated false-discovery rate to determine statistically significant lncRNAs, TE transcripts or circRNAs. gpplot2 (v3.3.5) [240] was used to make the volcano plots.

Gene Ontology analysis was performed with the GO Enrichment Analysis tool accessed at http://geneontology.org. Bedtools was used to obtain a list of protein-coding genes overlapping with all our detected long noncoding RNAs, which was used as the reference list. We ran a PANTHER Overrepresentation Test (Release 20210224) with the latest gene ontology database (DOI: 10.5281/zenodo.5228828, released on 2021-08-18). The statistical testing was performed with a binomial test using False Discovery Rate to correct for multiple comparisons [259–261]. Transposable element expression analysis was performed using TEtranscripts (v2.2.1) with its default parameters [262]. circRNAs were analysed with CIRI2 (v2.0.6) [263,264], using the filtering parameters min.count=3 and min.total.count=18.

We used bedtools to assess the abundance of transposable elements in the promoter region of sperm long noncoding RNAs, by intersecting the coordinates of

transposable elements from the mm10 RepeatMasker reference (Dec 2011, RepeatMasker open-4.0.6 - Dfam 2.0), and the coordinates of the region located 1kb upstream from the transcription start site (TSS) of the first exon of each lncRNA. Statistical test was performed with corrplot (v0.92).

4.4. Results

4.4.1. Voluntary exercise induces many changes in sperm long noncoding RNA expression

To assess the sperm long noncoding RNA expression profile after exposure to voluntary running wheel exercise, we deployed CaptureSeq for a sensitive sequencing. CT and RW groups separated distinctly from each other, as shown in the heatmap with the 500 most variable lncRNAs, which shows a different expression pattern between CT and RW groups (Fig. 4.24A). The CaptureSeq detected 10816 reads in total, with 7552 reads after excluding lowly expressed reads. Statistical testing with edgeR identified 506 differentially expressed lncRNAs, with 32 upregulated and 474 downregulated (Fig. 4.24B).

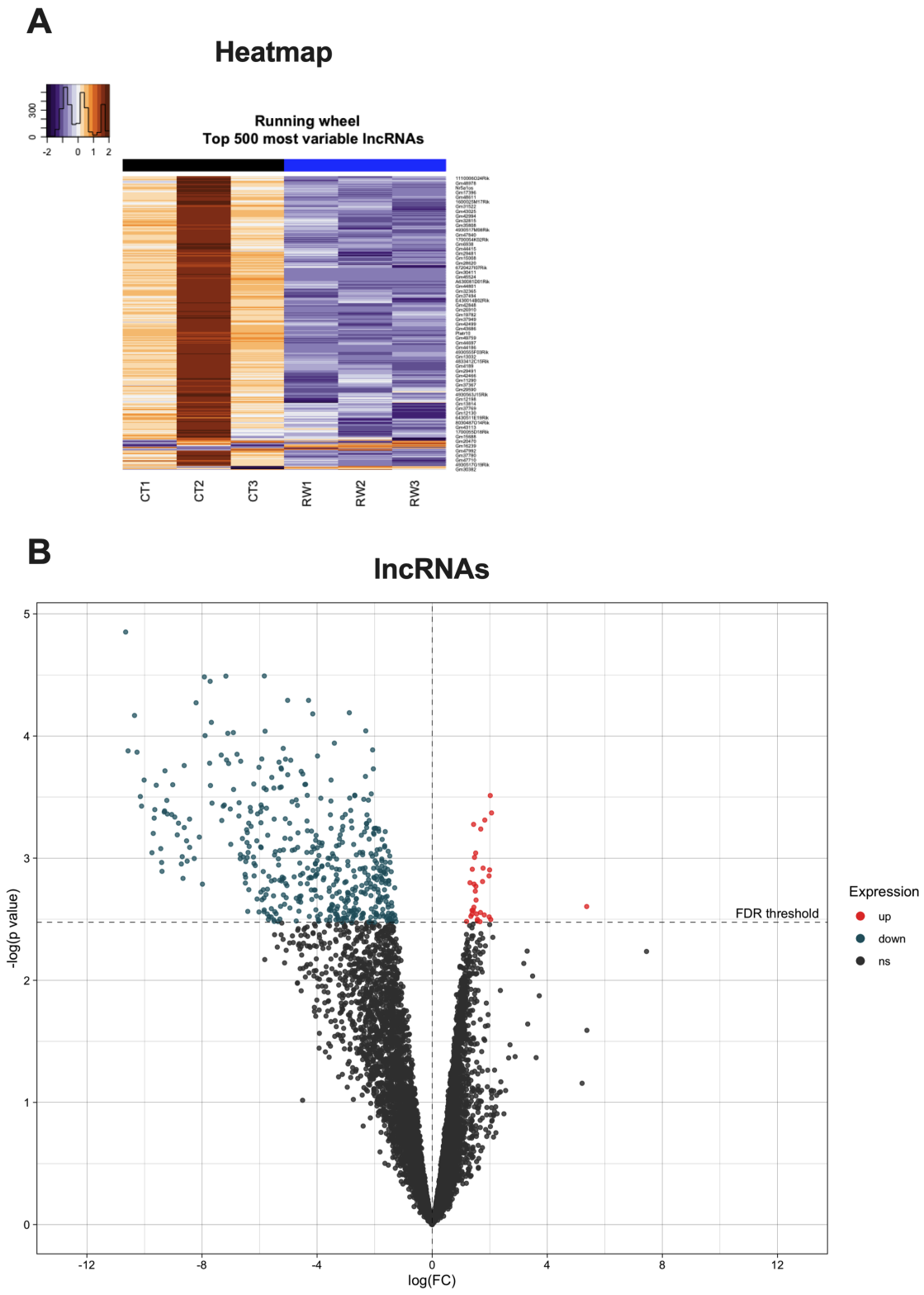


Fig. 4.24. Long noncoding RNA capture sequencing data analysis. (A) Heatmap of the top 500 most variable lncRNAs, reflecting a clear distinction between CT and RW samples. (B) Volcano plot of the lncRNA analysis. Up: upregulated lncRNAs. Down: downregulated lncRNAs. ns: non-significant. Significance was determined based on False-Discovery Rate threshold.

We then assessed the expression of other types of sperm long noncoding RNAs. First, we analysed transposable elements (TEs) using the tool TEtranscripts. We detected 1092 TEs, out of these 201 were differentially expressed, with 3 upregulated and 198 downregulated TEs (Fig. 4.25A). One of the downregulated transposable elements is L1Md_Gf, which is a member of the LINE class and L1 family. This is one of the few TEs that are mobile in the mouse genome, alongside L1Md_A and L1Md_Tf [284]. The downregulation of a mobile and autonomous L1 element could imply that the offspring of RW fathers are less prone to retrotransposition events, thus protecting them from mutations.

Lastly, we investigated the expression of circRNAs using CIRI2. We detected 10683 circRNAs and did not find any differentially expressed circRNAs in our dataset (Fig. 4.25B).

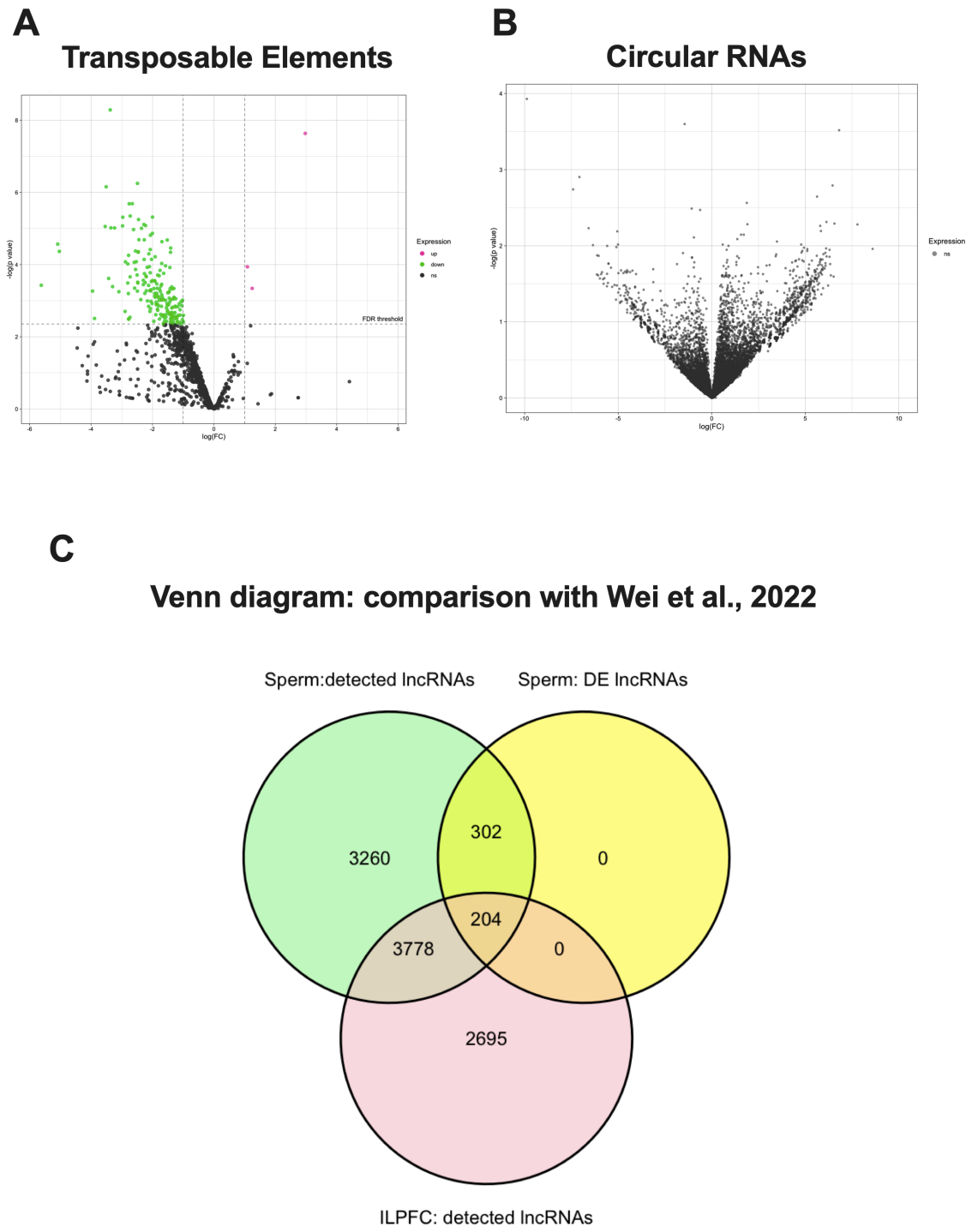


Fig. 4.25. Investigation of other long RNA types, and cis-target prediction. (A) Volcano plot of the Transposable Element transcript analysis. Up: upregulated TE transcripts. Down: downregulated TE transcripts. Ns: non-significant transcripts. Significance was determined based on False-Discovery Rate threshold. (B) Volcano plot of the circRNA analysis. ns: non-significant circRNAs. Significance was determined based on False-Discovery Rate threshold. (C) Venn diagram of comparison to ILPFC CaptureSeq.

4.4.2. The differentially expressed lncRNAs likely influence brain function and cell-adhesion processes

Although we observed significant differences between CT and RW groups, it is difficult to attribute a biological significance to these results due to the poor functional annotation of long noncoding RNA types. To overcome this, we used bioinformatic approaches to predict their function. It is known that lncRNAs have their expression correlated with genes located in proximity to their chromosomal location, called cis-action [84]. We used bedtools to identify 4240 protein-coding genes overlapping with the 7552 lncRNAs we detected, and 161 protein-coding genes overlapping with the 506 differentially expressed lncRNAs. Gene Ontology analysis revealed that those 161 genes are associated with the cell adhesion (GO: 0007155) pathway.

We next compared our detected lncRNAs to those of a study that performed CaptureSeq in the mouse Infralimbic Prefrontal Cortex (ILPFC) [256]. They detected 6677 unique lncRNAs, compared to 7544 unique lncRNAs that we detected. Out of these, 3982 were shared between both lists (52.78% of the sperm lncRNAs, 59.64% of the ILPFC lncRNAs), and 204 of them were differentially expressed in the sperm, which corresponds to 40.32% of the differentially expressed sperm lncRNAs also being expressed in the ILPFC (Fig. 4.25C). Due to their expression in the ILPFC, this suggests that some of the lncRNAs present in the sperm could also have an effect in brain function.

4.4.3. Up and downregulated lncRNAs have a different profile of transcription enhancers

Endogenous retroviruses function as active enhancers and drive the expression of the germline transcriptome during spermatogenesis [266]. To investigate whether up and downregulated lncRNAs have different profiles of endogenous retroviruses in their promoter regions, we used bedtools to identify transposable elements located 1kb upstream the transcription start site of the first exon of each differentially expressed lncRNA. 15 TEs were found in the promoter region of upregulated lncRNAs, all of them being part of the Alu family. On the other hand, 749 TEs were found in the promoter region of downregulated lncRNAs, with elements from the L1 family being the most abundant, with 220 elements, followed by the ERVL-MaLR family with 128 elements, and the Alu family with 94 elements (Fig. 4.26A). After statistical testing using correlation plots, we found that there is a statistically significant underrepresentation of the Alu and B2 families in downregulated lncRNAs, and an overrepresentation of L1

elements in downregulated lncRNAs (Fig. 4.26B, $\chi^2_{(78)}$=242.72, p<2.2e-16). This statistical result is mostly driven by the reduction of Alu and B2 elements and an increase in L1 elements in downregulated lncRNAs (Fig. 4.26C).

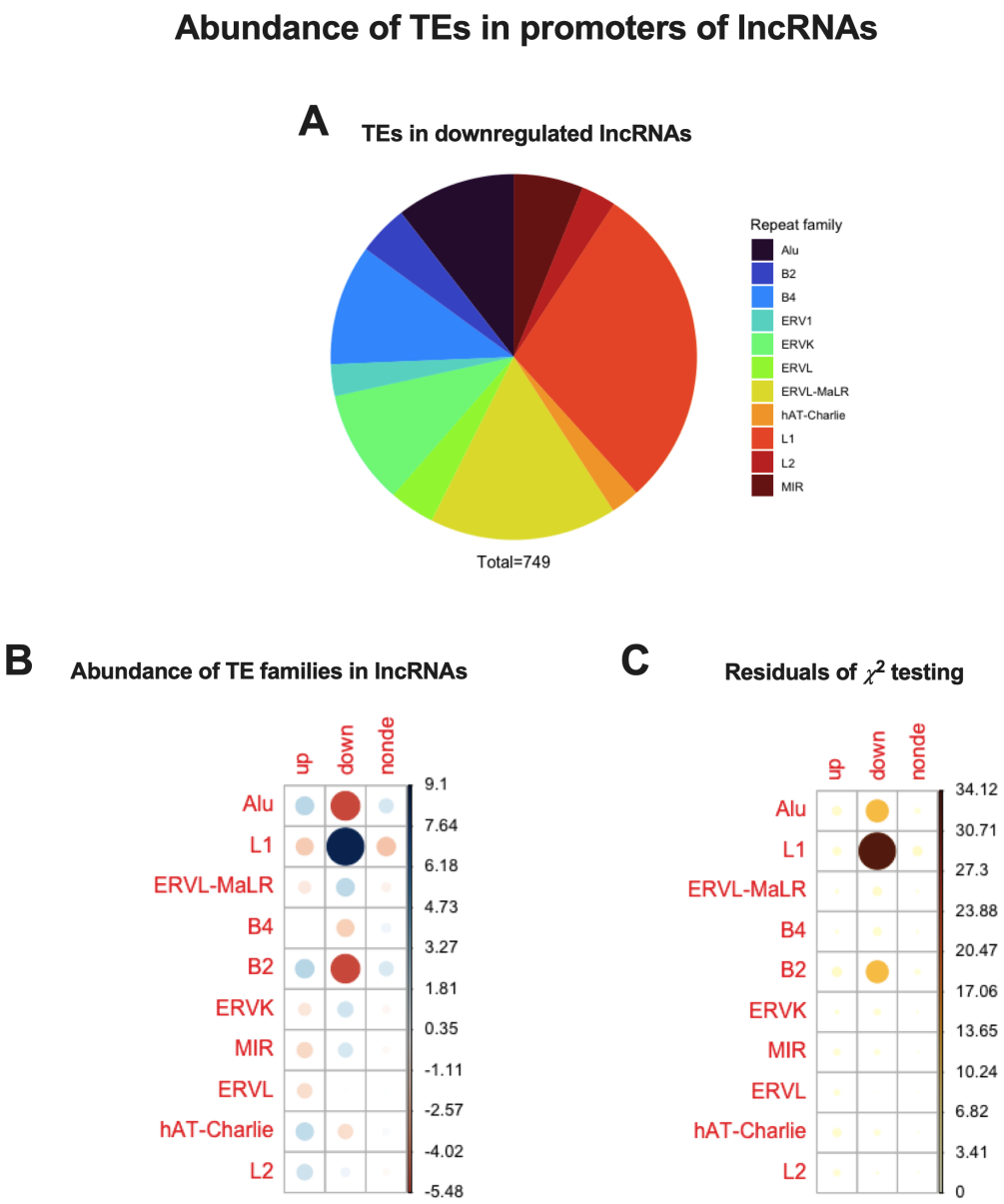


Fig. 4.26 Analysis of transposable elements in the promoter regions of up and downregulated lncRNAs. (A) Pie chart showing abundance of transposable elements in the promoter region of upregulated sperm lncRNAs. Only families with more than 10 elements are shown. (B) Pie chart showing abundance of different families of transposable elements in the promoter region of downregulated sperm lncRNAs. Only families with more than 10 elements are shown. (C) Representation of statistical results of abundance of each family of transposable elements. Blue: overrepresented. Red: underrepresented. (D) Representation of the contribution of each residual to the χ^2 value.

4.5. Discussion

In this study we exposed male mice to voluntary running wheel exercise and performed CaptureSeq to sequence their sperm lncRNAs. We found downregulation of sperm lncRNAs and transposable elements, and bioinformatic approaches indicated that almost half of these lncRNAs are expressed in the brain and are potentially biologically active. To our knowledge this is the first study to show that physical exercise alters the sperm lncRNA profile, and it expands the understanding of the physiological effects of exercise, as well as informing potential mechanisms underlying the intergenerational epigenetic effects of paternal exercise on offspring phenotypes.

lncRNAs have a robust expression in the testes, an organ that shows the most variability in its lncRNA repertoire [285]. The brain is also transcriptionally complex [286] and expresses around 40% of the known mammalian lncRNAs [287], which are thought to be involved in brain evolution [288] and brain disease [289]. The complex and extensive lncRNA expression in the testes can also be tied to evolutionary processes, as it has been suggested that the rapid acquisition of new noncoding genes in the testes leads to gain of function in other tissues, which could ultimately be one of the major forces driving evolution and diversity [290]. Therefore, it is not surprising that we found a high overlap between sperm and brain lncRNA expression, and it reinforces the many potential biological functions of lncRNAs in the brain.

We also found that downregulation of specific lncRNAs were associated with specific transposon element families (Alu, B2 and L1, more specifically) in their promoter region. This indicates that different promoters and enhancers are activated after voluntary exercise, which drive the differential expression of up and downregulated lncRNAs that we observed. It is known that physical activity regulates gene expression in skeletal muscle, which is first signalled by the muscle contraction and signalling kinases and their downstream pathways [291] and microRNAs [292]. How physical exercise induces specific changes in testes gene expression remains to be clarified, but circulating factors liberated in the bloodstream after exercise that can act in the testes, such as testosterone [293,294], may be involved.

Our bioinformatic target prediction analysis revealed that the differentially expressed sperm lncRNAs have as their potential cis targets protein-coding genes involved in the cell-adhesion gene ontology pathway (GO: 0007155). This pathway is defined as “the attachment of a cell, either to another cell or to an underlying substrate such as the extracellular matrix, via cell adhesion molecules”, a biological process that also occurs during implantation. There is one study that investigated the relationship between paternal exercise and blastocyst implantation, and they found that paternal exercise increases cell to cell contacts in the early embryo, as well as the blastocyst trophectoderm and inner cell mass; additionally, they reported an improvement in

implantation rates [295]. It should also be noted that lncRNAs can also have action in trans, when their targets are in distant chromosomal locations, and these effects are more difficult to predict *in silico*. Therefore, it is expected that these differentially expressed lncRNAs have additional unreported biological effects.

We also observed downregulation of transposon elements, and special attention should be given to the downregulation of the Line1 retrotransposon L1Md_Gf, which could result in a reduction in retrotransposition events and subsequent mutations. Previous research has shown that L1 insertion rates happen at ≥1 events per eight births [296], thus it is of interest to investigate whether this rate is affected by paternal exercise. Line1 expression is ubiquitous in germ cells and across early embryonic stages compared to somatic cells, and this expression is essential for preimplantation development [297], due to their effect on initiating early embryonic transcriptional processes [266]. However, it has been predicted that conditions involving reduced suppression of L1 in germ cells or early embryos could lead to excessive retrotransposition events and thus reduced embryonic survival [298].

Overall, the changes in sperm lncRNA due to physical exercise indicate the porosity of the Weismann barrier, which is a theoretical barrier separating the germline from the soma that was once assumed to be a biological dogma [299]. How exactly such permeability happens has yet to be determined; however, as we have presented here, the lncRNAs affected by paternal exercise potentially have biological functions relevant to intergenerational epigenetic inheritance, including roles in embryonic development, neurodevelopment and brain function. These are predictions based on *in silico* approaches that require additional *in vitro* and *in vivo* experiments to investigate whether sperm lncRNAs affect blastocyst cell adhesion and implantation rates, brain development and function, but also whether paternal exercise changes L1 insertion rates. Lastly, microinjection studies are paramount to determine the involvement of sperm lncRNAs in the epigenetic inheritance of paternal exercise.

4.6. Declarations

Conflict of interest

The authors declare no conflict of interest.

Author contributions

L.B.H. contributed to planning the bioinformatic experiments, conducted all analyses, and wrote the manuscript. Q.Z. and W.W. performed the CaptureSeq gene expression analysis. L.J.L. conducted the capture sequencing. T.W.B. supervised the

CaptureSeq and gave critical feedback to its analysis. T.Y.P. envisioned the study, performed the tissue collection, supervised study design and statistical analyses, and reviewed the manuscript. A.J.H. envisioned and funded the study, contributed to planning experiments, provided critical feedback throughout the experiments, and reviewed the manuscript.

Data availability

Data is in the process of being uploaded. Its accession number will be made available soon.

Acknowledgements

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Chapter 5

General discussion

5.1. Summary

Briefly, the studies conducted in this thesis have found that:

- Corticosterone-treatment preconception has an intergenerational effect on mouse offspring social behaviour, but this effect does not persist transgenerationally (Chapter 2).
- Stress-induced changes to the mouse sperm epigenome include alterations to long noncoding RNAs, transposable elements and circular RNAs. lncRNAs have a role in the intergenerational adaptations of offspring affective behaviour (Chapter 3).
- Voluntary exercise affects the mouse sperm epigenome, including a majority downregulation of lncRNAs and transposable elements (Chapter 4).

In the paragraphs below I will discuss the broader implications of this work for our understanding of epigenetic inheritance and human health.

5.2. Fickle nature of paternal stress studies and claimed impacts on offspring behaviour

The behavioural results we observed in the male offspring in chapters 2 and 3 differ, as well as they differ from the results previously obtained by Short et al., 2016 and Rawat et al., 2018, which showed anxiety-like behaviour in the male offspring. Although there are experimental differences that add confounding factors between each of these studies, such as a different mouse substrain that was used for chapter 3, or a potential modified epigenetic background that might have arisen due to the time difference between the studies of Short et al. and chapter 2 (which are at least 4 years apart), a new hypothesis that can be explored is that how epigenetic inheritance affects behaviours is not necessarily predictable. For instance, even though there is enough evidence that the paternal corticosterone model will likely affect anxiety-like behaviours, the direction of such change might not be constant between studies. This is also observed in studies from other groups that investigate epigenetic inheritance, where the different parameters of anxiety- or depressive-like behaviours are not necessarily consistent, as it can be seen in the publications from the Mansuy group [27,31,180]. If that was the case, it suggests a probabilistic - rather than deterministic - nature of the epigenetic inheritance [300]. We could think of the effects of the epigenetic inheritance as inducing variation in the expression of genes that are modulated by epigenetic mechanisms, with the overall result in increased phenotypic variability through the modulation of certain behaviours (such as anxiety- and depression-like behaviours, as observed in this study). For instance, different mathematical models have

suggested that environmental pressures could drive adaptation faster if epigenetic and genetic variation are taken into consideration, leading to adaptive phenotypes before genetic changes; as well as the generation of stochastic population diversity represents a way to adapt to fluctuating environments [142,301]. Therefore, we suggest that the epigenetic inheritance induces variability in conditions of stress to increase the likelihood that offspring individuals will be well equipped to deal with the parental environment challenge.

5.3. Corticosterone exposure had a larger effect size on the sperm lncNA profile than paternal running

We observed a much higher number of differentially expressed long noncoding RNA types in the sperm of corticosterone-treated than running wheel exercised males. This follows the results observed from Short et al., 2016, and Short et al., 2017, which showed that corticosterone treatment also affects sperm noncoding RNAs at a higher degree, as well as induces more pronounced behavioural effects in the male offspring. One explanation for these results is that the protocol of paternal corticosterone exposure is more intense than the protocol of paternal voluntary running wheel exercise, despite both lasting for 4 weeks during early adulthood. Different paternal stress exposure paradigms could have different intensities by applying stresses of varying degrees, or by exposing the males during development stages or adulthood. For instance, the number of differentially expressed sperm miRNAs found in a mouse model of unpredictable post-natal maternal separation combined with unpredictable maternal stress was 43 [31]; in a mouse model of chronic variable stress during puberty there were 9 [29]; and in our model of corticosterone exposure during adulthood, 101 were found [28]. However, these differences can be, to some degree, due to the sequencing platform and bioinformatic analyses that differed between these studies. If sperm lncRNAs play a role in reprogramming the early embryo, which would ultimately result in the observed offspring behavioural differences, then the sperm long noncoding RNA profile induced by corticosterone exposure would have more intense effects in the embryo than those induced by the running wheel exercise. Therefore, an alternative hypothesis is that the evolutionary pressures that shaped the epigenetic inheritance might have favoured adaptation through stressful environments rather than positive ones. Additionally, if the informational function of the epigenetic inheritance is true, which posits that it allows cues about the paternal environment to be passed on to the next generation to increase adaptation to it, then paternal running wheel exercise could be used to counteract the consequences of stressful paternal environmental effects on sperm, as it has been attempted [82], but a positive environment in itself would not be as impactful because it would inform the next generation that there are no specific challenges to which it needs adaptation.

Nevertheless, it is intuitive to investigate whether the 'negative' effects on the offspring induced by paternal stress exposures can be countered with 'positive' paternal environmental exposures, such as exercise or environmental enrichment. Accordingly, it has been reported that the transgenerational effects resulting from the exposure to post-natal maternal separation can be prevented when these stressed mice are reared in an enriched environment with access to running wheels from weaning until adulthood [82]. Interestingly, in our dataset we observed that corticosterone exposure and running wheel affect the long noncoding RNA profile in a similar way, despite the former having a larger effect size. In Fig. 5.27, most of the upregulated lncRNAs induced by exercise are also upregulated after corticosterone exposure, and the same effect is seen for the downregulated lncRNAs. Therefore, taking this finding into consideration, in our model the running wheel voluntary exercise might not be able to rescue the effects caused by corticosterone exposure.

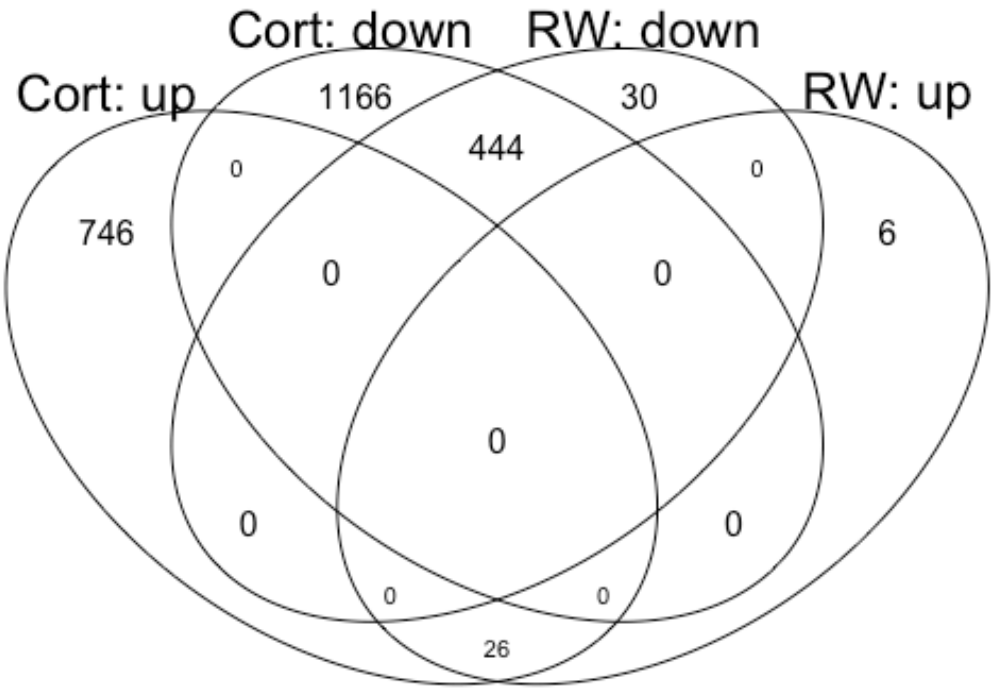

Fig. 5.27. Venn diagram showing the intersection of differentially expressed long noncoding RNAs. Many upregulated lncRNAs are shared between corticosterone exposure and running wheel voluntary exercise, and the same effect can be seen for downregulated lncRNAs. Cort: corticosterone. RW: running wheel.

5.4. How does the environment induce changes to the sperm lncRNA profile?

There is plenty of evidence that different environmental exposures modulate the sperm RNA profile, but it is still not clear how this happens. Small noncoding RNAs have

been shown to be delivered to sperm cells by epididymosomes [302], which are exosomes secreted by the epididymal cells. During sperm maturation along the epididymides, sperm cells are continuously exposed to epididymosomes and absorb them, as well as their RNA content [303]. Epididymal cells show a high expression of glucocorticoid receptors [304], therefore the sperm noncoding RNA content is likely to be a direct consequence of the glucocorticoid receptor activity in epididymal cells. On the other hand, long noncoding RNAs are produced in spermatids and are preserved across maturation into fully mature sperm cells, although the long RNA profile changes during this process, which is associated to an enrichment in their expression in sperm cells, compared to mRNAs [305]. Therefore, as long noncoding RNAs are expressed in spermatids and are modulated by corticosterone exposure, this raises the question on how the spermatids contained within the testes are exposed to circulating factors, considering the presence of the blood-testes barrier. In the testes there are mainly 3 cell types: the Leydig cells that produce hormones, the spermatogonia that further mature into sperm cells, and the Sertoli cells that offer structural support, nutrients and envelop the spermatogonia (Fig. 5.28). The Sertoli cells have been shown to have their mitochondrial membrane potential decreased after 4 weeks of corticosterone treatment [306], and have their transcriptome and mitochondrial electron transport chain affected by early postnatal stress, an effect that was mediated by serum factors [307], and they also express glucocorticoid receptors, making them responsive to stress hormones [308,309]. Therefore, it is reasonable to hypothesise that Sertoli cells would be responsible for responding to circulating factors and inducing the differential long noncoding RNA expression observed in the sperm, especially when considering that genetic manipulation of Sertoli cells can greatly affect spermatogonium viability [310]. However, there still needs to be determined to which types of circulating factors the Sertoli cells respond, and whether corticosterone exposure and voluntary running wheel exercise exert their effect through them. Recently, it has been shown that circulating factors, more specifically lipid-derived metabolites, can activate their receptors in the spermatogonia and induce offspring phenotype changes [311].

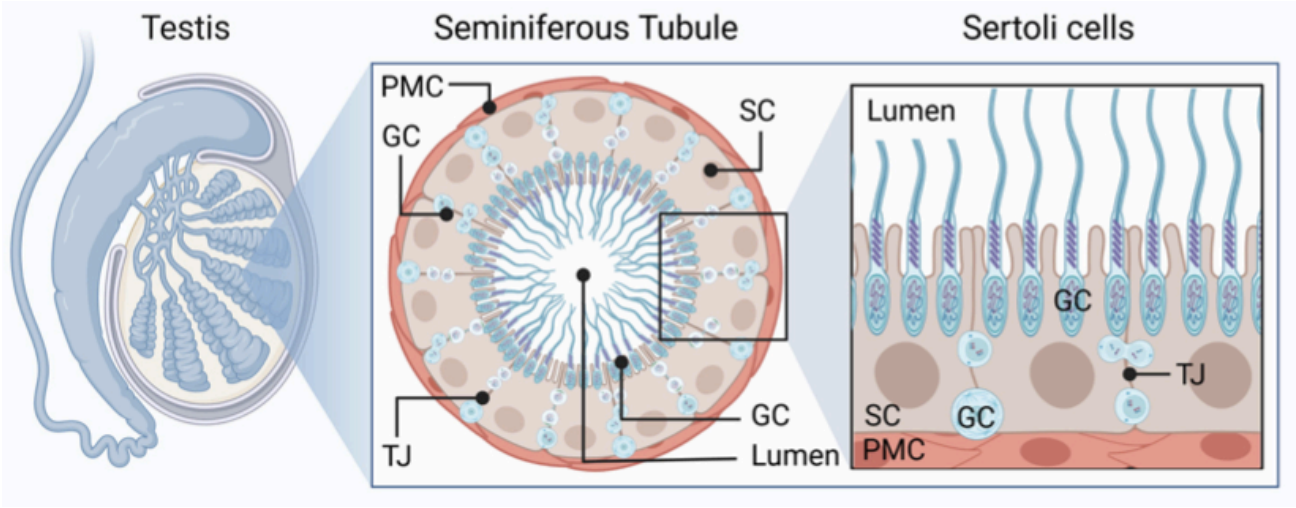

Fig. 5.28. Schematic of a cross section of the seminiferous tubule. Germ cells (GC) are enveloped and nurtured by Sertoli cells (SC), which are surrounded by the peritubular myoid cells (PMC). Sertoli cells form tight junctions (TJ) with each other, which comprises the blood-testis barrier. Figure extracted from Washburn and Dufour, 2023.

5.5. How do sperm RNAs acting on the embryo modulate adult phenotype?

Our microinjection study and others have shown that sperm RNAs can induce altered adult phenotype [26,31]. A few others have also investigated the effects of such procedure on the early embryo transcriptome [126,313]. Even though determining the mechanisms that bridge these two distinct timepoints is essential to the understanding of the mechanisms underlying the epigenetic inheritance, this is a very challenging venture. For instance, the embryonic transcriptional landscape is very different to somatic cells, and this adds an inherent potential misinterpretation to noncoding RNA target prediction, which are based on studies in somatic cells. Additionally, the embryonic processes, such as the epigenetic reprogramming and the maternal to zygotic transition, which could be targeted by sperm RNAs, are highly complex and unique to the early developmental stage. Collecting, handling, and cultivating embryos is difficult, expensive, and potentially ethically problematic; therefore, alternatives need to be considered, such as using induced pluripotent stem cells, which conserve the same totipotency. They could be used to study the effect of sperm RNAs on development, by differentiating them into cell types of interest.

Another challenge is that sperm RNA sequencing studies have shown multiple sperm RNAs to be modified by parental exposures, and it is likely that a combination of both the multiple small and long noncoding RNAs is needed for the complete epigenetic inheritance effect [126]. Therefore, the attempt to pinpoint a single sperm RNA as being responsible for major effects in the embryo is very likely counterproductive, and this issue is accentuated when determining the effect of long noncoding RNAs, as their

functional annotation is extremely poor. However, with the aid of bioinformatic tools to predict trans targets, a study has shown the contribution of a single lncRNA to the cardiac mesoderm development by using antisense oligonucleotides during the differentiation of human pluripotent stem cells into cardiomyocytes, and assessing mRNA expression of their targets predicted with bioinformatic tools [121].

Sperm RNAs might also influence adult phenotype by affecting the placenta [10]. The placenta is formed by the expression of factors from both the embryo and the mother, and due to its role in nourishing the embryo, it can determine how many resources are delivered to it. Consequently, the placenta can have a great effect in the embryonic development, as it can regulate access to nutrients that are vital for growth and brain development, such as growth factors and methyl donor nutrients [10]. These potential mechanisms could be studied by investigating placental histology and gene expression after the paternal corticosterone exposure.

Our current working hypothesis on how the microinjection of long noncoding RNAs induced altered adult phenotype, given that in a separate study we did not observe changes in the prefrontal cortex transcriptome (chapter 2), is that sperm long noncoding RNAs act as guides to epigenetic marks (DNA methylation and histone modifications) in genes involved in neurogenesis and brain function. Across development, at the crucial timepoints when such genes should be expressed for proper development, those epigenetic marks instruct how these genes will be expressed and influence neurogenesis trajectory, such as through axon guidance and synapse formation. The result is a modulation of brain connectivity, in such a way that the brain transcriptome does not show overt changes, but instead subtle changes in circuitry function could be in place (Fig. 5.29). Such changes in brain function could be investigated by mapping c-Fos activation in response to stress, for instance. This type of study has been done in a mouse model of paternal exposure to maternal separation, with their offspring showing reduced c-Fos activation in fear-regulating brain regions in response to predator odour [268]. To assess the contribution of long noncoding RNAs for brain function, in particular to anxiety- or depressive-like phenotypes, a study similar to Wei et al., 2022 could be done, where they performed capture Seq to identify long noncoding RNAs in a brain region of interest after a relevant behavioural testing, and also performed ATAC-sequencing to assess genome-wide chromatin accessibility in the same brain region. By performing bioinformatic analyses to link the detected lncRNAs with chromatin accessibility, they identified one lncRNA that was later shown to contribute to the behaviour performance. Lastly, to investigate if stable epigenetic marks inherited by the offspring, such as histone modifications, can cause altered neurogenesis, one could use induced pluripotent stem cells. By deriving them from fibroblasts collected from adult offspring, and then differentiating the induced pluripotent stem cells into neurons, changes in their differentiation and synaptogenesis could be investigated.

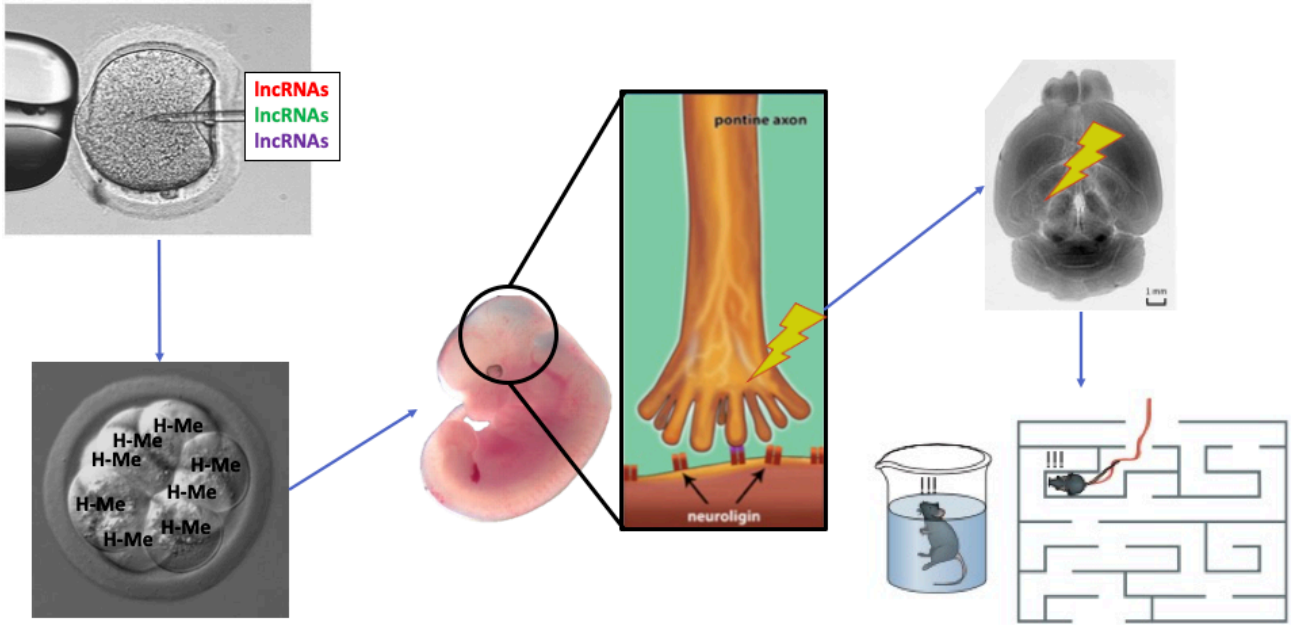

Fig. 5.29. Visual representation of our working hypothesis. (Top left) Differentially expressed long noncoding RNAs resulting from an altered paternal environmental exposure are injected into the fertilised oocyte, either through natural fertilisation or through a microinjection procedure. (Bottom left) By inducing differential histone modifications (H-Me) on their target genes, the information the long noncoding RNAs convey is "transferred" into a stable code. (Middle) During embryonic brain development, the differential histone modifications result in differential expression of relevant genes that will affect neurodevelopmental processes, such as axon guidance during synaptogenesis. (Top right) As a result, the adult offspring will show signs of altered brain activity. (Bottom right) These differences in brain function reflect on altered behaviour. Figure adapted from Bohacek and Mansuy, 2015; Li et al., 2016a, and https://www.neuroskills.com/brain-injury/neuroplasticity/synaptogenesis/.

5.6. Epigenetic inheritance modulates transposable elements

It is remarkable that different paternal environmental exposures modulate the degree of expression of transposable elements. Spermatogenesis is a process that involves the physiological expression of transposable elements due to the reprogramming of somatic cells into haploid germ cells, and accordingly, there are mechanisms in place to keep deadly transpositions at bay, such as the expression of piRNAs and the large amount of sperm cells being produced, as well as the competitive nature of fertilisation, which weeds out unfit sperm cells. However, if the expression of transposable elements is affected by the paternal environment, it can result in modulation of the retrotransposition rates in the germ cells and inheritance by the offspring. Assuming that the epigenetic inheritance and its underlying mechanisms were selected due to their beneficial effects on fitness, this could represent a mechanism to jump-start evolutionary processes when stressful paternal environmental conditions

happen, through loosening the control over retrotransposition rates and increasing genomic variability at random [315]. Lethal transpositions would be selected out before fertilisation, during spermatogenesis, with the death of sperm cells carrying lethal insertions. Interestingly, transposable elements are also highly expressed in neuron progenitors during brain development, when an excess of synapses is created that will be pruned later during the first years of life, according to their connectivity and activity [316]. This process shares some resemblance to the spermatogenesis, because when lethal insertions happen, they would be pruned and, on the other hand, neutral or beneficial DNA insertions would be kept, which might result in an adaptive function. Indeed, it has been shown that brain neurons show genetic mosaicism, which is a consequence of transposition events [131]. Therefore, both spermatogenesis and neurogenesis seem to be perfect developmental timepoints for a physiological experimentation with retrotransposition for the generation of DNA variability, due to their common feature of producing a cell excess. Even though this process harbours an adaptive potential, it has also been associated with neuropsychiatric disorders [317,318] and cancer [319]. Therefore, these diseases can also be involved with the epigenetic inheritance and investigating the incidence of cancer after generational stressful exposures could help shed a light into this hypothesis.

5.7. Creating biological novelty with lncRNAs

Due to the close association between transposable elements and long noncoding RNAs, spermatogenesis and neurogenesis could also represent a potential for generation of new lncRNAs. When transposable elements insert into “junk” DNA, they can originate and lend their functions to lncRNAs from a premade transposition “toolkit”, which as a consequence makes them bioactive. Indeed, this is most commonly how lncRNAs are formed [320]. Among their functions are those of enhancer, promoter, exon, intron and polyA site [173]. For this reason, transposable elements and lncRNAs are active players in evolution, and due to their potential regulatory effect, lncRNAs are thought to be associated with complex diseases [321], and thus their heritability. Therefore, the physiological expression of transposable elements and lncRNAs during spermatogenesis and neurogenesis could also function as an attempt to create biological novelty.

5.8. What is the overall purpose of the epigenetic inheritance?

Does the epigenetic inheritance happen in all organisms? If it does, did different evolutionary pressures create different forms of epigenetic inheritances in different

organisms? Based on the literature and the studies conducted in this thesis, I would like to speculate and propose that the epigenetic inheritance in mice might modulate specific effects, aimed at modifying certain offspring behaviours to attempt an increase in adaptiveness to the paternal environment, but might also be geared towards increasing variability in the offspring by modulating retrotransposition rates (Fig. 5.30). It is possible that different mechanisms underlie each of these epigenetic inheritance "modes". Determining how and why different paternal environmental conditions engage these "modes" and how they affect the missing heritability problem are also highly relevant. Answering these questions is extremely challenging. For instance, even determining what is an adaptive behaviour is challenging because these qualities are ultimately determined by natural selection. Nonetheless, the research on the epigenetic inheritance has shown us so far that embryo development is vulnerable to the effects of the external world, also impacting adulthood, which can represent immense consequences. For instance, it informs us on risk considerations for *in vitro* fertilisation techniques, especially when considering that an increasing number of babies will be born through such techniques due to the reduced fertility in the general population [322,323].

Fig. 5.30. Diagram picturing the proposed concept of epigenetic inheritance determining adaptive function. On the left, the black figures represent a hypothetical environment in which a perfectly adapted individual fits perfectly in the white centre. On the right, each coloured figure represents an individual offspring, with their shape representing their fitness, and the red 'block' symbol indicating they are not adapted to that environment. In environment 1, most of the offspring is adapted, except for those presenting some kind of maladaptive natural variation. In environment 2, the previous environment has changed, and a new selective pressure is presented. This condition is modelled by the epigenetic inheritance studies. The offspring shows increased variability (due to increased transposition rates), as well as overall variation that attempts to adapt to the environment (due to the epigenetic inheritance). In environment 3, a new environment is presented, in which only one of the individuals is somewhat adapted to it, due to the increased variability that the selective pressure in environment 2 induced.

5.9. Limitations of the study

An important limitation of the studies conducted in this thesis is that the exploration of lncRNA function was done *in silico* only. As stated previously, lncRNAs have extremely poor functional annotation, and only few have been studied individually

through biomolecular tools, which are needed to determine the genes that lncRNAs regulate. This also reflects on few bioinformatic resources that can be used to infer their functional annotation, as well as the limited ability of these tools to do so. In the present study, even though we explored the potential *cis* action of lncRNAs *in silico*, we could only go as far as to determine their gene ontology terms. Biomolecular experiments are needed to confirm these results, but designing such experiments is no short of challenges either.

As sperm lncRNAs are expected to have a short window of action during early-embryogenesis, when they would induce epigenetic modifications to their target genes, the actual changes in gene expression are hypothesised to be observed during embryonic development only, such as brain development. However, even if brain tissue were to be extracted from foetuses sired by control or corticosterone-treated fathers, its gene expression determined by RNA sequencing, and differential gene expression determined, it would be impossible to attribute such changes to the action of sperm lncRNAs. An alternative experiment could be, instead, to conduct the microinjection of sperm lncRNAs into fertilised oocytes and perform single-cell RNA sequencing to determine their effect on gene expression, or even perform another technique to investigate changes in the epigenome. However, these experiments would still not explain the behavioural differences that are observed in adulthood. If we were to approach this question by following up on behavioural differences observed in the adult offspring, an outstanding challenge that arises is determining the genes that underlie such behavioural changes. If we were to find a good single gene candidate, it would be possible to analyse its epigenetic state (eg, histone modifications) during offspring development, since early-life. However, determining lncRNAs that regulate the epigenetic state of this gene would also incredibly challenging.

Therefore, without clear answers on how to follow up on the experiments described in this thesis, or how to determine how lncRNAs affect gene expression, or even how a hypothesised differential gene expression during early-embryo stages modulate behaviour in later adulthood, we are restricted to only providing results that suggest the involvement of sperm lncRNAs in the epigenetic inheritance.

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