Elevated paternal glucocorticoid exposure prior to conception causes intergenerational shifts in offspring social and sexual behaviours and expression of major urinary protein pheromone

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ABSTRACT

Studies have shown that paternal stress prior to conception can influence the innate behaviours of their offspring. The evolutionary benefits of such intergenerational adaptations continue to be speculated upon. Our group previously showed that glucocorticoid treatment of adult male 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 adaptive fitness. 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 purpose. 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. Overall, our findings highlight the contribution of moderate paternal stress to an intergenerational adaptive response, informing future studies of adaptive fitness in rodents, humans and other species.

Keywords: paternal stress, epigenetic inheritance, social dominance, mate choice, major urinary protein, adaptive fitness

INTRODUCTION

Paternal environment preconception can affect offspring behaviour via intergenerational epigenetic inheritance (Chan et al., 2020; Crews et al., 2007; Gapp et al., 2014b; Hoffmann et al., 2020; Zhang et al., 2018). Various animal models have been developed to study this phenomenon, with effects described most commonly in the offspring of these animals, but also up to the third generation (van Steenwyk et al., 2018). Many of these studies have reported an influence on affective function and suggest that this type of inheritance could be a contributor to the 'missing heritability' problem associated with anxiety, depression and other psychiatric disorders (Yeshurun and Hannan, 2019). Additionally, some speculate about the evolutionary advantages that such heritability could confer (Badyaev, 2014; Richards, 2006).

Our previous work on the paternal corticosterone-treatment model of mild to moderate daily stress preconception had demonstrated the paternal influence on anxiety-like behaviours of male F_1

offspring (PatCort) and depressive-like behaviours of male F_2 grand-offspring (GPCort) (Short et al., 2016). Several independent preclinical studies have reported that small noncoding RNAs, especially miRNAs, are differentially expressed in the sperm of stressed fathers, and microinjection of sperm RNAs isolated from "stressed" sperm reproduces the offspring phenotypes observed through natural mating (Chen et al., 2016a; Gapp et al., 2014a; Rodgers et al., 2015). Affective disorders often associate with deficits in social behaviour (Chou et al., 2011), and social behaviour in mice is particularly important for reproduction and survival, thus determining adaptive fitness. Importantly, taking into consideration that the epigenetic inheritance can modulate endophenotypes that contribute to adaptiveness, this raises the possibility that this inheritance could regulate the inheritance rates of the acquired epigenetic marks, across different generations, with the consequence on adaptiveness of such generations and evolution of the species (Klironomos et al., 2013; Skinner, 2015).

Therefore, we sought to determine whether paternal corticosterone treatment prior to conception modulates social behaviours that are relevant to adaptive fitness. We hypothesised that male PatCort offspring would display lower levels of social reward and dominance, and be less attractive to females, consistent with their pro-anxiety behavioural phenotype. We tested 3 generations of mice in the social-conditioned place preference test to assess social reward, the social-dominance tube test to assess dominance hierarchies and the mate-choice test to assess male attractiveness. We also investigated gene expression in the offspring prefrontal cortex (PFC), a brain region implicated in such behaviours, through mRNA sequencing, and major urinary protein (MUP) pheromone levels in the urine. Here, we present the first evidence of altered adaptive fitness in offspring, attributed to paternal stress exposure.

MATERIALS AND 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. After the designated corticosterone treatment period, described below, 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 different parents 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 CT (GPCT) and grand-paternal Cort (GPCort); the procedure was the same as described above. 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:

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. 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. 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 (Figure 1B). The 'bedding only' set up consisted of presenting the soiled bedding from their home cage, to assess if pheromones alone determine attractiveness (Figure 1C), 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 (Figure 1D). Females were not tested because this test relies on behavioural responses linked to the development of male secondary sexual characteristics.

Social-dominance tube test: the protocol was adapted from Tada et al., 2016; Zhou et al., 2016. 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. 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

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 (Beynon et al., 2002). 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 (Beynon and Hurst, 2003), 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 as a normaliser.

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.

	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.

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) (Kim et al., 2019) using mm10 as the reference. Gene count matrix was generated with HTSeq-count (v0.9.1) (Anders et al., 2015) 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) (Chen et al., 2016b; Robinson et al., 2010). Differential analysis expression was done using edgeR, and the volcano plot was made with ggplot2 (v3.3.5) (Wickham, 2016). Gene Set Enrichment Analysis was performed using GSEA (v4.2.3) (Mootha et al., 2003; Subramanian et al., 2005), with the c2.cp.v7.5.1 gene set database.

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. 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.

RESULTS

Corticosterone-treatment alters male social dominance but not mate-choice attractiveness

4 weeks of corticosterone treatment significantly reduced the percentage of wins in the tube test (Figure 1A, χ^2=30.25, p<0.001).
No significant effect of Cort-treatment was observed in the mate-choice test. There were no differences between the groups in all 3 variations of the set up ('Standard' set up: Sum of Signed Ranks W=8.000, p=0.8603, n=16; 'Mouse only' set up: Sum of Signed Ranks W=32.00, p=0.4332, n=16; 'Bedding only' set up: Sum of Signed Ranks W=36.00, p=0.3755, n=16). The total percentage of time was comparatively similar between the groups (Figure 1E, 'Standard' set up: χ^2=0.2500, p=0.6171; Figure 1F, 'Mouse only' set up: χ^2= 1.000, p=0.3173; Figure 1G, 'Bedding only' set up: χ^2=0.2500, p=0.6171).

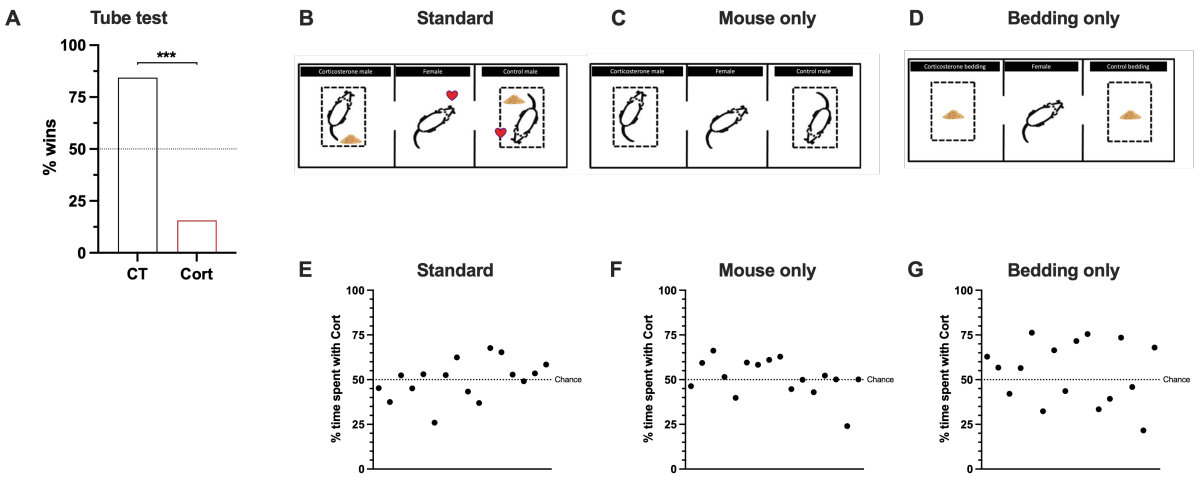


Figure 1. Assessing effects on social dominance and mate-choice attractiveness. (A), corticosterone treatment reduces male dominance as assessed by the percentage of wins in the tube test. % wins calculated as the number of wins per group in the total number of interactions. CT n=16, Cort n=16, total of 64 unique interactions. (B – D), figures adapted from Toth and Neumann, 2013. (E – G), male attractiveness was not affected by corticosterone-treatment. A, E – G: one-sample chi-squared test. *** p<0.001.

Paternal Cort-treatment affects selective aspects of adult male offspring social behaviour

Juvenile (PND 35) F_1 male and female offspring displayed clear preference for the social-conditioned bedding (Figure 2A, Males: $F_{(1,72)}$=12.76, p<0.001; Figure 2C, Females: $F_{(1,74)}$=8.227, p=0.0054). No effects of paternal treatment were found for males (Figure 2B, U=601, p=0.3811, n=39/35) nor for females (Figure 2D, U=640.5, p=0.4035, n=37/39).

Male PatCort F_1 offspring showed significantly decreased percentage of wins compared to the null hypothesis, indicative of a lower social dominance (Figure 2E, χ^2=15.75, p<0.001).

Male PatCort F_1 offspring were investigated by *naïve* females for a longer time than their PatCT counterparts (Sum of Signed Ranks W=241.0, p=0.0080, n=29), and showed significantly increased percentage of time being investigated (Figure 2F, 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, n=16; 'Bedding only': Sum of Signed Ranks W=-18.00, p=0.5186, n=12), and the total percentage of time was comparatively similar between the groups (Figure 2G, 'mouse only' set up, χ^2=1.000, p=0.3173; Figure 2H, 'bedding only' set up, χ^2=1.333, p=0.2482).

Additionally, analysis of the time each male interacted back with the female when she approached them revealed no differences (Figure 2I, $t_{(26)}$=0.8106, p=0.4249).

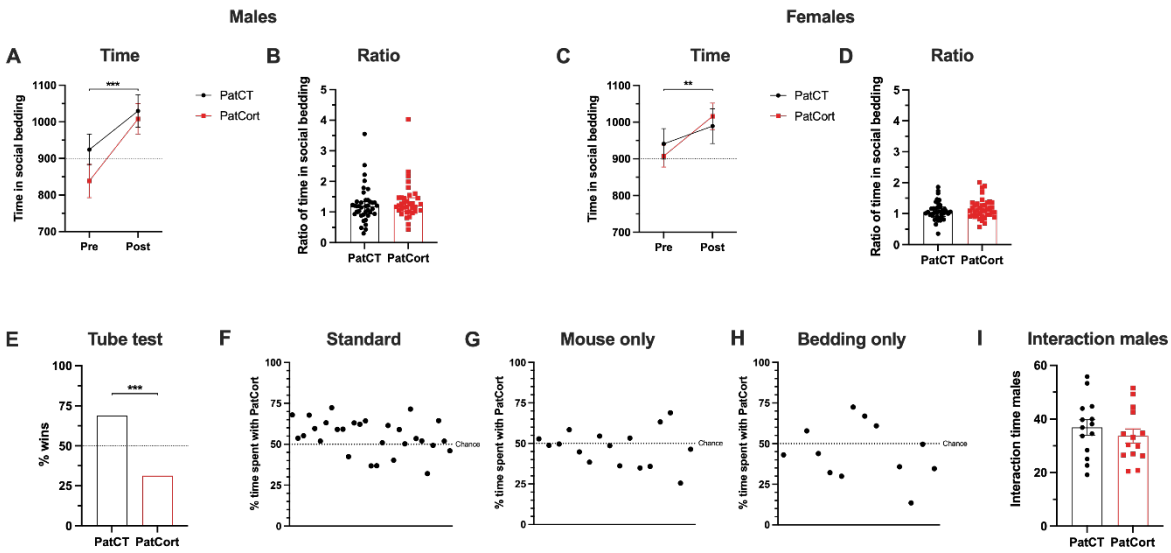


Figure 2. Assessing effects of paternal Cort-treatment on offspring social behaviour. (A – D), the degree of social reward in male or female offspring was not affected by paternal Cort-treatment. Time and ratio of the time spent in the social-conditioned bedding. (E), paternal Cort-treatment reduces male dominance in the tube test. % wins calculated as the number of wins per group in the total number of interactions. (F – H), paternal corticosterone treatment increases male attractiveness in the 'standard' set up only. (I), PatCort mice do not interact more with female mice, compared to PatCT. 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.

Paternal Cort-treatment does not exert a transgenerational influence on grand-offspring social behaviour

Juvenile F_2 male and female grand-offspring showed preference for the social-conditioned bedding (Figure 3A, Males: $F_{(1,76)}$=35.14, p<0.001; Figure 3C, Females: $F_{(1,80)}$=12.09, p<0.001). No differences in

the extension of social reward were found between the groups for males (Figure 3B, U=714.5, p=0.6527, n=40/38) nor for females (Figure 3D, U=755, p=0.4547, n=44/38).

In the social dominance tube test, male F_2 grand-offspring did not show differences in the total number of wins (Figure 3E, χ^2=2.667, p=0.1025).

No differences were observed between the groups for neither of the set ups (Sum of Signed Ranks W=-10.00, p=0.8209; n=16; 'Mouse only' set up: Sum of Signed Ranks W=40.00, p=0.3160; n=16; 'Bedding only' set up: Sum of Signed Ranks W=-40.00, p= 0.3225; n=16), and the total percentage of time was comparatively similar between the groups (Figure 3F, 'Standard' set up: χ^2= 1.000, p= 0.3173; Figure 3G, 'Mouse only' set up: χ^2= 1.000, p= 0.3173; Figure 3H, 'Bedding only' set up: χ^2= 0.2500, p= 0.6171).

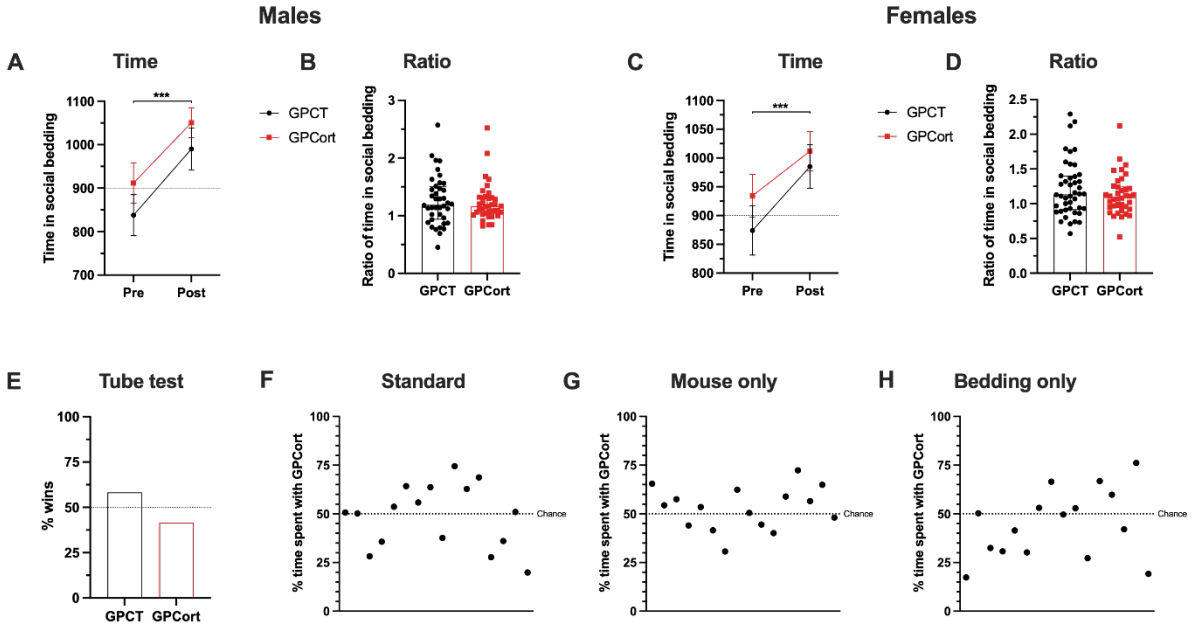


Figure 3. Assessing effects of grand-paternal Cort-treatment on grand-offspring social behaviour. (A – D), the degree of social reward in male or female grand-offspring was not affected by grand-paternal Cort-treatment. Time and ratio of the time spent in the social-conditioned bedding. (E), social dominance 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. (F – H), male attractiveness in male grand-offspring was not affected by grand-paternal Cort-treatment. 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.

Paternal Cort-treatment alters subpopulation of MUPs in F_1 offspring

Quantification of urinary creatinine revealed no difference between PatCT and PatCort male offspring (Figure 4A, $t_{(12.74)}$=1.704, p=0.1126, n=16/13). The relative levels of MUPs normalised to creatinine levels also did not differ between the groups (Figure 4B, $t_{(15.24)}$=1.792, p=0.0930, n=16/13).

Three different MUP bands were separated with SDS-PAGE (Figure 4C), in agreement with previous publications (Nelson et al., 2013; Roberts et al., 2010). Semi-quantification of the protein bands revealed that PatCort males have reduced concentrations of the small MUP band (Figure 4D,

$t_{(15.41)}$=2.252, p=0.0393, n=16/13) and of Darcin (Figure 4F, U=53, p=0.0243, n=16/13), with no difference in the big MUP band (Figure 4E, $t_{(14.09)}$=1.904, p=0.0775, n=16/13).

Darcin is proposed to be the main MUP population to act as a pheromone (Roberts et al., 2010). We found that urinary Darcin in male mice does not correlate with total time being investigated by the female in the mate-choice test (Figure 4G, PatCT: R^2=0.1112, p=0.2068; PatCort: R^2=0.0921, p=0.3134).

Since MUP proteins are produced and secreted by the liver (Norstedt and Palmiter, 1984), we investigated hepatic gene expression of various Mup genes and Zhx2 (a transcript factor for Mup genes) (Jiang et al., 2017). No differences were observed in mRNA levels between the groups, showing that Mup gene expression in the PatCort mice does not differ from the controls (Figure 4H, Mup3: $t_{(21.88)}$=1.666, p=0.1100, n=16 per group; Figure 4I, Mup20: U=107, p=0.4393, n=16 per group; Figure 4J, MupB: $t_{(30)}$=1.235, p=0.2264, n=16 per group; Figure 4K, Zhx2: $t_{(30)}$=0.5436, p=0.5907, n=16 per group).

Mup genes, however, showed a very strong positive correlation with MUP protein populations in the PatCort offspring. MupB, which corresponds to every Mup gene belonging to the B-class, correlates very strongly with total MUP urinary concentration in the urine of PatCort mice (Figure 4L, R^2=0.9165, p<0.001), whereas this correlation was not observed in the PatCT mice (Figure 4L, R^2= 0.0001251, p=0.9672). Mup20, in its turn, correlates very strongly with the Darcin band population of MUPs in the urine of PatCort mice (Figure 4M, R^2=0.6823, p=0.0005), while it does not correlate in the PatCT (Figure 4M, R^2=0.04098, p=0.4521). The Darcin band has been attributed to the Mup20 gene in previous publications (Lee et al., 2017; Roberts et al., 2010).

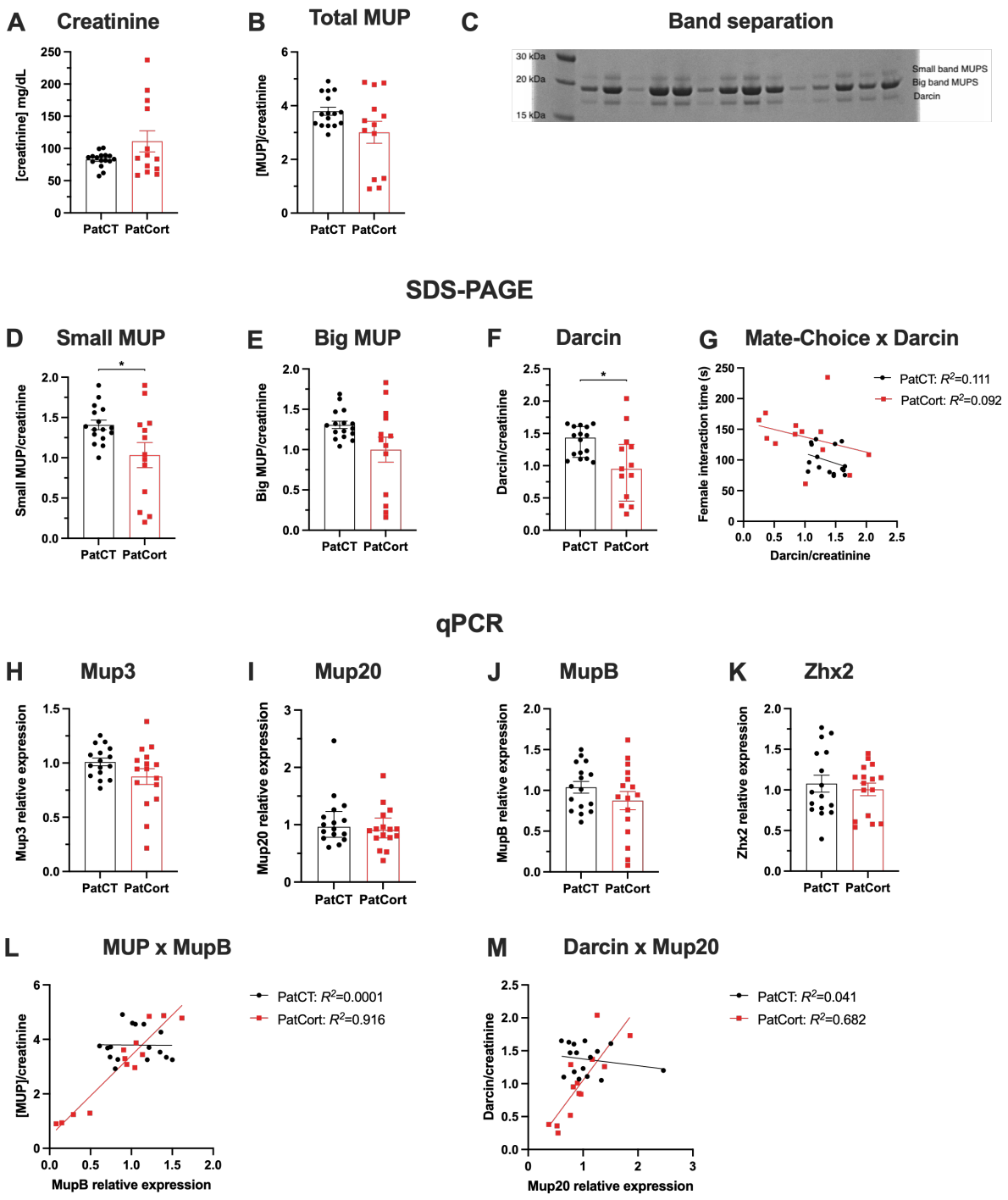


Figure 4. Assessing effects of paternal Cort-treatment on offspring urinary MUP levels and liver gene expression. (A – B), urinary creatinine or total MUP were not affected by paternal Cort-treatment. (C), after SDS-PAGE of mouse urine, three different MUP bands can be seen, whose molecular weight correspond 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. (G), time being investigated by the female does not correlate with urinary Darcin concentration. (H – K), Mup gene expression in the liver was not affected by paternal Cort-treatment. (L – M), Mup genes correlate with specific MUP populations in PatCort, but not in PatCT. A – B: t-test with Welch correction, data represented as mean ± SEM. D and E: unpaired t-test with Welch correction, data represented as mean ± SEM. F: Mann-Whitney test, data represented as median ± interquartile range. G: correlation of Pearson and simple linear regression. H: unpaired t-test with

Welch correction, data represented as mean ± SEM. I: Mann-Whitney test, data represented as median ± interquartile range. J and K: unpaired t-test, data represented as mean ± SEM. L – M: correlation of Pearson and simple linear regression. * $p<0.05$.

No evidence of transgenerational influences of paternal Cort-treatment on MUPs

Urinary creatinine and MUP protein concentration were assessed in F_2 male grand-offspring mice to investigate whether, following the results observed in the mate-choice test, no differences would be observed for the MUP levels. Urinary creatinine levels were not different between the groups (Figure 5A, U=101.5, p=0.8897, n=15/14), and MUP concentration was not different either (Figure 5B, $t_{(27)}$=0.9832, p=0.3343, n=15/14).

Additionally, we investigated the MUP band intensities, and no differences were found for neither of the bands Small MUP (Figure 5C, $t_{(27)}$=0.4547, p=0.6529, n=15/14), Big MUP (Figure 5D, $t_{(27)}$=0.7838, p=0.4400, n=15/14), or Darcin (Figure 5E, $t_{(27)}$=0.5951, p=0.5568, n=15/14).

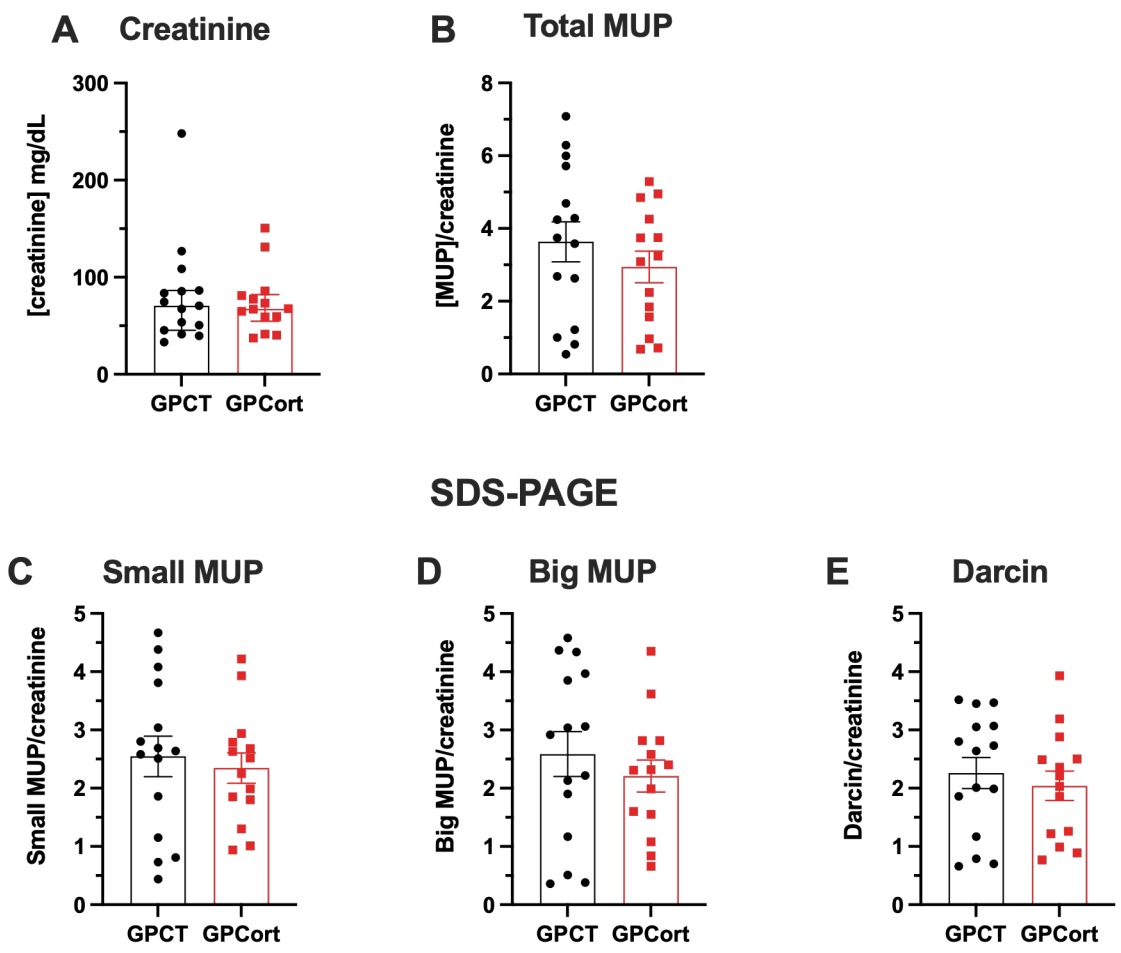
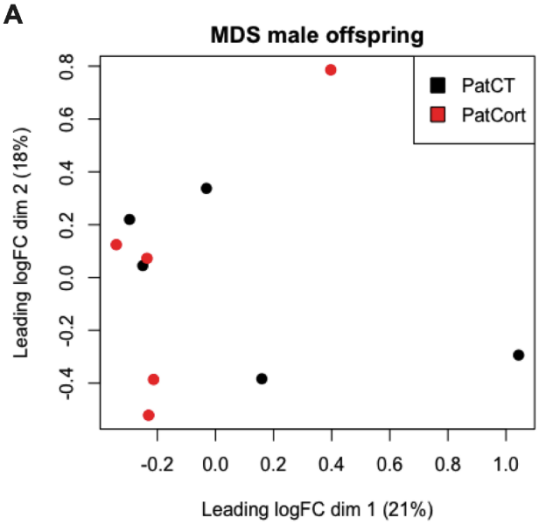


Figure 5. Assessing effects of grand-paternal Cort-treatment on grand-offspring urinary MUP levels. (A – B), urinary creatinine or total MUP were not affected by grand-paternal Cort-treatment. (C – E), specific MUP populations in the urine were not affected by grand-paternal Cort-treatment. A: Mann-Whitney test, data represented as median ± interquartile range. B: unpaired t-test, data represented as mean ± SEM. C – E: unpaired t-test, data represented as mean ± SEM.

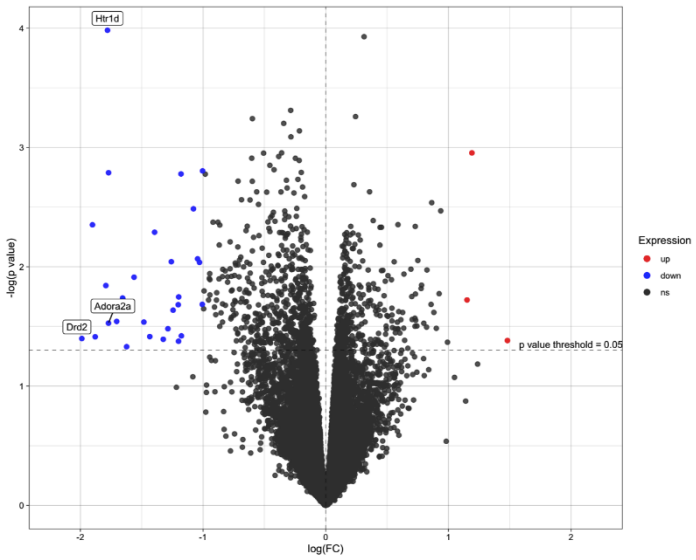
Adult male offspring prefrontal cortex gene expression is relatively unchanged by paternal Cort-treatment

We dissected prefrontal cortex tissue from adult male F_1 offspring and performed mRNA sequencing. This brain region was chosen because of its importance on signalling social dominance (Zhou et al., 2017) and anxiety-like behaviour (Calhoon and Tye, 2015). The MDS plot shows that the samples are overall very similar independently of the group (Figure 6A). Differential expression analysis with edgeR showed that 32 genes were found with $p < 0.05$ and logfold-change threshold of 1, being 3 upregulated and 29 downregulated in the PatCort male mice (Figure 6B). Some of the downregulated genes are of potential interest, such as Dopamine Receptor D2 (Drd2), Adenosine A2a Receptor (Adora2a) and 5-HT1D receptor (Htr1d). Gene Set Enrichment Analysis was performed on this dataset for an exploratory analysis and generation of new hypotheses. One gene set was found to be significantly downregulated in the PatCort male offspring at FDR < 25%, WP_PEPTIDE_GPCRS, which refers to G protein-coupled receptors (Figure 6C).

A


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

B


Htr1d
Adora2a
Drd2
p value threshold = 0.05
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Expression
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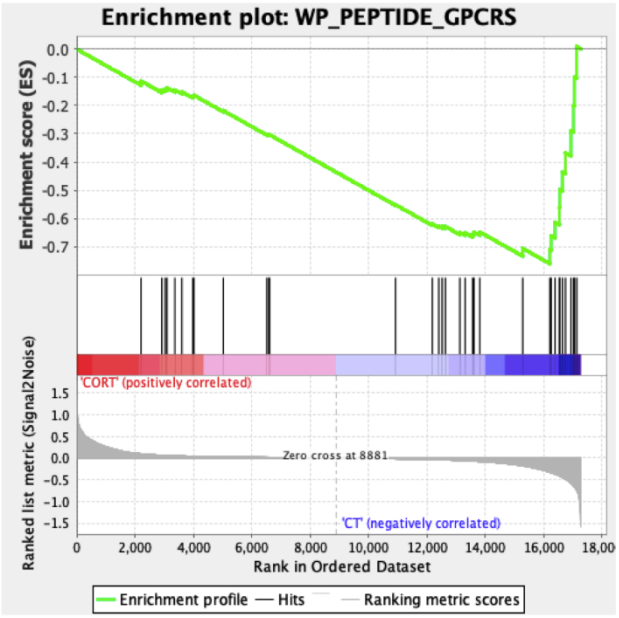
Enrichment plot: WP_PEPTIDE_GPCRS
Enrichment score (ES)
'CORT' (positively correlated)
Ranked list metric (Signal2Noise)
Zero cross at 8881
'CT' (negatively correlated)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores

Figure 6. 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. Red: genes upregulated in PatCort. Blue: genes downregulated in PatCort, compared to PatCT. (C), GSEA Enrichment plot of the statistically significant enrichment of the WP_PEPTIDE_GPCRS gene set.

DISCUSSION

This study has uncovered novel evidence of the paternal influence over offspring social behaviours. Paternal Cort-treatment was associated with reduced social dominance and increased attractiveness of their male F_1 offspring , in addition to elevated anxiety we previously reported (Short et al., 2016). Interestingly, these effects on social behaviours were restricted to the immediate generation (F_1 offspring), with no significant effects on the F_2 grand-offspring. Additionally, the male offspring also showed reduced MUP protein output in their urine, in particular the band that corresponds to the male pheromone Darcin. No differences were observed in Mup mRNA expression, despite the strong positive correlation with MUP protein levels. Additionally, the gene expression patterns in the PFC of adult PatCort F_1 offspring and control offspring were similar, as no differentially expressed genes remained after rigorous statistical correction for multiple comparisons.

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 great 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 (Sapolsky, 2005), as it is known that hierarchical rank modulates the HPA system reactivity (Yohn et al., 2019), 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 studied, but it has been reported that a cortico-hypothalamic circuit modulates social dominance (Padilla-Coreano et al., 2022) and maternal separation reduces adult dominance in a competition for water in water-restricted mice (Benner et al., 2014). Furthermore, chronic restraint decreases social dominance in the tube test (Park et al., 2018) and severe immobilisation stress heavily reduces social dominance in anxious mice (Lucion and Vogel, 1994). Another study reported the opposite effect of stress on dominance, with maternal separation resulting in increased social dominance in the tube test in rats (Tada et al., 2016). 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.

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 (Panksepp and Lahvis, 2007). Through the acquired social experiences resulting from this behaviour, the social reward would then influence the development of adult sociality (Yamamuro et al., 2020). Stress can modulate social reward, as it has been shown that foot-shock stress exposure reduces social reward expression (Daniels et al., 2021). However, such

differences 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.

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 (Allsop et al., 2014; Zhang et al., 2021), although other studies did not show association between anxiety-like behaviour and social rank in the tube test (Pallé et al., 2019). 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 or competition over mating opportunities, rendering them passive to the female mouse discretion. Therefore, the results in attractiveness could have been different if a competitive setup had been used.

Both MUPs and ultrasonic vocalisations (USVs) have been shown to attract females (Asaba et al., 2014; Egnor and Seagraves, 2016). When males are exposed to females, they emit USVs as part of a 'male song' (Yang et al., 2013). 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 (Beynon and Hurst, 2003). 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 (Chabout et al., 2012). MUPs and other olfactory-based signals, on the other hand, can travel long distances and have therefore been associated with long-distance communication (Asaba et al., 2014). 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.

F_2 male and female 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 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 (Yeshurun and Hannan, 2019).

F_1 mRNA sequencing does not show overt changes in gene expression

Following the reduced social dominance in the PatCort male offspring, we investigated 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 receptors in this region controls the expression of social dominance (Wang et al., 2011), 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 (Tada et al., 2016), and that social dominance is followed by differential gene expression in this region (Pallé et al., 2019). Lastly, neuronal population activity in the mPFC predicts social rank and success in competitive settings (Padilla-Coreano et al., 2022).

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.

Nonetheless, the investigation of the p values without FDR correction can still be valuable for an exploratory analysis to generate new hypotheses. We therefore conducted this exploratory analysis and found multiple genes that have lower expression in the adult PFC of PatCort offspring, (considering $p<0.05$ and a log-fold change threshold of 1). One of the differentially expressed candidates in the PFC of PatCort offspring is the dopamine receptor 2 (Drd2) gene. The dopamine receptor 2 gene (encoding the D2 receptor) has been shown to affect social dominance depending on the hierarchical rank (Yamaguchi et al., 2017), and D2-receptor-positive prefrontal cortex neurons signal submissiveness in mice (Xing et al., 2022). It is important to notice that oxytocin-induced long-term depression in D2-medium spiny neurons in the nucleus accumbens is necessary for the expression of social reward in adolescent mice (Dölen et al., 2013) and, although no changes in social reward were observed in the PatCort offspring, the investigation of dopamine receptor expression in these mice would be interesting. This is corroborated by the GSEA analysis, which indicates downregulation of the GPCR gene set in the PatCort offspring. Specific GPCR signalling disruption during critical periods of development (through early life stress, including *in utero*) has been proposed to lead to aberrant adult emotionality due to molecular, cellular and behavioural reprogramming (Tiwari et al., 2021). Therefore, future studies should focus on investigating GPCR signalling in the developing brain of the offspring of Cort-treated fathers.

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 concentration of total MUP proteins in their urine as analysed with a Bradford assay, and this effect is driven by reduced levels of specific MUP bands observed after separation in the 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 (Nelson et al., 2013; Roberts et al., 2010). 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' (Roberts et

al., 2010). 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 (Kumar et al., 2014; Roberts et al., 2010), 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 (Asaba et al., 2014). 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 (Armstrong et al., 2005), 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 (Thoß et al., 2019). Due to the reduced social dominance in the PatCort mice, it would be interesting to profile the volatile pheromone content in their urine.

F_1 male 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 MupB 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 (Stopka et al., 2007), pulsatile growth hormone (Norstedt and Palmiter, 1984), and circadian glucocorticoid (Cho et al., 2011). Social factors also modify the expression of MUPs, with social dominance proportionally affecting MUP production (Janotova and Stopka, 2011), possibly due to changes in testosterone levels (Stopka et al., 2007). The differential transcription of Mup gene paralogs results in the large diversity of MUP proteins observed in the urine (Sheehan et al., 2016), and one of their known transcription factors is Zhx2 (Jiang et al., 2017). There is no evidence of post-transcriptional processes regulating MUP protein concentration, as the mRNA expression predicts the urinary protein output (Sheehan et al., 2016), and MUPs are not reabsorbed in the kidney (Lehman-McKeeman and Caudill, 1992). Lastly, creatinine levels relate to the muscle mass and are a marker of glomerular filtration (Stopka et al., 2007), 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 (Janotova and Stopka, 2011). 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.

Male F_2 grand-offspring and no differences in total [MUP] or SDS-PAGE

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 offspring, particularly with respect to depression-like behaviour (Short et al., 2016), 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.

Final remarks

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 adaptive fitness in mice (Evans et al., 2020). More specifically, social hierarchy can affect survival due to differential access to resources (Strauss and Holekamp, 2019) and modulation of the HPA axis responsiveness (Sapolsky, 2012) and, together with male attractiveness, they can impact access to mating opportunities (Thonhauser et al., 2019; Zhou et al., 2018). 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, 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 (Thonhauser et al., 2013). These male offspring (whose fathers had elevated stress hormone levels) would also have a decline in their survival rates, with the overall effect of reducing their fitness.

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 (Tuscher and Day, 2019). However, the hypothesised decrease in fitness proposed above due to the reduced social dominance observed in the PatCort mice suggests that by modulating endophenotypes that determine fitness, epigenetic inheritance could heavily impact many generations, even though the changes in behaviour are observed in only one generation. This 'trans-populational impact' has been suggested in mice before (Crews et al., 2007), and has been shown to occur in *C. elegans* (Toker et al., 2022).

Another factor that can impact fitness 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 (Schmidt, 2011), 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 (De Miguel et al., 2019). 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 (Burton and Metcalfe, 2014; Nettle and Bateson, 2015).

Lastly, it has been suggested that male attractiveness could have evolved with the aid of epigenetic mechanisms and female mate choice (Fang et al., 2016). The evolution of the mouse Mup genes is recent (Charkoftaki et al., 2019), involving the presence of many pseudo-genes (Thoß et al., 2016), which indicates selective pressures shaping scent signals relevant for social communication (Sheehan et al., 2019). Interestingly, one of the first reports on epigenetic inheritance in mice showed differential methylation of Mup genes (Roemer et al., 1997), and it has been shown that different sociality levels can have transgenerational effects on MUP expression (Nelson et al., 2013). Therefore, it is possible that epigenetic inheritance could modulate the pattern of Mup expression and shape the evolution of such fast-evolving genes that have important impacts on mouse social recognition and attractiveness. Similar processes could happen in other species; for instance, epigenetic

reprogramming of reproductive function, driven by early-environment, has been shown in humans (Bar-Sadeh et al., 2020).

In conclusion, in this study we showed that epigenetic inheritance can modulate social behaviours that are important for determining fitness, thus potentially impacting many generations. The present findings, along with other studies investigating epigenetic inheritance, help shed light on the evolutionary advantage 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.

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. E.M. assisted in collecting the behavioural data for the offspring and grand-offspring. A.J.H. funded the study, contributed to planning experiments, provided critical feedback throughout the experiments, and reviewed the manuscript. T.Y.P. envisioned the study, funded the study, contributed to planning experiments, provided critical feedback throughout the experiments, and reviewed the manuscript.

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REFERENCES

Allsop, S. A., Vander Weele, C. M., Wichmann, R. and Tye, K. M. (2014). Optogenetic insights on the relationship between anxiety-related behaviors and social deficits. *Front. Behav. Neurosci.* **8**, 1–14.

Anders, S., Pyl, P. T. and Huber, W. (2015). HTSeq--a Python framework to work with high-throughput sequencing data. *Bioinformatics* **31**, 166–169.

Armstrong, S. D., Robertson, D. H. L., Cheetham, S. A., Hurst, J. L. and Beynon, R. J. (2005). Structural and functional differences in isoforms of mouse major urinary proteins: a male-specific protein that preferentially binds a male pheromone. *Biochem. J.* **391**, 343–350.

Asaba, A., Hattori, T., Mogi, K. and Kikusui, T. (2014). Sexual attractiveness of male chemicals and vocalizations in mice. *Front. Neurosci.* **8**,.

Badyaev, A. V. (2014). Epigenetic resolution of the 'curse of complexity' in adaptive evolution of complex traits. *J. Physiol.* **592**, 2251–2260.

Bar-Sadeh, B., Rudnizky, S., Pnueli, L., Bentley, G. R., Stöger, R., Kaplan, A. and Melamed, P. (2020). Unravelling the role of epigenetics in reproductive adaptations to early-life environment. *Nat. Rev. Endocrinol.* **16**, 519–533.

Benner, S., Endo, T., Endo, N., Kakeyama, M. and Tohyama, C. (2014). Early deprivation induces competitive subordinance in C57BL/6 male mice. *Physiol. Behav.* **137**, 42–52.

Beynon, R. J. and Hurst, J. L. (2003). Multiple roles of major urinary proteins in the house mouse, Mus domesticus. *Biochem. Soc. Trans.* **31**, 142–146.

Beynon, R. J., Veggerby, C., Payne, C. E., Robertson, D. H. L., Gaskell, S. J., Humphries, R. E. and Hurst, J. L. (2002). Polymorphism in major urinary proteins: molecular heterogeneity in a wild mouse population. *J. Chem. Ecol.* **28**, 1429–1446.

Burton, T. and Metcalfe, N. B. (2014). Can environmental conditions experienced in early life influence future generations? *Proc. R. Soc. B Biol. Sci.* **281**, 20140311.

Calhoon, G. G. and Tye, K. M. (2015). Resolving the neural circuits of anxiety. *Nat. Neurosci.* **18**, 1394–1404.

Chabout, J., Serreau, P., Ey, E., Bellier, L., Aubin, T., Bourgeron, T. and Granon, S. (2012). Adult Male Mice Emit Context-Specific Ultrasonic Vocalizations That Are Modulated by Prior Isolation or Group Rearing Environment. *PLoS One* **7**, e29401.

Chan, J. C., Morgan, C. P., Adrian Leu, N., Shetty, A., Cisse, Y. M., Nugent, B. M., Morrison, K. E., Jašarević, E., Huang, W., Kanyuch, N., et al. (2020). Reproductive tract extracellular vesicles are sufficient to transmit intergenerational stress and program neurodevelopment. *Nat. Commun.* **11**, 1499.

Charkoftaki, G., Wang, Y., McAndrews, M., Bruford, E. A., Thompson, D. C., Vasiliou, V. and Nebert, D. W. (2019). Update on the human and mouse lipocalin (LCN) gene family, including evidence the mouse Mup cluster is result of an "evolutionary bloom." *Hum. Genomics* **13**, 11.

Chen, Q., Yan, W. and Duan, E. (2016a). Epigenetic inheritance of acquired. *Nat. Publ. Gr.*

Chen, Y., Lun, A. T. L. and Smyth, G. K. (2016b). From reads to genes to pathways: differential expression analysis of RNA-Seq experiments using Rsubread and the edgeR quasi-likelihood pipeline. *F1000Research* **5**, 1438.

Cho, Y.-H., Kim, D., Choi, I. and Bae, K. (2011). Identification of transcriptional regulatory elements required for the Mup2 expression in circadian clock mutant mice. *Biochem. Biophys. Res. Commun.* **410**, 834–840.

Chou, K.-L., Liang, K. and Sareen, J. (2011). The Association Between Social Isolation and DSM-IV Mood, Anxiety, and Substance Use Disorders. *J. Clin. Psychiatry* **72**, 1468–1476.

Crews, D., Gore, A. C., Hsu, T. S., Dangleben, N. L., Spinetta, M., Schallert, T., Anway, M. D. and Skinner, M. K. (2007). Transgenerational epigenetic imprints on mate preference. *Proc. Natl. Acad. Sci.* **104**, 5942–5946.

Daniels, S., Lemaire, D., Lapointe, T., Limebeer, C., Parker, L. and Leri, F. (2021). Effects of inescapable stress on responses to social incentive stimuli and modulation by escitalopram. *Psychopharmacology (Berl).* **238**, 3239–3247.

De Miguel, Z., Haditsch, U., Palmer, T. D., Azpiroz, A. and Sapolsky, R. M. (2019). Adult-generated neurons born during chronic social stress are uniquely adapted to respond to subsequent chronic social stress. *Mol. Psychiatry* **24**, 1178–1188.

Dölen, G., Darvishzadeh, A., Huang, K. W. and Malenka, R. C. (2013). Social reward requires coordinated activity of nucleus accumbens oxytocin and serotonin. *Nature* **501**, 179–184.

Egnor, S. R. and Seagraves, K. M. (2016). The contribution of ultrasonic vocalizations to mouse courtship. *Curr. Opin. Neurobiol.* **38**, 1–5.

Evans, J. C., Liechti, J. I., Boatman, B. and König, B. (2020). A natural catastrophic turnover event: individual sociality matters despite community resilience in wild house mice. *Proc. R. Soc. B Biol. Sci.* **287**, 20192880.

Fang, Q., Zhang, Y. H., Shi, Y. L., Zhang, J. H. J. X. and Zhang, J. H. J. X. (2016). Individuality and Transgenerational Inheritance of Social Dominance and Sex Pheromones in Isogenic Male Mice.

J. Exp. Zool. Part B Mol. Dev. Evol. **326**, 225–236.

Gapp, K., Jawaid, A., Sarkies, P., Bohacek, J., Pelczar, P., Prados, J., Farinelli, L., Miska, E. and Mansuy, I. M. (2014a). Implication of sperm RNAs in transgenerational inheritance of the effects of early trauma in mice. *Nat. Neurosci.* **17**, 667–669.

Gapp, K., Soldado-Magraner, S., Alvarez-Sánchez, M., Bohacek, J., Vernaz, G., Shu, H., Franklin, T. B., Wolfer, D. and Mansuy, I. M. (2014b). Early life stress in fathers improves behavioural flexibility in their offspring. *Nat. Commun.* **5**, 5466.

Hoffmann, L. B., Rae, M., Marianno, P., Pang, T. Y., Hannan, A. J. and Camarini, R. (2020). Preconceptual paternal environmental stimulation alters behavioural phenotypes and adaptive responses intergenerationally in Swiss mice. *Physiol. Behav.* **223**, 112968.

Janotova, K. and Stopka, P. (2011). The Level of Major Urinary Proteins is Socially Regulated in Wild Mus musculus musculus. *J. Chem. Ecol.* **37**, 647–656.

Jiang, J., Creasy, K. T., Purnell, J., Peterson, M. L. and Spear, B. T. (2017). Zhx2 (zinc fingers and homeoboxes 2) regulates major urinary protein gene expression in the mouse liver. *J. Biol. Chem.* **292**, 6765–6774.

Kim, D., Paggi, J. M., Park, C., Bennett, C. and Salzberg, S. L. (2019). Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nat. Biotechnol.* **37**, 907–915.

Klironomos, F. D., Berg, J. and Collins, S. (2013). How epigenetic mutations can affect genetic evolution: Model and mechanism. *BioEssays* **35**, 571–578.

Kumar, V., Vasudevan, A., Soh, L. J. T., Le Min, C., Vyas, A., Zewail-Foote, M. and Guarraci, F. A. (2014). Sexual Attractiveness in Male Rats Is Associated with Greater Concentration of Major Urinary Proteins. *Biol. Reprod.* **91**, 1–7.

Lee, W., Khan, A. and Curley, J. P. (2017). Major urinary protein levels are associated with social status and context in mouse social hierarchies. *Proc. R. Soc. B Biol. Sci.* **284**, 20171570.

Lehman-McKeeman, L. D. and Caudill, D. (1992). Biochemical basis for mouse resistance to hyaline droplet nephropathy: Lack of relevance of the α2u-globulin protein superfamily in this male rat-specific syndrome. *Toxicol. Appl. Pharmacol.* **112**, 214–221.

Lucion, A. and Vogel, W. H. (1994). Effects of stress on defensive aggression and dominance in a water competition test. *Integr. Physiol. Behav. Sci.* **29**, 415–422.

Mitra, R. and Sapolsky, R. M. (2012). Short-Term Enrichment Makes Male Rats More Attractive, More Defensive and Alters Hypothalamic Neurons. *PLoS One* **7**, e36092.

Mootha, V. K., Lindgren, C. M., Eriksson, K.-F., Subramanian, A., Sihag, S., Lehar, J., Puigserver, P., Carlsson, E., Ridderstråle, M., Laurila, E., et al. (2003). PGC-1α-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat. Genet.* **34**, 267–273.

Nardou, R., Lewis, E. M., Rothhaas, R., Xu, R., Yang, A., Boyden, E. and Dölen, G. (2019). Oxytocin-dependent reopening of a social reward learning critical period with MDMA. *Nature* **569**, 116–120.

Nelson, A. C., Cauceglia, J. W., Merkley, S. D., Youngson, N. A., Oler, A. J., Nelson, R. J., Cairns, B. R., Whitelaw, E. and Potts, W. K. (2013). Reintroducing domesticated wild mice to sociality induces adaptive transgenerational effects on MUP expression. *Proc. Natl. Acad. Sci.* **110**, 19848–19853.

Nelson, A. C., Cunningham, C. B., Ruff, J. S. and Potts, W. K. (2015). Protein pheromone expression levels predict and respond to the formation of social dominance networks. *J. Evol. Biol.* **28**, 1213–1224.

Nettle, D. and Bateson, M. (2015). Adaptive developmental plasticity: what is it, how can we recognize it and when can it evolve? *Proc. R. Soc. B Biol. Sci.* **282**, 20151005.

Norstedt, G. and Palmiter, R. (1984). Secretory rhythm of growth hormone regulates sexual differentiation of mouse liver. *Cell* **36**, 805–812.

Padilla-Coreano, N., Batra, K., Patarino, M., Chen, Z., Rock, R. R., Zhang, R., Hausmann, S. B., Weddington, J. C., Patel, R., Zhang, Y. E., et al. (2022). Cortical ensembles orchestrate social competition through hypothalamic outputs. *Nature* **603**, 667–671.

Pallé, A., Zorzo, C., Luskey, V. E., Mcgreevy, K. R., Fernández, S. and Trejo, J. L. (2019). Social dominance differentially alters gene expression in the medial prefrontal cortex without affecting adult hippocampal neurogenesis or stress and anxiety-like behavior. *FASEB J.* **33**, 6995–7008.

Panksepp, J. B. and Lahvis, G. P. (2007). Social reward among juvenile mice. *Genes, Brain Behav.* **6**, 661–671.

Park, M.-J., Seo, B. A., Lee, B., Shin, H.-S. and Kang, M.-G. (2018). Stress-induced changes in social dominance are scaled by AMPA-type glutamate receptor phosphorylation in the medial prefrontal cortex. *Sci. Rep.* **8**, 15008.

Richards, E. J. (2006). Inherited epigenetic variation - Revisiting soft inheritance. *Nat. Rev. Genet.* **7**, 395–401.

Roberts, S. A., Simpson, D. M., Armstrong, S. D., Davidson, A. J., Robertson, D. H., McLean, L., Beynon, R. J. and Hurst, J. L. (2010). Darcin: A male pheromone that stimulates female memory and sexual attraction to an individual male's odour. *BMC Biol.* **8**,.

Robinson, M. D., McCarthy, D. J. and Smyth, G. K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**, 139–140.

Rodgers, A. B., Morgan, C. P., Leu, N. A. and Bale, T. L. (2015). Transgenerational epigenetic programming via sperm microRNA recapitulates effects of paternal stress. *Proc. Natl. Acad. Sci.* **112**, 13699–13704.

Roemer, I., Reik, W., Dean, W. and Klose, J. (1997). Epigenetic inheritance in the mouse. *Curr. Biol.* **7**, 277–280.

Sapolsky, R. M. (2005). The Influence of Social Hierarchy on Primate Health. *Science (80-.).* **308**, 648–652.

Sapolsky, R. M. (2012). Importance of a sense of control and the physiological benefits of leadership. *Proc. Natl. Acad. Sci.* **109**, 17730–17731.

Schmidt, M. V. (2011). Animal models for depression and the mismatch hypothesis of disease. *Psychoneuroendocrinology* **36**, 330–338.

Sheehan, M. J., Lee, V., Corbett-Detig, R., Bi, K., Beynon, R. J., Hurst, J. L. and Nachman, M. W. (2016). Selection on Coding and Regulatory Variation Maintains Individuality in Major Urinary Protein Scent Marks in Wild Mice. *PLOS Genet.* **12**, e1005891.

Sheehan, M. J., Campbell, P. and Miller, C. H. (2019). Evolutionary patterns of major urinary protein scent signals in house mice and relatives. *Mol. Ecol.* **28**, 3587–3601.

Short, A. K., Fennell, K. A., Perreau, V. M., Fox, A., O'bryan, M. K., Kim, J. H., Bredy, T. W., Pang, T. Y., Hannan, A. J., O'Bryan, M. K., et al. (2016). Elevated paternal glucocorticoid exposure alters the small noncoding RNA profile in sperm and modifies anxiety and depressive phenotypes in the offspring. *Transl. Psychiatry* **6**, e837–e837.

Skinner, M. K. (2015). Environmental epigenetics and a unified theory of the molecular aspects of evolution: A neo-Lamarckian concept that facilitates neo-Darwinian evolution. *Genome Biol. Evol.* **7**, 1296–1302.

Stopka, P., Janotova, K. and Heyrovsky, D. (2007). The advertisement role of major urinary proteins in mice. *Physiol. Behav.* **91**, 667–670.

Strauss, E. D. and Holekamp, K. E. (2019). Social alliances improve rank and fitness in convention-based societies. *Proc. Natl. Acad. Sci.* **116**, 8919–8924.

Subramanian, A., Tamayo, P., Mootha, V. K., Mukherjee, S., Ebert, B. L., Gillette, M. A., Paulovich, A., Pomeroy, S. L., Golub, T. R., Lander, E. S., et al. (2005). Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci.* **102**, 15545–15550.

Tada, H., Miyazaki, T., Takemoto, K., Takase, K., Jitsuki, S., Nakajima, W., Koide, M., Yamamoto, N., Komiya, K., Suyama, K., et al. (2016). Neonatal isolation augments social dominance by altering actin dynamics in the medial prefrontal cortex. *Proc. Natl. Acad. Sci.* **113**, E7097–E7105.

Thonhauser, K. E., Raveh, S., Hettyey, A., Beissmann, H. and Penn, D. J. (2013). Scent marking increases male reproductive success in wild house mice. *Anim. Behav.* **86**, 1013–1021.

Thonhauser, K. E., Raffetzeder, A. and Penn, D. J. (2019). Sexual experience has no effect on male mating or reproductive success in house mice. *Sci. Rep.* **9**, 1–11.

Thoß, M., Enk, V., Yu, H., Miller, I., Luzynski, K. C., Balint, B., Smith, S., Razzazi-Fazeli, E. and Penn, D. J. (2016). Diversity of major urinary proteins (MUPs) in wild house mice. *Sci. Rep.* **6**, 38378.

Thoß, M., Luzynski, K. C., Enk, V. M., Razzazi-Fazeli, E., Kwak, J., Ortner, I. and Penn, D. J. (2019). Regulation of volatile and non-volatile pheromone attractants depends upon male social status. *Sci. Rep.* **9**, 489.

Tiwari, P., Fanibunda, S. E., Kapri, D., Vasaya, S., Pati, S. and Vaidya, V. A. (2021). GPCR signaling: role in mediating the effects of early adversity in psychiatric disorders. *FEBS J.* **288**, 2602–2621.

Toker, I. A., Lev, I., Mor, Y., Gurevich, Y., Fisher, D., Houri-Zeevi, L., Antonova, O., Doron, H., Anava, S., Gingold, H., et al. (2022). Transgenerational inheritance of sexual attractiveness via small RNAs enhances evolvability in C. elegans. *Dev. Cell* **57**, 298-309.e9.

Toth, I. and Neumann, I. D. (2013). Animal models of social avoidance and social fear. *Cell Tissue Res.* **354**, 107–118.

Tuscher, J. J. and Day, J. J. (2019). Multigenerational epigenetic inheritance: One step forward, two generations back. *Neurobiol. Dis.* **132**, 104591.

van Steenwyk, G., Roszkowski, M., Manuella, F., Franklin, T. B. and Mansuy, I. M. (2018). Transgenerational inheritance of behavioral and metabolic effects of paternal exposure to traumatic stress in early postnatal life: evidence in the 4th generation. *Environ. Epigenetics* **4**, 1–8.

Wang, F., Zhu, J., Zhu, H., Zhang, Q., Lin, Z. and Hu, H. (2011). Bidirectional Control of Social Hierarchy by Synaptic Efficacy in Medial Prefrontal Cortex. *Science (80-.).* **334**, 693–697.

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York.

Xing, B., Mack, N. R., Zhang, Y.-X., McEachern, E. P. and Gao, W.-J. (2022). Distinct Roles for Prefrontal Dopamine D1 and D2 Neurons in Social Hierarchy. *J. Neurosci.* **42**, 313–324.

Yamaguchi, Y., Lee, Y.-A., Kato, A., Jas, E. and Goto, Y. (2017). The Roles of Dopamine D2 Receptor in the Social Hierarchy of Rodents and Primates. *Sci. Rep.* **7**, 43348.

Yamamuro, K., Bicks, L. K., Leventhal, M. B., Kato, D., Im, S., Flanigan, M. E., Garkun, Y., Norman, K. J., Caro, K., Sadahiro, M., et al. (2020). A prefrontal–paraventricular thalamus circuit requires juvenile social experience to regulate adult sociability in mice. *Nat. Neurosci.* **23**, 1240–1252.

Yang, M., Loureiro, D., Kalikhman, D. and Crawley, J. N. (2013). Male mice emit distinct ultrasonic vocalizations when the female leaves the social interaction arena. *Front. Behav. Neurosci.* **7**, 1–13.

Yeshurun, S. and Hannan, A. J. (2019). Transgenerational epigenetic influences of paternal environmental exposures on brain function and predisposition to psychiatric disorders. *Mol. Psychiatry* **24**, 536–548.

Yohn, C. N., Ashamalla, S. A., Bokka, L., Gergues, M. M., Garino, A. and Samuels, B. A. (2019). Social instability is an effective chronic stress paradigm for both male and female mice. *Neuropharmacology* **160**, 107780.

Zhang, Y., Zhang, X., Shi, J., Tuorto, F., Li, X., Liu, Y., Liebers, R., Zhang, L., Qu, Y., Qian, J., et al. (2018). Dnmt2 mediates intergenerational transmission of paternally acquired metabolic disorders through sperm small non-coding RNAs. *Nat. Cell Biol.* **20**, 535–540.

Zhang, G.-W., Shen, L., Tao, C., Jung, A.-H., Peng, B., Li, Z., Zhang, L. I. and Tao, H. W. (2021). Medial preoptic area antagonistically mediates stress-induced anxiety and parental behavior. *Nat. Neurosci.* **24**, 516–528.

Zhou, Y. Y., Kaiser, T., Monteiro, P., Zhang, X., Van der Goes, M. S., Wang, D., Barak, B., Zeng, M., Li, C., Lu, C., et al. (2016). Mice with Shank3 Mutations Associated with ASD and Schizophrenia Display Both Shared and Distinct Defects. *Neuron* **89**, 147–162.

Zhou, T., Zhu, H., Fan, Z., Wang, F., Chen, Y., Liang, H., Yang, Z., Zhang, L., Lin, L., Zhan, Y., et al. (2017). History of winning remodels thalamo-PFC circuit to reinforce social dominance. *Science (80-.).* **357**, 162–168.

Zhou, T., Sandi, C. and Hu, H. (2018). Advances in understanding neural mechanisms of social dominance. *Curr. Opin. Neurobiol.* **49**, 99–107.