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Haldane's rule in the developing brain: X-linked and imprinted genes contribute to male-biassed transgressive expression in hybrid mice

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The placenta and developing brains of eutherian mammals are intimately connected and enriched for imprinted gene (IG) expression. In hybrids, the placenta is a hotspot for genetic and epigenetic incompatibilities that preferentially affect males. Whether the hybrid brain is similarly affected is an open question. We evaluated the effects of hybridization and placental dysregulation on neural gene expression (embryonic, juvenile, adult) and behaviour (juvenile, adult) in a cross between the house mouse (*Mus musculus domesticus*) and Algerian mouse (*Mus spretus*), in which transgressive expression of imprinted and X-linked genes is a prominent feature of undersized F_1 male placentas. In hybrid hypothalamus, transgressive expression was mainly embryonic and highly male-biased. Although fewer genes were dysregulated in hybrid male embryonic hypothalamus relative to placenta, we infer a similar genetic architecture involving X–IG incompatibilities and incompatibilities between IGs and autosomal interaction partners. Hybrids had heightened anxiety-like behaviour and reduced activity relative to parental species, with a tendency towards larger behavioural differences in adult males. As the first study to our knowledge to characterize the effects of interspecific hybridization on neural gene expression and behaviour in a mammal, this work extends the scope of phenotypes impacted by intrinsic incompatibilities and demonstrates that the brain obeys Haldane's rule.

1. Introduction

A complex placenta and a highly developed forebrain are defining features of eutherian mammals. Beyond its essential functions in mediating maternal–fetal interactions and provisioning embryonic growth and development, the placenta is thought to directly promote and protect brain development [1–3]. Evidence for brain-specific placental effects includes placental synthesis of serotonin, essential to early forebrain development, production of an enzyme that buffers the developing brain from maternal glucocorticoid damage, and robust associations between placental dysfunction and neurodevelopmental disorders [2–6].

The functional connection between the placenta and developing brain is further supported by the enriched expression of imprinted genes (IGs) in both tissues [7]. This small (approx. 150 in mouse) but developmentally critical group of autosomal genes is characterized by parent-of-origin allelic expression, such that some IGs are expressed predominantly or exclusively from the maternally inherited allele (maternally expressed genes; MEGs) and

others from the paternally inherited allele (paternally expressed genes; PEGs). Therefore, similar to the X chromosome in males, IGs are hemizygous.

According to Moore & Haig [8], the evolutionary origin of imprinted expression is rooted in parental conflict over maternal investment which plays out in embryonic tissues during development [8,9]. Pregnancy and maternal care necessitate significant investment on the part of the mother, which is offset by costs to her own survival and her ability to invest in future offspring. For the father, there is no such trade-off, resulting in a conflict of interest. This is proposed to lead to intense antagonistic co-evolution on an intragenomic, allelic level: PEGs that promote embryonic growth and maternal resource transfer via the placenta and MEGs that restrict growth and nutrient transfer. In the brain, IGs are key regulators of multiple physiological, cognitive and emotional processes, including metabolism, circadian rhythms, learning and memory, and anxiety [10]. As in the placenta, the functions of IGs important to postnatal growth and maturation are suggestive of parental conflict, with PEGs promoting food intake, growth, reproductive maturation and maternal behaviours, and MEGs limiting food intake and growth and increasing metabolic rate [10,11].

Mammalian hybrids often exhibit striking asymmetries in their growth that depend on the direction of the cross (parent-of-origin effects) and hybrid sex. When present, these growth asymmetries are particularly pronounced in the placenta [12–15]. For example, in the cross between the Algerian mouse (*Mus spretus*) and house mouse (*Mus musculus domesticus*), hybrid placentas are over-sized when the father is a house mouse and undersized when an Algerian mouse is the father [12]. In both cases, hybrid growth abnormalities are more extreme in males than in females. Owing to their mode of expression and importance to embryonic growth and development, IGs are strong candidates for explaining hybrid growth asymmetries in reciprocal crosses. IGs are not, however, strong *a priori* candidates for sex-specific effects. Instead, the larger negative effects on F_1-hybrid males are consistent with Haldane's rule—the taxonomically widespread prevalence of hybrid sterility or inviability in the heterogametic (XY or ZW) sex [16–18]. The contribution of the X (or Z) chromosome to these sex-specific hybrid deficits is disproportionately large relative to the autosomes [17,19,20]. Indeed, quantitative trait locus (QTL) mapping in the Algerian–house mouse cross identified a significant association between a large interval on the X chromosome and variation in hybrid placental dysplasia [12,21]. Similarly, parent-of-origin-dependent hybrid growth abnormalities are associated with X-linked incompatibilities in crosses between deer mouse (*Peromyscus*) and dwarf hamster (*Phodopus*) species [22–24]. Notably, negative interactions between X-linked and autosomal imprinted loci are implicated in both systems, with male-biased hybrid mortality and extreme overgrowth in deer mice, and more subtle male-biased growth effects in dwarf hamsters [22–24].

These efforts to uncover the genetic basis and gene regulatory underpinnings of growth abnormalities in rodent hybrids have logically focused on the placenta, the tissue most consistently affected and most enriched for IG-regulated functions. In contrast, we know very little about how hybrid incompatibilities affect the developing brain and, ultimately, hybrid behaviour. We recently explored the molecular basis of undersized placentas in *M. m. domesticus*/*M. spretus* hybrids [25]. We found that transgressive hypermethylation in distal mouse chromosome 7 imprinting cluster leads to misexpression of several IGs in the cluster, including *Ascl2* and *Phlda2*, which are key regulators of placental development in mice [26,27]. Importantly, we found that hypermethylation and IG misexpression are more severe in males, in accordance with Haldane's rule [25]. With this detailed knowledge of the effect of hybridization on placental gene expression and phenotype, we now leverage the *M. m. domesticus*/*M. spretus* cross to explore the effects of hybridization on neural gene expression and behaviour, and the effects of placental dysregulation on the embryonic brain.

Neural patterns of IG expression are specific to both developmental timepoint and brain region, with particular enrichment in the developing cortex and hypothalamus [7]. Here, we measure hypothalamic gene expression in *M. m. domesticus*/*M. spretus* hybrids across three developmental timepoints (embryo, juvenile, adult), and assay activity and anxiety-like behaviours in juveniles and adults. Given evidence for brain-specific effects of placental dysfunction (e.g. [3]), we expected to find an association between transgressive expression in hybrid placenta and embryonic hypothalamus. Based on the enrichment of IG expression in the developing relative to adult brain, we also expected to find the highest levels of transgressive expression in the embryonic hypothalamus. Given that both placental dysfunction and IG misexpression can have profound effects on cognition and behaviour [3,6], we expected that hybrid behaviours would be altered relative to both parental species. Finally, we expected that altered gene expression and behaviour in hybrids would be more extreme in males, in line with prior work on placental abnormalities in the *M. m. domesticus*/*M. spretus* cross [12,25], and the widespread pattern described by Haldane's rule.

2. Methods

(a) Animals and tissue collection

Mice used in this study were maintained on a 12 : 12 light : dark cycle with lights on at 09.00 and were provided with 5001 Rodent Diet (LabDiet, Brentwood, MO, USA) and water ad libitum. *M. m. domesticus* (hereafter, *Dom*) was represented by the wild-derived inbred strain WSB/EiJ (Jackson Laboratory) and *M. spretus* (hereafter, *Spret*) was represented by the wild-derived inbred strain SFM/Pas (Montpellier Wild Mice Genetic Repository). We conducted three crosses, *Dom* × *Dom*, *Dom* × *Spret* and *Spret* × *Spret* (female listed first). The reciprocal cross (*Spret* × *Dom*) was attempted 25 times but was never successful. For embryonic tissue collections, we used timed mating to establish embryonic age ±24 h as described previously [28]. Pregnant females (n = 5 per cross) were euthanized by cervical dislocation between 10.00 and 11.00 on embryonic days 17 or 18 (e17.5). Tissues (maternal brain, embryo heads, placentas) were collected into RNAlater (Thermo Fisher, USA), kept at 4°C overnight to allow RNAlater perfusion, and stored at −20°C until processing. Embryo sex was determined by polymerase chain reaction (PCR) for the Y-linked gene *Zfy1*, and the hypothalamus was dissected from one female and one male embryo per litter per

genotype with the exception of one all-male hybrid litter (n = 5 males per genotype, n = 5 *Dom* females, n = 5 *Spret* females, n = 4 hybrid females; see [25] for litter sizes, sex ratio and viability). Brains from juvenile and adult animals were collected into RNAlater at day 21 (average 21.3, s.d. 0.73, n = 5 per sex per genotype), and day 71 (average 70.9, s.d. 2.09, n = 5 per sex per genotype), respectively. The hypothalamus was dissected and stored at −20°C in RNAlater until processing. Adult animals were sexually naive and singly housed for at least 24 h prior to euthanasia.

(b) Sample preparation and sequencing

Total RNA was extracted from hypothalamus using the AllPrep RNA/DNA Mini Kit (Qiagen), and was stored at −80°C until sequencing. Illumina library preparation was done at the sequencing facility (Novogene, Sacramento, CA) using the NEB Next Ultra RNA library prep kit for Illumina. Individual libraries were sequenced on the Illumina HiSeq 4000 platform, producing >40 million, 150 bp paired-end reads per sample.

(c) Transcriptome data processing and analysis

Quality control, trimming of raw sequencing reads, filtering and mapping were performed as described in Arévalo *et al*. [25]. Please see electronic supplementary material, methods, for details. The list of imprinted genes was assembled using the *geneimprint* database, excluding genes with a 'predicted' imprinted status (https://www.geneimprint.com/site/genes-by-species.Mus+musculus), with additional information from Andergassen *et al*. [29].

(d) Gene set analyses

We performed Gene Ontology (GO) term and pathway over-representation analyses on relevant lists of genes from differential expression (DE), Allele-specific expression (ASE) and allele bias analyses using the ShinyGO gene list analysis tool [30]. We used String (v. 11 [31]), to construct gene networks for the set of transgressively expressed genes in hybrid male embryonic hypothalamus. Parameters for STRING were set to a minimum required interaction score of 0.4, including all available sources of evidence.

(e) Behavioural assays and data analysis

We used open field tests to evaluate activity and anxiety-like behaviour in juvenile (day 21) and adult (day 70) hybrids, *Spret* and *Dom*. The open field apparatus was a 16-square grid enclosed by a clear Plexiglas box with no lid (60.96 cm^3). To ensure that all animals started in the same location, mice were placed in a vertically oriented opaque polyvinyl chloride (PVC) tube in the centre of the arena, and each 5 min trial began after the mouse left the centre of the grid following removal of the tube. Trials were video-recorded and activity (total number of gridlines crossed), time spent in the four central squares of the grid, and time spent frozen at the start of the trial were scored. Because the two parental species and their hybrid are phenotypically distinct, trials could not be scored blind to genotype.

To obtain a measure of activity levels in a familiar environment, we used an automated monitoring system (VitalView Animal Monitoring Software, v. 5.0) to measure home cage activity in adult (68–75 days) hybrid, *Spret* and *Dom*. Activity was measured as the number of times a mouse crossed an infrared beam per 5 min over the course of 72 h. For analysis, these values were averaged across days in four 6 h blocks: first half of the light period, second half of the light period, first half of the dark period and second half of the dark period.

Open field behaviour was analysed with linear mixed models (LMMs) with genotype (hybrid, *Spret* and *Dom*), sex and their interaction as fixed effects. Because some of the animals were full siblings we included litter ID as a random effect. Home cage activity was analysed with repeated measures ANOVA with genotype, sex and their interaction as fixed effects. For both datasets, Tukey's honestly significant difference (HSD) tests were used for *post hoc* evaluation of significant effects.

3. Results

(a) Pseudohybrid genome mapping efficiency and hybrid allelic expression distribution

Overall mapping rates of samples to the pseudo-hybrid genome were similar between *Dom* (average 91.31%), *Spret* (average 84.81%) and hybrid (average 89.09%). Both the mean and median of the proportion of reads assigned to the *Dom* allele in hybrid samples are very close to 0.5 and slightly right-skewed (e.g. female embryonic hypothalamus (ehypo): median: 0.496, mean: 0.512, skewness: 0.383; for all sample data, see electronic supplementary material, table S1 and figure S1), indicating an almost even distribution of expression from the maternal and paternal alleles.

(b) Only the hybrid male embryonic hypothalamus has high levels of transgressive expression comparable to the placenta

In order to identify general patterns of altered expression in hybrid hypothalamus throughout development, we analysed overall expression in e17.5 embryonic hypothalamus (ehypo), day 21 juvenile hypothalamus (jhypo) and day 70 adult hypothalamus (ahypo). For each developmental stage, neural gene expression in hybrids was compared with that in both parental species in analyses split by sex (figure 1A). We also compared the level of DE in the hybrid hypothalamus with that previously found in the placenta [25]. Total numbers of DE genes are not directly comparable between this and the placental dataset because the latter was analysed with an earlier version of DESeq2 than that used here. We therefore compared percentages of DE genes between neural and placental datasets.

Total DE between parental species, compared with the number of all assessed genes, differs slightly between timepoints (males: ehypo, 3169/19 626; jhypo, 4214/20 507; ahypo, 3965/20 277; females: ehypo, 3481/19 819; jhypo, 3590/19 895; ahypo, 3823/19 859). When looking at hybrid total DE as percentage of total DE between the parental species, overall hybrid total DE is larger in the placenta compared with the hypothalamus in both sexes (figure 1B). Only in the hybrid male placenta does total DE surpass the level of DE between parental species. Hybrid hypothalamus DE is highest in the embryo and decreases throughout development in males, whereas female hybrid hypothalamus DE is lowest in the embryo and increases slightly in juvenile and adult hypothalamus (figure 1B). In line with our observations for the placenta [25], overall expression patterns in the hybrid hypothalamus are more similar to *Dom*, the maternal species in the cross, than to *Spret* (figure 1E; electronic supplementary material, datasets S1–S3).

The level of transgressive expression in hybrid male ehypo is at a comparable level to the placenta when calculated as percentage of total DE, whereas in female hybrids transgressive expression is substantially reduced in ehypo compared with the placenta (figure 1C). In fact, transgressive expression in the hybrid brain is minimal in females at all stages, and in juvenile and adult males (figure 1A; electronic supplementary material, dataset S1). Notably, 91% (150/165) of transgressively expressed genes in male hybrid ehypo have reduced expression relative to both parental species (figure 1A). In contrast, there is a moderate bias (63%; 283/451) towards increased expression among genes with transgressive expression in hybrid male placenta [25].

(c) Hybrid hypothalamus X-chromosomal differential expression is higher in males than in females, but lower than in the placenta for both sexes

Given the pronounced male bias in hybrid transgressive expression in this cross, we were especially interested in X-chromosomal DE. We find that the highest level of X-chromosomal DE is detected in the placenta, where between 20 and 25% of all X-chromosomal genes are differentially expressed in both sexes of hybrid [25]. Fewer than 10% of X-chromosomal genes are differentially expressed in hybrid male ehypo, with an even lower percentage in jhypo and ahypo. In hybrid females, X-chromosomal DE is lowest in ehypo at fewer than 2% of all X-linked genes, but increases to more than 5% throughout hypothalamus development (figure 1D; electronic supplementary material, datasets S1–S3). We detected four X-chromosomal genes that are transgressively expressed in the hybrid male ehypo (androgen receptor (*Ar*), NHS-like 2 (*Nhsl2*), diacylglycerol kinase kappa (*Dgkk*) and glypican 3 (*Gpc3*)). *Gpc3* had transgressively lower expression while *Ar*, *Nhsl2* and *Dgkk* were expressed transgressively higher. No X-chromosomal genes were transgressively expressed in female ehypo, or in jhypo for either sex. In ahypo, we found only one transgressive X-chromosomal gene in males (SRY (sex-determining region Y)-box 3 (*Sox3*)) and two in females (G-protein coupled receptor 34 (*Gpr34*), protocadherin-19 (*Pcdh19*)) (electronic supplementary material, datasets S1–S3). In contrast, in hybrid male placenta, 29 X-chromosomal genes were transgressively expressed, including another glypican, *Gpc4*, located immediately upstream of *Gpc3*. In hybrid female placenta, 17 X-chromosomal genes were transgressive [25].

(d) Hypothalamus–placenta coexpression does not explain transgressive expression in hybrids

To test for an association between transgressive expression in hybrid male placenta and embryonic hypothalamus, we looked for overlap in the identity of transgressively expressed genes and assessed overall levels of coexpression between placenta and hypothalamus. Only 11 genes are transgressively expressed in both tissues in hybrid males (figure 1F). However, there are more than twice as many genes that are coexpressed at similar levels (hybrid hypothalamus versus placenta: LFC <0.2) in placenta and ehypo relative to placenta and postnatal hypothalamus. This pattern is consistent across genotypes and sexes (figure 1F). Enriched GO-terms for placenta–ehypo coexpressed genes are related to methylation, histone modification, transcription and cell cycle (electronic supplementary material, dataset S9). The subset of genes that are highly expressed at similar levels in hybrid placenta and ehypo but have lower expression in postnatal hypothalamus (DESeq normalized read counts in hybrid ehypo >1000; hybrid ehypo versus placenta: LFC <0.2; hybrid j-ahypo versus ehypo: LFC <−1) is significantly enriched for mammalian phenotype ontology terms [32] related to placental morphology and embryonic lethality in both sexes (figure 1F; electronic supplementary material, dataset S9). These coexpression patterns highlight the connection between placenta and developing brain but do not suggest a direct effect of placental transgressive expression on expression in hybrid male ehypo.

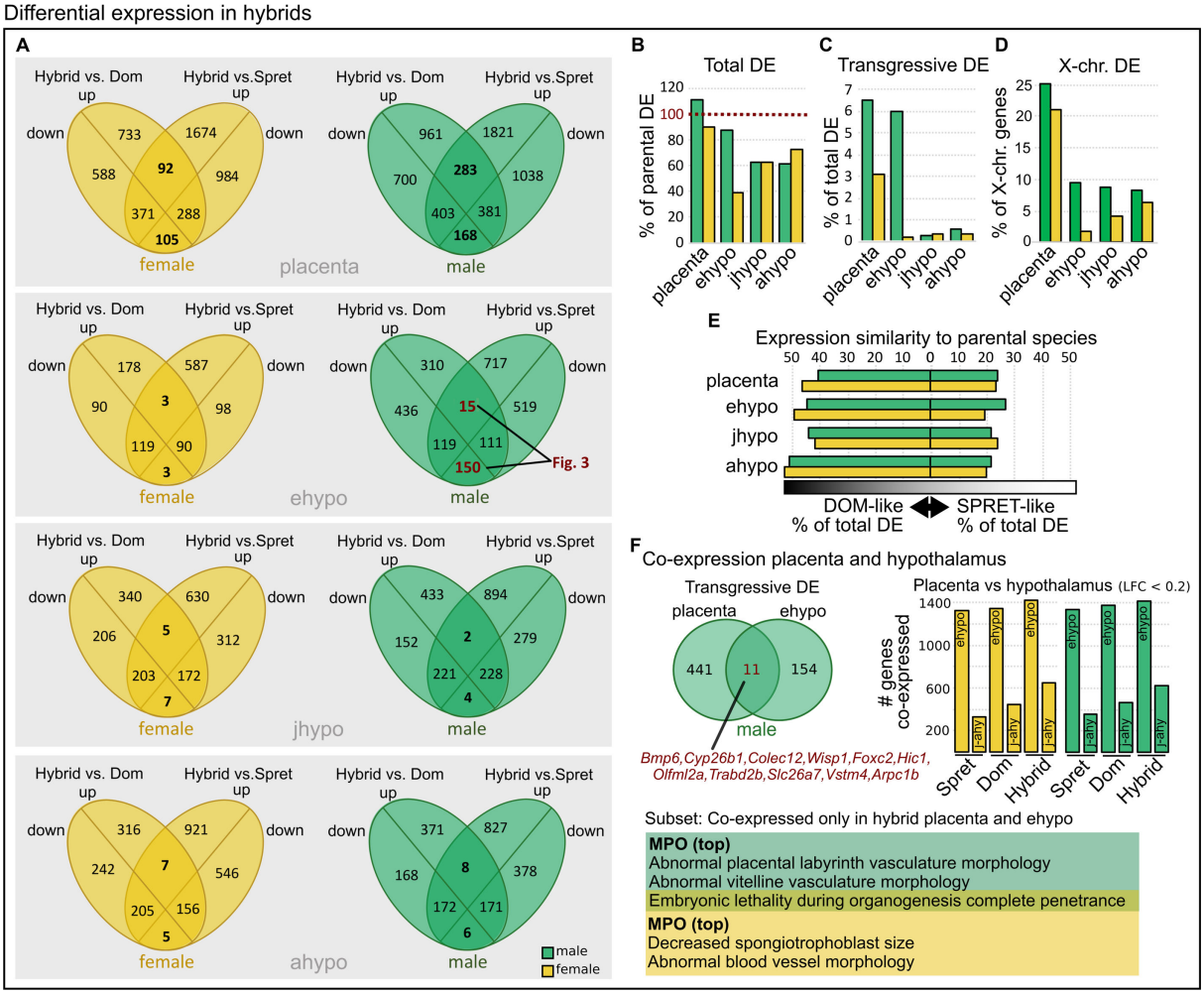

Figure 1. Differential expression (DE) in hybrids compared with *Mus musculus domesticus* (*Dom*) and *Mus spretus* (*Spret*). (A) Venn diagrams depicting the overlap between DE genes between hybrid versus *Dom* and hybrid versus *Spret* in placenta (data from [25]), embryo (17.5 days post-fertilization) hypothalamus (ehypo), juvenile (postnatal day 21) hypothalamus (jhypo) and adult (postnatal day 70) hypothalamus (ahypo). DE: up, genes expressed higher in hybrids compared with parental species (false discovery rate (FDR) ≤0.05 and $\log_2$-fold change (LFC) ≥0.5); down, genes expressed lower in hybrids compared with parental species (FDR ≤0.05 and LFC ≤−0.5); transgressively expressed genes (up or down in hybrids compared with both parental species) are indicated in bold. The list of transgressively expressed genes in male hybrid ehypo (bold red) was further analysed using the STRING database [31]. (B–D) Comparison of DE levels between male (green) and female (yellow) in the different tissues. (B) Hybrid DE as percentage of DE between parental species. Parental DE: total number of genes differentially expressed between *Dom* and *Spret*. Total DE: total number of differentially expressed genes in hybrids compared with *Dom* and/or *Spret*. (C) Hybrid transgressive expression as percentage of hybrid total DE. Transgressive DE: number of genes expressed significantly higher or lower in hybrids compared with both parental species. (D) Percentage of X-chromosomal genes differentially expressed in hybrids (percentage of all expressed/assessed X-chromosomal genes). X-chr. DE: X-chromosomal genes differentially expressed in hybrids compared with *Dom* and/or *Spret*. (E) Expression similarity to parental species. DOM-like: genes that are differentially expressed in hybrid compared with *Spret* but not *Dom*. SPRET-like: genes that are differentially expressed in hybrid compared with *Dom* but not *Spret*. (F) Coexpression between placenta and hypothalamus. Overlap between placenta and ehypo male hybrid transgressively expressed genes is shown in the Venn diagram and gene symbols of overlapping genes are listed. The bar graph shows the level of coexpression with placenta in ehypo and j-ahypo (jhypo and ahypo jointly) in all three genotypes and both sexes. Top overrepresented mammalian phenotype ontology (MPO) terms [32] for genes exclusively coexpressed in placenta and ehypo in all genotypes (DESeq normalized read counts in ehypo >1000; ehypo versus placenta: LFC <0.2; j-ahypo versus ehypo: LFC <−1).

(e) Imprinted genes are transgressively expressed in the hybrid male embryonic hypothalamus

Eight IGs are transgressively expressed in the hybrid male ehypo. *Cdkn1c*, *Dcn*, *Peg12*, *Slc22a18*, *Igf2* and *Igf2os* have transgressively lower expression, whereas *Zim1* and *Kcnk9* expression is transgressively higher (electronic supplementary material, datasets S1–S3). Notably, *Cdkn1c* and *Slc22a18* are neighbouring genes in the same IG cluster on distal chromosome 7 that is strongly associated with hybrid male placental disorganization and undergrowth [25].

Since loss of imprinting (i.e. biallelic expression) is a widely proposed mechanism to explain parent-of-origin growth effects in mammalian hybrids, we determined the allelic expression of transgressively expressed imprinted genes in the hypothalamus datasets. Similar to our findings for the placenta, global loss of imprinting was not detected in the hybrid hypothalamus at any stage of development (electronic supplementary material, datasets S4–S6). Of the transgressively expressed IGs, *Igf2* is biallelically expressed in both sexes (figure 2; electronic supplementary material, datasets S4–S6). However, biallelic expression of the Igf2/H19 IG cluster is a normal occurrence in mouse brain [33]. *Dcn* is biallelically expressed in both sexes but slightly paternally biased (electronic supplementary material, datasets S1–S6). *Igf2os* is strongly maternally biased in male ehypo and

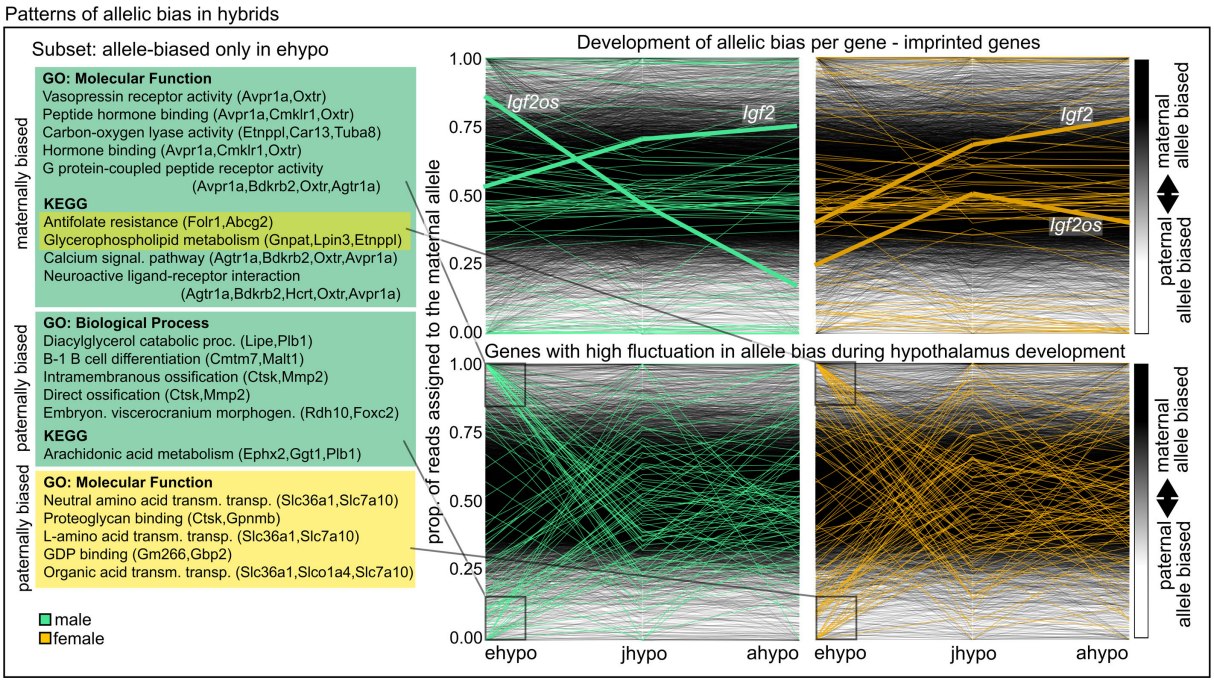

Figure 2. Patterns of allelic bias in hybrids. Line plots show the development of allelic bias per gene in hybrid hypothalamus from embryo (ehypo) to juvenile (jhypo), and adult (ahypo). Each line represents a gene, showing the proportion of reads assigned to the *Dom* (maternal) allele for the three timepoints. In the upper two plots, imprinted genes are marked in green (male) or yellow (female). *Igf2* and *Igf2os* are bold and labelled. The lower line plots highlight genes with an overall high fluctuation in allelic bias between developmental timepoints, defined as a switch from strong allele bias (proportion of reads assigned to maternal allele ≥0.8 or ≤0.2) to not biased (proportion of reads assigned to maternal allele 0.4–0.6) one or more times during development. Top overrepresented Gene Ontology (GO) terms and pathways (KEGG) for genes with a strong allelic bias in ehypo only. transm. transp., transmembrane transporter.

switches to paternal expression in adult. In females, *Igf2os* is moderately paternally biased in ehypo and is biallelic in later stages (figure 2).

(f) Allele-biased expression is stronger in the embryonic hypothalamus than at later developmental stages

Allele-specific or allele-biased expression is not restricted to imprinted genes. A gene's expression can be allele-biased based on developmental stage, tissue-specific regulation or random allelic expression [34]. We therefore compared autosomal patterns of allele-bias in hybrids between ehypo, jhypo and ahypo and between sexes. In ehypo only, we found a large number of genes with strong allelic bias (proportion of reads assigned to the maternal allele >0.8 or <0.2; figure 2; electronic supplementary material, dataset S7). Overrepresentation results for these subsets of genes are listed in figure 2. Interestingly, in hybrid male ehypo only, several important neuroactive receptors are strongly biased towards the *Dom* allele (angiotensin II receptor, type 1a (*Agtr1a*), arginine vasopressin receptor 1A (*Avpr1a*), bradykinin receptor, beta 2 (*Bdkrb2*), oxytocin receptor (*Oxtr*)) (figure 2; electronic supplementary material, datasets S7 and S8). In the absence of data from the reciprocal cross, we cannot discriminate directly between parent-of-origin allelic bias and allelic expression differences between species. However, none of the four receptors is differentially expressed between *Dom* and *Spret* (electronic supplementary material, dataset S1) and the *Dom* allelic bias is only detected in the embryonic hypothalamus of male hybrids, both of which suggest a true sex- and developmental stage-limited maternal allelic bias in this direction of the cross rather than a species effect.

(g) Hybrid male embryonic hypothalamus transgressive expression network is centred on imprinted and X-linked genes

Since the hybrid male ehypo is the only tissue in which we found transgressive expression at comparable levels to the placenta, we further investigated the transgressively expressed gene list. A strong bias towards transgressive expression in one direction (in this case, lower expression) is suggestive of a common regulatory mechanism affecting a gene expression network. Indeed, based on evidence for functional interactions from the STRING database [31], most of the transgressively expressed genes in the hybrid male ehypo are included in a single highly interconnected network (figure 3A; STRING network: https://version-12-0.string-db.org/cgi/network?networkId=bXs85IBT9rm9). Transgressive imprinted (*Igf2*, *Cdkn1c*, *Zim1*, *Slc22a18*, *Dcn*) and X-chromosomal genes (*Gpc3*, *Ar*) are functionally interconnected and occupy central points between the network's two clusters of hub genes (identified in the STRING database using *k*-means clustering; figure 3A; electronic supplementary material, dataset S10). Cluster 1 (grey) contains genes involved in cell adhesion and extracellular matrix structure (e.g. *Nid2*, *Postn*, *Cdh5*, *Fbln1*, *Efemp1*, multiple collagens) and regulation of cell proliferation and growth (*Sparc*, *Ctsk*, *Fstl1*, *Ccn1*); Cluster 2 (white) comprises multiple cell cycle genes (e.g. *E2f1*, *Asf1b*, *Ube2c*, *Dlgap5*, *Ccnb2*, *Ska1*, *Melk*, *Gtse1*, *Esco2*, *Ckap2*, *Kif22*) (figure 3A; electronic supplementary material, dataset S10).

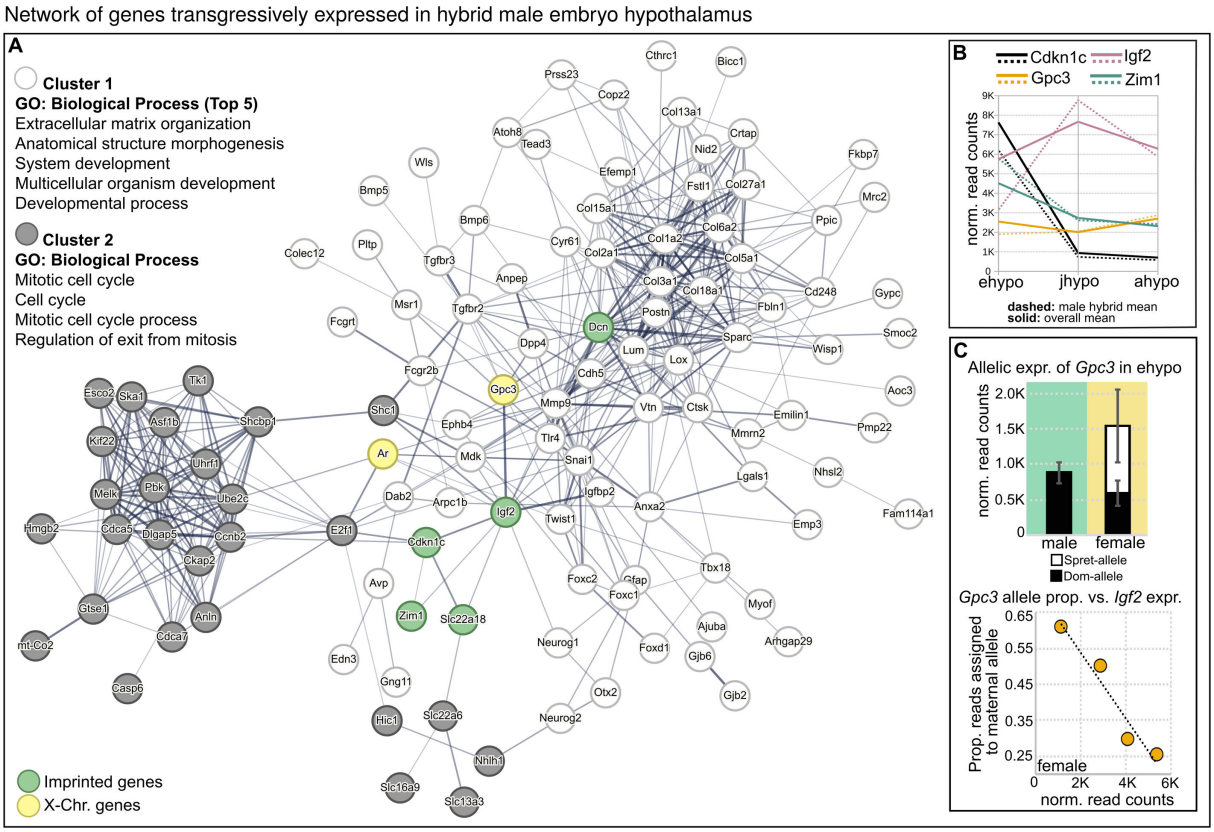

Figure 3. Network of transgressively expressed genes in hybrid male embryonic hypothalamus and expression patterns of central genes. (A) STRING database network (network statistics: no. nodes: 161, no. edges: 489, average node degree: 6.07, average local clustering coefficient: 0.45, expected no. edges: 111, PPI (protein-protein interaction) enrichment *p*-value: $<1.0 \times 10^{-16}$). Nodes are labelled with gene symbols; the thickness of the network edges indicates the strength of support for a functional interaction (nodes not connected to the main network were deleted). Clusters were defined in the STRING database using *k*-means clustering (Cluster 1, grey; Cluster 2, white). Imprinted genes are indicated in green, X-chromosomal genes in yellow. Top overrepresented Biological Process Gene Ontology (GO) terms for clusters are shown. (B) Line plot shows average DESeq2 normalized counts in male hybrids and across datasets for highly expressed imprinted genes in the network and *Gpc3*. (C) Potential effects of paternal *Gpc3* on *Igf2* expression. Bar plot shows total DESeq2 normalized counts of *Gpc3* in male (green) and female (yellow) hybrid embryo hypothalamus (ehypo). DESeq2 normalized counts of *Gpc3* reads assigned to the maternal X (*Dom*-allele) are depicted in black; reads assigned to the paternal X (*Spret*-allele) are in white. Scatterplot shows the relationship between proportional *Gpc3* expression from the maternal X and expression levels of *Igf2* in hybrid female ehypo.

Of the transgressively expressed imprinted genes in this network, *Igf2* and *Cdkn1c* are especially highly expressed in general and have important functions during brain development [10]. Notably, *Cdkn1c* expression is high in the ehypo and strongly reduced in jhypo and ahypo, indicating a less prominent role in the postnatal hypothalamus (figure 3B).

(h) Paternal X expression levels of *Gpc3* correlate with *Igf2* expression levels in hybrid female embryonic hypothalamus

To further explore possible causes for hybrid male-limited transgressive expression, we concentrated on *Gpc3* and *Igf2*. X-chromosomal *Gpc3*, which has transgressively lower expression in hybrid male ehypo, interacts with *Igf2* [35] and both genes have central positions in the male hybrid ehypo transgressive gene network (figure 3A; electronic supplementary material, dataset S1). Since *Gpc3* is X-linked, we assessed allelic expression in hybrid female ehypo, in which this gene is not differentially expressed relative to either parental species. Whereas *Gpc3* is not differentially expressed between *Dom* and *Spret* in the ehypo in either sex (electronic supplementary material, dataset S1), 61% of *Gpc3* reads were assigned to the paternal X in hybrid female ehypo (figure 3C). Moreover, the proportion of *Gpc3* expression from the maternal allele is negatively correlated with *Igf2* expression level (R^2(adj) = 0.91, F_2 = 31.75, p = 0.03, n = 4), indicating that allelic expression of *Gpc3* might affect *Igf2* expression (figure 3C).

(i) Evidence for reduced activity and increased anxiety-like behaviours in hybrids

As a first step to connecting transgressive expression in the developing hypothalamus to potential behavioural consequences in hybrids, we assayed activity and anxiety-like behaviours in juveniles and adults. As juveniles, hybrids were less active in the open field test than age-matched *Dom* or *Spret* and remained frozen longer at the start of the trial. Specifically, genotype had a significant effect on number of lines crossed (LMM, F = 25.48, $p < 0.0001$; figure 4A(a)) and on time spent frozen at the start of the trial (LMM, F = 7.98, p = 0.0084; figure 4A(c)). For both variables, the effect was driven by the difference between hybrids and both *Dom* and *Spret* (lines crossed: hybrid versus *Dom*, p = 0.0003, hybrid versus *Spret*, $p < 0.0001$, *Dom* versus *Spret*, p = 0.44; time frozen at start: hybrid versus *Dom*, p = 0.017, hybrid versus *Spret*, p = 0.013, *Dom* versus *Spret*, p = 1.0). There was no

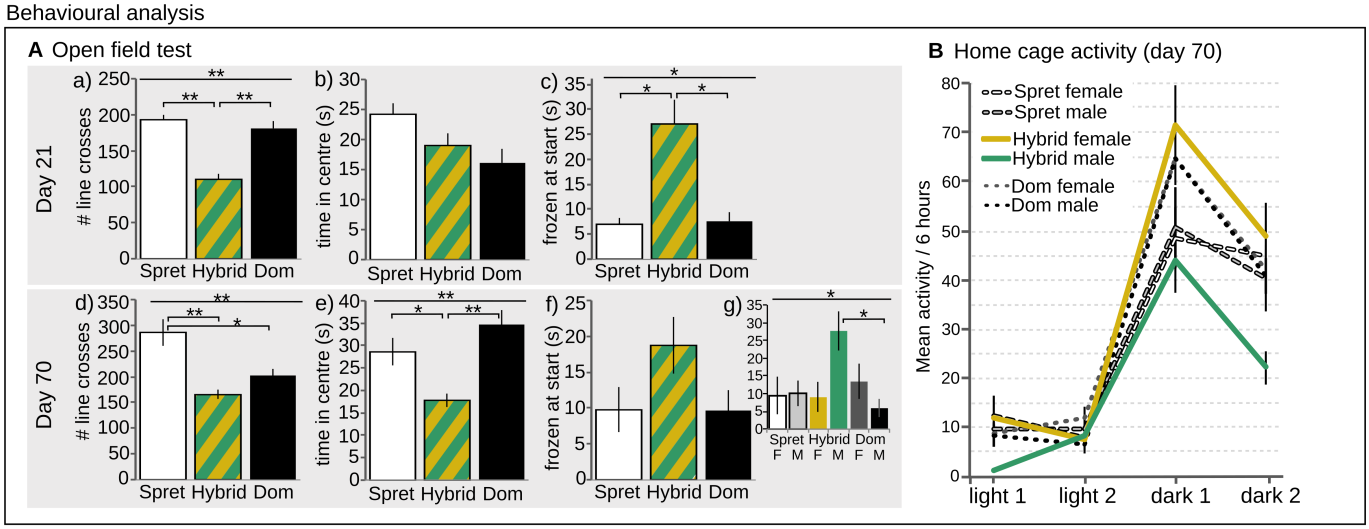

Fig.4

Figure 4. Hybrid, *Dom* and *Spret* behaviour. Results of (A) the open field test measured at 21 days (a–c) and 70 days (d–g), and (B) home cage activity measured at 70 days. Means ± s.e. M, male; F, female. Statistical significance is indicated by asterisks: *$p < 0.05$, **$p < 0.01$.

effect of sex and no interaction between genotype and sex for either variable (all $p \geq 0.1$). Juvenile hybrids spent an intermediate amount of time in the centre squares of the open field apparatus relative to *Dom* and *Spret* (figure 4A(b)) and there was no effect of genotype, sex or their interaction (all $p > 0.08$).

As adults, hybrids spent significantly less time in the centre of the open field than either *Dom* or *Spret* (LMM, $F = 10.71$, $p = 0.0016$; hybrid versus *Dom*, $p = 0.0013$, hybrid versus *Spret*, $p = 0.026$, *Dom* versus *Spret*, $p = 0.28$; figure 4A(e)). There was also a significant effect of genotype on number of lines crossed (LMM, $F = 7.74$, $p = 0.0034$; figure 4A(d)) but this was driven by higher activity in *Spret* relative to both hybrid and *Dom* (hybrid versus *Dom*, $p = 0.49$, hybrid versus *Spret*, $p = 0.0027$, *Dom* versus *Spret*, $p = 0.03$). Neither sex, nor the interaction between genotype and sex, was significant for either variable (all $p \geq 0.1$). There was no effect of genotype or sex on the amount of time mice spent frozen at the start of the trial (all $p > 0.17$) but the interaction between genotype and sex was significant (LMM, $F = 4.27$, $p = 0.019$; figure 4A(f,g)). Across all genotypes split by sex, male hybrids and male Dom remained frozen for the most and least amount of time, respectively (males: hybrid versus *Dom*, $p = 0.02$; all other comparisons, $p > 0.06$; figure 4A(g)).

Adult hybrid activity in the home cage followed the same general pattern as that of both parental species, with reduced activity during the light cycle and highest activity levels during the first part of the dark cycle (figure 4B). The overall model was significant (repeated measures ANOVA, $F_{5,84} = 2.52$, $p = 0.035$), with significant effects of sex ($F_{1,84} = 5.01$, $p = 0.028$) and the interaction between sex and genotype ($F_{2,84} = 3.11$, $p = 0.05$), but not genotype ($F_{5,84} = 0.7$, $p = 0.5$). *Post hoc* pairwise tests for differences between genotypes split by sex indicated that hybrid males were less active than hybrid females during the dark cycle (dark 1, $p = 0.042$; dark 2, $p = 0.012$) and less active than *Spret* females during the second part of the dark cycle (dark 2, $p = 0.048$). All other comparisons were non-significant ($p > 0.12$).

4. Discussion

Mammalian hybrid abnormalities are often characterized by both parent-of-origin and sex-specific effects, the latter in accordance with Haldane's rule. We investigated *M. m. domesticus*/*M. spretus* hybrid neural gene expression and effects on hybrid behaviour across development, and compared expression in the hypothalamus with our previous findings for the placenta. Consistent with the expectation that neural dysregulation should be most evident during prenatal development, substantial transgressive gene expression was limited to the embryonic hypothalamus. In line with our previous findings in the placenta, we found an excess of neural transgressive expression in male as compared with female hybrids. Hybrids were generally less active and exhibited higher levels of anxiety-like behaviours relative to both parental species. However, contrary to expectations, we did not find an association between placental misexpression and observed patterns of transgressive expression in the embryonic brain of male hybrids. Evidence rather points towards X-chromosomal–IG incompatibilities, and the critical roles of the affected IGs and non-imprinted interacting genes in embryonic brain development.

(a) The developing brain obeys Haldane's rule

The testes and the placenta are hotspots for the expression of genomic incompatibilities that preferentially affect the fertility and viability of male hybrids [24]. Consistent with Haldane's rule, F_1 male hybrids between *M. m. domesticus* and *M. spretus* are completely sterile whereas their sisters are not, and placental size dysplasia, cellular disorganization and transgressive expression are all more extreme in male relative to female hybrids [12,25]. Our results extend this pattern to the developing brain: transgressive expression in hybrid hypothalamus is both highly male-biased and concentrated in the prenatal period. We found no evidence for a relationship between expression in the developing brain and placenta that was unique to male hybrids.

Thus, transgressive expression in embryonic hypothalamus is better explained by genomic incompatibilities that manifest in the brain than by downstream effects of placental abnormalities. For example, transgressive expression of different genes within the same chromosome 7 imprinting cluster in placenta (*Phlda2* and *Ascl2*; [25]) and brain (*Cdkn1c* and *Slc22a18*) suggests a single epigenetic incompatibility with tissue-specific manifestation.

The exposure of X-linked incompatibilities in the heterogametic sex is the most widely supported genetic basis for Haldane's rule, and the X chromosome is disproportionately associated with hybrid male sterility in *Drosophila* and mammals [17,36,37]. The X chromosome is similarly implicated in hybrid male-biased placental dysfunction in multiple interspecific crosses in rodents, including the one studied here [21,23,24]. Whereas the X chromosome is enriched for transgressive expression in hybrid male placenta in the *M. m. domesticus*/*M. spretus* cross [25], in hybrid male embryonic hypothalamus transgressive X-linked expression was limited to just four genes. Two of these (*Ar* and *Gpc3*), however, occupy central positions in the network constructed from functional associations between transgressively expressed genes and are interconnected with the five imprinted genes in the network, particularly *Igf2*.

Gpc3 regulates prenatal growth, in large part by negatively regulating the activity of Igf2, which promotes growth [35]. Because Gpc3–Igf2 interactions are well documented and neither is transgressively expressed in hybrid female ehypo, we evaluated the relationship between *Gpc3* and *Igf2* expression in our four hybrid female ehypo samples. We recovered the expected negative correlation between *Gpc3* and *Igf2* expression and found that normal *Gpc3* expression levels in hybrid females are largely explained by elevated expression from the paternal *Spret* X chromosome. This pattern suggests that regulatory incompatibilities between imprinted genes and genes expressed from the maternally inherited X chromosome can catalyse transgressive expression in the developing hypothalamus of hybrid males. Consistent with Haldane's rule, hybrid females are less affected, potentially owing to the rescue effects of their paternally inherited X chromosome (electronic supplementary material, figures S2 and S3).

Whereas *Igf2* is paternally expressed in placenta and has biallelic expression in the brain, five of eight IGs with transgressive expression in hybrid male embryonic brain are normally maternally expressed (*Cdkn1c*, *Slc22a18*, *Zim1*, *Kcnk9*, *Dcn*). It is not intuitively obvious how incompatibilities between the maternally inherited X chromosome and maternally expressed IGs would arise. However, theory shows that sexually antagonistic alleles that benefit males (in this case fathers) at a cost to females (in this case mothers) should accumulate more readily on X chromosomes than female-benefit/male-cost alleles [38]. If true empirically then we might expect X–IG incompatibilities to involve an excess of MEGs.

To our knowledge, this is the first genome-scale study to explore the regulatory consequences of interspecific hybridization in the mammalian brain (but see [39] on subspecific crosses). However, the inference that X–IG incompatibilities are key contributors to regulatory abnormalities in the embryonic hypothalamus is broadly consistent with recent work in dwarf hamster (*Phodopus*) hybrids. In the cross between *Phodopus sungori* and *Phodopus campbelli*, placental overgrowth phenotypes are strongly associated with a single large effect X-linked QTL and transgressive downregulation of multiple known and candidate imprinted genes [14,24]. X–IG incompatibilities are also strong candidates for the genetic basis of placental overgrowth in deer mouse (*Peromyscus*) hybrids [22,23].

Because QTL mapping studies of both *Phodopus* and *Mus* hybrid growth abnormalities have focused primarily on the over-grown direction of each cross and only on the placenta, it remains to be seen whether the association between X chromosome genotype and neurodevelopmental dysregulation in under-grown hybrids is equally strong. While a complete understanding of the genetic architecture of hybrid male dysgenesis in the *M. m. domesticus*/*M. spretus* cross direction studied here awaits genotypic and expression (e)QTL mapping in placenta and brain, current results suggest that the genetic architecture of hybrid incompatibilities is similar in placenta and embryonic brain, the two tissues with the highest enrichment for IG expression [7].

(b) Hybrid male transgressive gene network recapitulates the cellular functions of imprinted genes

Regulation of transitions between cellular proliferation and quiescence during mammalian development is a unifying function of imprinted genes [40]. Specifically, Al Adami *et al*. [40] proposed that the large phenotypic effects of altered expression of one or a few imprinted genes are achieved via IG-regulated expression of a larger network of autosomal genes enriched for functions in the extracellular matrix, growth factor signalling and cell–cell adhesion. Moreover, experimental manipulation of IG expression in cell culture impacted both proliferation and cell cycle exit [40]. Notably, the network of transgressively expressed genes in hybrid male ehypo features two clusters that represent the critical roles of IGs in regulation of both extracellular matrix structure and cell proliferation and growth via the cell cycle. We therefore speculate that misregulation of a small number of imprinted genes contributes disproportionately to transgressive expression in the developing hypothalamus of hybrid males.

In addition to these general roles in growth and development, several transgressively expressed IGs and non-imprinted interaction partners have critical functions specific to brain development. Among IGs, *Cdkn1c* negatively regulates neural growth and, together with *Kcnk9*, positively regulates neural migration (reviewed in [10]). As noted above, X-linked *Gpc3* regulates growth via suppression of growth-promoting *Igf2*. In the prenatal brain, *Gpc3* is involved in neural differentiation and migration [41,42]. Although less studied than *Igf2*, the long non-coding RNA antisense transcript *Igf2os* is highly expressed in the embryonic brain and is implicated in suppression of neural growth and promotion of apoptosis [43,44]. In contrast, non-imprinted genes *Neurog1* and *Neurog2* generally promote neurogenesis and *Neurog2* specifically regulates neurogenesis and neural differentiation in the hypothalamus [45]. These neuron-specific functions of transgressively expressed genes, together with transgressive expression of multiple regulators of cellular growth and extracellular structure identified in the gene

network, suggest that neuronal density and perhaps connectivity should be altered in the brains of hybrid males. Testing this hypothesis will require detailed neuroanatomical analysis of hybrid and parental species' brains.

(c) Developmental and sex differences in allelic expression in hybrids

Beyond the strong male bias in transgressive expression in embryonic hypothalamus, we found intriguing sex differences in allelic expression that shifted across development. Among autosomal genes with no prior evidence of imprinting, strong allelic bias (>80% expression from one allele) was concentrated in the embryonic brain relative to postnatal timepoints, a pattern observed in both sexes but more pronounced in males. For example, we found a strong maternal allelic bias unique to male hybrid ehypo for a suite of neuromodulatory receptors, including the oxytocin and vasopressin receptors. Biased allelic expression is a widespread feature of mammalian genomes [46] and there is evidence for sex differences in the identity ([47]; but see [48]), epigenetic regulation [49] and behavioural effects [50] of genes with parent-of-origin allelic bias in the brain. Therefore, our result might reflect a general effect of the maternal genome on expression in the hypothalamus of male embryos. Alternatively, male-limited maternal-biased expression is unique to hybrids, possibly owing to *trans*-acting effects of a single *Dom*-derived X chromosome.

Many known imprinted genes vary across tissues and timepoints in degree of parent-of-origin allelic bias [7,10]. However, we are not aware of prior reports of an imprinted gene with sex-dependent expression from different parental alleles. We found this to be the case for *Igf2os*, which is transgressively expressed in hybrid male ehypo with a strong maternal allelic bias in males and a moderate paternal bias in females. Given that *Igf2os* is normally paternally expressed and is co-regulated with *Igf2* [51], we infer that the switch to maternal expression and transgressive downregulation of both genes in male hybrids reflects epigenetic perturbation at this locus as a consequence of incompatibilities between parental genomes and/or epigenetic states.

In a prior study that was part of this same experiment, we evaluated potential effects of hybrid placental dysregulation on gene expression in the brains of otherwise normal *Dom* females [28]. We found a handful of genes with altered expression in mothers carrying hybrid litters and a strong effect of the hybrid placenta's paternally inherited genome on placenta–maternal brain coexpression of maternal–fetal communication genes [28]. Although we found no overlap in the identity of transgressive genes in hybrid male ehypo and genes with altered expression in their mothers, the apparent reciprocal sensitivity of the maternal brain to their offspring's paternal genome and of male embryonic brains to their mother's genome is intriguing.

(d) Hybrid brain–behaviour associations

As a first step to connecting gene regulatory phenotypes to organismal phenotypes, we evaluated a suite of anxiety- and activity-associated behaviours in juvenile and adult hybrids relative to parental species. Hybrids were less active as juveniles (fewer lines crossed in open field) and exhibited heightened anxiety-like behaviours as both juveniles (longer freeze duration at start of open field trial) and adults (less time in the centre squares of the open field). Whereas prenatal transgressive expression was strongly male-biased, transgressive behaviour was observed in both sexes. However, only male hybrids exhibited longer freeze duration in the open field trial and reduced home cage activity as adults.

Several genes with transgressive expression in the developing brain of male hybrids impact anxiety-like behaviour or activity patterns in genetically modified lab mice. For example, *Kcnk9* knockout mice exhibit increased nocturnal activity [52], a result consistent with reduced nocturnal activity and transgressive upregulation of *Kcnk9* in hybrid male ehypo. Similarly, upregulation of *Cdkn1c* in adult lab mice increases motor activity and reduces anxiety-like behaviour [53] whereas transgressive downregulation of this gene in hybrid male ehypo is associated with elevated anxiety-like behaviour. Finally, transgressive upregulation of the androgen receptor in hybrid male ehypo suggests greater sensitivity to testosterone released from the embryonic testes. In rodents, elevated androgen exposure during brain development is associated with increased anxiety-like behaviours in later life [54].

Although the behavioural effects of developmental silencing or overexpression of *Kcnk9*, *Cdkn1c* and *Ar* are broadly consistent with the direction of transgressive behaviours in hybrid males, similar patterns of transgressive behaviour in hybrid females suggest underlying incompatibilities that are at least partially shared between the sexes. None of the transgressively expressed genes in embryonic, juvenile or adult hybrid female hypothalamus appears to be a strong candidate for affecting activity or anxiety. Therefore, we can only speculate that brain regions not evaluated in this study are comparably dysregulated in both sexes of hybrids and/or that shared and female-specific transgressive expression in some hypothalamic nuclei is masked by our use of whole hypothalamus transcriptomes.

5. Conclusions

Here we report for the first time to our knowledge the effects of interspecific hybridization on gene expression in the mammalian brain and make preliminary connections between transgressive gene expression and behaviour. We find highly male-biased and embryo-specific dysregulation of gene expression in the hybrid hypothalamus. This pattern parallels but is not explained by dysregulated expression in hybrid placenta from the same cross. Whereas different sets of genes are dysregulated in hybrid male embryonic hypothalamus and placenta, we infer a shared genetic and/or epigenetic architecture, in which incompatibilities between imprinted and X-linked genes play prominent roles. Importantly, our results extend the widespread pattern described by Haldane's rule to the developing brain, and identify this tissue as a novel hotspot for genomic incompatibilities that involve

imprinted genes. This study lays the foundation for future genomic dissection of X–IG incompatibilities in hybrid brains, and isolation of downstream effects on behaviour.

Ethics. All animal procedures were approved by the Oklahoma State University IACUC under protocol 141-AS.

Data accessibility. RNA-seq data from this study are deposited on NCBI GEO: GSE311072 https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE311072.

Supplementary material is available online [55].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. L.A.: conceptualization, data curation, formal analysis, investigation, methodology, visualization, writing—original draft, writing—review and editing; P.C.: conceptualization, funding acquisition, investigation, methodology, project administration, supervision, writing—original draft, writing—review and editing.

Both authors gave final approval for publication and agreed to be held accountable for the work performed herein.

Conflict of interest declaration. We declare we have no competing interests.

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