LETTER

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# Distinct physiological and behavioural functions for parental alleles of imprinted *Grb10*

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**Imprinted genes, defined by their preferential expression of a single parental allele, represent a subset of the mammalian genome and often have key roles in embryonic development[1], but also postnatal functions including energy homeostasis[2] and behaviour[3,4]. When the two parental alleles are unequally represented within a social group (when there is sex bias in dispersal and/or variance in reproductive success)[5,6], imprinted genes may evolve to modulate social behaviour, although so far no such instance is known. Predominantly expressed from the maternal allele during embryogenesis, *Grb10* encodes an intracellular adaptor protein that can interact with several receptor tyrosine kinases and downstream signalling molecules[7]. Here we demonstrate that within the brain *Grb10* is expressed from the paternal allele from fetal life into adulthood and that ablation of this expression engenders increased social dominance specifically among other aspects of social behaviour, a finding supported by the observed increase in allogrooming by paternal *Grb10*-deficient animals. *Grb10* is, therefore, the first example of an imprinted gene that regulates social behaviour. It is also currently alone in exhibiting imprinted expression from each of the parental alleles in a tissue-specific manner, as loss of the peripherally expressed maternal allele leads to significant fetal and placental overgrowth. Thus *Grb10* is, so far, a unique imprinted gene, able to influence distinct physiological processes, fetal growth and adult behaviour, owing to actions of the two parental alleles in different tissues.**

To characterize expression and investigate functions of the two parental *Grb10* alleles we have generated a mutant mouse strain (*Grb10KO*), derived by insertion of a *LacZ:neomycin*$^r$ gene-trap cassette within *Grb10* exon 8 (Fig. 1a). Transmission of the *Grb10KO* allele separately through the two parental lines generated heterozygous progeny in which either the maternal (*Grb10KO*$^{m/+}$) or paternal (*Grb10KO*$^{+/p}$) *Grb10* allele was disrupted by the *β-geo* cassette and allowed us to examine *Grb10* expression in an allele-specific manner. Northern blot analysis of RNA samples prepared from whole fetuses (Fig. 1b) showed that endogenous *Grb10* transcripts were readily detected in wild-type animals and in heterozygotes that inherited a mutant *Grb10KO*$^{+/p}$ allele. In contrast, *Grb10* transcripts were found at relatively low levels in heterozygous animals with a mutant *Grb10KO*$^{m/+}$ allele, an observation consistent with previous demonstrations that most *Grb10* expression is maternally derived (for example, ref. 8). We next conducted more refined *in situ* analyses of allele-specific expression, using the integrated *LacZ* reporter gene. During fetal development, *LacZ* expression from the maternal allele was widespread in tissues of mesodermal and endodermal origin, but absent from the central nervous system (CNS) proper (Fig. 1d, f). At embryonic day (E) 14.5, expression of the maternal *Grb10* allele within the brain was seen only in the ventricular ependymal layers, the epithelium of the choroid plexus and the meninges, presumably identifying sources of maternal brain expression that have been reported by others[9–13] (Supplementary Fig. 1a, b). In contrast, expression from the paternal allele was predominant within the developing CNS, with only a few discrete sites of relatively low-level expression seen in other tissues (Figs 1e, g, 2a and Supplementary Fig. 1c, d). The CNS expression starts between E11.5 and E14.5, consistent with the onset of neurogenesis, and correlates with the brain-specific loss of a repressive histone modification (H3K27me3) from the paternal *Grb10* allele during development and during neural precursor cell differentiation *in vitro*[14]. This loss of H3K27me3 from the promoter region of the *Grb10* paternal allele-specific transcripts (see Fig. 1a) leaves a permissive histone mark on the paternal allele (H3K4me2), whereas this region of the maternal allele is constitutively associated with two repressive histone modifications (H3K9me3 and H4K20me3)[14].

Our analysis showed paternal allele expression within the developing CNS was restricted to specific regions of both the brain and spinal cord, with reporter signal identified within select areas of the diencephalon, ventral midbrain and the medulla oblongata extending caudally along the ventral spinal cord. There was no expression detected within the presumptive neocortex, dorsal midbrain or the cerebellar primordium (Fig. 2a). Embryonic *Grb10* expression within the CNS proper was entirely paternal in origin, a fact that was not evident from previous expression studies that identified a promoter and brain-specific transcripts associated with the paternal allele, but relied on techniques involving RNA extraction from tissue homogenates[9–12]. Thus our *Grb10* expression analysis provides striking evidence of reciprocal imprinted expression from the two parental alleles in different tissues. Several imprinted genes exhibit tissue-specific and/or temporal regulation, such that their expression is biallelic (non-imprinted) at some of their sites of expression. However, the reciprocal parent-of-origin expression described here is unprecedented, suggesting new and intriguing possibilities for imprinted gene function and evolution.

Consistent with our previous studies of *Grb10Δ2–4* mice[8,15], *Grb10KO*$^{m/+}$ animals displayed a disproportionate overgrowth phenotype apparent from E12.5 onwards (Fig. 1h, i and Supplementary Fig. 2). At birth, the mean body weight of *Grb10KO*$^{m/+}$ pups was $25 \pm 2.5\%$ greater than that of wild-type littermates. The liver was disproportionately enlarged ($117 \pm 9.8\%$ heavier), but there was sparing of the brain and kidney, such that the weights of these organs were not significantly different to those of wild types (Fig. 1i). The cranial sparing is consistent with limited *Grb10* maternal allele expression within the developing CNS. Body weight and proportions of *Grb10KO*$^{+/p}$ mutants did not differ from wild-type controls and no function has yet been ascribed to the paternally inherited *Grb10* allele, despite evidence of its expression within the neonatal brain[9]. Both *Grb10KO*$^{m/+}$ and *Grb10KO*$^{+/p}$ mutants were present at the expected Mendelian frequencies ($\chi^2$ values, $P = 0.737$ and $P = 0.395$, respectively) when animals were genotyped at 3–4 weeks of age, indicating that survival to weaning was unimpaired. Observations of *Grb10KO*$^{+/p}$ pups before weaning, including analysis

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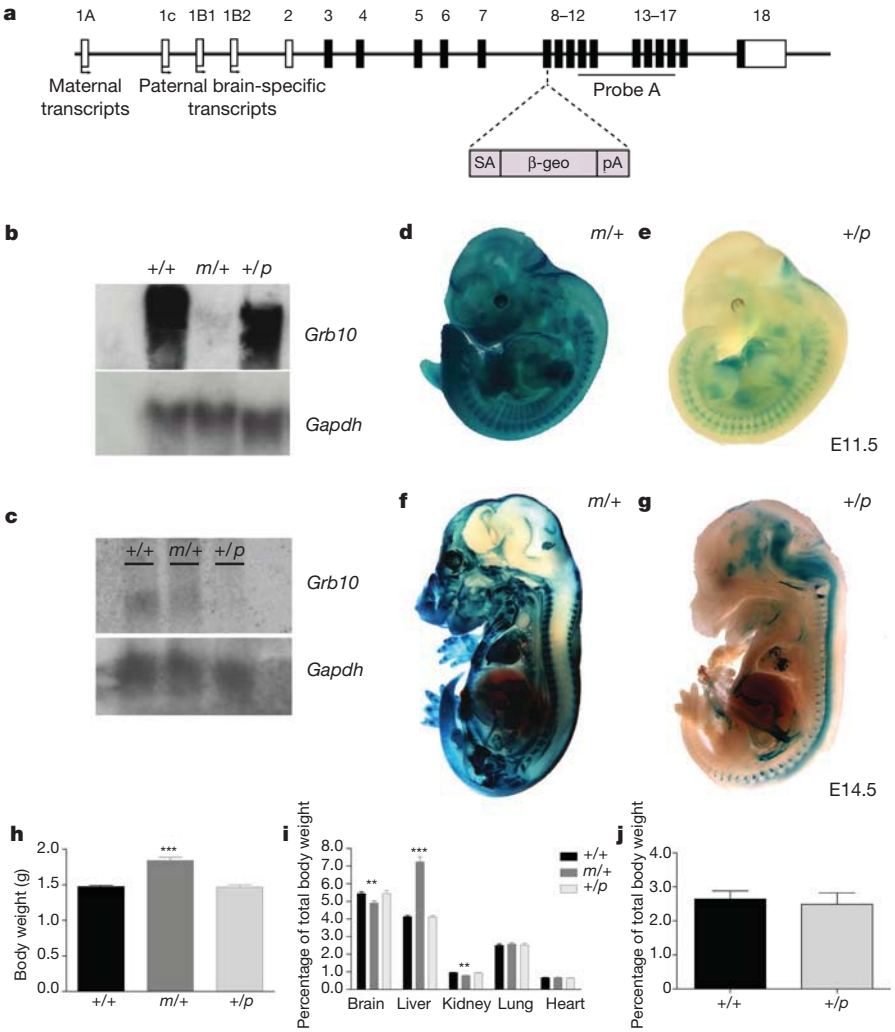


**Figure 1 | Generation and characterization of *Grb10KO* mutants.** **a**, The mouse *Grb10* locus showing the *LacZ:neomycin*$^{r}$ (*β-geo*) insertion in the *Grb10KO* allele (not to scale), showing transcriptional start-sites (arrows), numbered exons (boxes), translated regions (filled boxes) and heterologous splice acceptor (SA) and polyadenylation (pA) signals. **b**, Northern (RNA) blot analysis of *Grb10* expression in whole E14.5 embryos. *Grb10* transcripts (approximately 5.5 kilobases) were readily detected in wild-type (+/+) and *Grb10KO*$^{+/p}$ (+/*p*) but not *Grb10KO*$^{m/+}$ (*m*/+) samples. **c**, Northern blot analysis of *Grb10* expression in adult brain. *Grb10* transcripts were evident in wild-type (+/+) and *Grb10KO*$^{+/m}$ (*m*/+) but not *Grb10KO*$^{+/p}$ (+/*p*) samples. **d**–**g**, *LacZ* reporter expression in E11.5 (**d**–**e**) and E14.5 (**f**–**g**) heterozygous *Grb10KO* embryos showing opposite imprinting of the two parental alleles in different tissues. **h**, Neonatal body weight analysis revealed that *Grb10KO*$^{m/+}$, but not *Grb10KO*$^{+/p}$, neonates were overgrown compared with wild-type littermates on the day of birth ($F_{(2,90)} = 41.69$; $P < 0.001$). **i**, Analysis of dissected organs showed that growth enhancement in *Grb10KO*$^{m/+}$ neonates was proportional within most tissues, except the liver, which was disproportionately overgrown ($F_{(2,87)} = 118.60$; $P < 0.001$), and the kidneys and brain, which were spared. **j**, Stomach weights of wild-type and *Grb10KO*$^{+/p}$ neonates were not significantly different, suggesting that feeding was not impaired in the mutants ($t_{(36)} = 0.38$; $P = 0.70$). **h**–**j**, Values represent means ± s.e.m.

of stomach weights (Fig. 1j), suggested that suckling behaviour was normal.

*Grb10* expression in the adult brain was consistent with that observed during embryogenesis in being predominately paternally derived. Northern blot analysis demonstrated the presence of *Grb10* transcripts in the wild-type and *Grb10KO*$^{m/+}$ brains but not in the *Grb10KO*$^{+/p}$ brain (Fig. 1c), with no effects observed on expression of the adjacent *Ddc* gene (data not shown). Consistent with this, expression of the *LacZ* reporter was derived exclusively from the *Grb10KO*$^{+/p}$ allele (Fig. 2a–d and Supplementary Fig. 3). Histological analysis of *LacZ* expression in the adult *Grb10KO*$^{+/p}$ brain revealed a discrete pattern of paternal allele expression (Fig. 2k–m). Reporter expression was observed within thalamic, hypothalamic, midbrain and hindbrain nuclei, with no cortical expression detected at any point throughout the brain. Forebrain expression was also evident within the septal nuclei and specifically the cholinergic inter-neurons of the caudate putamen. Within the midbrain and hindbrain sites of expression included almost all monoaminergic cell populations (for a complete list of sites of paternal allele expression see Supplementary Table 1). *In situ* hybridization analysis of endogenous *Grb10* messenger RNA (mRNA) expression was in close accordance with the observed *LacZ* expression profile, indicating that it was not an artefact of reporter insertion (Fig. 2e–j and Supplementary Fig. 4). *Grb10* maternal allele expression is dramatically downregulated from late gestation and persists postnatally in only a subset of peripheral tissues[16]. Consistent with this, ependymal and choroid plexus epithelial expression observed in the embryo was no longer apparent in the *Grb10KO*$^{m/+}$ adult. *In situ* hybridization analysis of *Grb10KO*$^{+/p}$ brains revealed an almost complete absence of maternal *Grb10* expression. A low level of maternal allele expression was detected in a few brain regions, including the median preoptic nucleus, medial habenular, medial amygdaloid nuclei and ventromedial hypothalamus (Supplementary Fig. 4), therefore representing sites of biallelic *Grb10* expression. These sites within the brain mirror the situation outside the CNS where expression from

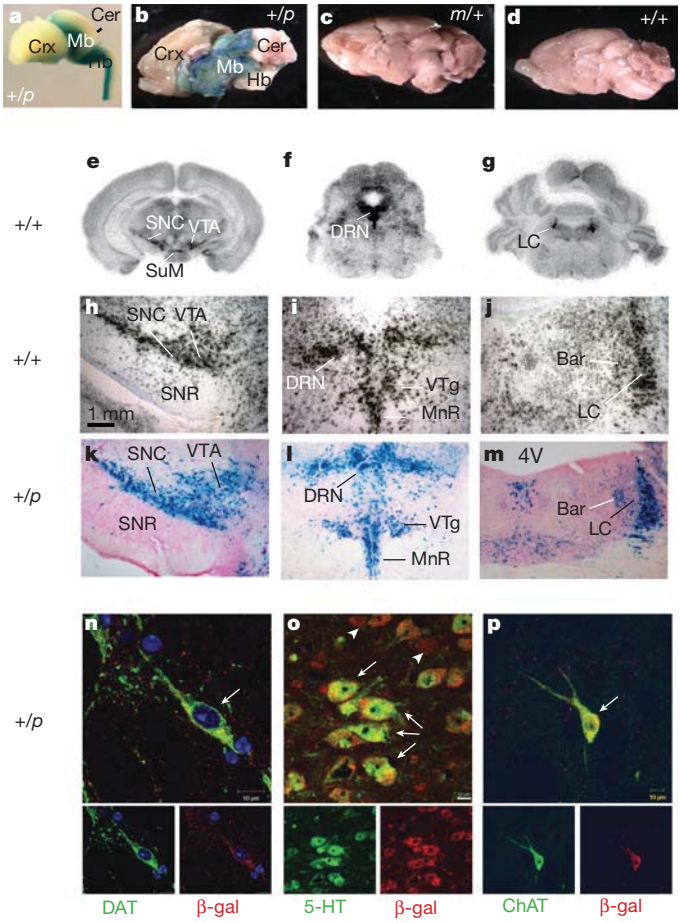


**Figure 2 | *Grb10* expression in the mouse brain.** **a**, A *Grb10KO*$^{+/p}$ brain dissected from an E14.5 embryo, showing *LacZ* reporter expression within the ventral midbrain (Mb), hindbrain (Hb) and ventral spinal cord, but not cortex (Crx) or cerebellum (Cer). **b**–**d**, Adult brains, bisected longitudinally, showing *LacZ* reporter expression, in *Grb10KO*$^{+/p}$ (**b**), but not *Grb10KO*$^{m/+}$ (**c**) and wild-type (**d**) samples, demonstrating exclusively paternal *Grb10* allele expression. **e**–**g**, *In situ* hybridization autoradiographs of endogenous *Grb10* mRNA expression (using probe A, see Fig. 1a) within the substantia nigra pars compacta (SNC), supramammillary nucleus (SuM), ventral tegmental area (VTA) (**e**), dorsal raphe nucleus (DRN) (**f**) and locus coeruleus (LC) (**g**) of the adult brain. **h**–**j**, Higher-resolution microscope images from hybridized slides dipped in photographic emulsion; positive signal (black grains) demonstrates cellular localization of *Grb10* mRNA at the level of **e**–**g**. **k**–**m**, *LacZ* expression from the paternal *Grb10* allele in the brain of *Grb10KO*$^{+/p}$ mice faithfully recapitulates endogenous *Grb10* expression as reported by *in situ* hybridization (in **h**–**j**; 4V indicates fourth ventricle). **n**–**p**, Histological sections of *Grb10KO*$^{+/p}$ adult brain showing co-localization of immunofluorescence staining for β-galactosidase (β-gal), expressed from the *Grb10* paternal allele, with markers specific for dopaminergic neurons (dopamine transporter; DAT) within the SNC (**n**), serotonergic neurons (serotonin; 5-HT) within the DRN (**o**) and cholinergic inter-neurons (choline acetyltransferase; ChAT) within the caudate putamen (**p**). Arrows indicate co-localized cells. Arrowheads indicate cells positive for β-galactosidase but negative for the neurochemical marker. SNR, substantia nigra pars reticulata; Bar, Barrington's nucleus; MnR, median raphe nucleus; VTg, ventral tegmental nucleus.

the maternal allele predominates, but there are also discrete sites of biallelic expression (Fig. 1e, g). Expression from the paternal allele in the CNS followed a pattern suggesting that expression established during embryogenesis was maintained into adulthood, as demonstrated by analysis of endogenous *Grb10* expression in *Grb10KO*$^{m/+}$ brains (Supplementary Fig. 4).

The distribution of *LacZ*-positive cells within expressing regions of the adult brain suggested expression from the paternal allele was predominantly neuron specific. Supporting this, immunofluorescent co-localization experiments performed within three discrete brain regions, the substantia nigra pars compacta, dorsal raphe nucleus and caudate putamen, demonstrated that paternal *Grb10* expression was detectable in dopaminergic (Fig. 2n), serotonergic (Fig. 2o) and cholinergic (Fig. 2p) neurons, respectively.

Despite having established a role for maternal *Grb10* as a major regulator of both fetal and placental growth[8,15] (Fig. 1 and Supplementary Fig. 2), we found no evidence of brain overgrowth in neonatal *Grb10KO*$^{+/p}$ mutant animals (Fig. 1i). Several genes show imprinted expression in the brain and knockout mouse studies have shown that some of these genes regulate specific behaviours. Notably, these include paternally expressed genes important for maternal nurturing of young (*Mest* and *Peg3*, reviewed in ref. 4), but also genes regulating other behavioural functions, including exploratory behaviour (maternally expressed *Nesp55* (ref. 17)) and circadian rhythm output (paternally expressed *Magel2* (ref. 18)). We therefore sought to assay *Grb10KO*$^{+/p}$-mutant animals by using standard tests of different behavioural parameters. In most of these assays *Grb10KO*$^{+/p}$ mice were essentially indistinguishable from wild-type littermate controls, including tests of anxiety-related behaviour, locomotor activity, olfaction and aggression (Supplementary Fig. 5). However, in an assay of social dominance in which a forced encounter was observed between two unfamiliar animals, using the tube test[19], *Grb10KO*$^{+/p}$ mutants were found to be significantly less likely to back down than their wild-type 'opponents' (Fig. 3a). This was not the case for *Grb10KO*$^{m/+}$ mice (Supplementary Fig. 5i). In the context of all of our behavioural testing, the outcome of the tube test was interpreted as a specific change in the behaviour of *Grb10KO*$^{+/p}$-mutant animals. Moreover, this behavioural change was found to correlate with observations of socially housed mice, where there was a significantly elevated incidence of facial barbering in cages containing at least one *Grb10KO*$^{+/p}$ mutant (Fig. 3b, c). Typically, these cages contained a single unbarbered *Grb10KO*$^{+/p}$ mutant (81% of cages), suggesting this animal was responsible for allogrooming of cage-mates. Consistent with this, isolation of barbered animals facilitated complete regrowth of missing hair and vibrissae. Barbering was observed in both male and female animals (Supplementary Fig. 5j). Allogrooming is regarded as a robust correlate of social dominance, as its assessment is independent of exogenous confounds[20]. Rigorous testing of additional aspects of social behaviour in *Grb10KO*$^{+/p}$ mice revealed no further differences in comparison with wild-type littermate controls (Fig. 3d, e). Specifically, habituation–dishabituation studies designed to probe aspects of social recognition, pertinent to the interpretation of the tube-test data, indicated that *Grb10KO*$^{+/p}$ animals reacted normally by exhibiting a general habituation to the olfactory cue, urine, followed by subsequent dishabituation when presented with a novel odour. Consistent with this outcome, *Grb10KO*$^{+/p}$-mutant mice exhibited normal olfactory responses when tested in their latency to investigate two different odours (Supplementary Fig. 5e, f).

Our study identifies *Grb10* as the first imprinted gene to have a role in the modulation of a specific social behaviour (as distinct from parental care). This function is predicted to be subject to the effects of intragenomic conflict within social groups when the two parental alleles are unequally represented, notably when there is sex bias in dispersal and/or variance in reproductive success[5,6]. In mice, as in other mammals[6],there is probably greater variance in reproductive success in males than in females and unequal representation is thus very likely. However, whether the association of the phenotypes with the sex-of-origin that we observe is consistent with the theory is unclear. For species such as humans, in which there is greater variation between males in reproductive success and (most probably) female dispersal, the involvement of paternally derived genes in promoting more altruistic behaviours is expected[6]. For mice, however, the necessary parameters are not well enough described to enable confident prediction as to whether paternal or maternal alleles should be the more 'altruistic'[6]. We note that our finding might also be considered consistent with the co-adaptation theory of imprinted gene evolution[21,22]. Similarly, the

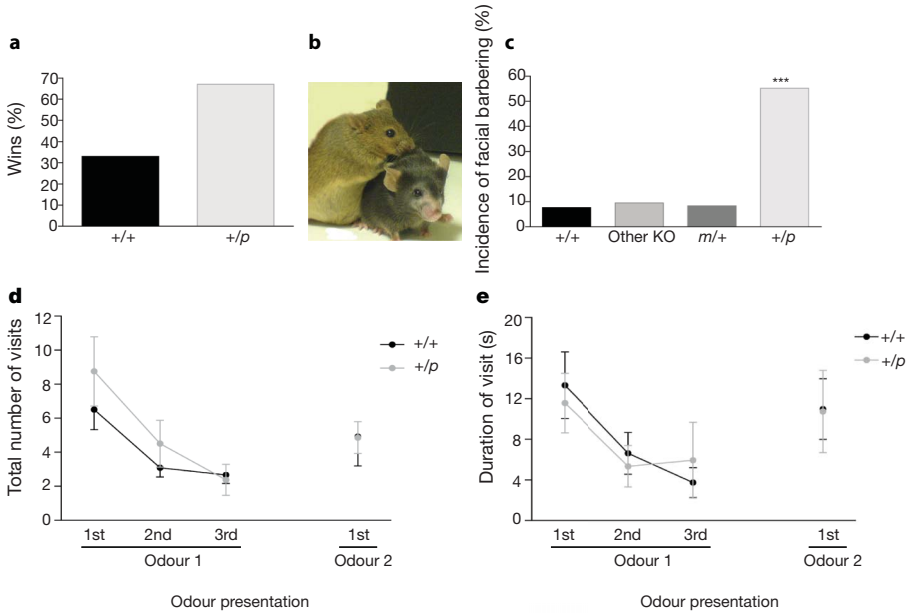


**Figure 3 | Increased social dominance in *Grb10KO*$^{+/p}$ mice.** **a**, Assessment of social dominance using the tube test revealed that *Grb10KO*$^{+/p}$ mutants were significantly more likely to prevail in a forced encounter than wild-type controls ($P < 0.05$, significance determined by non-parametric Monte Carlo permutation test at the individual level: see Methods for details). **b**, A typical example of a barbered animal (black mouse), pictured with an unbarbered cage-mate. **c**, The incidence of facial barbering within cages containing one or more *Grb10KO*$^{+/p}$ (+/*p*) mutants was significantly greater than in control cages containing wild-type animals (+/+), other genetically modified strains (Other KO) or *Grb10KO*$^{m/+}$ mutants (*m*/+) ($\chi^2 = 24.86$; $P < 0.001$). **d**, **e**, Assessment of social recognition used a urinary odour habituation–dishabituation test. *Grb10KO*$^{+/p}$ mutants were indistinguishable from wild-type controls in the number of visits made to the odour source (**d**; ANOVA, no effect of genotype $F_{(1,10)} = 0.513$, $P = 0.49$) and duration of these investigations (**e**; ANOVA, no effect of genotype $F_{(1,10)} = 0.011$, $P = 0.92$). Mutant and control animals exhibited the significant but comparable increases in both measures on presentation of a second novel odour (visits, $P = 0.015$; duration, $P = 0.032$). **d**, **e**, Values represent means ± s.e.m.

effect of *Grb10* on placental growth[8,15] is potentially consistent both with the parental conflict and co-adaptation theories.

It has also been suggested that differences in parental genome representation within social groups could engender differential tolerance to risk-taking behaviours[4]. Tempering of socially dominant behaviour can be viewed as a risk-averse phenotype aimed at maximizing reproductive success by avoiding the potentially detrimental consequences of challenging for social status. Expression of *Grb10* within several monoaminergic nuclei may be relevant to the possible underlying mechanism, as cerebrospinal fluid levels of serotonin and dopamine metabolites have been independently correlated with dominant/submissive behaviour[23,24]. However, no changes in the levels of dopamine, serotonin, noradrenalin and acetylcholine (and associated metabolites) were detected from macro-dissected brain regions of *Grb10KO*$^{+/p}$ mice (Supplementary Fig. 6). The imprinted *Nesp55* gene has been associated with the promotion of risk tolerance, notable because *Nesp55* is expressed from the maternal allele within discrete brain regions that overlap sites of *Grb10* paternal allele expression, including the serotonergic raphe nucleus and noradrenergic neurons of the locus coeruleus[4,17]. This raises the possibility that these two genes might represent antagonistic components within the same neurological systems. Moreover, a recent genome-wide screen has indicated that over 1,300 loci could be subject to parent-of-origin allelic expression bias within the mouse brain[13], suggesting the influence of genomic imprinting within the brain may be much greater than previously thought. However, verification of this will require extensive validation of allelic expression bias together with functional testing of the identified genes.

Many imprinted genes are found in clusters that can contain genes expressed from either parental allele as well as non-imprinted genes[25]. However, the demonstration of opposite imprinting within a single mouse gene, most likely conserved in humans[26], represents a highly provocative situation, whereby the two parental alleles of *Grb10* have evolved distinct patterns of imprinted expression according to their functions in different tissues.

## METHODS SUMMARY

***Grb10KO* mice.** Chimaeric animals were generated by microinjection of a gene-trap ES cell line (XC302; Baygenomics) into $F_2$ (C57BL/6 × CBA) strain blastocysts, using standard methods[27]. Mice were maintained on a C57BL/6:CBA mixed genetic background and kept as previously described[28]. Behavioural phenotyping and statistical methods are detailed in Methods.

**Northern blot analysis.** Total RNA was extracted using TRI Reagent (Sigma Aldrich), with 20–50 μg run on denaturing agarose gels and transferred to a nylon membrane for hybridization with a Grb10-specific radiolabelled probe[8].

***In situ* hybridization.** Adult brain tissue was collected from animals transcardially perfused with 4% paraformaldehyde, cryoprotected in 20% sucrose and sectioned at 30 μm on a freezing microtome. Tissue was processed for *in situ* hybridization[29] and a [$^{35}$S]riboprobe specific to exons 11–16 of the mouse *Grb10* mRNA sequence was used to detect endogenous Grb10 expression.

***LacZ* expression analysis.** Dissected embryos were fixed in 2% formaldehyde/0.2% glutaraldehyde in PBS for 2 h at room temperature, stained at 37 °C for approximately 2 h in freshly prepared X-gal solution, post-fixed overnight at 4 °C using 4% paraformaldehyde in PBS, then cleared in 80% glycerol. Adult brains were longitudinally bisected and stained, as above, without fixation. For adult brain sections, animals were first perfused with chilled 9.25% (w/v) sucrose solution, followed by approximately 100 ml of chilled 3% paraformaldehyde in 0.1% PBS. Brains were sectioned at 50 μm on a vibratome (VT1000S; Leica), with the tissue kept ice-cold. Free-floating sections were collected and immersed in X-gal staining solution at 28 °C overnight. *Grb10KO* samples were coetaneously stained alongside wild-type controls.

**Immunofluorescent analysis of adult brain sections.** Brain sections (50 μm) were collected from animals perfused with 4% paraformaldehyde and the sections post-fixed in 4% paraformaldehyde at 4 °C overnight before antibody staining, as described[30].

**Full Methods** and any associated references are available in the online version of the paper at www.nature.com/nature.

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**Supplementary Information** is linked to the online version of the paper at www.nature.com/nature.

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**Author Contributions** A.W. and A.S.G. conceived the project and interpreted the data, with input from L.D.H., A.R.I. and L.S.W.; K.M. and J.E.S.-C. generated the *Grb10KO* mice; A.S.G. performed most of the experiments with contributions from M.C., J.W.D., S.B., K.G., A.R.I., F.M.S., J.X. and A.W.; A.S.G. and A.W. jointly wrote the manuscript.

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## METHODS

***Grb10KO* mice.** Chimaeric animals were generated by microinjection of a gene-trap ES cell line (XC302; Baygenomics) into $F_2$ (C57BL/6 × CBA) strain blastocysts, using standard methods[27]. Mice were maintained on a C57BL/6:CBA mixed genetic background and kept as previously described[28]. We note that *Grb10KO* mice were derived using the XC302 gene-trap ES cell line and are distinct from our previously published *Grb10Δ2–4* model[8]. The XC302 *β-geo* gene-trap insertion has replaced 12 base pairs (bp) of endogenous *Grb10* sequence (the 3′ terminal 11 bp from exon 8 plus the 5′-most 1 bp from intron 8; data not shown). The mutant allele might, therefore, express the amino (N)-terminal 248 amino acids of Grb10 that would include two of the five recognized domains of the Grb10 adaptor protein (the Ras-association-like and proline-rich domains). Using an antibody raised to the N terminus of Grb10, we have been unable to detect any novel protein products in *Grb10KO* tissue (we examined protein from E14.5 embryo and placenta when Grb10 expression is strong and widespread; data not shown), either in the form of a distinct short peptide or a long *Grb10:β-geo* fusion product. *Grb10KO* mice were derived in addition to our previously published *Grb10Δ2-4* model[8] which did not recapitulate fully the expression profile of endogenous *Grb10*, most likely because of the loss of a brain-specific enhancer element within a 36-kilobase deletion mapped to the *Grb10Δ2-4* allele (ref. 8 and M.C., A.S.G. and A.W., unpublished observations).

**Mouse genotyping and weight analyses.** $Grb10KO^{m/+}$ and $Grb10KO^{+/p}$ study animals were generated separately by transmission of the *Grb10KO* locus from heterozygous females and males, respectively. Animals were genotyped by PCR using primers specific to the *β-geo* insertion (βgeoF2 5′-CCGAGCGAAAACGG TCTGCG-3′ and βgeoR2 5′-CTTCCGCTTAGTGACAACG-3′). Analysis of neonatal body and dissected organ weights was performed on postnatal day 1 ($Grb10KO^{+/+}$ $n = 43$; $Grb10KO^{m/+}$ $n = 23$; $Grb10KO^{+/p}$ $n = 27$), as was analysis of stomach weight ($Grb10KO^{+/+}$ $n = 23$; $Grb10KO^{+/p}$ $n = 16$).

**Probes for northern blot analysis.** Total RNA was extracted using TRI Reagent (Sigma Aldrich), with 20–50 μg run on denaturing agarose gels and transferred to a nylon membrane for hybridization with a radiolabelled Grb10 probe. Grb10 probes were generated from mouse complementary DNA (cDNA) by PCR with reverse transcription using primers specific to *Grb10* (probe A, Fig. 1) (F, 5′-CTG ACCTGGAAGAAAGCAGC-3′ and R, 5′-GATCCTGTGAGACTCCTCGC-3′). The Grb10 probe corresponds to nucleotides 1,353–1,858 of the mouse *Grb10* gene (accession number NM_010345). Purified PCR products were cloned into pGEM-T Easy (Promega), excised from the vector backbone and gel-purified. Isolated probes were labelled with [α-$^{32}$P]dCTP and hybridized to blots, as previously described[8].

***In situ* hybridization.** Adult brain tissue was collected from animals transcardially perfused with 4% paraformaldehyde, cryoprotected in 20% sucrose and sectioned at 30 μm on a freezing microtome. Tissue was processed for *in situ* hybridization as previously described[29]. A [$^{35}$S]riboprobe specific to exons 11–16 of the mouse *Grb10* mRNA sequence was used to detect endogenous Grb10 expression (probe A, Fig. 1). The Grb10 riboprobe was synthesized by PCR using cDNA obtained from normal mouse brain and was the same as described above. The recombinant plasmid was linearized by restriction digest and subjected to *in vitro* transcription with a T7 RNA polymerase (antisense) or SP6 RNA polymerase (sense) in the presence of $^{35}$S-labelled UTP, according to the manufacturer's instructions (Ambion). The [$^{35}$S]Grb10 riboprobe was diluted to $2 \times 10^7$ c.p.m. ml$^{-1}$ in a hybridization solution composed of 50% formamide, 20 mM Tris-HCl pH 7.5, 0.02% sheared single-stranded DNA (Sigma), 0.1% total yeast RNA (Sigma), 0.01% yeast tRNA (Gibco), 20% dextran sulphate, 0.3 M NaCl, 2 mM EDTA pH 8.0, Denhardt's solution (Sigma), 100 mM DTT, 0.2% SDS and 0.2% sodium thiosulphate (Sigma). Before hybridization, brain sections were fixed in 4% formaldehyde in DEPC-treated PBS for 20 min at 4 °C, dehydrated in ascending concentrations of ethanol, cleared in xylene for 15 min, rehydrated in descending concentrations of ethanol and permeabilized by heating in prewarmed sodium citrate buffer (95–100 °C, pH 6.0), before being dehydrated in ascending concentrations of ethanol, and air-dried. Hybridization solution (containing radiolabelled riboprobe) and a coverslip were applied to each slide, and sections were incubated for 12–16 h at 57 °C. After this time the coverslips were removed, and slides were washed with 2× sodium chloride/sodium citrate buffer (SSC). Sections were then incubated in 0.002% RNase A (Qiagen) for 30 min, followed by sequential washes in decreasing concentrations of SSC. The sections were dehydrated in ascending concentrations of ethanol with 0.3 M ammonium acetate ($NH_4OAc$) followed by 100% ethanol. Slides were air-dried and placed in X-ray film cassettes with BMR-2 film (Kodak) for 72 h. Films were developed on an OPTIMAX X-ray film processor (Protec). Slides were subsequently dipped in photographic emulsion (GE Healthcare) and stored at 4 °C for 2 weeks before being developed in D-19 developer and fixer (Kodak).

***LacZ* expression analysis.** Dissected embryos were fixed in 2% formaldehyde/0.2% glutaraldehyde in PBS for 2 h at room temperature, stained at 37 °C for approximately 2 h in freshly prepared X-gal solution[31], post-fixed overnight at 4 °C using 4% paraformaldehyde in PBS, then cleared in 80% glycerol. Adult brains were longitudinally bisected and stained, as above, without fixation. For adult brain sections, animals were first perfused with chilled 9.25% (w/v) sucrose solution, followed by approximately 100 ml of chilled 3% paraformaldehyde in 0.1% PBS. Brains were sectioned at 50 μm on a vibratome (VT1000S; Leica), with the tissue kept ice-cold. Free-floating sections were collected and immersed in X-gal staining solution at 28 °C overnight. *Grb10KO* samples were coetaneously stained alongside wild-type controls.

**β-galactosidase immunohistochemistry.** Free floating 50-μm sections from *Grb10KO* mice transcardially perfused with 4% paraformaldehyde were processed for immnohistochemistry analysis of β-galactosidase expression. Tissue was washed extensively in PBS and endogenous peroxidases quenched with a 30 min 0.3% $H_2O_2$ incubation. Sections were blocked in 10% normal donkey serum, 0.5% BSA and 0.5% Tirton-X 100 in PBS for 1 h at room temperature. The β-galactosidase antibody (α-rabbit, Cappel) was diluted 1/10,000 in antibody buffer (1% normal donkey serum, 0.5% BSA and 0.5% Triton-X 100 in PBS) overnight at 4 °C. Sections were washed in PBS and a biotinylated donkey anti-rabbit secondary antibody (Vector Laboratories) applied at 1/1,000 in antibody buffer for 1 h at room temperature. Sections were then washed in PBS and incubated for 1 h at room temperature in VectaStain ABC Reagent (Vector Laboratories) according to the manufacturer's instructions. Chromogenic detection of positive labelling was conducted using 3,3′-diaminobenzidine (DAB) reagent (Vector Laboratories) according to the manufacturer's instructions. Sections were then rinsed extensively in PBS, mounted onto glass microscope slides, dehydrated along an ascending series of alcohol, cleared in xylene and coverslipped for viewing.

**Immunofluorescence histochemistry.** Brain sections (50 μm) were collected from animals perfused with 4% paraformaldehyde and the sections post-fixed in 4% paraformaldehyde at 4 °C overnight before antibody staining, as described[30]. Adult brain sections were processed as described above with the exclusion of the hydrogen peroxide step. Primary antibodies were diluted as follows in antibody buffer: β-galactosidase (α-rabbit, 1/500, Cappel), choline-acetyltransferase (α-mouse, 1/1,000)[32], dopamine transporter (α-rat, 1/1000, Abcam), serotonin (1/1,000, Immunostar). Secondary antibodies (Alex Fluor, Molecular Probes) were diluted to 1/750 in antibody buffer and applied to the tissue for 1 h. Sections were then immediately viewed and images captured under a confocal microscope (Zeiss, LSM-510).

**Behavioural phenotyping.** Male wild-type and $Grb10KO^{+/p}$ littermates between the ages of 10 and 12 months and $Grb10KO^{m/+}$ at 3 months were tested. We note that although $Grb10KO^{m/+}$ mice are a good genetic control for $Grb10KO^{+/p}$ mice, their altered body size and proportions may confound behavioural comparisons with wild-type and $Grb10KO^{+/p}$ littermates. Thus wild-type littermates were the preferred controls in most tests and the use of younger $Grb10KO^{m/+}$ mice allowed body size to be better matched across all of the tested genotypes. Unless otherwise stated, tests were performed during daylight hours using socially housed animals that were removed from their home cages, and therefore isolated, only for the duration of each test. Where possible, tests and subsequent analyses were performed blind for animal genotype.

Light/dark box. Dimensions were 46 cm × 27 cm × 30 cm (length × breadth × height) with the dark compartment comprising one-third of the length. Animals were placed in the light compartment facing away from the dark side and allowed to explore freely for 10 min ($Grb10KO^{+/+}$ $n = 16$; $Grb10KO^{+/p}$ $n = 16$).

Open field test. Arena diameter was 76 cm, with eight central and eight peripheral segments demarked with non-tactile paint. Animals were individually placed in the same segment facing towards the centre and allowed to explore freely for 10 min ($Grb10KO^{+/+}$ $n = 15$; $Grb10KO^{+/p}$ $n = 16$).

Locomotor activity (beam-break apparatus). Animals were socially isolated and allowed to acclimatize to test cages/apparatus (Linton Instrumentation) for 1 week before analysis. Daytime (08:00–18:00) and night-time (19:00–05:00) readings were made on three consecutive days with data recorded in 5 min bins by a computer running AmLogger software (Linton Instrumentation; $Grb10KO^{+/+}$ $n = 15$; $Grb10KO^{+/p}$ $n = 15$).

Assessment of olfaction. Small squares of filter paper soaked with 10 μl of vanilla extract or urine (collected from four 8-week-old $F_1$ females and pooled) were attached to one corner of the 14 test cages at a height that required animal rearing to be reached. Socially housed animals were placed in the test cage and allowed to explore freely for 2 min ($Grb10KO^{+/+}$ $n = 8$; $Grb10KO^{+/p}$ $n = 8$). The time for each animal to touch its snout to the filter paper was recorded.

Resident–intruder test. Socially isolated $Grb10KO^{+/p}$ animals (residents) were confronted with the introduction of a weight matched, socially housed wild-type male of the same genetic background (intruders). Each $Grb10KO^{+/p}$ mutant was

assessed against three different wild-type animals on three consecutive days. Animals were allowed to explore freely for 10 min ($Grb10KO^{+/+}$ $n = 8$; $Grb10KO^{+/p}$ $n = 8$).

Tube test. Test apparatus comprised a 30-cm smooth, transparent acrylic tube with an internal diameter of 3.5 cm. Socially isolated *Grb10KO* animals were challenged with at least three different socially isolated wild-type animals on consecutive days. Animals were placed at opposite ends of the tube and released simultaneously. Losers were scored as those animals that retreated from the tube; a full retreat was determined by the absence of any paws within the tube. Tests in which crossing over or turning of animals occurred were not scored ($Grb10KO^{+/+}$ $n = 7$; $Grb10KO^{+/p}$ $n = 8$). Light/dark box, open field and resident–intruder tests were videotaped for analysis.

Urinary odour habituation–dishabituation test. The test protocol was adapted from Isles *et al.*[33] and took place in a Perspex testing arena divided into two equal-sized chambers and linked by a small opening. The arena was lit in low-level white light (15 lux). Male animals were habituated to the whole arena for 10 min ($Grb10KO^{+/+}$ $n = 12$; $Grb10KO^{+/p}$ $n = 8$). The test period was divided into four separate 5-min trials, with a gap of 2 min between each trial during which the test animals were placed in a separate holding cage. During a trial, 15 µl of urine was applied to a disc of filter paper in one of the two chambers (alternated between subjects). This was secured to the arena floor with a 4-cm plastic washer, but the test animal could have contact with the urine. Contact with the urine was deemed important as both volatile and involatile urinary cues are thought to be important for recogntion[34]. The animal was placed in the chamber not containing the urine, and the time an animal spent in either chamber and investigated the odour cue itself was measured using Ethovision software (Noldus) linked to a digital camera. Several other measures indicative of locomotor activity were also obtained. In trials 1–3 the same odour cue was presented (habituation); however, in trial 4, urine from a different individual animal, of differing background strain, was presented (dishabituation). The urine was collected from individual female mice, and within any given test the two odour cues were matched for oestrus cycle, which was determined by vaginal smear[33].

**Neurochemical analysis.** Animals were killed by exposure to a rising concentration of carbon dioxide and cervical dislocation ($Grb10KO^{+/+}$ $n = 5$; $Grb10KO^{+/p}$ $n = 5$). The brains were rapidly removed and dissected according to four key regions of interest: the frontal cortex, striatum, hypothalamus and brainstem. Tissue aliquots were derived from both hemispheres and homogenized in 200 µl of 0.2 M perchloric acid by an ultrasonic cell disruptor (Microson). Levels of noradrenaline, dopamine, 5-hydroxytryptamine and acetylcholine and associated metabolites (DOPAC and 5HIAA) were determined in the supernatant by reversed-phase, high-performance liquid chromatography (HPLC), as described previously[35]. Levels of ACh were assessed by microbore HPLC with an acetlcholinesterase/choline oxidase IMER and a wired peroxidase glassy carbon electrode[36].

**Statistical analyses.** Body and organ weights (Fig. 1h, i) were analysed using one-way ANOVA with Tukey's post-hoc test. Stomach weights (Fig. 1j), HPLC analysis of neurotransmitter levels (Supplementary Fig. 6) and most of the behavioural tests (Supplementary Fig. 1a–h) were analysed using the two-tailed Student's *t*-test. *Grb10KO* survival and whisker barbering were analysed using $\chi^2$ tests.

Non-parametric statistical analysis assessed the significance of winning tendency in the tube test (Fig. 3a) and was based on assigning a +1 value for animals that won more than 50% of their bouts, −1 for animals that lost more than 50% of their bouts and 0 for those animals that won as many as they lost. Next, the net effect of each genotype was determined by addition of the individual scores ($+/+ = -4$, $+/p = +4$) and the difference between the genotypes calculated from the difference between these summed values (+8). The same number of animals tested in reality ($Grb10KO^{+/+}$ $n = 7$, $Grb10KO^{+/p}$ $n = 8$) was used to run 100,000 randomizations so as to estimate significance of the observed data, by estimating how commonly, if the null of no difference between genotypes was correct, we would expect to see a deviation as large as or larger than that observed. In each randomization the number of winners, losers and neutrals was held constant, the same as in the observed data, with the first seven results assigned to a pseudo-wild-type class and the last eight to a pseudo-$Grb10KO^{+/p}$ class. We asked, across these 100,000 randomizations, whether a difference of +8 or greater was seen between the summed genotypic scores. Three thousand one hundred and thirty-seven randomizations with a difference of +8 or greater were observed. If this value is assigned as $r$ and $N$ is the number of randomizations (in this case 100,000), then the unbiased estimator of $P = r+1/N+1$.

For the urinary odour habituation–dishabituation test (Fig. 3d, e), a repeated-measure ANOVA determined an interaction between odour and genotype.


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