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

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Abstract

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 post-natal 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 to date no such instance is known. Predominantly expressed from the maternal allele during embryogenesis, *Grb10* encodes an intracellular adapter protein that can interact with a number of 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 to date a unique imprinted gene, able to influence distinct physiological processes, fetal growth and adult behaviour, due to actions of the two parental alleles in different tissues.

To characterise 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 (Figure 1a). Transmission of the *Grb10KO* allele separately through the two parental lines generated heterozygous progeny in which either

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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 (Figure 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 the majority of *Grb10* expression is maternally derived (e.g. Ref [8]). We next conducted more refined *in situ* analyses of allele-specific expression, utilising 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 (Figure 1d, f). At e14.5 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] (Supplemental Figures 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 (Figures 1e, g, 2a and Supplemental Figures 1c, d). The CNS expression commences 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 Figure 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 (Figure 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. A number of 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 (Figure 1h,i and Supplementary Figure 2). At birth, the mean body weight of *Grb10KO*$^{m/+}$ pups was 25±2.5% greater than that of wild type littermates. The liver was disproportionately enlarged (117±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 (Figure 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 (χ^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 prior to weaning, including analysis of stomach weights (Figure 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 (Figure 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 (Figure 2a-d and Supplemental Figure 3). Histological analysis of *LacZ* expression in the adult *Grb10KO*$^{+/p}$ brain revealed a discrete pattern of paternal allele expression (Figure 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-neurones 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* hybridisation analysis of endogenous *Grb10* mRNA expression was in close accordance with the observed *LacZ* expression profile, indicating that it was not an artefact of reporter insertion (Figure 2e-j and Supplemental Figure 4). *Grb10* maternal allele expression is dramatically downregulated from late gestation and persists post-natally 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* hybridisation 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 small number of brain regions, including, the median preoptic nucleus, medial habenular, medial amygdaloid nuclei and ventromedial hypothalamus (Supplemental Figure 4), therefore representing sites of biallelic *Grb10* expression. These sites within the brain mirror the situation outside the CNS where expression from the maternal allele predominates, but there are also discrete sites of biallelic expression (Figure 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 (Supplemental Figure 5).

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 colocalisation experiments carried out within three discrete brain regions, substantia nigra pars compacta, dorsal raphe nucleus and caudate putamen, demonstrated that paternal *Grb10* expression was detectable in dopaminergic (Figure 2n), serotonergic (Figure 2o) and cholinergic (Figure 2p) neurons, respectively.

Despite having established a role for maternal *Grb10* as a major regulator of both fetal and placental growth[8], [15] (Figure 1 and Supplementary Figure 2), we found no evidence of brain overgrowth in neonatal *Grb10KO*$^{+/p}$ mutant animals (Figure 1i). A number of 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*; [17]) and circadian rhythm output (paternally expressed *Magel2*; [18]). We therefore sought to assay *Grb10KO*$^{+/p}$ mutant animals 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 Figure 5). However, in an assay of social dominance in which a forced encounter was observed between two unfamiliar animals, using the tube-test paradigm[19], *Grb10KO*$^{+/p}$ mutants were found to be significantly less likely to back down than their wild-type "opponents" (Figure 3a). This was not the case for *Grb10KO*$^{m/+}$ mice (Supplemental Figure 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 made of socially housed mice, where there was a significantly elevated incidence of facial barbering in cages containing at least one *Grb10KO*$^{+/p}$ mutant (Figure 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 cages (Supplemental Figure 5j). Allogrooming is regarded to be 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 (Supplemental Fig 4e, 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 intra-genomic 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 are 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 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 maximising reproductive success by avoiding the potentially detrimental consequences of challenging for social status. Expression of *Grb10* within a number of 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 (Supplemental Figure 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 1300 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, although 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

Chimeric animals were generated by microinjection of a gene-trap ES cell line (XC302; Baygenomics, California, USA) into F2 (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 the online Methods section.

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 hybridisation with a Grb10 specific radiolabelled probe[8].

In situ hybridisation

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* hybridisation[29] and a [^{35}S] radiolabelled 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 hours at room temperature, stained at 37°C for approximately 2 hours 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

50 μm brain sections were collected from animals perfused with 4% paraformaldehyde and the sections post-fixed in 4% paraformaldehyde at 4°C overnight prior to antibody staining, as described[30].

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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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 carried out the bulk 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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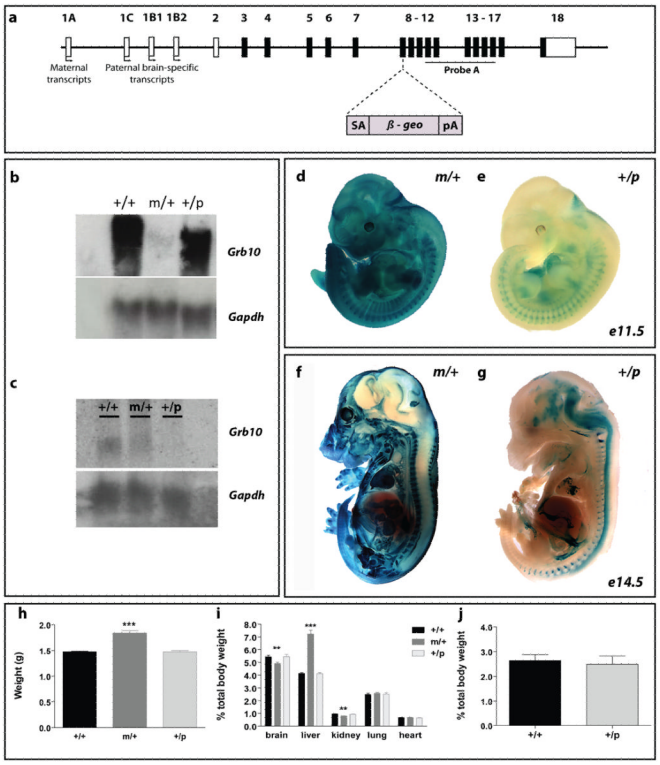

Figure 1. Generation and characterisation of *Grb10KO* mutants

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 (approx 5.5Kb) 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 to 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 ($t(36) = 0.38$; $p = 0.70$).

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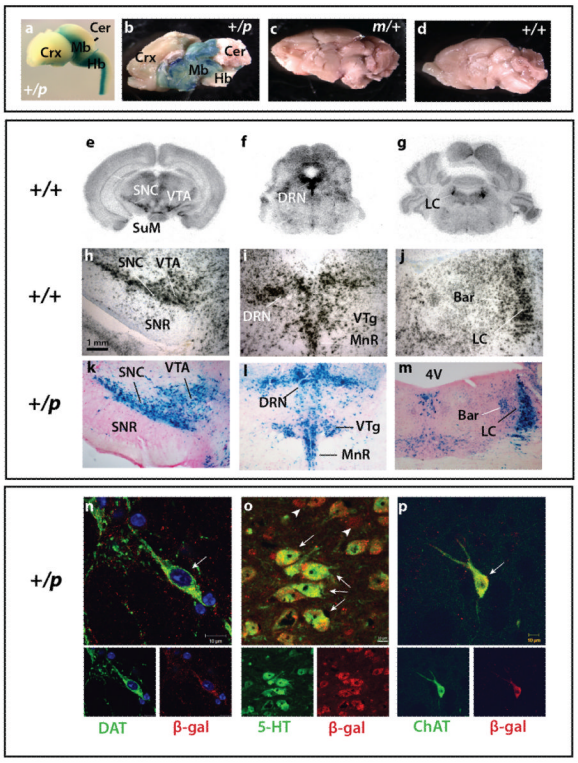

Figure 2. *Grb10* expression in the mouse brain

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* hybridisation 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 hybridised slides dipped in photographic emulsion; positive signal (black grains) demonstrates cellular localisation of *Grb10* mRNA at the level of figures 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* hybridisation (in h-j; 4V indicates fourth ventricle). **n-p**, Histological sections of *Grb10KO*$^{+/p}$ adult brain showing colocalisation of immunofluorescence staining for β-galactosidase, expressed from the *Grb10* paternal allele, with markers specific for dopaminergic neurones, (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 colocalised cells. Arrow heads indicate cells positive for β-gal but negative for the neurochemical marker. Abbreviations: 4V, fourth ventricle; SNR, substantia nigra pars reticulate; Bar, Barrington's nucleus; SuM, supramammillary nucleus; MnR, median raphe nucleus; VTg, ventral tegmental nucleus.

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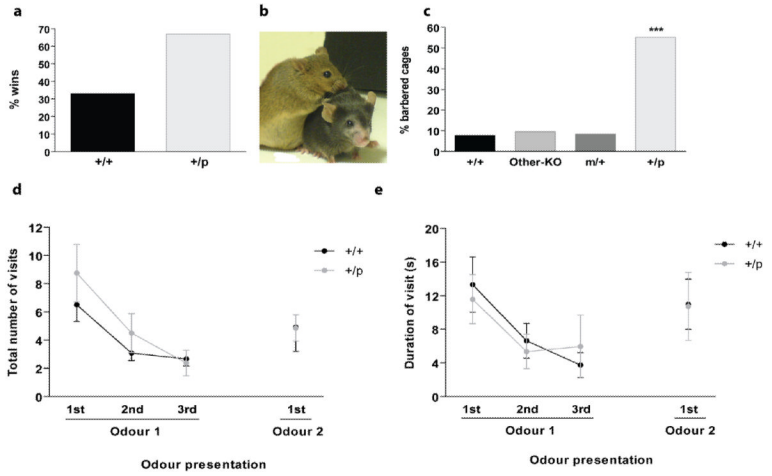

Figure 3. Increased social dominance in *Grb10KO*$^{+/p}$ mice

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 were wild type controls ($p < 0.05$, significance determined by non-parametric Monte Carlo permutation test at the individual level: see supplementary 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) mutant 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 was conducted using a urinary odour habituation-dishabituation test. *Grb10KO*$^{+/p}$ mutants were indistinguishable from wild type controls in regard to 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, $p = 0.032$).

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