Wild-type microglia do not reverse pathology in mouse models of Rett syndrome

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Rett syndrome is a severe neurodevelopmental disorder caused by mutations in the X chromosomal gene *MECP2* (ref. 1), and its treatment so far is symptomatic. *Mecp2* disruption in mice phenocopies major features of the syndrome[2] that can be reversed after *Mecp2* re-expression[3]. Recently, Derecki *et al.*[4] reported that transplantation of wild-type bone marrow into lethally irradiated *Mecp2*-null

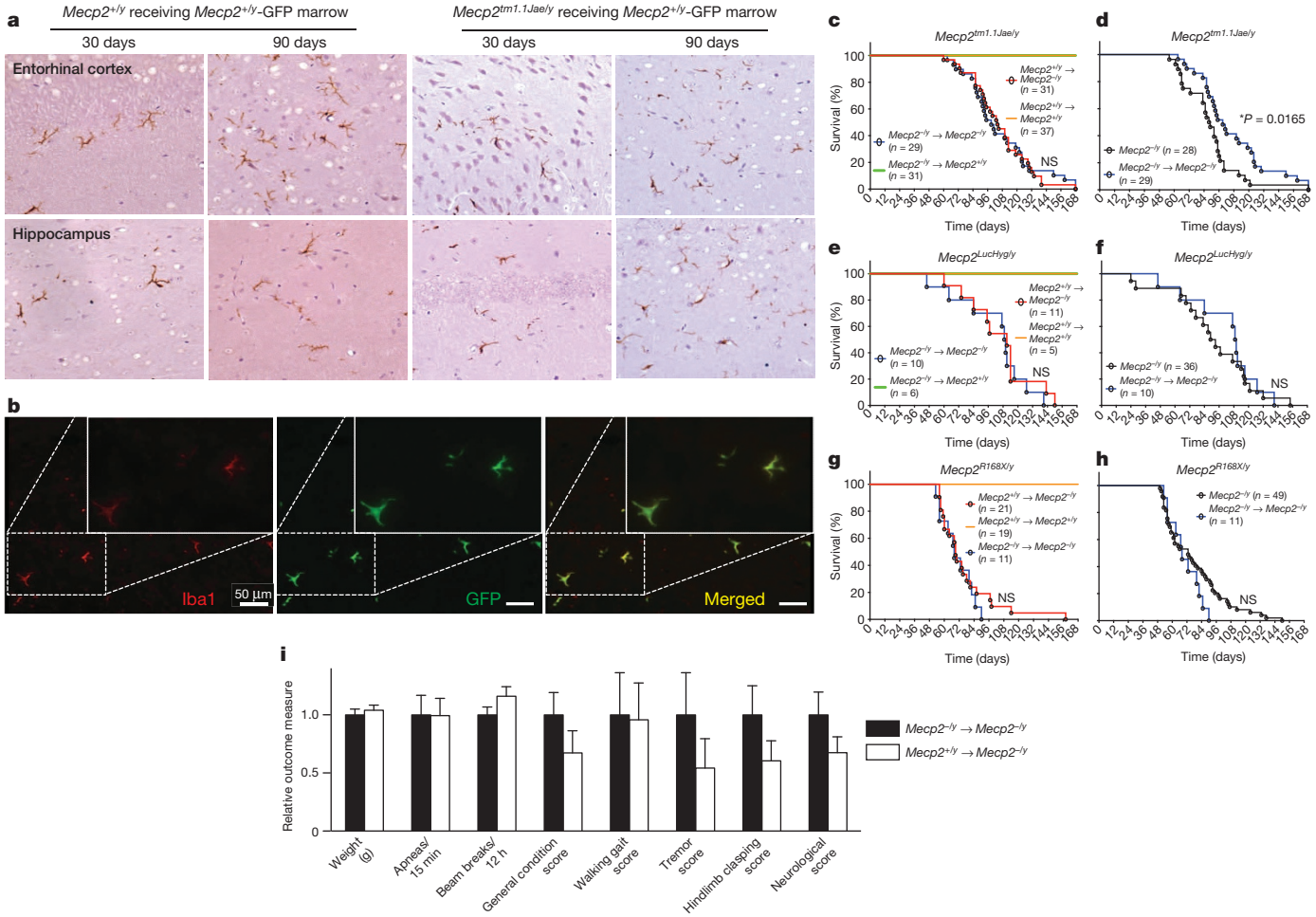

Figure 1 | Early transplantation of wild-type microglia into the brain does not rescue *Mecp2*-null mice. a, Transplantation of bone marrow from C57BL/6 ($Mecp2^{+/y}$-GFP marrow) mice with ubiquitous GFP transgene expression into $Mecp2^{tm1.1Jae/y}$ mice confers robust donor engraftment at the indicated time after transplant (30 or 90 days) shown via immunohistochemical detection of GFP-positive cells of microglial morphology in entorhinal cortex and hippocampus, in both the $Mecp2^{+/y}$-GFP donors to $Mecp2^{tm1.1Jae/y}$ recipient (right panels) and to $Mecp2^{+/y}$ recipient (left panels) groups. Original magnification, ×400. **b**, Double immunofluorescent labelling with GFP and the microglia marker Iba1 in cerebellar tissue from $Mecp2^{+/y}$-GFP donors to $Mecp2^{tm1.1Jae/y}$ recipient mice examined 30 days after BMT confirms early microglial engraftment in brain parenchyma. Quantification of microglial engraftment is shown in Extended Data Fig. 1c. **c**–**h**, No differences in survival were noted between transfer of $Mecp2^{+/y}$ to *Mecp2*-null ($Mecp2^{-/y}$) mice and of *Mecp2*-null to *Mecp2*-null mice, indicating that engraftment of wild-type microglia into the brains of *Mecp2*-null mice did not protect *Mecp2*-null mice from premature death. In addition, no differences in survival were noted between transfer of *Mecp2*-null to $Mecp2^{+/y}$ mice and of $Mecp2^{+/y}$ to $Mecp2^{+/y}$ mice, indicating that engraftment of *Mecp2*-null microglia into the brains of wild-type mice does not shorten survival as seen in *Mecp2*-null mice. NS, not significant. **i**, No differences were seen in other outcome measures at 12 weeks of age (8 weeks after BMT) between $Mecp2^{+/y}$ to $Mecp2^{tm1.1Jae/y}$ (also termed $Mecp2^{-/y}$) mice ($n = 31$) and $Mecp2^{tm1.1Jae/y}$ to $Mecp2^{tm1.1Jae/y}$ mice ($n = 25$), including weight, frequency of breathing apneas, locomotor activity (beam breaks), general condition, walking gait, tremor, hindlimb clasping or neurological score. Here, data are presented as relative outcome measure (mean and s.d. for each measure were calculated, and values were divided by the mean value for the $Mecp2^{tm1.1Jae/y}$ to $Mecp2^{tm1.1Jae/y}$ transplantation mice). Specific values obtained for $Mecp2^{tm1.1Jae/y}$ to $Mecp2^{tm1.1Jae/y}$ mice and $Mecp2^{+/y}$ to $Mecp2^{tm1.1Jae/y}$ mice are as follows: weight (in g) (18.22 ± 0.93 versus 18.96 ± 0.8); apneas per 15 min (35.4 ± 5.96 versus 35.2 ± 5.24); beam breaks per 12 h (3,729.2 ± 253.3 versus 4,330.2 ± 305.2); general condition score (0.52 ± 0.1 versus 0.35 ± 0.1); walking gait score (0.24 ± 0.087 versus 0.23 ± 0.076); tremor score (0.24 ± 0.087 versus 0.13 ± 0.061); hindlimb clasping score (0.48 ± 0.12 versus 0.29 ± 0.083); neurological score (1.48 ± 0.29 versus 1.0 ± 0.2). None of the differences were statistically significant.

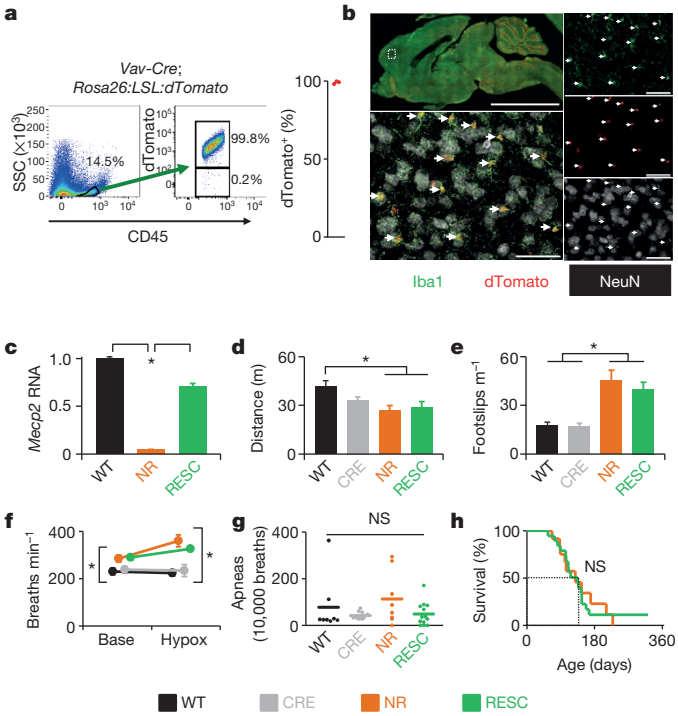

Figure 2 | Genetic reconstitution of *Mecp2* in microglia does not rescue *Mecp2*-null mice. **a**, **b**, Evaluation of efficiency and specificity of *Vav1-Cre* in microglia. **a**, Representative flow sorting of microglia derived from brains from *Vav1-Cre; Rosa26:LSL:tdTomato* mice shown on left, with quantification ($n = 3$) of cells that express both fluorescent reporter (tdTomato) and microglia marker (CD45) shown on right. SSC, side scatter. **b**, Histological characterization of tdTomato expression in brain from *Vav1-Cre; Rosa26:LSL:tdTomato*. Top left, low-power image of a mid-sagittal section; bottom left, higher power image of cortex. Right, individual colour channels contributing to merged image (bottom left). Arrows indicate microglia expressing tdTomato ($Iba1^+$/$tdTomato^+$). Scale bars, 5 mm (low power) and 50 μm (high power). **c**, Quantitative PCR (qPCR) results of *Mecp2* expression from flow-sorted microglia derived from wild-type (WT; $n = 2$), $Mecp2^{LSL/Y}$ (NR; $n = 2$) and $Vav1\text{-}Cre^{Tg/+}$; $Mecp2^{LSL/Y}$ (RESC; $n = 3$) animals. **d**, Distance travelled in open field assay. CRE denotes $Vav1\text{-}Cre^{Tg/+}$ mice. **e**, Number of footslips per distance travelled on parallel rods. **f**, Breathing rate at baseline and during hypoxia challenge. **g**, Number of apneas per 10,000 breaths. **h**, Survival curve. In **d**–**h**, WT $n = 8$; CRE $n = 10$; NR $n = 12$; RESC $n = 13$. Data are mean and s.e.m. $^*P < 0.05$. Statistical analyses in **c**–**g** analysed by one-way analysis of variance (ANOVA) with post-hoc pairwise *t*-test with Bonferroni correction, and in **h**, Kaplan–Meier survival analysis was used.

($Mecp2^{tm1.1Jae/y}$) mice prevented neurological decline and early death by restoring microglial phagocytic activity against apoptotic targets[4], and clinical trials of bone marrow transplantation (BMT) for patients with Rett syndrome have thus been initiated[5]. We aimed to replicate and extend the BMT experiments in three different Rett syndrome mouse models, but found that despite robust microglial engraftment, BMT from wild-type donors did not prevent early death or ameliorate neurological deficits. Furthermore, early and specific *Mecp2* genetic expression in microglia did not rescue *Mecp2*-deficient mice.

We first sought to replicate BMT-mediated rescue of male mice derived from the same $Mecp2^{tm1.1Jae/y}$ colony used in the original report[4], implementing established standards for conducting preclinical studies[2,6]. Mice were maintained on a C57Bl/6J background, which was confirmed in recipient animals by genome scanning (see Supplementary Information). Four-week-old $Mecp2^{tm1.1Jae/y}$ mice and wild-type littermates were subjected to the same protocol of lethal split-dose γ-irradiation. Mice were then randomized to receive tail vein injection of bone marrow from *Mecp2*-deficient male littermates or from *Mecp2*-proficient animals, including C57Bl/6J male mice ubiquitously expressing green fluorescent protein (GFP) and $Mecp2^{+/y}$ littermates of the recipients. All animals achieved multilineage peripheral blood engraftment as judged by the fraction of donor-derived GFP-expressing cells in peripheral blood 4 and 8 weeks after transplant (Extended Data Fig. 1a). PCR analysis of blood and tail tissue 4 weeks after transplant also confirmed expression of the appropriate mutant or wild-type variant of *Mecp2* in blood in all groups (Extended Data Fig. 1b). Microglial engraftment in brain parenchyma 30 and 90 days after transplant was similar in mutant and wild-type recipients engrafted with marrow from wild-type mice ubiquitously expressing a GFP transgene (Fig. 1a, b and Extended Data Fig. 1c), and comparable to engraftment observed by Derecki *et al.*[4] and others[7].

Contrary to our expectation, $Mecp2^{tm1.1Jae/y}$ mice that received $Mecp2^{+/y}$ marrow had no extension of lifespan compared to $Mecp2^{tm1.1Jae/y}$ marrow recipients (Fig. 1c). No difference in survival was observed in mutant animals that received $Mecp2^{+/y}$ marrow from wild-type littermates or C57Bl/6J animals ubiquitously expressing GFP (Extended Data Fig. 1d). We also observed no benefit in outcome measures at 12 weeks of age, 8 weeks after transplant, including weight, breathing, locomotion, general condition, walking gait, tremor, hindlimb clasping or neurological score (Fig. 1i). Thus, the same BMT procedure with substantially greater numbers of animals, randomly assigned to treatment group, with mice from the same $Mecp2^{tm1.1Jae/y}$ colony did not replicate any aspects of the protective effect reported by Derecki *et al.*[4]. Furthermore, histological analysis blind to genotype and treatment group showed no neuropathological evidence of differential apoptosis, microglial response, or tissue degeneration between experimental groups (Extended Data Fig. 1e). There was also no protective effect on survival after BMT in two additional mouse models of Rett syndrome (Fig. 1e, g): $Mecp2^{LucHyg/y}$ mice that contain a *Mecp2*-firefly luciferase/hygromycin-resistance gene fusion (Extended Data Fig. 2a–e) and $Mecp2^{R168X/y}$ mice[8], despite excellent engraftment after BMT (Extended Data Fig. 2f–h). Experiments with these two models were performed in independent laboratories following the same BMT protocol[4].

In all models, wild-type mice transplanted with wild-type bone marrow showed no mortality, indicating that the procedure was well tolerated (Fig. 1c, e, g). Likewise, BMT was well-tolerated by mutant animals, as *Mecp2* mutant animals receiving mutant marrow exhibited either no change ($Mecp2^{LucHyg/y}$ and $Mecp2^{R168X/y}$ mice), or, surprisingly, slightly reduced mortality ($Mecp2^{tm1.1Jae/y}$ mice) compared to naive mice not subjected to BMT (Fig. 1d, f, h). The small survival extension may be related to a salutary effect of post-irradiation antibiotic treatment of transplanted animals, to which naive animals were not exposed, or to differences in animal handling[9].

To address the role for microglia in Rett syndrome reported by Derecki *et al.*[4] further, we used the Cre/*lox* system and a *lox-stop-lox* allele of *Mecp2* ($Mecp2^{LSL}$, referred to as $Mecp2^{lox\text{–}stop/y}$ in ref. 4) to examine the effect of genetically driven expression of *Mecp2* in microglia during development (see Supplementary Information for full Methods details). First, we analysed the suitability of the *LysM-Cre* transgene ($Lysm^{cre}$ in ref. 4; *Lysm* is also known as *Lyz2*), which was used by Derecki *et al.*[4] in their genetic $Mecp2^{LSL/y}$ rescue experiments[4], to drive efficient microglia-specific gene restoration. As previously reported[10], *LysM-Cre*-driven dTomato reporter cells accounted for less than 25% of microglia, as assessed using flow cytometry of microglia derived from mice containing the *LysM-Cre* transgene and a transgene expressing Cre-dependent dTomato (Extended Data Fig. 3a). Furthermore, when

we generated *LysM-Cre; Mecp2*$^{LSL/Y}$ mice (termed *Mecp2*$^{lox-stop/y}$*Lysm*^{cre} in ref. 4), we observed MeCP2 expression in neurons (large NeuN^{+} cells) in many brain regions (Extended Data Fig. 3b).

To identify a Cre transgenic line that drives efficient expression within microglia, we next evaluated the *Vav1-Cre* transgene, which selectively expresses throughout the haematopoietic compartment[11]. In contrast to *LysM-Cre*, the *Vav1-Cre* transgene targeted microglia with high efficiency (Fig. 2a) and specificity (Fig. 2b). As *Vav1-Cre*-driven expression in brain proved to be efficient and restricted to microglia, we applied this system to test whether expression of *Mecp2* in microglia rescues *Mecp2*-null mice. To quantify *Mecp2* restoration in microglia, we used the *fms-GFP* transgene, the expression of which within brain is restricted to microglia, for flow sorting[11] (Extended Data Fig. 3c). Microglia derived from *Vav1-Cre; Mecp2*$^{LSL/Y}$ animals expressed *Mecp2* messenger RNA at 75% of the level of *Mecp2* mRNA in microglia derived from *Mecp2*$^{+/Y}$ animals (Fig. 2c). Similar to other *Mecp2*-null mouse models, *Mecp2*$^{LSL/Y}$ animals showed hypoactivity, poor motor coordination on parallel rod walking, increased basal and hypoxia breathing rate, increased frequency of apneas, and early death, none of which was improved by *Mecp2* expression in microglia of *Vav1-Cre; Mecp2*$^{LSL/Y}$ animals (Fig. 2d–h). We thus conclude that driving *Mecp2* expression developmentally in microglia did not ameliorate the phenotype of *Mecp2*-null mice, in contrast to the data reported by Derecki *et al.*[4].

In conclusion, our experiments do not support BMT as therapy for Rett syndrome. We observe no benefit of BMT-mediated delivery of wild-type microglia into the brains of three different preclinical models of Rett syndrome, nor do we observe a causative role of microglia in the disease process. Our BMT studies included large numbers of mice derived from the same parent colony used in the original report[4], with treatment assigned randomly and analysis conducted blind to genotype and treatment group. Finally, we showed that even early and highly efficient genetically driven *Mecp2* expression in the microglia of *Mecp2*-null mice conferred no protective effect. Restoration of MECP2 in microglia through either BMT or genetics did not rescue the major observed phenotypes in Rett syndrome, which argues against the previously proposed therapeutic potential of BMT in patients with Rett syndrome[4].

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Supplementary Information is available in the online version of the paper.

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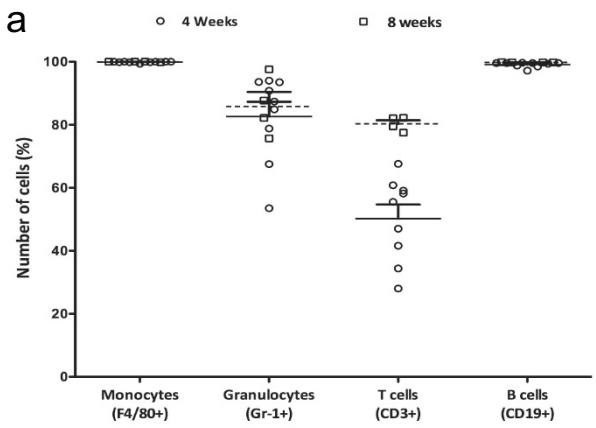
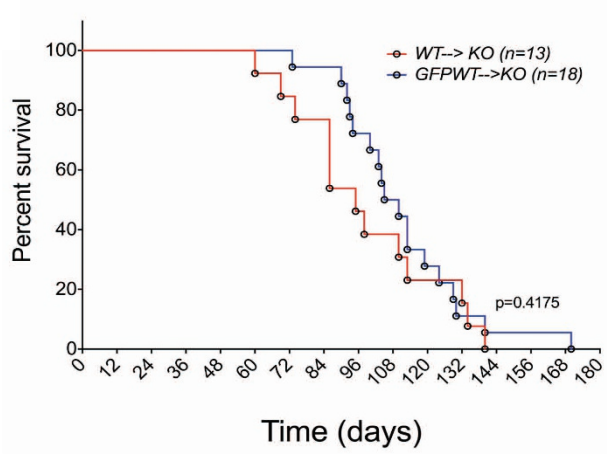
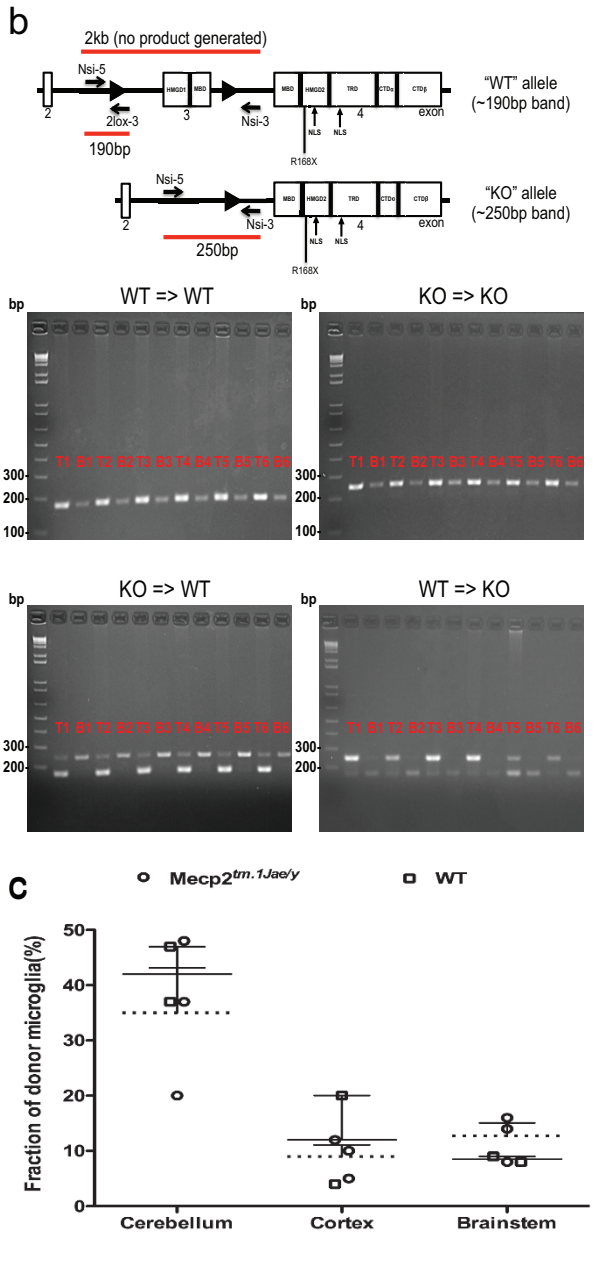
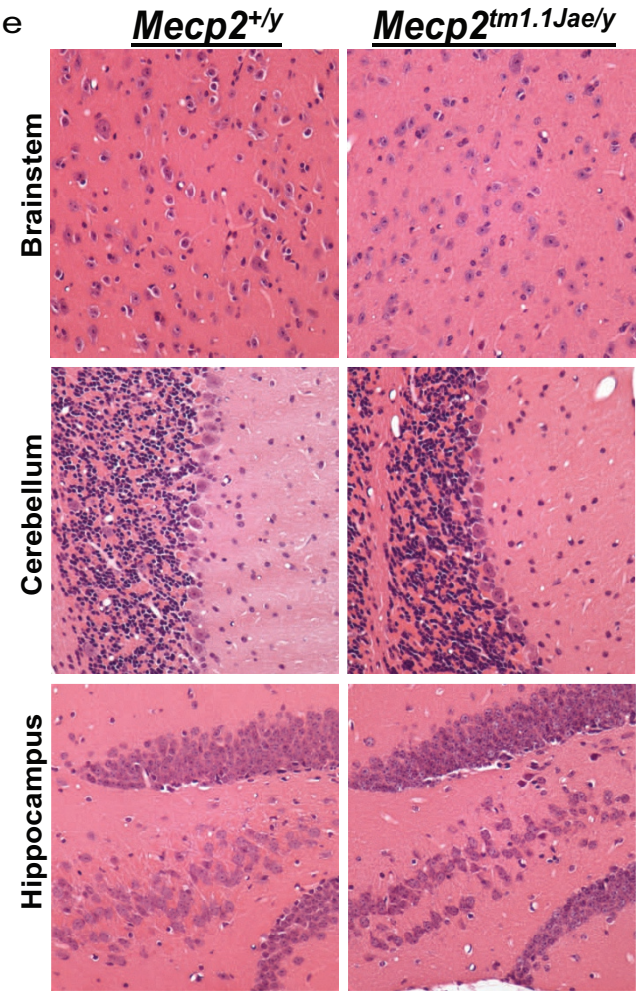
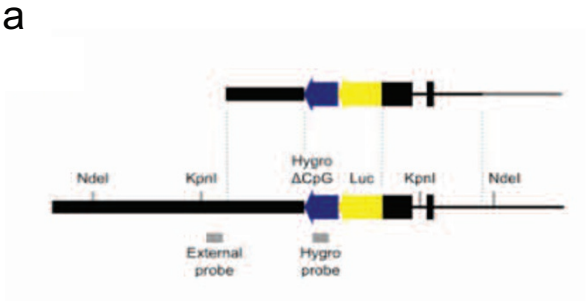


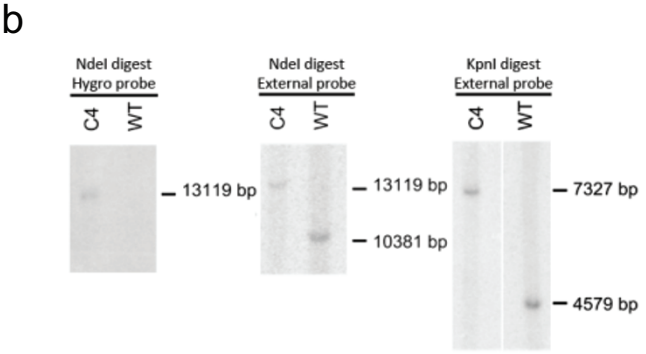


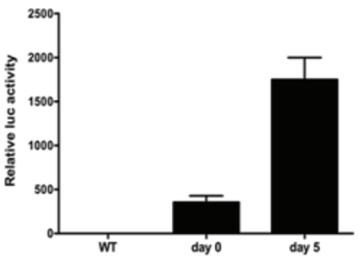


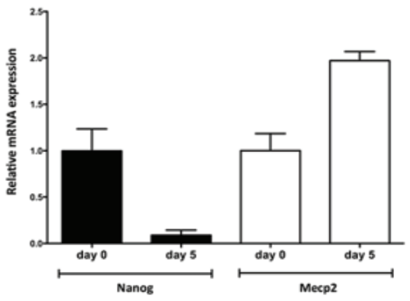


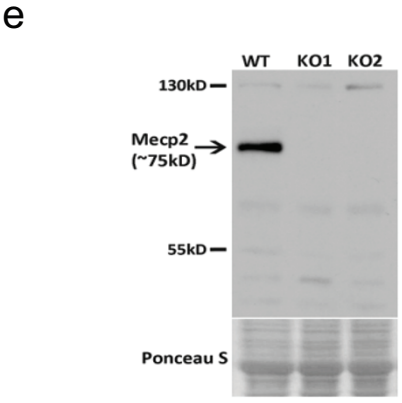
Extended Data Figure 1 | Engraftment with donor cells after bone marrow transplantation, and lack of evidence of neuropathology in *Mecp2*-null animals. **a**, Multilineage peripheral blood engraftment with donor cells in *Mecp2*$^{tm1.1Jae/y}$ and wild-type mice. Wild-type and *Mecp2*$^{tm1.1Jae/y}$ animals received transplant from wild-type animals ubiquitously expressing GFP (Jackson Labs, C57BL/6-Tg(UBC-GFP)20Scha/J, stock 004353). Peripheral blood engraftment in indicated blood lineages was measure by flow cytometry (GFP) 4 and 8 weeks after transplant. **b**, PCR analysis of blood and tail tissue 4 weeks after transplant. Expression of only the appropriate mutant or wild-type variant of *Mecp2* from the donor in blood in all four groups is shown, with retention of the original genotype in tail tissue as expected. Specifically, *Mecp2*$^{+/y}$ to *Mecp2*$^{+/y}$ mice show only the wild-type allele at 190 base pairs (bp), whereas *Mecp2*$^{tm1.1Jae/y}$ to *Mecp2*$^{tm1.1Jae/y}$ mice show only the mutant allele at 250 bp, as previously described in the original report of generation of these mice[12] . *Mecp2*$^{tm1.1Jae/y}$ to *Mecp2*$^{+/y}$ mice, however, show only the mutant allele in blood tissue and retention of host wild-type allele in tail tissue. Accordingly, *Mecp2*$^{+/y}$ to *Mecp2*$^{tm1.1Jae/y}$ mice show only the wild-type allele in blood tissue, with retention of the host mutant allele in tail tissue. Tail tissue in these latter two groups shows some of the allele from the donor as well, presumably owing to blood contained within the tail clips used for analysis. Notably, the *Mecp2* allele expressed in blood is always restricted to the donor genotype, indicating successful transplantation with complete replacement of the haematopoietic system in the host. Samples are labelled with a 'T' for tail and 'B' for blood, followed by the number of the animal, indicating that six different animals were analysed for each condition. CTD, C terminus domain α and β; HMGD1/2, high mobility group protein-like domain 1/2; MBD, methyl binding domain; NLS, nuclear localization signal; TRD, transcription repression domain. **c**, Robust and early microglial engraftment of donor cells after BMT in *Mecp2*$^{tm1.1Jae/y}$ and wild-type mice. Microglial engraftment was visualized using double immunofluorescence staining in sections quenched for autofluorescence by incubation in Sudan black solution. All sections were stained with an anti-Iba1 primary with CY-3 secondary and an anti-GFP primary with CY-5 secondary. All microglia are Iba1-positive, and thus successfully engrafted GFP-expressing donor-derived microglia were observed as GFP$^+$/Iba1$^+$, whereas native microglia were only Iba1$^+$. Engraftment of microglia into wild-type and *Mecp2*$^{LucHyg/y}$ mice was determined by dividing the GFP$^+$/Iba1$^+$ cells by the number of total Iba1$^+$ cells. Cell counts were performed in cerebellum, cortex and brainstem from mice. Percentage engraftment in wild-type and *Mecp2*$^{tm1.1Jae/y}$ mice yielded similar results to previously published engraftment results at 30 days after transplantation[9]. **d**, BMT was well-tolerated in animals. No difference in survival was observed in mutant animals that received *Mecp2*$^{+/y}$ marrow from their wild-type littermates ($n = 13$) and C57Bl/6J animals ubiquitously expressing GFP ($n = 13$). KO, knockout. **e**, Representative haematoxylin-and-eosin-stained sections of cerebellum, brainstem and hippocampus from age-matched wild-type and *Mecp2*$^{tm1.1Jae/y}$ mice killed at 7 weeks of age. Original magnification, ×400. Sections demonstrate comparable histological features between wild-type and *Mecp2*$^{tm1.1Jae/y}$ brains, and a lack of gliosis, cell loss, cellular debris, microglia or macrophages in *Mecp2*$^{tm1.1Jae/y}$ brains.

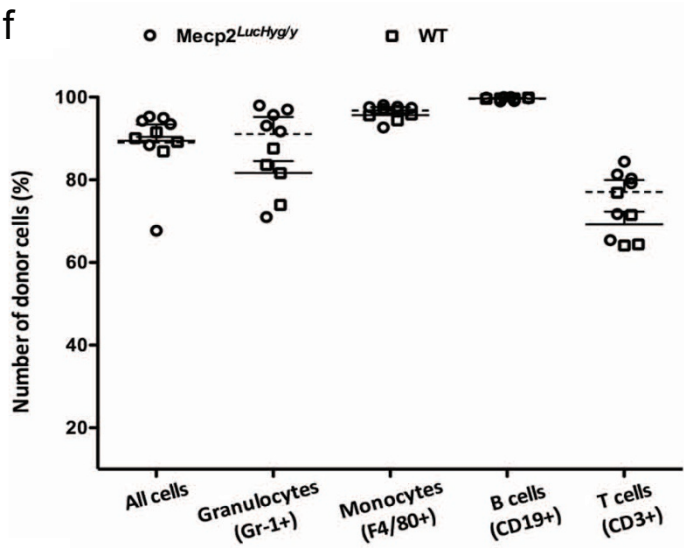


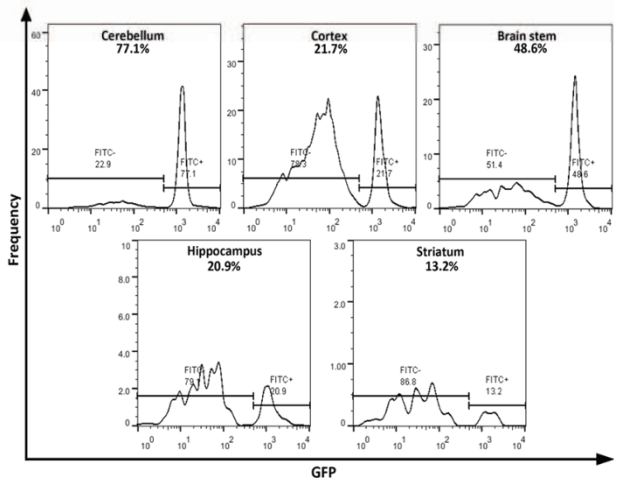


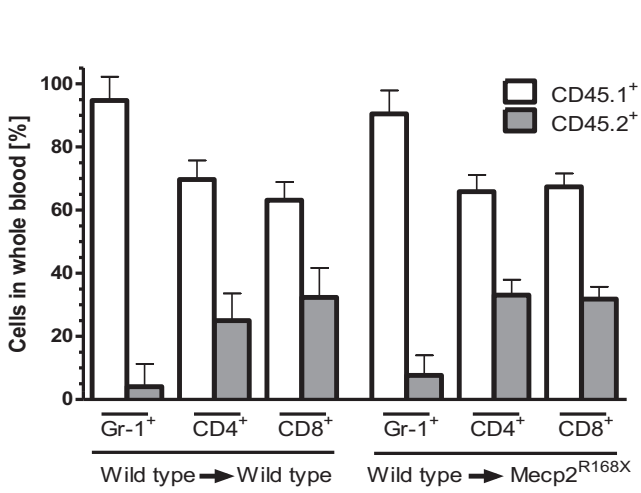




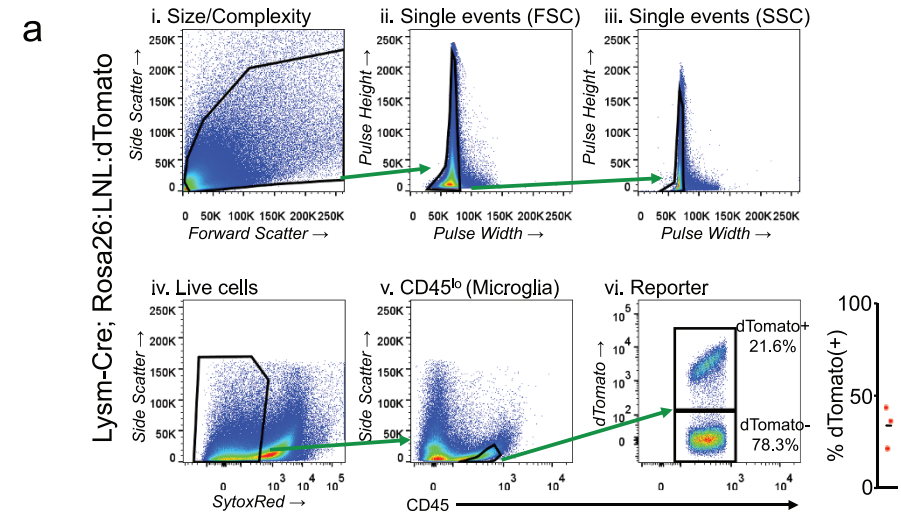


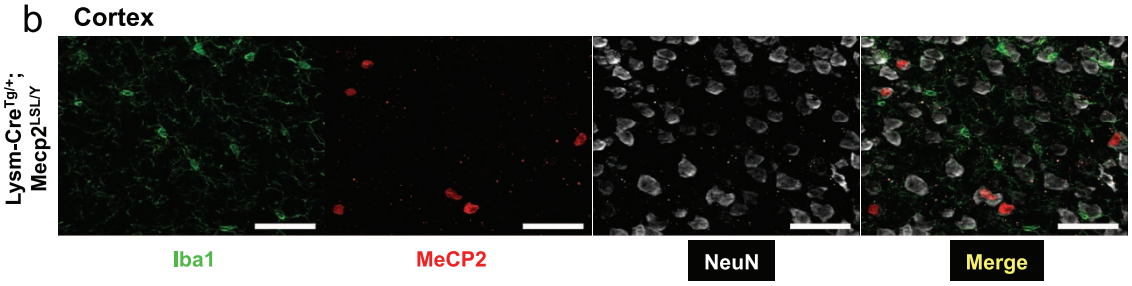


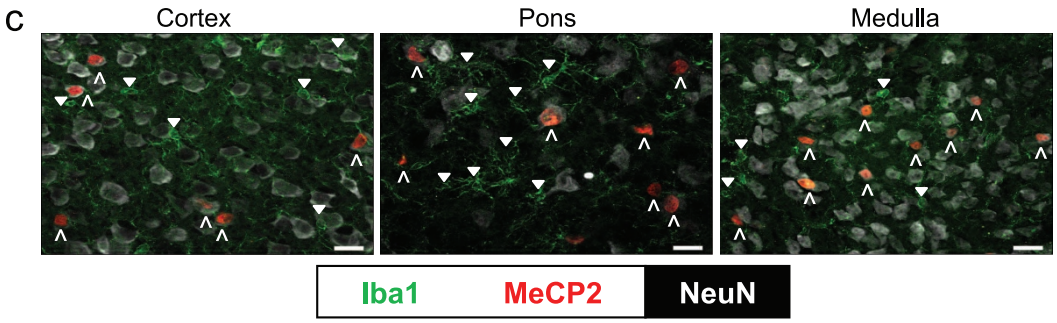


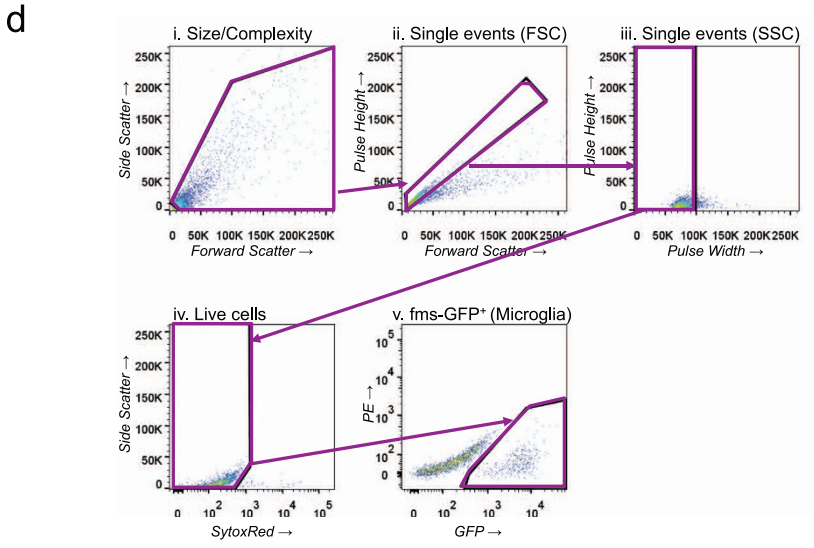


Extended Data Figure 2 | Early transplantation of wild-type microglia into the brain does not rescue additional models of *Mecp2*-null mice: *Mecp2*^{LucHyg} mice and C57Bl/6J *Mecp2*^{R168X} mice. **a**, Generation of *Mecp2*^{LucHyg} mice. Luciferase/hygromycin (LucHyg) fusion gene vector correctly targeted to the *Mecp2* locus in embryonic stem cells. Positions of the probes and enzyme restriction sites are indicated. The homology arms of the targeting vector are depicted in black, and its backbone in grey. **b**, Confirmation of genetic targeting for *Mecp2*^{LucHyg} mice. Southern blotting of NdeI- or KpnI-digested DNA extracted from clone C4 cells, used for blastocyst injections, hybridized with either the hygromycin or external probe confirms correctly targeted event. **c**, Luciferase activity in clone C4 cells before (day 0) or after (day 5) subjecting cells to retinoic-acid-induced differentiation. After adsorption to eliminate feeder mouse embryonic fibroblasts, clone C4 embryonic stem cells were treated with retinoic acid (100 nM) in differentiation medium for 5 days, and luciferase activity was measured before and after retinoic acid treatment. Mean values are plotted relative to that of the wild-type cells ($n = 3$, error bars denote s.d.). Retinoic-acid -induced differentiation leads to an increase in luciferase activity consistent with an increase in *Mecp2* expression level as measured in **d**. **d**, mRNA levels of *Mecp2* increased and of embryonic stem-cell marker *Nanog* decreased in clone C4 cells subjected to retinoic-acid-induced differentiation. mRNA levels were measured before and after treatment by qPCR. Mean values plotted relative to day 0 for each mRNA ($n = 3$, error bars denote s.d.). **e**, Western blot analysis of MECP2 expression in brains of wild-type and *Mecp2*^{LucHyg} male mice. MECP2 protein is not detected in MECP2 luciferase males. Ponceau S staining serves as a loading control. **f**, **g**, Robust peripheral blood and microglial engraftment of donor cells after BMT in *Mecp2*$^{LucHyg/y}$ mice. Wild-type and *Mecp2*$^{LucHyg/y}$ mice received wild-type bone marrow marked with GFP or CD41.1. Peripheral blood engraftment was measured by flow cytometry (GFP or CD45.1) in the indicated lineage 4–8 weeks after transplantation. For central nervous system engraftment, flow cytometry was performed on isolated mononuclear cells from the cortex, brainstem, cerebellum, hippocampus and striatum. Engraftment of BMT-derived cells was determined by dividing the $CD11b^+CD45^+GFP^+$ cell population by total $CD11b^+CD45^+$ monocytes/microglia. **h**, Robust peripheral blood engraftment of donor cells 7 weeks after BMT in *Mecp2*^{R168X} mice. Reconstitution of bone marrow from *B6.SJL-Ptprc*^{a} *Pepc*^{b}*/BoyJ* mice into wild-type mice and C57Bl/6J *Mecp2*^{R168X} mice showed robust engraftment in peripheral blood. Reconstitution of bone marrow was determined by FACS analysis of peripheral blood using anti-GR-1, anti-CD4 and anti-CD8 antibodies and CD45.1 for the donor cells (*B6.SJL-Ptprc*^{a} *Pepc*^{b}*/BoyJ* mice, white bars) and CD45.2 for host cells (wild-type and C57Bl/6J *Mecp2*^{R168X} mice, grey bars).









Extended Data Figure 3 | Flow sorting and histological characterization of *LysM-Cre* or *Vav1-Cre* transgenic mice. **a**, Stepwise process to characterize the amount of microglia (CD45 lo expressing) cells that also express tdTomato in a *LysM-Cre*-dependent fashion. **b**, High power images of cortex from *LysM-Cre*$^{Tg/+}$; *Mecp2*$^{LSL/Y}$ animals. Scale bars, 50 μm. **c**, Merged high power images from cortex, pons and medulla from *LysM-Cre*; *Mecp2*$^{LSL/Y}$ animal. Circumflex (^) symbols identify large NeuN staining cells that express MECP2 (NeuN^{+}/MECP2^{+}); downward-facing triangles mark microglia not expressing MECP2 (Iba1^{+}/MECP2^{-}). Scale bars, 20 μm. **d**, Gating strategy for microglia sorting for *Mecp2* expression quantification in *Vav1-Cre*; *Mecp2*$^{LSL/Y}$ and control animals is presented: (i) size/complexity (size/cytoplasmic granularity for cells but not debris); (ii) forward scatter pulse height/area (eliminates doublet cells); (iii) side scatter pulse height/width (eliminates doublet cells); (iv) SYTOX red staining; dead cells are SYTOX-red-positive and removed from the following analysis; (**v**) *fms-GFP* expression analysis enables the purification of microglia.

CORRIGENDUM

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Corrigendum: Wild-type microglia do not reverse pathology in mouse models of Rett syndrome

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In this Brief Communication Arising, the first name of author Sébastien Vingeau was misspelled 'Sebastian'. In addition, the labels ('WT→KO' and 'KO→WT') of the two bottom panels in Extended Data Figure 1b were swapped. Both errors have been corrected online.