LETTER

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Autism-like behaviours and germline transmission in transgenic monkeys overexpressing MeCP2

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Methyl-CpG binding protein 2 (MeCP2) has crucial roles in transcriptional regulation and microRNA processing[1–4]. Mutations in the *MECP2* gene are found in 90% of patients with Rett syndrome, a severe developmental disorder with autistic phenotypes[5]. Duplications of *MECP2*-containing genomic segments cause the *MECP2* duplication syndrome, which shares core symptoms with autism spectrum disorders[6]. Although *Mecp2*-null mice recapitulate most developmental and behavioural defects seen in patients with Rett syndrome, it has been difficult to identify autism-like behaviours in the mouse model of MeCP2 overexpression[7,8]. Here we report that lentivirus-based transgenic cynomolgus monkeys (*Macaca fascicularis*) expressing human MeCP2 in the brain exhibit autism-like behaviours and show germline transmission of the transgene. Expression of the *MECP2* transgene was confirmed by western blotting and immunostaining of brain tissues of transgenic monkeys. Genomic integration sites of the transgenes were characterized by a deep-sequencing-based method. As compared to wild-type monkeys, *MECP2* transgenic monkeys exhibited a higher frequency of repetitive circular locomotion and increased stress responses, as measured by the threat-related anxiety and defensive test[9]. The transgenic monkeys showed less interaction with wild-type monkeys within the same group, and also a reduced interaction time when paired with other transgenic monkeys in social interaction tests. The cognitive functions of the transgenic monkeys were largely normal in the Wisconsin general test apparatus, although some showed signs of stereotypic cognitive behaviours. Notably, we succeeded in generating five F_1 offspring of *MECP2* transgenic monkeys by intracytoplasmic sperm injection with sperm from one F_0 transgenic monkey, showing germline transmission and Mendelian segregation of several *MECP2* transgenes in the F_1 progeny. Moreover, F_1 transgenic monkeys also showed reduced social interactions when tested in pairs, as compared to wild-type monkeys of similar age. Together, these results indicate the feasibility and reliability of using genetically engineered non-human primates to study brain disorders.

We first co-injected lentivirus expressing synapsin-promoter-driven[10] haemagglutinin (HA)-tagged human MeCP2 and green fluorescence protein (GFP) and lentivirus expressing mCherry into the perivitelline space of 94 mature oocytes of cynomolgus monkeys (Fig. 1a). We found that 61 out of 88 (69%) of the surviving oocytes became zygotes after intracytoplasmic sperm injection (ICSI), and 53 embryos were then transferred into 18 surrogate monkeys. Nine surrogates (9 out of 18, 50%) became pregnant and produced eight live births (3 male, 5 female; Fig. 1b) and four stillbirths, all carrying human *MECP2*, GFP and mCherry transgenes, as determined by PCR (Fig. 1c). The AccuCopy assay showed that the copy numbers of *MECP2* transgenes in 8 live (T04–T11) and 2 aborted (T01 and T02) transgenic (TG) monkeys varied from 1.0 to 7.3 (Extended Data Table 1a). In the second experiment, we injected 264 mature oocytes with lentivirus carrying the hSynapsin-HA-hMECP2-2a-GFP cassette, and transferred 105 embryos after ICSI into 36 surrogates. Owing to unfavourable seasonal conditions, only 7 pregnant surrogates gave birth to 9 monkeys (T13–T21), and only 2 survived (Supplementary Table 1).

Western blotting of tissues of stillbirth TG monkey T14 showed expression of GFP and HA–MeCP2 proteins in the cortex and cerebellum, but not in non-neural tissues, confirming specific transgene expression under the synapsin promoter (Fig. 1d). Levels of MeCP2 protein were also significantly higher than that found in an aborted wild-type (WT) monkey of a similar age (Fig. 1e, f). Transgenic integration was confirmed by Southern blotting using a probe targeting the HA-hMECP2-2a-GFP transgene (Extended Data Fig. 1a). We next analysed genomic integration sites of lentiviral cassettes containing HA-hMECP2-2a-GFP and mCherry transgenes by a deep-sequencing-based method (Extended Data Fig. 1b). All transgenes were located in genomic loci distant from known coding exons, and thus unlikely to interfere with endogenous genes (Fig. 1g and Supplementary Table 2), and insertion numbers were largely consistent with the copy numbers identified by AccuCopy (Extended Data Fig. 1c). Therefore, the human *MECP2* transgene was successfully incorporated into the monkey genome and specifically expressed in the monkey's brain.

Compared to WT monkeys of similar ages, the body weight and abdominal circumference of the TG group (T04–T11) was slightly lower before 20 months of age, whereas no difference was found for head–truck length, heart and respiratory rates or body temperature (Extended Data Fig. 2a–g). We did not observe in TG monkeys any seizure phenotype, which was associated with *MECP2* duplication syndrome patients[6], perhaps owing to the young age of the monkeys. Interestingly, mass spectrometry of blood metabolites at ~18 and ~36 months suggested metabolic abnormalities in the TG group, with significantly higher levels of some short- and long-chain fatty acids (Extended Data Fig. 3a, b), reminiscent of some human autistic patients[11].

Despite their generally normal early development, one TG monkey (T05) showed severe weight loss and head circumference reduction after 15 months (Extended Data Fig. 4a–c), and was unable to complete behavioural tests. Monkeys T09 and T07 became severely sick at 43 and 46 months, respectively, after behavioural tests. The sickness of these TG monkeys echoed the severe phenotypes of human patients with the *MECP2* duplication syndrome[6]. The euthanasia procedure was performed, and their brain tissues were collected for further analysis with western blotting, immunostaining and RNA-sequencing (RNA-seq). We found that HA–MeCP2 and GFP were expressed in the brain

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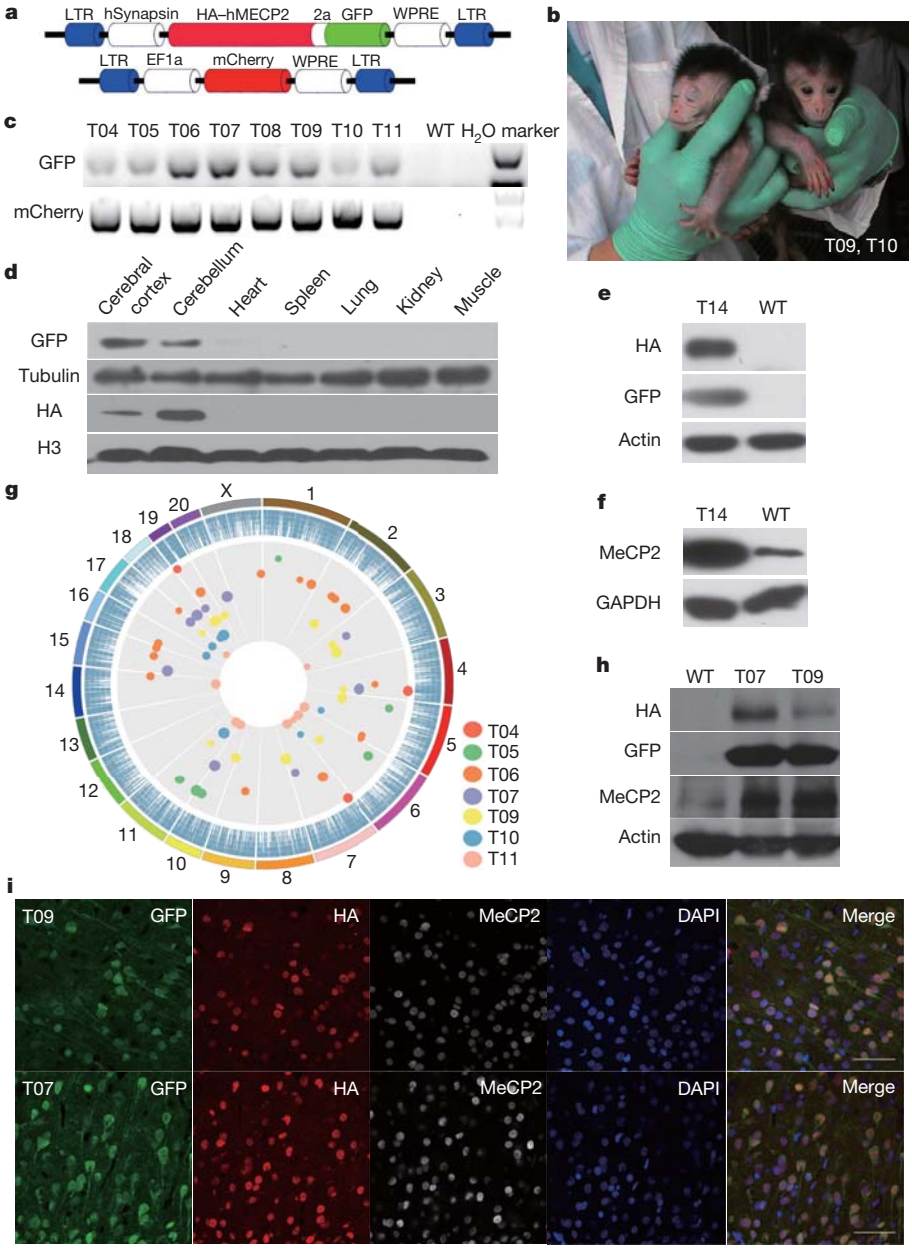

Figure 1 | Construction of *MECP2* transgenic monkey and brain-specific expression of transgenes. a, Top, lentiviral HA-hMECP2-2a-GFP cassette. Bottom, the EF1a-mCherry cassette. **b**, Image of newborn *MECP2* transgenic (TG) monkeys (T09 and T10). Photo credit: Y.W. **c**, PCR analysis showing the presence of transgenes (GFP, top; mCherry, bottom) in 8 live TG monkeys' genomes. **d**, Brain-specific transgene expression, shown by western blots of different tissues of T14. Top two panels: cytosolic fractions; bottom two panels: nuclear fractions, stained with antibodies indicated. Note the exposure times for transgene and the loading control were different (see Supplementary Fig. 1 for further comparison). H3, histone 3. **e**, **f**, **h**, Western blots showing expression of HA–MeCP2 and GFP in brain tissues of TG (T07, T09 and T14) and wild-type (WT) monkeys. For gel source data, see Supplementary Figs 1–3. **g**, Genome-wide distribution of transgenes in F_0 TG monkeys. Insertion sites (dots) distribute on various chromosomes (outermost circle). Sizes of dots are proportional to reads identified by deep-sequencing, colour-coded and aligned circularly for different monkeys (sample for T08 absent owing to preparation failure). **i**, Immunostaining of cortical sections of brains of T07 and T09 for GFP, HA, MeCP2 and DAPI. Scale bars, 50 μm.

lysates of T07 and T09 (Fig. 1h). Immunostaining of cortical slices of T07 and T09 showed that the MeCP2 and HA signals were co-localized (Fig. 1i), indicating expression of the *MECP2* transgene in the TG monkeys' brain.

Further transcriptome-wide analysis of the brain tissues was performed on four deceased TG (T14, T05, T07 and T09) and four WT monkeys using RNA-seq, based on the whole-genome sequencing data for cynomolgus monkeys[12–14]. We found 105 upregulated and 209 downregulated genes in TG monkeys (Extended Data Fig. 4d, e), with ≥2-fold change as compared to WT monkeys. Among them, 13 upregulated and 3 downregulated genes were also reported to exhibit similar changes in the *MECP2* transgenic mice[15].

Motor functions and responses to stress[16–18] were examined for 8 TG (T04–T11) and 8 WT monkeys (aged 12–18 months). First, we video-recorded the locomotion of each monkey alone for 20 min per day for 5 days, and found that four TG (T04, T05, T06 and T09) and two WT monkeys exhibited repetitive circular locomotion (in the same direction, at least three times; Supplementary Videos 1 and 2). The total time spent in circular locomotion during the observation period (average over 5 days) for all eight transgenic monkeys was significantly higher than that of eight WT monkeys (Fig. 2a, b). This difference

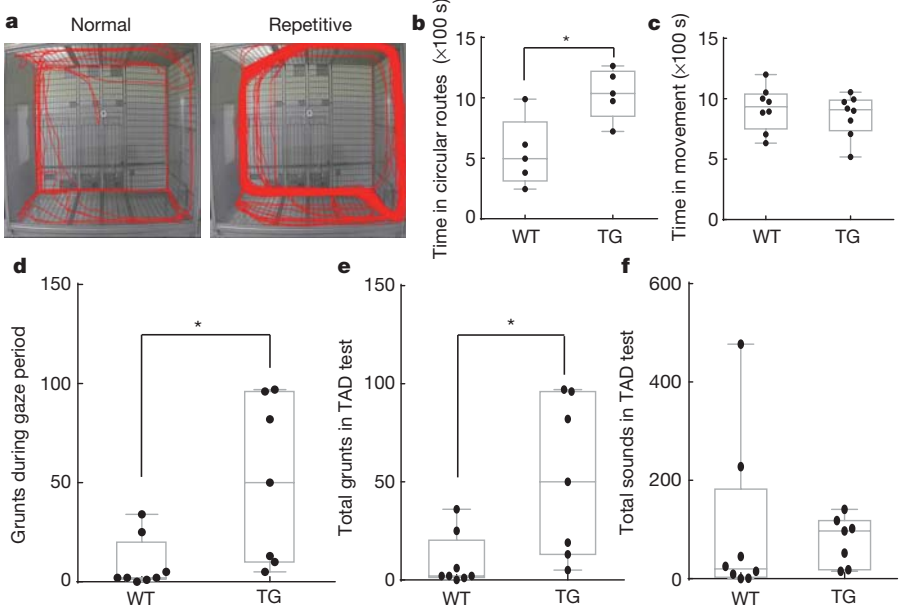

Figure 2 | Alterations in locomotion activity and increased anxiety in *MECP2* TG monkeys. a, Examples of movement trajectories (red traces) viewed from cage-top, showing normal activity (left) and repetitive circular routing (right). **b**, Boxplots of time spent in repetitive routing of TG and WT monkeys ($n = 8$ each), monitored for 20 min daily for 5 days. Each dot depicts data from 1 day (*$P = 0.014$, Student's t-test). **c**, Average total time spent in movements in 20-min period ($n = 8$ each). **d**, Results from threat-related anxiety and defence (TAD) test. Boxplots of numbers of grunts during the gaze period ($n = 8$, WT; $n = 7$, TG; *$P = 0.009$, Mann-Whitney U test) at 18 months of age. **e**, **f**, Total number of grunts (**e**) and all sounds (**f**) during the entire TAD tests (same monkey sets as in **d**; *$P = 0.014$, Mann–Whitney U test). Ends of whiskers represent the minimum and maximum of data points. The line within box represents the median (odd numbers of data points) or second quartile (even number of data points). The bottom and top edge of box represents the first and the third quartile, respectively.

was not due to hyperactivity of the TG monkeys, because the total time spent in locomotion was similar between the two groups during observation (Fig. 2c).

Anxiety-associated behaviours were found in autism patients and mouse models of MeCP2 overexpression[6,7]. We used the threat-related anxiety and defensive (TAD) behavioural test[9] to assay the vocalization responses of the monkeys to human gaze (Extended Data Fig. 5a). Typical sounds include grunt, coo and scream (Supplementary Audio 1–3), each was identified by its distinct signature in the sound spectrograph (Extended Data Fig. 6a–c). Notably, we found that at ~18 months of age, the total number of grunts made by the TG group during the gaze period and the entire TAD test was significantly higher than that of the WT group (Fig. 2d, e), with similar total numbers of sounds (grunt, coo and scream) per session produced by both groups (Fig. 2f and Supplementary Table 3). This increase in anxiety-related grunts was also found at 36 months of age (Extended Data Fig. 5b–d). Thus, *MECP2* TG monkeys showed increased levels of anxiety.

Impairment of social interaction is a hallmark of autism and *MECP2*-associated disorders. We examined the time monkeys sat together with apparent interactions, a prominent social behaviour in monkey colonies[19–21]. First, three groups of monkeys (at ~18 months of age, two TG and three WT in each group) were reared together for over 6 months (Supplementary Table 4a). We found that the average time a TG monkey sat together with another WT monkey within the group was significantly lower than that of the WT monkey (60-min daily observation for 5 days, Fig. 3a and Supplementary Video 3; no data for TG–TG interaction owing to the limited number of TG monkeys). The total time all TG monkeys sat with any other monkey (either TG or WT) was also slightly lower than for WT monkeys (Fig. 3a). Next (at ~24 months of age), we paired two female monkeys from different groups in a single cage (60 min daily for 5 days), and found that the interaction time of TG–TG pairs was significantly lower than that of WT–WT pairs (Fig. 3b, Supplementary Video 4 and Supplementary Table 4b). Pairing of unfamiliar male monkeys was not performed owing to their aggressive behaviours near adolescence. Finally (at 36 months of age), we paired familiar female and male monkeys from the same group, in which male pairs showed no aggressive interaction, and found that the male TG–TG pairs interacted less than that of the TG–WT pairs, whereas the difference between female TG–TG pairs and TG–WT pairs was not significant (Fig. 3c, d, Extended Data Fig. 7a–f and Supplementary Table 4c). All of these social interaction tests were performed with observers blinded to monkey genotypes. This apparent difference between male and female TG monkeys is reminiscent of the finding that *MECP2* duplication syndrome show more severe autism-related symptoms in male patients[6].

Cognitive function tests were performed using the Wisconsin general test apparatus (WGTA)[22–26]. During adaptation, discrimination and reversal steps of black/white tests, both the WT and TG groups passed each step with a similar average time course, but the TG monkeys exhibited much larger variability and one (T11) failed to pass the black/white reversal step (and was thus dropped from subsequent tests) (Extended Data Fig. 8a, b and Supplementary Table 5). In the Hamilton searching tests (adaptation, searching, set-breaking and forced set-breaking), the TG group showed a slightly slower learning in the forced set-breaking step (Extended Data Fig. 8c, d). Finally, the two groups showed no significant difference in the average performance in the reward-shape association learning test (Extended Data Fig. 9a–c and Supplementary Video 5). However, three out of seven TG monkeys showed marked left-side preference, regardless of the left or right location of the reward, a behaviour that was not observed in the WT monkeys (Fig. 3e, f and Supplementary Video 6). Thus, *MECP2* transgene expression resulted in some abnormalities in cognitive behaviours.

We further examined the germline transmission of *MECP2* TG monkeys, in view of previous lentiviral-based transgenic monkey experiments[27,28]. To facilitate the reproduction of TG monkeys, we used a recently developed testicular tissue xenografting method (see Methods). In brief, one testicle was obtained from T07 at 27 months of age, and pieces of the testicle tissue were xenografted subcutaneously in nude mice. Mature motile sperm were obtained from the xenografts after 10 months and used for ICSI on 176 monkey oocytes.

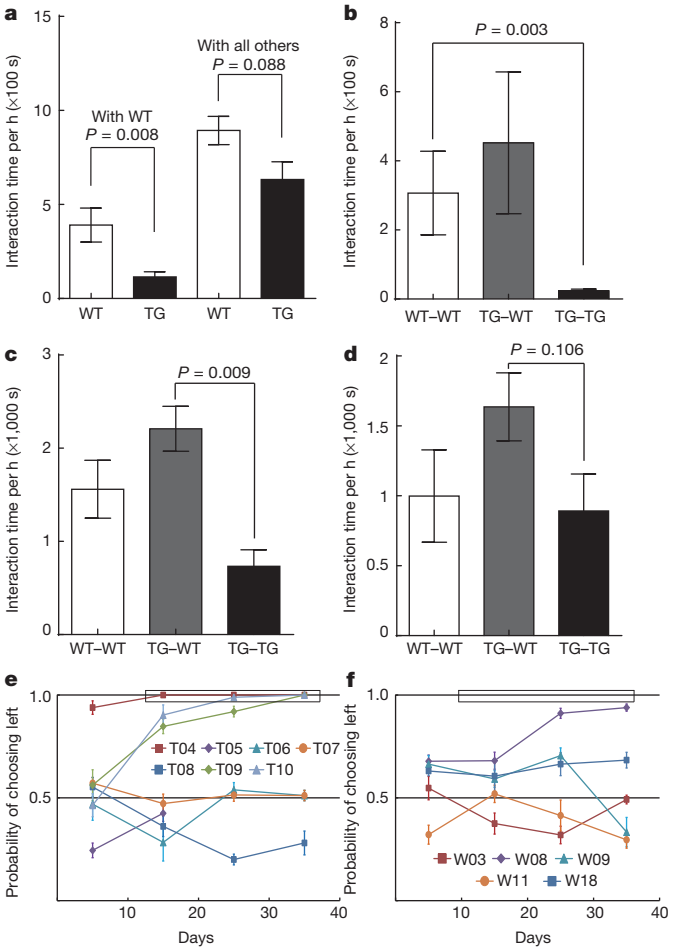

Figure 3 | Impaired social interaction and cognitive functions in *MECP2* TG monkeys. a, Interaction at 18 months of age among monkeys within the same group ('familiar', reared together for 6 months), defined by the average time a WT or a TG monkey sat together (for >3 s) with a WT monkey from the same group (left), or with any other monkey (right) during a 60-min observation period ($n = 6$, TG; $n = 9$, WT). **b**, Interaction time between a pair of female monkeys from a different group ('unfamiliar', reared separately for 12 months) at 24 months of age, for WT–WT, WT–TG and TG–TG pairs ($n = 4$, TG; $n = 12$, WT). **c**, **d**, Interaction time between familiar monkey pairs at 36 months of age (**c**, male pairs, $n = 3$, TG; $n = 8$, WT; **d**, female pairs, $n = 4$, TG; $n = 11$, WT) from the same group reared together for 6 months. All *P* values are from Mann–Whitney *U* test. **e**, **f**, Data from WGTA test ($n = 7$, TG; $n = 5$, WT), showing distinct left bias in the probability of left or right choice in three TG monkeys. Boxed areas denote >95% probability of choosing left side. Error bars denote s.e.m.

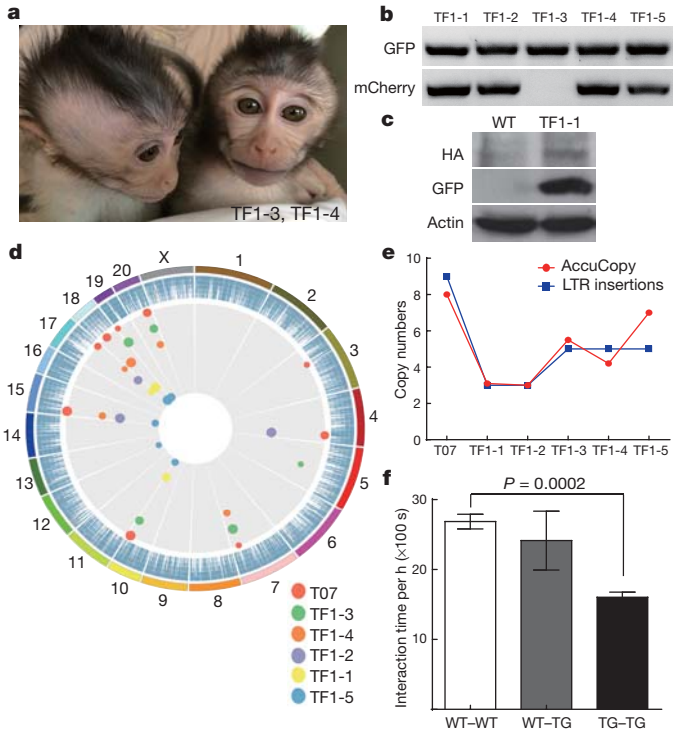

Figure 4 | Generation of F_1 progeny of *MECP2* TG monkey. a, Image of two newborn F_1 TG monkeys (TF1-3 and TF1-4). Photo credit: Y.-H.N. **b**, PCR analysis showing the presence of transgenes (GFP and mCherry) in five F_1 monkeys' genomes. **c**, Western blots showing expression of the HA–MeCP2 and GFP in F_1 monkeys' brain. For gel source data, see Supplementary Fig. 4. **d**, Genomic-wide distribution of transgenes in T07 and five F_1 offspring. Insertion sites (dots) distribute on various chromosomes (outermost circle). Sizes of dots are proportional to reads identified by deep-sequencing, colour-coded and aligned circularly for different monkeys. **e**, Copy numbers obtained with AccuCopy (red, *MECP2* and mCherry) are consistent with LTR insertion numbers identified by deep-sequencing (blue). **f**, Social interaction time between *MECP2* TG F_1 and WT monkeys from a different group ('unfamiliar pairing') at 11 months of age, for WT–WT, WT–TG and TG–TG pairs (*P* value, Student's *t*-test) ($n = 4$, TG; $n = 6$, WT). Error bars denote s.e.m.

Implantation of 95 fertilized zygotes into 22 surrogates resulted in five F_1 offspring (TF1-1 to TF1-5; 4 live births; Fig. 4a and Extended Data Table 1b). The PCR analysis showed that all five F_1 monkeys carried the human *MECP2* transgene (Fig. 4b), and western blotting showed the expression of HA–MeCP2 and GFP in brain lysates of TF1-1 (deceased 3 days after birth) (Fig. 4c). Thus, the transgenes were expressed in the F_1 offspring. Deep-sequencing further showed the genomic integration sites of the HA-hMECP2-2a-GFP and mCherry transgenes in F_1 TG monkeys (Fig. 4d and Supplementary Table 6). As expected, transgenes in F_1 TG monkeys (TF1-1 to TF1-5) were mostly distributed in chromosomes that were a subset of transgene-containing chromosomes of the paternal monkey T07 (Fig. 4d), showing Mendelian segregation of transgenes among the F_1 progeny. Moreover, long-terminal repeat (LTR) insertion sites were consistent with AccuCopy-identified copy numbers, confirming the germline transmission of transgenes (Fig. 4e).

Finally, we examine whether F_1 TG monkeys may also show defects in social interaction. We set up two groups of monkeys at 11 months of age (two TG and three WT in each group; Supplementary Table 7), then paired two monkeys from two different groups in a single cage (60 min daily for 5 days), observed in a blinded manner. We found that the TG–TG pairs showed a significantly lower total interaction time than the WT–WT pairs (Fig. 4f), although in general young monkeys exhibited more frequent interaction than older monkeys. Thus, defective social behaviours were inherited in the F_1 generation of *MECP2* TG monkeys.

In summary, we have generated transgenic cynomolgus monkeys by using lentiviral infection of monkey oocytes. These TG monkeys showed an increased frequency of repetitive circular locomotion, increase anxiety, reduced social interaction and relatively weak cognitive phenotypes. Overall, we found no evidence of correlation between the copy number of transgenes and the extent of behavioural abnormalities, presumably owing to the low sample number with each copy number, and the possibility of nonspecific effects of gene transfer on behaviours could not be excluded. Importantly, we generated five F_1 TG offspring from one founder TG monkey, confirming the feasibility of germline transmission of lentiviral-based genetic engineering in monkeys. Together with recent progress in applying new gene-editing methods in monkey[29,30], our findings pave the way for the efficient use of genetically engineered macaque monkeys for studying brain disorders.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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

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Author Contributions Z.Q. and Q.S. conceived and supervised the project. T.-L.C. constructed the lentiviral constructs. Q.S. and Z.L. performed the cynomolgus oocytes preparation and injection. Y.-J.C., Y.W., C.-C.Z., Y.-H.N. and Z.L. contributed to monkey reproductive experiments. Y.-F.Z. performed PCR-based genotyping experiments. Z.-F.C., W.-J.B., X.-D.Z. and X.Y. performed immunohistochemistry and AccuCopy experiments. C.C., B.L., X.S. and Z.-Q.X. performed western blot experiments. X.L. and J.-J.W. performed behavioural analysis. J.-T.Z. and N.G. performed WGTA tests. W.-H.Z. and X.X. contributed to metabolic measurements and behavioural analysis. T.-L.C. and X.L. performed genomic integration sites analysis based on deep-sequencing. J.X., L.Z. and F.Z. helped with identification of genomic integration sites of transgenes. Z.Q. wrote the manuscript.

Author Information The raw sequence and processed data have been submitted to the NCBI Gene Expression Ominbus (GEO) under accession number GSE57974. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to Z.Q. (zqiu@ion.ac.cn) or Q.S. (qsun@ion.ac.cn).