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General Article

GENERAL ARTICLE

CRISPR/Cas9-mediated disruption of *SHANK3* in monkey leads to drug-treatable autism-like symptoms

Zhuchi Tu[1,†], Hui Zhao[2,3,†], Bang Li[1,†], Sen Yan[1], Lu Wang[4], Yongjin Tang[4], Zhujun Li[1], Dazhang Bai[1], Caijuan Li[1], Yingqi Lin[1], Yuefeng Li[5], Jianrong Liu[6], Hao Xu[4], Xiangyu Guo[1], Yong-hui Jiang[7,*], Yong Q. Zhang[2,*] and Xiao-Jiang Li[1,8,*]

[1]Guangdong-Hongkong-Macau Institute of CNS Regeneration, Ministry of Education CNS Regeneration Collaborative Joint Laboratory, Jinan University, Guangzhou 510632, China, [2]State Key Laboratory of Molecular Developmental Biology, CAS Center for Excellence in Brain Science and Intelligence Technology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China, [3]Key Laboratory of Regenerative Biology, South China Institute for Stem Cell, Biology and Regenerative Medicine, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou 510530, China, [4]Department of Nuclear Medicine and PET/CT-MRI Center, the First Affiliated Hospital of Jinan University & Institute of Molecular and Functional Imaging, Jinan University, Guangzhou 510630 China, [5]Guangdong Landau Biotechnology Co. Ltd., Guangzhou 510555, China. [6]Yuanxi Biotech Inc., Guangzhou 510663 China, [7]Department of Pediatrics and Department of Neurobiology, Duke University, Durham, NC 27710, USA and [8]Department of Human Genetics, Emory University School of Medicine, Atlanta, GA 30322, USA

*To whom correspondence should be addressed at: Xiao-Jiang Li, Guangdong-Hongkong-Macau Institute of CNS Regeneration, Ministry of Education CNS Regeneration Collaborative Joint Laboratory, Jinan University, Guangzhou 510632, China. Tel: +86 02085221351; Fax: +86 02085221351; Email: xli2@emory.edu; Yong Q. Zhang, State Key Laboratory of Molecular Developmental Biology, CAS Center for Excellence in Brain Science and Intelligence Technology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China. Tel: +86 1064807393; Fax: +86 01064806219; Email: yqzhang@genetics.ac.cn; Yong-hui Jiang, Department of Pediatrics and Department of Neurobiology, Duke University, Durham, NC 27710, USA. Tel: +919 6812789; Email: yong-hui.jiang@duke.edu

Abstract

Monogenic mutations in the *SHANK3* gene, which encodes a postsynaptic scaffold protein, play a causative role in autism spectrum disorder (ASD). Although a number of mouse models with *Shank3* mutations have been valuable for investigating the pathogenesis of ASD, species-dependent differences in behaviors and brain structures post considerable challenges to use small animals to model ASD and to translate experimental therapeutics to the clinic. We have used clustered regularly interspersed short palindromic repeat/CRISPR-associated nuclease 9 to generate a cynomolgus monkey model by disrupting *SHANK3* at exons 6 and 12. Analysis of the live mutant monkey revealed the core behavioral abnormalities of ASD, including

[†]The first three authors contributed equally to this work.

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impaired social interaction and repetitive behaviors, and reduced brain network activities detected by positron-emission computed tomography (PET). Importantly, these abnormal behaviors and brain activities were alleviated by the anti-depressant fluoxetine treatment. Our findings provide the first demonstration that the genetically modified non-human primate can be used for translational research of therapeutics for ASD.

Introduction

Mutations in *SHANK3* gene, which encodes a scaffolding protein in the postsynaptic density of excitatory synapses, contribute to approximately 1–2% of autism spectrum disorder (ASD) cases (1–4). *SHANK3* also plays a key role in the clinical presentations of Phelan–McDermid syndrome, a chromosome 22q13.3 deletion syndrome (5). Haploinsufficiency of SHANK3 is thought to be the mechanism responsible for the frequent presentation of ASD and other neurological comorbidity. Because of the well-established connection of SHANK3 to the synaptic development and function, SHANK3 causing ASD offers one of the best opportunities to model human ASD in model organisms.

Mutations in *SHANK3* have been introduced and characterized in Drosophila, zebrafish, mouse and rat. Remarkably, a total of 14 lines of *SHANK3* mutant mice have been reported (2,4). Analyses of these *Shank3* isoform or complete knock-out mutant mice have offered many novel insights regarding the role of SHANK3 in synapses and the pathophysiology caused by *SHANK3* mutations. However, one of notable findings is that, in contrast to severe clinical presentation in humans with heterozygous *SHANK3* mutations, there is no or only very mild phenotypes in heterozygous *Shank3* mutant mice (2,4,6). This observation indicates that differences between human and rodent brains influence their response to the deficiency of SHANK3 protein. Indeed, our recent report has discovered that SHANK3 protein is the most abundant in the prefrontal cortex (PFC) in monkey but is expressed at the highest level in striatum (Str) in mice (4,7,8). Furthermore, it has been well recognized that replication or translation of the therapeutic effects on rodent models to human clinical trials is often difficult (9,10). While there are many contributing factors for this difficulty, one of frequently debated subjects is whether an alternative model closer to human is needed for a preclinical study before launching an expensive clinical trial. Given those differences described, the importance of using non-human primates to model human diseases, especially psychiatric disorders, is clear.

We recently used clustered regularly interspersed short palindromic repeat (CRISPR)/CRISPR-associated nuclease 9 (Cas9) to disrupt exons 6 and 12 of *SHANK3* gene in cynomolgus monkeys (8). Our recent studies found that the complete loss of *SHANK3* in the PFC results in a significant neuronal loss which probably leads to the observed late embryonic lethality (8). However, one male monkey with a 2 bp deletion in exon 12 of *SHANK3* (*SHANK3*^{M3}) has lived for 26 months now. Longitudinal investigation over the last 2 years revealed that the *SHANK3*^{M3} monkey developed the core features of behavioral phenotypes of ASD, including impaired social interaction and apparent stereotypical locomotion. Furthermore, after treatment with fluoxetine, a selective serotonin reuptake inhibitor commonly prescribed for major depression and obsessive–compulsive disorder (OCD) (11), the impaired social interaction and stereotypical behaviors were markedly improved. Thus, by generation of *SHANK3* mutant monkey, we demonstrate that the non-human primate could faithfully recapitulate ASD-like behaviors, which holds a great potential for the development of effective therapeutics for ASD.

Results

Delayed growth and vocalization in *SHANK3* mutant monkey

In our previous study, we used the Cas9/sgRNA method to target exons 6 and 12 of *SHANK3* in cynomolgus monkeys (*Macaca fascicularis*), which resulted in different mutations including a large deletion (11 456 bp) and small indels of −2, −5, −8, −22 and +1 bp in the *SHANK3* gene (8). We observed a higher than expected embryonic and perinatal lethality associated with *SHANK3* targeting, suggesting that SHANK3 is important for early development in primates. Nevertheless, we obtained a single male cynomolgus monkey (*SHANK3*^{M3}) that carries a 2 bp deletion in peripheral tissues (8) and has survived for 26 months up to now. Due to the mosaic nature of CRISPR/Cas9-mediated mutations and the lack of brain samples, the exact nature and extent of *SHANK3* mutation in the brain of *SHANK3*^{M3} monkey remain to be determined. We examined the behaviors of *SHANK3*^{M3} monkey longitudinally after birth but more extensively after 12 months of age when abnormal behaviors started to emerge.

Although the fact of a single mutant monkey and the choice of control have posed a challenge for the experimental design, we believe that a longitudinal and repeated study of behavioral phenotypes could still offer valuable data as described in other behavioral studies using non-human primates (12,13) and case reports of human patients. Three age- and gender-matched (male) monkeys were used as controls, and all the monkeys for examination were housed in the same facility under the same living conditions. Newborn monkeys were kept together with mothers until 12 months of age. The first noticeable difference was that *SHANK3*^{M3} monkey did not vocalize until 18 months, whereas three control monkeys did so immediately after birth (Fig. 1A). The delayed vocalization in mutant monkey may be analogous to the delayed or no speech phenotype in young patients carrying *SHANK3* mutations (14,15).

We measured the growth parameters every 6 months after birth. The body weight and body length of *SHANK3*^{M3} monkey were significantly smaller and shorter, respectively, than those of control monkeys (Fig. 1B–D). However, the head circumference of *SHANK3*^{M3} monkey is comparable to the controls (Fig. 1E). No other behavioral difference between *SHANK3*^{M3} monkey and controls was noticed before they became one year old. We also noted enamel dysplasia, which had been reported in some autistic children with *SHANK3* mutations (16), in *SHANK3*^{M3} monkey (Fig. 1F).

Repetitive and anxiety-like behaviors in *SHANK3* mutant monkey

Video recording of daily activity is considered a simple and effective method to assess the behaviors of monkeys (17). We assessed the activity of monkeys in the home cage and the adaptability in a new cage using video recording of two half-hour daily sessions for six consecutive days. The behaviors were scored by three trained experimenters unaware of animal identity. Compared with control monkeys, *SHANK3*^{M3} monkey

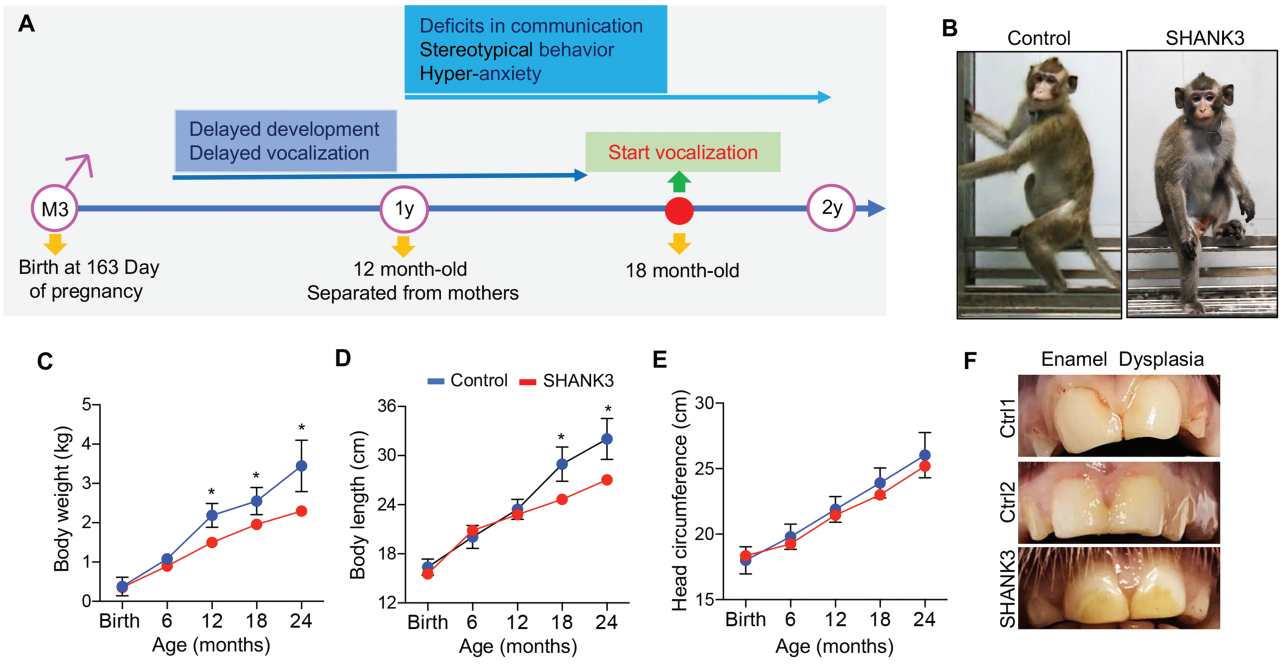

Figure 1. Delayed development and vocalization in *SHANK3* mutant monkey. **(A)** Schematic diagram summary of the development of behavioral phenotypes in *SHANK3*^{M3} monkey. **(B)** Representative pictures of *SHANK3*^{M3} monkey and an age- and gender-matched control monkey at the age of 20 months. **(C)** Body weight, **(D)** body length and **(E)** head circumference of *SHANK3*^{M3} monkey compared to 15 wild-type control monkeys. Statistical analyses were performed using GraphPad Prism 6.0. Data are presented as mean ± SEM. **(F)** Abnormal dental development of enamel dysplasia in *SHANK3*^{M3} monkey as compared to a control monkey.

exhibited significant stereotypic and anxiety-like behaviors in both home and new cages, which is evident by more frequent circling behavior (Supplementary Material, Video S1). This typical behavior has been reported to associate with anxiety in non-human primates (18,19). The stereotypical behavior was aggravated in the new cage (Supplementary Material, Video S2). Quantification of the duration of inactivity, exploration and stereotypical behaviors verified that *SHANK3*^{M3} monkey displayed stereotypical behavior. Compared to individual control monkey, *SHANK3*^{M3} monkey displayed significantly reduced time on environment exploration in the home cage (*SHANK3*^{M3} 2.2 ± 0.50 min; Ctrl1 12.89 ± 1.47 min, $^{**}P = 0.0012$; Ctrl2 17.10 ± 2.66 min, $^{**}P = 0.0038$; Ctrl3 10.92 ± 2.38 min, $^{*}P = 0.0124$) and in a novel cage (*SHANK3*^{M3} 7.71 ± 1.34 min; Ctrl1 28.05 ± 2.06 min, $^{***}P = 0.0006$; Ctrl2 21.38 ± 1.48 min, $^{***}P = 0.0007$; Ctrl3 18.59 ± 2.11, $^{*}P = 0.0169$). For the locomotion activity, no significant differences were found between *SHANK3*^{M3} monkey and the control monkeys in both the home cage (*SHANK3*^{M3} 2.16 ± 0.62 min, Ctrl1 1.16 ± 0.62 min, $P = 0.4469$; Ctrl2 4.90 ± 1.67 min, $P = 0.2273$; Ctrl3 11.96 ± 1.57 min, $^{**}P = 0.0016$) and the novel cage (*SHANK3*^{M3} 14.56 ± 2.41 min, Ctrl 6.60 ± 2.80 min, $P = 0.1479$; Ctrl2 8.88 ± 3.03 min, $P = 0.1425$; Ctrl3 5.81 ± 2.71 min, $P = 0.0623$; Fig. 2A). However, *SHANK3*^{M3} monkey exhibited stereotypical behavior more frequently than control monkeys in both the home cage (*SHANK3*^{M3} 13.23 ± 3.16 min, Ctrl1 0.00 ± 0.00 min, $^{**}P = 0.0085$; Ctrl2 1.78 ± 0.87 min, $^{*}P = 0.0172$; Ctrl3 7.58 ± 1.52 min, $P = 0.1931$) and the novel cage (*SHANK3*^{M3} 9.08 ± 1.50 min, Ctrl1 0.91 ± 0.49 min, $^{**}P = 0.0065$; Ctrl2 0.97 ± 0.83 min, $^{**}P = 0.0019$; Ctrl3 0.23 ± 0.08 min, $^{**}P = 0.002$; Fig. 2B).

Crook-tail is a behavior that also associates with anxiety or fearfulness in a juvenile monkey (20). *SHANK3*^{M3} monkey exhibited significantly longer time of crook-tail behavior than the control monkeys in the home cage (*SHANK3*^{M3} 34.28 ± 5.17 s, Ctrl1 1.67 ± 0.78 s, $^{*}P = 0.045$; Ctrl2 1.30 ± 0.79 s, $P = 0.1571$; Ctrl3 1.12 ± 0.72 s, $^{**}P = 0.0013$) and the novel cage (*SHANK3*^{M3} 56.75 ± 21.08 s, Ctrl1 2.80 ± 1.26 s, $^{*}P = 0.0106$; Ctrl2 3.37 ± 0.59 s, $P = 0.1364$; Ctrl3 1.85 ± 0.61 s, $^{**}P = 0.0035$; Fig. 2D). The mutant monkey also exhibited significantly high frequency of crook-tail in the home cage (*SHANK3*^{M3} 15.17 ± 2.89, Ctrl1 1 ± 0.37, $^{*}P = 0.0117$; Ctrl2 0.83 ± 0.31, $^{**}P = 0.0088$; Ctrl3 0.5 ± 0.34, $^{**}P = 0.0090$) and the novel cage (*SHANK3*^{M3} 26.83 ± 11.54, Ctrl1 2.5 ± 0.76, $^{**}P = 0.0074$; Ctrl2 2.3 ± 0.42, $^{**}P = 0.0070$; Ctrl3 2.56 ± 0.73, $^{**}P = 0.0078$; Fig. 2E).

Impaired social interaction in *SHANK3* mutant monkey

We next examined social interaction by performing a one-to-one interaction test using a protocol previously described (21), in which one monkey (*SHANK3*^{M3} or its age- and gender-matched control monkey) was paired with a sociable wild-type monkey. A sociable monkey was selected based on its frequent interactions with others (22). *SHANK3*^{M3} monkey did not actively initiate or respond to social interaction, as shown by the significantly less frequent and shorter duration of interactions with its partner in both active and passive social interactions compared with control monkeys. *SHANK3*^{M3} monkey initiated social interaction with a significantly lower frequency (*SHANK3*^{M3} 1.17 ± 0.47, Ctrl1 11 ± 2.90 $^{*}P = 0.0176$; Ctrl2 4.17 ± 0.87 $^{*}P = 0.0130$; Ctrl3 24.67 ± 3.95, $^{***}P = 0.0001$; Fig. 2F) and shorter duration (*SHANK3*^{M3} 0.13 ± 0.10 min, Ctrl1 5.91 ± 1.36 min, $^{*}P = 0.0104$; Ctrl2 6.65 ± 1.55 min, $^{**}P = 0.0086$; Ctrl3 13.43 ± 1.36 min, $^{***}P = 0.0002$; Fig. 2G). Consistently, *SHANK3*^{M3} mutant monkey received fewer interactions (*SHANK3*^{M3} 0.67 ± 0.33, Ctrl1 6.3 ± 1.65, $^{**}P = 0.0048$; Ctrl2 6.83 ± 1.35, $^{*}P = 0.0108$; Ctrl3 9.33 ± 1.59, $^{*}P = 0.0283$) with shorter duration (*SHANK3*^{M3} 0.03 ± 0.01 min, Ctrl1 11.35 ± 2.83 min, $^{**}P = 0.0033$; Ctrl2

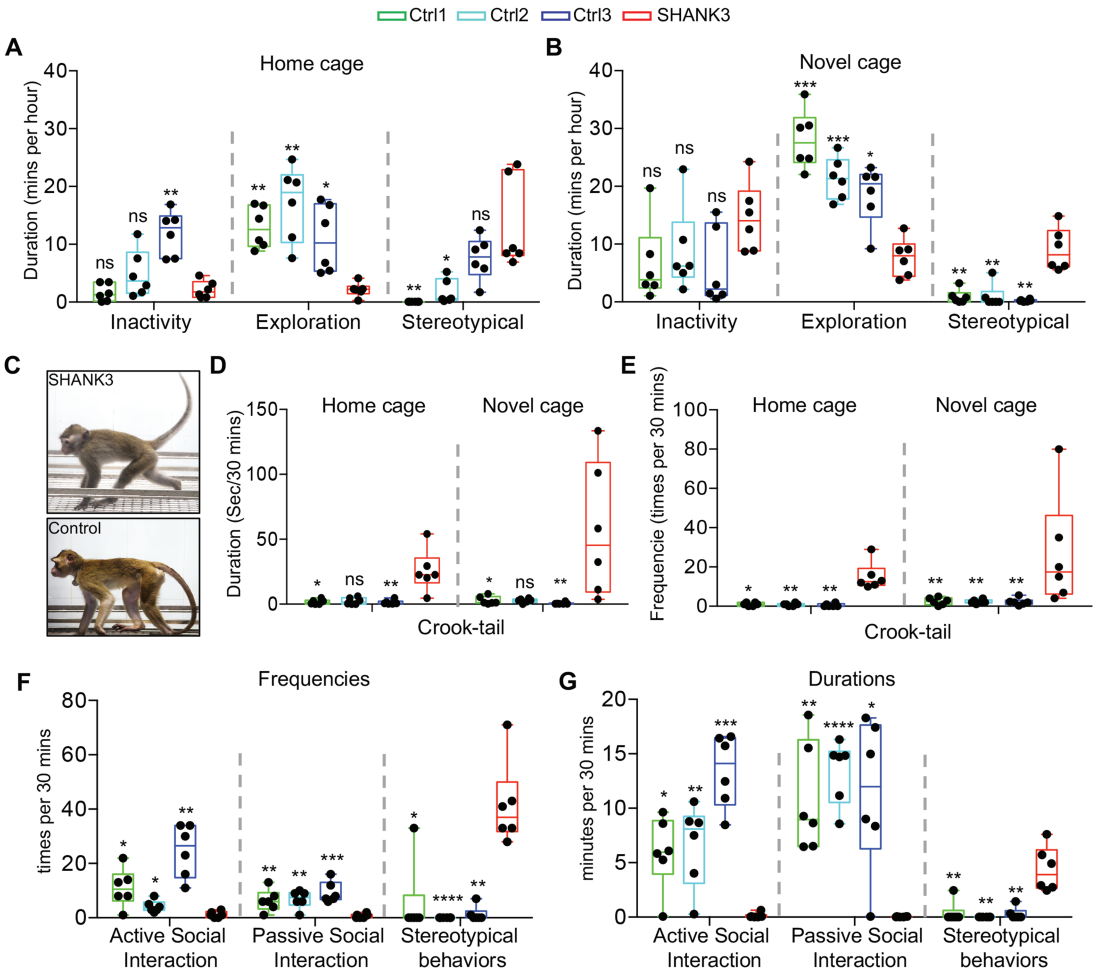

Figure 2. Impaired social and more repetitive behaviors in *SHANK3* mutant monkey. (**A**) Solo behaviors of *SHANK3*^{M3} and wild-type control monkeys in a familiar home cage and (**B**) a novel cage. Compared to each control monkey, *SHANK3*^{M3} monkey displayed significantly lower environment exploration in home cage and significantly higher stereotypical behavior than control monkeys in both home and new cage. (**C**) Representative pictures of *SHANK3*^{M3} monkey with crook-tail, a posture suggesting anxiety and fearfulness in juvenile monkeys. (**D**) The frequency and (**E**) duration of crook-tails in both home and novel cages. Three wild-type control monkeys were examined. The data are presented as mean ± SEM. NS, $^{*}P < 0.05$, $^{**}P < 0.01$, Mann–Whitney *U* test. (**F**) The frequency and (**G**) duration of active social interaction, passive social interaction and stereotypical behaviors when a test monkey of *SHANK3*^{M3} or wild-type control was paired with a sociable monkey. Data are analyzed by one-way ANOVA and presented as mean ± SEM. NS, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. See detailed statistic results in the text.

13.41 ± 1.19 min, $^{****}P < 0.0001$; Ctrl3 10.83 ± 2.05 min, $^{*}P = 0.0105$) than each control monkey. Instead, *SHANK3*^{M3} monkey displayed a greater frequency (*SHANK3*^{M3} 41.5 ± 6.3, Ctrl1 5.5 ± 5.5, $^{**}P = 0.0016$; Ctrl2 0 ± 0, $^{****}P < 0.0001$; Ctrl3 1.33 ± 1.15, $^{**}P = 0.0012$; Fig. 2F) and longer duration (*SHANK3*^{M3} 4.39 ± 0.83 min, Ctrl1 0.41 ± 0.41 min, $^{**}P = 0.0039$; Ctrl2 0.00 ± 0.00 min, $^{**}P = 0.0033$; Ctrl3 0.29 ± 0.24 min, $^{**}P = 0.0010$; Fig. 2G) of stereotypical behaviors, including circling in the cage than each control monkey. When grouped with other sociable control monkeys, *SHANK3*^{M3} monkey displayed social interaction deficits that prevented it from developing a close relationship with the control monkeys (Supplementary Material, Video S3).

Reduced eye contact in *SHANK3* mutant monkey

Eye contact is considered to be essential for communication and social interaction in both humans and other primate species (17,23). Compared with control monkeys, *SHANK3*^{M3} monkey displayed significantly less gazing with shorter time (*SHANK3*^{M3} 18.99 ± 4.038 s, Ctrl1 69.81 ± 5.017 s, $^{**}P = 0.0015$; Ctrl2 68.38 ± 2.910 s, $^{***}P = 0.0001$; Ctrl3 88.60 ± 13.42 s, $^{**}P = 0.0047$; Fig. 3A) and lower frequency of eye contact with the person who was examining the monkey (*SHANK3*^{M3} 9.833 ± 1.537, Ctrl1 35.67 ± 3.148, $^{**}P = 0.0045$; Ctrl2 35.33 ± 5.364, $^{***}P = 0.0004$; Ctrl3 43.83 ± 11.44, $^{***}P = 0.0001$; Fig. 3B). Instead, the mutant monkey showed intense anxiety and fear by frequently circling in the cage (Supplementary Material, Video S4).

Reduced brain activity in *SHANK3* mutant brain

Positron emission computed tomography (PET) is a non-invasive *in vivo* molecular imaging technique that is widely used in clinical diagnosis and treatment evaluation (24–26). With the assistance of the most common PET tracer [^{18}F] fluorodeoxyglucose ([^{18}F]FDG), as well as the exquisite information on brain substructures provided by magnetic resonance imaging (MRI), PET can help us identify brain endophenotype associated with *SHANK3* mutations. In order to investigate possible structural and functional changes of mutant brain, both MRI and PET/computerized tomography (CT) were conducted

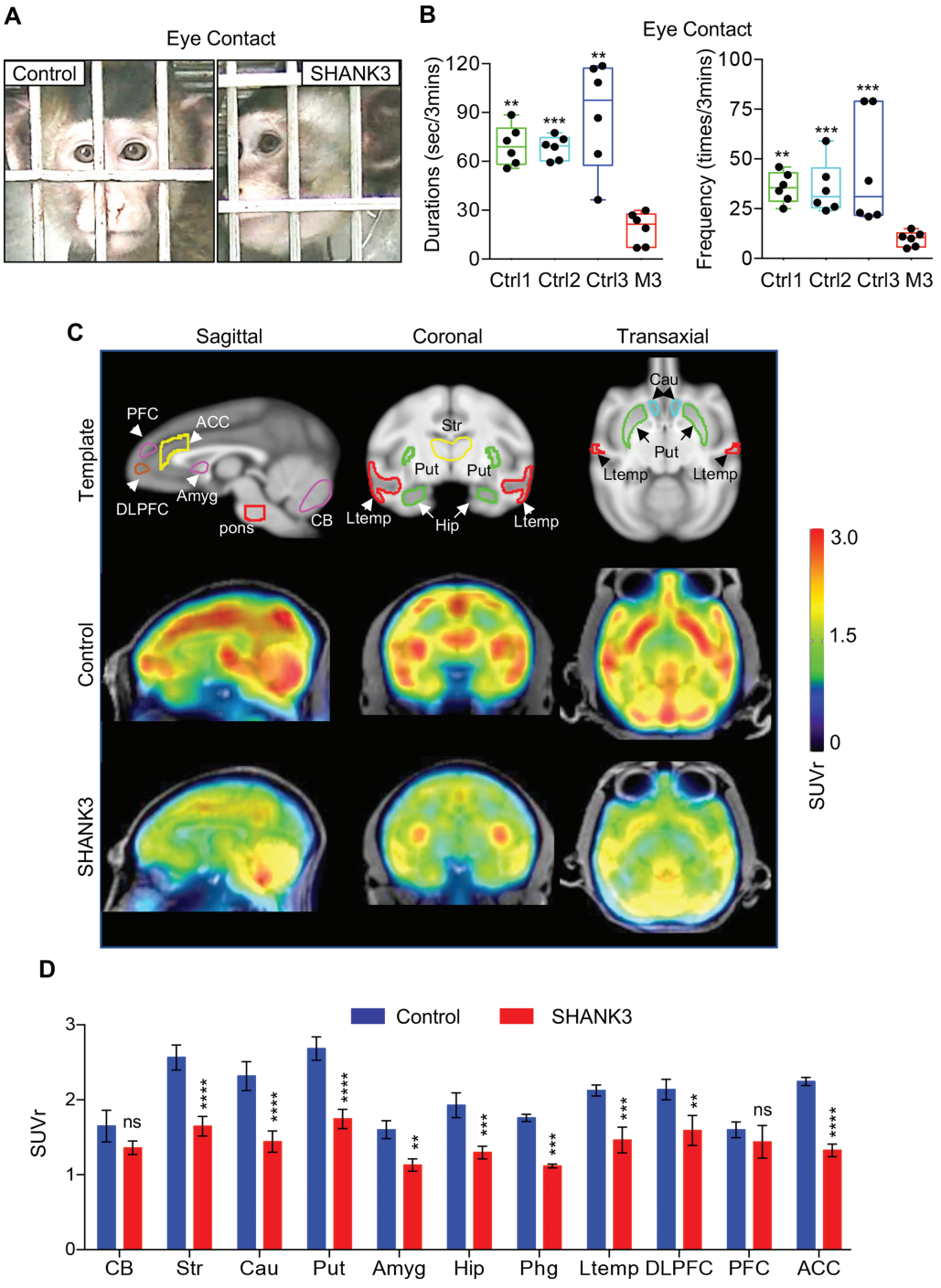

Figure 3. Impaired eye contact and decreased glucose metabolism in *SHANK3* mutant monkey. (**A**) Representative photos of eye contact of monkeys at 24 months old. (**B**) *SHANK3*^{M3} monkey displayed significantly shorter eye contact duration with less frequency than control monkeys. (**C**) PET-MRI imaging evaluation of [^{18}F]FDG in control and *SHANK3*^{M3} monkey. Patterns of brain regions (the upper panel) and representative brain PET images overlaid on their individual MRI (control monkey in the middle panel and *SHANK3*^{M3} monkey in the bottom panel) are presented. Regional radioactivity was normalized to the injected radioactivity and body weight and expressed as the SUVr with Pon uptake as reference. (**D**) Regional SUVr values in different brain regions in control ($n = 3$) and *SHANK3*^{M3} monkeys. Data were obtained from three PET-MRI imaging tests, presented as mean ± SEM and are analyzed by two-way ANOVA. NS, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ (see detailed statistic results in the text).

in mutant and control monkeys. Morphological T1-weighted 3-dimensional MRI demonstrated no obvious structural abnormality in *SHANK3*^{M3} mutant. However, the [^{18}F]FDG-PET study revealed that the glucose metabolism in *SHANK3*^{M3} monkey brain tissues was significantly lower than that observed in normal controls. As shown in Figure 3C and D, using pons (Pon) as the reference region (27,28), we observed that the regional brain standardized uptake value ratio (SUVr) values for *SHANK3* were specifically reduced in a variety of brain regions when compared with the control [cerebellum (CB) *SHANK3*^{M3}

1.36 ± 0.05, Ctrl 1.65 ± 0.21, P = 0.0756; Str *SHANK3*^{M3} 1.65 ± 0.07, Ctrl 2.6 ± 0.1672, ****P < 0.0001; caudate (Cau) *SHANK3*^{M3} 1.442 ± 0.08192, Ctrl 2.3 ± 0.19, ****P < 0.0001; putamen (Put) *SHANK3*^{M3} 1.75 ± 0.07, Ctrl 2.69 ± 0.16, ****P < 0.0001; amygdala (Amyg) *SHANK3*^{M3} 1.13 ± 0.05, Ctrl 1.6 ± 0.12, **P = 0.0049; hippocampus (Hip) *SHANK3*^{M3} 1.29 ± 0.05, Ctrl 1.93 ± 0.17, ***P = 0.0002; parahippocampal gyrus (Phg) *SHANK3*^{M3} 1.12 ± 0.01, Ctrl 1.76 ± 0.05, ***P = 0.0002; temporal lobe (Ltemp) *SHANK3*^{M3} 1.46 ± 0.10, Ctrl 2.12 ± 0.07, ***P = 0.0001; dorsal lateral prefrontal cortex (DLPFC) *SHANK3*^{M3} 1.59 ± 0.12, Ctrl 2.14 ± 0.14, **P = 0.0013; PFC *SHANK3*^{M3} 1.44 ± 0.13, Ctrl 1.60 ± 0.10, P = 0.3170; anterior cingulate cortex (ACC) *SHANK3*^{M3} 1.33 ± 0.05, Ctrl 2.23 ± 0.05, ****P < 0.0001]. These results showed significant decrease of glucose metabolism in the mutant monkey brain, which is consistent with the lower glucose metabolic activity in the brains of some ASD patients (29,30).

Improved ASD core symptoms and brain metabolism in *SHANK3* mutant monkey upon fluoxetine treatment

The robust autism-like behaviors observed in *SHANK3*^{M3} monkey prompted us to test whether this monkey model can be used for future drug development. Currently, there is no effective drug treatment for the core symptoms of ASD and *SHANK3*-related disorders. However, many clinicians have tried different anti-psychotic medications for managing the various behavioral issues associated with ASD. Fluoxetine, a commonly prescribed medication for major depression and OCD, has been used to treat repetitive behaviors in ASD patients (31,32). We treated *SHANK3*^{M3} and control monkeys with fluoxetine at 2.5 mg/kg per day, a dose used in previous studies for non-human primate and ASD patients (32,33). The behaviors of animals were then monitored by video recording daily. To better assess the therapeutic effects, we measured animal behavioral changes after fluoxetine treatment by comparing fold changes over the pre-treatment. We found that the treatment for 2 weeks markedly alleviated the repetitive behaviors and significantly increased active social interaction duration (*SHANK3*^{M3} 15.21 ± 1.38, Ctrl1 1.50 ± 0.48, ***P = 0.002; Ctrl2 2.07 ± 1.39, **P = 0.0062; Ctrl3 0.59 ± 0.07, ***P = 0.0003) and frequency (*SHANK3*^{M3} 15.53 ± 1.46, Ctrl1 3.89 ± 1.41, **P = 0.0028; Ctrl2 2.35 ± 0.29, **P = 0.001; Ctrl3 0.92 ± 0.18, ***P = 0.0006) for *SHANK3*^{M3} monkey (Fig. 4A; Supplementary Material, Video S5). Fluoxetine also significantly prolonged passive interaction duration (*SHANK3*^{M3} 10.98 ± 1.12; Ctrl1 1.26 ± 0.26, ***P = 0.0006; Ctrl2 1.44 ± 0.25, **P = 0.0011; Ctrl3 1.39 ± 0.49, **P = 0.002) but moderately increased passive social interaction frequency (*SHANK3*^{M3} 4.58 ± 1.29; Ctrl1 1.60 ± 0.31, P = 0.32; Ctrl2 2.85 ± 1.05, P = 0.99; Ctrl3 2.57 ± 0.79, P = 0.98). In addition, fluoxetine treatment alleviated stereotypical behavior by reducing its duration (*SHANK3*^{M3} 0.14 ± 0.06; Ctrl1 0.93 ± 0.05, ***P = 0.004; Ctrl2 1.0 ± 0.0, ***P = 0.0001; Ctrl3 0.90 ± 0.09, **P = 0.0017) and frequency (*SHANK3*^{M3} 0.05 ± 0.02; Ctrl1 0.78 ± 0.14, *P = 0.0112; Ctrl2 1.0 ± 0.0, ****P < 0.0001; Ctrl3 0.88 ± 0.12, **P = 0.0026; Fig. 4C). Moreover, fluoxetine treatment increased eye contact duration (*SHANK3*^{M3} 3.41 ± 1.04; Ctrl1 0.65 ± 0.07, **P = 0.004; Ctrl2 1.07 ± 0.11, *P = 0.0148; Ctrl3 0.60 ± 0.07, **P = 0.0034) and frequency of *SHANK3*^{M3} monkey (*SHANK3*^{M3} 4.55 ± 0.79; Ctrl1 0.88 ± 0.16, *P = 0.0247; Ctrl2 1.21 ± 0.17, *P = 0.0201; Ctrl3 0.91 ± 0.11, *P = 0.0219; Fig. 4D; Supplementary Material, Video S6). Also, *SHANK3*^{M3} monkey became less frequently to stay or play alone by itself (Supplementary Material, Video S5).

Furthermore, we also observed that brain glucose metabolism in *SHANK3*^{M3} monkey was increased to the level as the control monkeys, as indicated by PET imaging results (Fig. 4E). Regional brain SUVr value changes in *SHANK3*^{M3} monkey after fluoxetine treatment was increased while those of controls remained unaltered (CB, *SHANK3*^{M3} 1.21, Control 1.16 ± 0.08; Str, *SHANK3*^{M3} 1.43, Control 1.647 ± 0.07; Cau, *SHANK3*^{M3} 1.41, Control 0.92 ± 0.13; Put, *SHANK3*^{M3} 1.45, Control 0.96 ± 0.10; Amyg, *SHANK3*^{M3} 1.34, Control 0.91 ± 0.11; Hip, *SHANK3*^{M3} 1.37, Control 0.94 ± 0.11; Phg, *SHANK3*^{M3} 1.40, Control 0.97 ± 0.09; Ltemp, *SHANK3*^{M3} 1.48, Control 0.93 ± 0.08; DLPFC, *SHANK3*^{M3} 1.49, Control 0.97 ± 0.09; PFC, *SHANK3*^{M3} 1.23, Control 0.94 ± 0.13; ACC, *SHANK3*^{M3} 1.66, Control 0.97 ± 0.11). These results indicate that fluoxetine treatment corrected the network activities in various brain regions in *SHANK3*^{M3} monkey, consistent with the previous finding that the PET imaging functionality in ASD patients could be improved by fluoxetine (29). Taken together, these findings demonstrate that ASD-related abnormalities in *SHANK3*^{M3} monkey are reversible and would allow future studies to identify therapeutics that can be efficient to treat ASD.

Discussion

ASD represents one of the most important and common neurodevelopmental and neuropsychiatric diseases. Accordingly, there are considerable research efforts to tackle ASD from many different aspects. However, our understanding of the pathogenesis of ASD remains poor, and the effective treatment of ASD remains to be developed. These is an apparent question of whether many currently well-studied rodent models are sufficient to model the complex social behavior associated with ASD (34,35). *SHANK3* mutant monkeys generated by us (8) and other recently established *MECP2* mutant monkeys using transgenic or TALEN-mediated methods (21,36) have demonstrated the value of genetically modified non-human primate models for ASD research.

Using the CRISPR/Cas9-mediated genetic manipulation, we obtained a live mutant monkey that carries a 2 bp deletion in exon 12 of the *SHANK3* gene. This mutation is predicted to result in a frameshift at Q419 (Q419fsX486) between ANK and SH3 domains for the majority of *SHANK3* isoforms. It should be noted that a single base-pair insertion in an autism patient (37), which leads to a premature stop codon at the residue 503 (A447fsX503), is close to the 2 bp deletion of *SHANK3*^{M3} monkey. Because we were unable to analyze the brain tissues of this live *SHANK3*^{M3} monkey, we could not determine the *SHANK3* mutation types and mosaicism in its brain. However, the robust core features of ASD and abnormal brain activity in *SHANK3*^{M3} monkey lend the support for SHANK3 haploinsufficiency, which is well established in ASD patients with *SHANK3* mutations (3,15).

Through longitudinal and repeated studies over 14 months by three experimenters and the comparison with three age- and gender-matched control monkeys, we found that *SHANK3*^{M3} monkey displayed abnormal behaviors that recapitulate core ASD features of impaired social interaction and repetitive behaviors. The delayed vocalization in *SHANK3*^{M3} monkey is noteworthy. Although the exact meaning of delayed vocalization is not clear, it is possible that monkeys communicate via vocalization, and the defect in vocalization may be analogous to the delayed or absent speech in many young patients with *SHANK3* mutations (15,38). The impaired social interaction of *SHANK3*^{M3} monkey is apparent and constant, which is in clear contrast to the subtle or

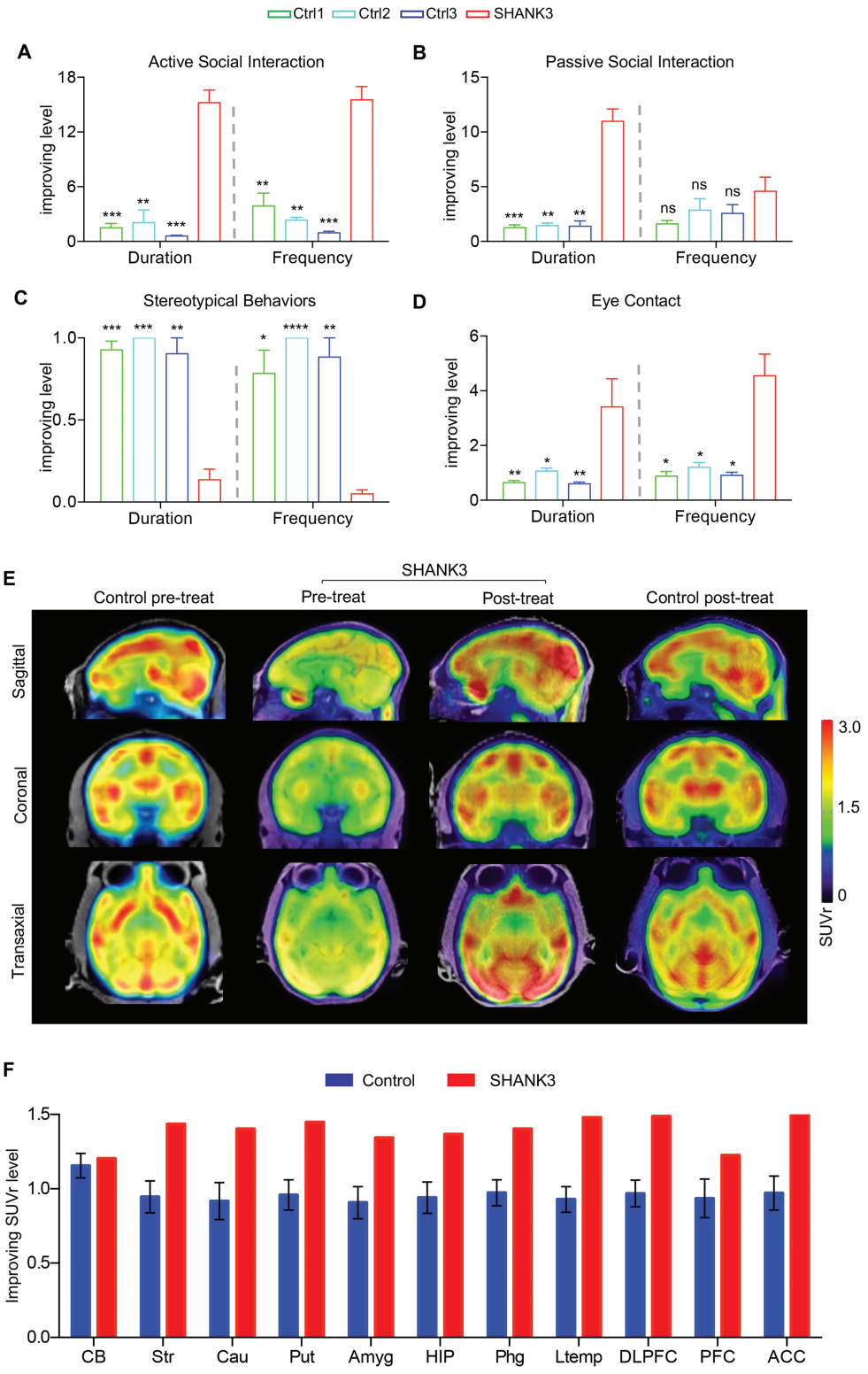

Figure 4. Fluoxetine robustly improves autism symptoms and increased glucose metabolism to a normal level in *SHANK3* mutant monkey. (**A**, **B**) Fluoxetine treatment at 2.5 mg/kg/per day significantly increased active (A) and passive (B) social interaction duration and frequency of *SHANK3*^{M3} monkey, while control monkeys remained the same level of interaction with drug treatment. The results are presented as fold changes of post-treatment compared with pre-treatment. Fluoxetine also significantly prolonged passive interaction duration but moderately increased passive social interaction frequency. (**C**) Fluoxetine alleviated stereotypical behavior by reducing its duration and frequency. (**D**) Fluoxetine increased eye contact duration and frequency of *SHANK3*^{M3} monkey. (**E**) Representative brain PET [^{18}F]FDG images of pre- and post-fluoxetine treatment in a normal control or *SHANK3*^{M3} monkey. (**F**) Regional brain SUVr value changes in control and *SHANK3*^{M3} monkey after fluoxetine treatment. Data are presented as mean ± SEM ($n = 3$ for control for comparing to *SHANK3*^{M3} monkey) and analyzed by one-way ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

no behavioral phenotypes of *Shank3* heterozygous mutant mice (6). Similarly, the circling behavior of *SHANK3*^{M3} monkey in cage is apparent and reproducible. The robust behavioral phenotypes of *SHANK3*^{M3} monkey strongly indicate that the non-human primate offers a good alternative to model ASD.

Despite the similarity in the phenotypes of the *SHANK3*^{M3} monkey, there are some nontrivial caveats that need to be considered and discussed. First, our findings are based on the results from one mutant monkey. Due to the lower than expected rate of newborn *SHANK3* mutant monkeys and the critical function of SHANK3 for early development of the monkey brain (8), it is difficult to obtain a large number of live founder animals by CRISPR/Cas9 gene editing. In addition, the genotype and phenotype correlation in humans has not been firmly established because of molecular heterogeneity of *SHANK3* mutations (3,15). Thus, it is expected to see a wide range of behavioral phenotypes in individual CRISPR/Cas9-targeted monkeys if their mutations are not identical. In the future, non-human primate cloning technique may facilitate the generation of monkeys with the same genetic modification (39). While it is reasonable to be cautious in drawing a conclusion from a single case study, like other typical case studies in clinic, our longitudinal observational studies presented here provided clear evidence for the abnormal behaviors in *SHANK3*^{M3} monkey.

From the studies of *SHANK3* in both rodent and non-human primate models, we have observed clear and significant differences among them. In humans, there are no reports on any case with homozygous *SHANK3* mutation, but patients with heterozygous *SHANK3* mutation are typically severely affected. In rodents, a complete depletion of *Shank3* in homozygotes does not affect the survival and early development. Heterozygotes of *Shank3* mutant mice show very mild or no abnormal behavioral phenotypes (6). In contrast, the complete depletion of the *SHANK3* gene in the certain monkey brain region results in significant neuronal loss and late embryonic lethality (8). This may suggest that complete loss of *SHANK3* in human is likely early lethal, and the live *SHANK3* mutant monkey is more likely to have a partial loss or haploinsufficiency of *SHANK3*.

Our findings also demonstrate that the ASD-like symptoms in *SHANK3* mutant monkey can be effectively alleviated by the treatment with the antidepressant fluoxetine. Fluoxetine is a member of the class of selective serotonin reuptake inhibitors drug commonly prescribed as an antidepressant. Empirically, many clinicians prescribe fluoxetine to treat repetitive behaviors in ASD children, including *SHANK3* mutation-causing ASD (Joan Jasien, personal communication). In a small scale of double-blind placebo-controlled fluoxetine trial, Holland *et al.* (32) reported a significant improvement for repetitive behavior in adult individuals with ASD. Although the mechanism underlying the efficacy of fluoxetine treatment in *SHANK3*^{M3} monkey remains to be investigated, the strong response to fluoxetine treatment provides the first example that the non-human primate model may be valuable for future drug development of ASD.

Materials and Methods

Animals

SHANK3^{M3} monkey and age- and gender-matched healthy control cynomolgus monkeys (*M. fascicularis*) were housed in cages and examined for their behaviors at Guangdong Landau Biotechnology Co. Ltd., which is an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility. All animal-related protocols were approved in advance by the Institutional Animal Care and Use Committee of Guangdong Landau Biotechnology Co. Ltd and Jinan University. Commercial monkey diet (Ke-Ao, #HFZ-15kg, Beijing) was provided *ad libitum* twice per day. Health and behavior of the monkeys were monitored daily by the husbandry staff and veterinarians. All animals completed the whole study.

Measurement of physical characteristics

Body weight, length and head circumference of *SHANK3*^{M3} monkey and 15 age- and gender-matched control monkeys, which were housed in the same condition, were measured at the first day of every 6 months.

Behavioral observations

Direct observation or recording of monkey behaviors when they were housed individually was performed following previously published protocols (40). *SHANK3*^{M3} monkey and three age- and gender-matched control monkeys were observed in both the home cage (L60 × W70 × H80 cm) and the novel cage (L120 × W85 × H140 cm) to assess solo behavioral manifestations in the absence of social stimulation. The behavioral categories of inactivity, environment exploration and stereotypical behaviors were statistically analyzed. The inactivity is defined as not engaged in any behaviors with open or close eyes. The environment exploration is defined as a monkey manipulates objects in the cage with hands and/or mouth non-repetitively and investigates cage (searches, sniffs bars) (41). The stereotypical behaviors are defined as repetitive and consistent actions with no apparent purposes, including pacing (repetitive, ritualized movement usually involving circling in the cage), self-grasping (grabbing or holding onto part of their own body), rocking (a back and forth movement of the upper body with still feet), bouncing (jumping up and down on all four legs) and cage shaking (any vigorous shaking of the cage) (41). The tested monkeys were recorded twice daily (half an hour a section) at the same time of the day (0900–1130; 1400–1630) for six consecutive days.

Social interaction test

Social interaction test was performed in the set of one-to-one interaction using the previously reported protocol for monkeys (21). *SHANK3*^{M3} or its age- and gender-matched control monkey was paired with one wild-type sociable monkey, a monkey with good social skills and high tendency to interact with others (22). The measured behaviors included active social interaction, passive social interaction, staying alone and stereotypical behaviors. Active social interaction is defined as initiating a play, sharing toys, grooming for others and sitting together (within another monkey's arms' reach or in contact), while passive social interaction is defined as receiving aforesaid social interactions from the sociable monkey. The monkey is considered as staying alone when there is no other monkey within its arms' reach. The stereotypical behaviors are defined earlier. In our experiment, the tested monkeys were recorded once daily (half an hour a day) at the same time of the day (1400–1630) for six consecutive days.

Each video recording was analyzed simultaneously by three trained observers unaware of animal identity. During the scoring, the observers recorded the frequency and duration of a specific behavior by manually starting and stopping the video under the condition that they all agreed on the classification of the

observed behavior. The inter-observer correlation coefficient was found to be >0.90 after training by SPSS (Chicago, IL, USA) statistical analysis. The frequency and duration of each behavior were statistically compared between *SHANK3*^{M3} and control monkeys.

Eye contact

Eye contact assay was carried largely following a previous protocol (17). During the test, the experimenter familiar to the tested monkeys sat opposite the monkey at a 1 m distance, and the experimenter was directly looking at the monkey for 3 min per section, once a day (0900–1030), and each monkey was tested for six consecutive days. The monkeys' (*SHANK3*^{M3} and three controls) responses were videotaped by a video camera that was positioned behind the experimenter. The frequency and duration of eye contact between the tested monkey and the experimenter were statistically analyzed.

PET-MR scan and imaging data process

We performed non-invasive multi-modality brain imaging study of *SHANK3*^{M3} and three control monkeys on a 3.0 T scanner (GE Discovery 750, Milwaukee, USA) and a PET/CT system (GE Discovery Elite 690, Waukesha, USA) at the PET/CT-MRI center at the First Affiliated Hospital of Jinan University. The PET tracer [^{18}F]FDG was obtained from Guangzhou HTA Co., Ltd. Animals were anesthetized by ketamine (10 mg/kg; i.m.) and placed into the scanner in the supine position for T1-weighted 3-dimensional MR scan. A circular 32-channel array head coil was placed on top of the monkey's head. Parameters for T1 sequence were as follows: repetition time (TR) = 8.4 ms, echo time (TE) = 3.4 ms, inversion time (TI) = 450 ms, slice thickness = 1.1 mm, matrix size = 256 × 256 and field of view (FOV) = 18 × 18 cm. For PET/CT section, animals were deprived of food for 12–15 h before [^{18}F]FDG injection but allowed to drink water at any time. Each monkey was intravenously injected ca. 1.0 mL of [^{18}F]FDG (18.5 MBq/kg), then kept in a shielded room with minimal ambient noise and light. After 50 min, the subject was immobilized with ketamine (10 mg/kg; i.m.), placed into the scanner in the supine position and maintained under anesthesia with 1–3% isoflurane and 98.4% oxygen. Head position was fixed with a stereotactic frame. CT scan was performed first, followed by eight-minute static positron emission data collection at 60 min post-injection. CT data were acquired in breath-hold with 140 kV, 230 mA modulated using the GE AutomA technique (GE Medical System, Milwaukee, USA) with a noise index of 30, slice thickness of 3.75 mm, slice interval of 3.27 mm, matrix size of 512 × 512 and scan FOV of 50 cm. PET data were acquired in 3-dimensional time-of-flight (TOF) mode with a slice thickness of 3.27 mm, slice interval of 3.75 mm, matrix size of 256 × 256 and scan FOV of 70 cm. The PET data were attenuation-corrected by the integrated CT attenuation-corrected (CTAC). The CT data were reconstructed in standard mode with display field of view (DFOV) of 30 cm and window width/window level of 100/45, advanced statistical iterative reconstruction 40%. The PET data were then reconstructed in terms of the point spread function together with TOF technology with DFOV of 30 cm.

Data analysis was performed using PMOD 3.9 (Pmod Technologies LLC, Zürich, Switzerland). The co-registration of PET image to individual MR image, which was transformed into brain template MR images, was carried out following published protocols (26,42). Volume of interests were extracted from the CB, Str, Cau, Put, Amyg, Hip, Phg, Ltemp, DLPFC, PFC, ACC and Pon. The uptake of [^{18}F]FDG in Pon was used as reference for SUVr analysis as previously described (42). Statistical analyses were performed with GraphPad Prism 6.0 (GraphPad Software, La Jolla, USA).

Fluoxetine treatment

Fluoxetine hydrochloride (20 mg/tablet, approved for human use; Patheon France), was grounded into powder and mixed with food for oral taking. The dosing regimen was chosen based on previous studies of fluoxetine in non-human primates and ASD patients (32,33). Over two-year-old monkeys (one mutant and three controls) were orally given fluoxetine hydrochloride for 2 weeks with a dosing began at 1.6 mg/kg/day for the first 3 days and adjusted to 2.5 mg/kg/day for the rest (11 days). Phenotypes, including stereotypical behaviors and eye contact, were examined daily 1 week after the drug administration for 2 weeks. Tested monkeys were recorded once daily (30 min a day) at the same time of the day (1400–1630) for six consecutive days.

Statistical analyses

Data analysis was conducted using the SPSS version 19.0 software package. The normality of the data was analyzed by Kolmogorov–Smirnov tests (Kolmogorov–Smirnov tests: $P > 0.05$). All the data except the stereotypical behavior (frequencies: $P = 0.002$ and durations: $P = 0.006$) were normally distributed. One-way ANOVA was used to analyze the differences between the mutant and control monkeys in active social interaction, passive social interaction and staying alone. One-way ANOVA was used to perform all pairwise comparisons between the mutant and each control monkey. Mann–Whitney *U* tests were used to analyze the frequency and duration of stereotypical behavior. Regional brain SUVr data were analyzed by two-way ANOVA. The alpha level was set at $P = 0.05$ (not significant (NS), $P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$). For body weight, body length and head circumference, statistical analyses were performed with GraphPad Prism 6.0 (www.graphpad.com). All *P*-values were generated using two-sided tests, and all the data were presented as the mean ± standard error of the mean (SEM).

Supplementary Material

Supplementary Material is available at *HMG* online.

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