Whole-brain in vivo base editing reverses autistic-like behaviors in mice

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Abstract

Autism spectrum disorder (ASD) is a highly heritable neurodevelopmental disorder with deficits in social communication and stereotypical behaviors. Whole-brain genome editing to correct single-base mutations and alleviate autistic-like behaviors in animal models has not been achieved. Here we developed an APOBEC-embedded cytosine base editor (AeCBE) system, for converting C·G to T·A base pairs. We demonstrate the effectiveness by targeting AeCBE to an ASD-associated mutation of the *MEF2C* gene (c.104T>C, p.L35P) *in vivo*. We constructed a *Mef2c* L35P heterozygous mouse, which exhibited autistic-like behavioral deficits. We programmed AeCBE to edit the mutated C·G base pairs of *Mef2c* in the mouse brain, via the intravenous injection of blood brain barrier (BBB)-crossing AAV. This treatment restored MEF2C protein levels and reversed impairments in social interactions and repetitive behaviors in *Mef2c* mutant mice. This work presents an *in vivo* base editing paradigm in which a single-base mutation in the brain could be successfully corrected.

One-Sentence Summary

Base editing *in vivo* in the mouse brain corrects autistic-like behaviors.

Introduction

Autism Spectrum Disorder (ASD) is a highly heritable neurodevelopmental disorder, characterized by deficits in social interaction and stereotypic behaviors ^{1–3}. Rare *de novo* variants, including single nucleotide variants (SNVs) and copy number variants (CNVs), have been confirmed as important contributors to the pathogenesis of ASD ^{4, 5}.

Myocyte-specific enhancer factor 2C (MEF2C), a member of the MEF2 transcriptional factor family, was reported to be implicated in ASD, as recurrent *de novo* variants of the *MEF2C* gene were found in people diagnosed with ASD ^{6, 7}. MEF2C is abundantly expressed in the cortex, hippocampus and amygdala of adult mice ⁸ and plays a vital role in neuronal differentiation, neural development and synaptic plasticity ^{9–14}. Microdeletions of the *MEF2C*-containing chromosomal segment (5q14.3-q15) causes developmental deficits in children, including intellectual disability (ID), poor reciprocal behaviors, lack of speech, stereotypic and repetitive behavior, and epilepsy, suggesting that *MEF2C* haploinsufficiency causes severe defects of brain development.

The Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) system has been widely used for genome editing ^{15, 16}. With well-designed single guide RNA (sgRNA), double-stranded DNA breaks (DSBs) are introduced into genomic targets via Cas9-mediated cleavage, followed by either non-homologous end-joining (NHEJ) or homology-directed repair (HDR) pathway ¹⁷. Although several approaches increasing HDR efficiencies have been developed to repair diseases-causing genetic mutations ¹⁷, induced DSBs may lead to unexpected genomic instability. Meanwhile, base editors (BEs), which edit single base pairs precisely without generation of DSBs, have

been developed through fusion of Cas9 nickase with various deaminases^{18,19}. Cytidine and adenosine deaminases have been used to generate cytosine base editors (CBEs) for C-T conversion and adenine base editors (ABEs) for A-G conversion, respectively²⁰. Take advantage of adeno-associated virus (AAV)-mediated delivery, *in vivo* base editing has been applied to various disease models^{21,22}. Nevertheless, whether base editors can be applied for neurodevelopmental disorders remains to be addressed.

In this work, we identified a *de novo* SNV in the *MEF2C* gene (c.104T > C, p.L35P) from people with ASD. We constructed *Mef2c*-L35P knock-in mouse and observed that *Mef2c*-L35P heterozygous mice displayed autistic-like behaviors, such as social deficits and repetitive behavior. *Mef2c* protein expression in the brain of *Mef2c*-L35P heterozygous mice was markedly reduced compared to WT mice, suggesting that the L35P mutation may decrease protein structural stability or accelerate *Mef2c* protein degradation.

We designed an APOBEC-embedding CBE system (AeCBE) to edit the mutated mouse *Mef2c* 104C back to 104T efficiently^{23,24}. Taken advantage of the blood brain barrier (BBB)-crossing AAV-PHP.eB system, we delivered AeCBE into the mouse brain by intravenous injection^{23,24}. We showed that the newly designed AeCBE-mediated base editing *in vivo* was sufficient to restore MEF2C protein levels and correct the behavioral defects in social interactions and repetitive behaviors of *Mef2c*-L35P heterozygous mice.

Results

Identification of a *de novo* SNV in the MEF2C gene in a Chinese patient with ASD

We performed whole-exome sequencing of one ASD patient with unaffected parents collected in the Xinhua hospital affiliated to Shanghai Jiao Tong University School of Medicine and identified a *de novo* SNV in the *MEF2C* gene (c.104T > C, p.L35P) (Fig. 1a and Fig. S1a), which we validated by Sanger sequencing (Fig. 1b). The amino acid change (L35P) caused by the *de novo* variant is located in the MCM1, AGAMOUS, DEFICIENS, and SRF (MADS) domain of MEF2C protein (Fig. 1c, S1b). This variant (*MEF2C*, chr5: 88804752) is not present in over 18,800 genomes from East Asian populations in the gnomAD database (<http://gnomad.broadinstitute.org>), indicating that it is a rare variant. In a recent report including 112 Chinese patients with intellectual disability and Rett-like symptoms, researchers identified numerous *de novo* and inherited variants in *MEF2C*, suggesting that *MEF2C* is a critical risk gene for developmental disorders in the Chinese population²⁵. A schematic illustration of various locations of genetic mutations in the MEF2C protein is shown in Fig. 1c^{26–30}.

To investigate whether the L35P mutation may affect the proper function of the MEF2C protein, we examined the expression level of flag-tagged wild-type (WT) and L35P MEF2C protein in both mouse cortical neurons and HEK293 cells. Intriguingly, we found that the protein level of MEF2C-L35P was significantly lower compared to MEF2C-WT (Fig. 1d-1i), whereas mRNA levels of WT and L35P MEF2C remained the same (Fig. 1e, 1h), suggesting that the L35P mutation may affect the level of MEF2C protein.

To further investigate the importance of L35 for MEF2C protein stability, we tested the impact of other hydrophobic amino acids such as isoleucine or phenylalanine (I/F), as well as alanine (A). L35F, L35I and L35A all led to decreased levels of MEF2C proteins, indicating that L35 is critical for MEF2C protein stability (Fig. S1c, S1d). After cycloheximide (CHX) treatment, an inhibitor of protein translation³¹, levels of MEF2C-L35P decreased faster than MEF2C-WT, indicating that the L35P mutation accelerated MEF2C protein degradation (Fig. 1j, 1k). Treatment with the proteasome inhibitor bortezomib (BTZ) restored levels of MEF2C-L35P, providing evidence that the rapid degradation of MEF2C-L35P was mediated by the ubiquitin-dependent pathway (Fig. 1l, 1m). Finally, to investigate whether the MEF2C-L35P protein may affect the protein level of MEF2C-WT, we co-transfected MEF2C-WT along with MEF2C-L35P into HEK293 cells. Surprisingly, we found that co-expression of MEF2C-L35P with MEF2C-WT led to marked reduction of total MEF2C protein level (Fig. 1n, 1o), suggesting that the L35P mutation exhibits a dominant negative effect on MEF2C.

Mef2c-L35p Leads To Aberrant Neuronal Dendritic And Axonal Development

To investigate the impact of MEF2C-L35P on neurons, we constructed a short hairpin RNA (shRNA) specifically targeting the mouse *Mef2c* gene. With two designed shRNA candidates (Fig. S2a), we assessed the knock-down efficiency by examining endogenous *Mef2c* mRNA levels in mouse cortical neurons (Fig. S2b). We found that expression of endogenous Mef2c protein was effectively reduced by sh*Mef2c*-1 (Fig. S2c, S2d).

Consistent with a previous report³², we found that knockdown of *Mef2c* led to decreased dendritic length and branch numbers, as well as axon length (Fig. S3a-f), which could be fully restored by co-transfection of a shRNA-resistant MEF2C-WT construct, but not MEF2C-L35P (Fig. S3a-f). These results suggest that the L35P mutation impairs MEF2C function in neurons.

Abnormal neural development and autistic-like behaviors in Mef2c-L35P knock-in mice

To investigate the role of MEF2C-L35P in ASD pathogenesis, we constructed *Mef2c* L35P knock-in mice by CRISPR/Cas9-mediated gene targeting (Fig. S4a). MEF2C protein levels of *Mef2c* L35P^{+/-} mice were markedly decreased compared to WT mice (Fig. 2a-2c). Since MEF2C is widely expressed in the cortex, hippocampus and amygdala^{8,33}, we performed immunohistochemical staining to examine the expression of MEF2C in the brain of *Mef2c* L35P^{+/-} mice. We found that the fluorescence intensity of MEF2C signals in *Mef2c* L35P^{+/-} outer cortex, dentate gyrus and amygdala were reduced compared to WT mice (Fig. 2d-2g).

Multiple ASD animal models exhibit aberrant inhibitory interneuron development³⁴, and it was reported that the population of parvalbumin (PV) positive interneurons decreased in the hippocampus of *Mef2c*^{+/-} mice^{32,35,36}. Thus, we examined PV-positive GABAergic neurons in the brain of *Mef2c* L35P^{+/-} mice.

By immunohistochemical staining of parvalbumin, we found that there was a prominent reduction of PV-positive interneurons in the retrosplenial cortex (RSC), dentate gyrus (DG), somatosensory cortex (SC) and visual cortex (VC) of *Mef2c* L35P^{+/-} mice compared to WT mice (Fig. S4b-4f). In contrast, *Mef2c* L35P^{+/-} mice showed normal populations of somatostatin positive interneurons in RSC, Hip and SC, suggesting that MEF2C dysfunction specifically impaired development of PV-positive interneurons (Fig. S4g-S4j). The aberrant development of PV-positive GABAergic neurons suggests that an imbalance of excitatory/inhibitory (E/I) synaptic transmission may exist in the brain of *Mef2c* L35P^{+/-} mice, which may contribute to ASD pathogenesis^{37,38}.

***Mef2c* -L35P^{+/-} mice display autistic-like and *Mef2c* haploinsufficiency syndrome-like behaviors**

We next examined whether MEF2C-L35P mutation affected the gross development of the mice by measuring body weights of WT and *Mef2c*-L35P^{+/-} mice from birth to 9 weeks old. We found there was no difference in body weights between WT and *Mef2c*-L35P^{+/-} mice (Fig. S5a). Previous reports have shown that *Mef2c*^{+/-} mice exhibited various abnormal behaviors, including deficits in social interaction, repetitive behaviors and hyperactivity^{13,32}. Interestingly, using the classic three-chamber test, we found that *Mef2c*-L35P^{+/-} mice exhibited normal social approach but abnormal performance in the social novelty test compared to WT mice (Fig. 2h-2n). In the novel object recognition test, *Mef2c*-L35P^{+/-} mice showed similar preference for novel object over familiar object compared to WT mice, suggesting that *Mef2c*-L35P^{+/-} mice have a specific defect in recognizing novel partners (Fig. S5b-d).

Mef2c-L35P^{+/-} mice did not display anxiety-like phenotypes, but exhibited remarkable hyperactivity in the open field test (Fig. 2o-2r). In the elevated plus maze test, we found that *Mef2c*-L35P^{+/-} mice exhibited more preference for open arms rather than closed arms, suggesting that *Mef2c*-L35P^{+/-} mice showed hyperactivity rather anxiety-like phenotype (Fig. S5h-S5j). *Mef2c*-L35P^{+/-} mice also exhibited prominent repetitive behavior, showing significantly more self-grooming and scratching than WT mice (Fig. S5k).

Lastly, we examined whether *Mef2c* L35P^{+/-} mice have normal spatial learning and memory capability with Barnes maze. We found that *Mef2c* L35P^{+/-} mice exhibited the same learning curve during training session and cumulative duration within the target zone in the test session compared to WT mice, indicating that *Mef2c* L35P^{+/-} mice have normal ability for learning and memory for spatial information (Fig. S5e-S5g).

Establishment of the APOBEC-embedding CBE for correcting the *Mef2c*-L35P mutation

To correct the *Mef2c* L35P mutation (c.104T > C), CBEs are required to convert mutated C·G base pairs to T·A. Based on the existing CBEs^{18,19}, we designed a new CBE tool derived from SpG, a *Streptococcus pyogenes* Cas9 variant targeting NGN protospacer-adjacent motif (PAM)³⁹, with human cytidine deaminase APOBEC3A-Y130F with minimal RNA off-targeting effects^{40,41}, and uracil glycosylase inhibitor (UGI) (Fig. 3a). Previously, we and others reported that fusing adenosine deaminase inside Cas9

may improve base editing efficiency of ABE^{23,42}. In order to locate the position to fuse cytidine deaminase (APOBEC3A-Y130F) inside of Cas9 nickase, we performed a series of screening and found that fusing APOBEC3A-Y130F in the 1249 a.a. of nCas9 yield best editing efficiency and accuracy in the range of C8-C14 (Fig. S6a, S6b)⁴³. This new CBE system is thereby named as APOBEC-embedding CBE (AeCBE).

Then we designed two sgRNAs (sgRNA-C8 and sgRNA-C15) targeting the mutation site 104C of *Mef2c* gene in the C8 and C15 site, respectively (Fig. 3b). To evaluate the efficiency of the newly developed AeCBE system in post-mitotic neurons, we co-transfected AeCBE with two sgRNAs into cultured primary cortical neurons from *Mef2c* L35P^{+/-} mice. Neurons transfected with both sgRNA and AeCBE were collected with fluorescence activated cell sorting (FACS) to evaluate the mutation status (c.104T > C) by PCR and Sanger sequencing (Fig. 3c). We found that the wild-type T percentage (86%) edited with sgRNA-C8 (SgC8) and AeCBE is significantly higher than that in the negative control (49%), while the T percentage edited with sgRNA-C15 and AeCBE remained similar (55%) to negative control (51%) (Fig. 3d). Therefore, sgRNA-C8 was chosen for subsequent *in vivo* therapeutic base editing of *Mef2c* L35P^{+/-} mice.

Next, we wonder how AeCBE compares to the available CBE systems such as the classic APOBEC-Y130F-SpG, as well as the Anc689-SpG system⁴⁴ (Fig. 3e). After transfection of various constructs into cultured *Mef2c* (c.104T > C) neurons, we collected transfected neurons using FACS and subject DNA samples to Sanger sequencing (Fig. 3f) and next-generation sequencing (Fig. 3g). We found that the C104T editing rate was significantly higher with the AeCBE system, comparing to the classic CBEs (Fig. 3f, 3g).

In vivo base editing mediated by AAV in *Mef2c* L35P^{+/-} mice

Because of the limited packaging capacity of AAV, we used an intein-mediated split strategy to generate dual-AAV system^{21,45}, with one AAV containing the N-terminus of SpG (a.a. 1-793) and sgRNA-C8 expression cassette, and the other AAV containing the C-terminus of SpG-APOBEC3A-UGI (a.a. 794–1368) (Fig. 4a). To deliver the AeCBE system into the mouse brain, we used the BBB-crossing adeno-associated virus (AAV-PHP.eB) and delivered the AAVs by tail veins injection in 1-month old *Mef2c* WT or L35P^{+/-} mice (Fig. 4a)^{21,24}.

We first evaluated the delivery efficiency of AAV-PHP.eB vectors using AAV-hSyn-EGFP, and immunohistochemical analysis 6–8 weeks after intravenous injection. We found that the expression of EGFP was widely distributed in the mouse brain, especially in cortical, hippocampal and midbrain regions (Fig. 4b). To assess the expression efficiency of the dual-AAV AeCBE system in the brain of *Mef2c* L35P^{+/-} mice, we performed immunohistochemical staining with an antibody against SpCas9, which could recognize the SpCas9 variant SpG, and found that SpG was expressed in the cortical regions as well as hippocampus (Fig. 4c).

We next investigate whether the decreased MEF2C protein level in *Mef2c* L35P^{+/-} mice could be rescued after *in vivo* base editing by AeCBE, we performed immunoblot with brain lysates collected from WT or

Mef2c L35P^{+/-} mice injected with either AAV-EGFP or AAV-AeCBE. We found that dual-AAV AeCBE successfully restored MEF2C protein in prefrontal cortex and hippocampus of *Mef2c* L35P^{+/-} mice to levels comparable to WT mice (Fig. 4d-g). Immunohistochemistry also demonstrated that the endogenous MEF2C protein level was significantly rescued in cortical regions and amygdala of *Mef2c* L35P^{+/-} mice (Fig. 4h-k).

Since AAV infections in after BBB-crossing are likely not ubiquitously distributed in the brain, it is difficult to determine the gene editing efficiency *in vivo*. Thus we designed a new approach to examine the editing efficiency of the AeCBE system on the *Mef2c* (c.104T > C) site *in vivo*. First, we performed immunostaining against endogenous MEF2C protein on the prefrontal brain slices. Then we using the laser-mediated microdissection system to remove small parts of slices containing positive MEF2C signals (150µm diameter, 30µm thick), followed by PCR to amplify the target segments containing *Mef2c* (c.104T/C) and potential off-target sites, which were subject to next-generation sequencing (Fig. 4l), as well as Sanger sequencing (Fig. S6c). We found that the C104T editing efficiency was around 20% in various animals (Fig. 4l). Since AeCBE elements are driven by the human synapsin 1 promoter (*hSyn*), base editing may preferentially function within the neurons in the mouse brain. We further performed the immunostaining against SpCas9 and NeuN, a pan-neuronal marker, and found that SpCas9 was expressed in over 50% NeuN positive neurons, suggesting the potential high efficiency of base editing in the mouse brain (Fig. S6d, S6e). We further evaluated the off-targeting effects by performing next-generation sequencing of PCR-amplified segments from laser-dissected prefrontal cortex samples from *Mef2c* L35P^{+/-} mice injected with AAV-AeCBE (Fig. S6f). Among potential off-target sites (OT1-OT11), some off-target editing events were found in 2 sites (OT2-C7, OT5-C8) (Fig. S6f), suggesting that off-targeting effects in the AeCBE system *in vivo* are minimal.

Furthermore, PV-positive neurons in various brain regions, such as retrosplenial cortex and dentate gyrus of *Mef2c* L35P^{+/-} mice were also restored after *in vivo* base editing with AeCBE, indicating that the imbalance of excitatory/inhibitory synaptic transmission in mutant mice may be recovered (Fig. 4m-n, Fig. S7a-b)⁴⁶, even in adult mice. Intriguingly, the decreased level of PV-positive neurons in the visual cortex and somatosensory cortex were not restored after AAV-AeCBE injection, suggesting that the restoration of excitatory/inhibitory synaptic functions in the brain of *Mef2c* L35P^{+/-} mice might be region-specific (Fig. S7c-e).

To assess whether injection of AeCBE could rescue synaptic function in *Mef2c* L35P^{+/-} mice, we used a sparse labeling method to label neurons in medial prefrontal cortex (mPFC) of WT and *Mef2c* L35P^{+/-} mice with injection of GFP or AeCBE (Fig. S8a, b). We found that the density of mushroom spines and total spines in the mPFC of *Mef2c* L35P^{+/-} mice were markedly decreased compared to WT mice, which was significantly rescued by AeCBE, suggesting that impaired excitatory synaptic functions are largely rescued in the *Mef2c* L35P^{+/-} mice with the dual-AAV AeCBE system (Fig. S8c-e)

In vivo base editing corrects autistic-like behaviors of *Mef2c* L35P^{+/-} mice

Finally, to test if autistic-like behaviors of *Mef2c* L35P^{+/-} mice could be rescued by *in vivo* base editing with the dual-AAV AeCBE system, we performed behavioral analysis. We conducted the three-chamber test for WT or *Mef2c* L35P^{+/-} mice four weeks after AAV injection. In general, AAV injection did not affect the social approach for WT or *Mef2c* L35P^{+/-} mice (Fig. 5a-e), however, the impaired social novelty observed in *Mef2c* L35P^{+/-} mice was fully rescued as compared to WT mice (Fig. 5f-i). We conducted another paradigm of social interaction behaviors, the social intruder test, for WT or *Mef2c* L35P^{+/-} mice treated with either AAV-AeCBE or AAV-GFP. This test measures reciprocal interaction time of mice actively interacting with a strange partner for 4 consecutive trials, followed by interacting with a new strange partner for the 5th trial (Fig. 5j, 5k). We found that the decreased initial social interaction time during the first trial between *Mef2c* L35P^{+/-} mice and partners was fully rescued by the dual-AAV AeCBE system, further demonstrating that behavioral impairments can be rescued by *in vivo* AeCBE postnatally (Fig. 5j). On the fifth trial, we found that *Mef2c* L35P^{+/-} mice injected with AAV-EGFP had severe defects in recognizing familiar or stranger partners, compared to WT mice injected with AAV-EGFP (Fig. 5k-o). However, the abnormal social interaction of *Mef2c* L35P^{+/-} mice was fully rescued after delivery of *in vivo* AeCBE (Fig. 5k-o), indicating that correction of the *Mef2c* mutations *in vivo* can restore the normal social behaviors in mice.

Finally, we tested if *in vivo* base editing could rescue the hyperactive phenotypes of *Mef2c* L35P^{+/-} mice. In the open field test, we found that *Mef2c* L35P^{+/-} mice treated with dual-AAV AeCBE displayed similar locomotion activity to WT mice (Fig. 5p-r). We also observed that the increased self-grooming behaviors in *Mef2c* L35P^{+/-} mice were rescued by AeCBE (Fig. 5s). Taken together, these results indicate that the dual-AAV AeCBE strategy can potentially rescue behavioral deficits in a mouse model of ASD, even if administered postnatally.

Discussion

Previous reports indicate that *Mef2c*^{+/-} mice exhibit behavioral deficits mimicking people with ASD, thus serving as a faithful animal model³². Interestingly, NitroSynapsin, a new dual-action compound similar to the FDA approved drug memantine, is able to rescue the behavioral deficits, E/I imbalance, and histological abnormalities of *Mef2c*^{+/-} mice, suggesting that modulating synaptic activity may be able to reverse the autistic-like phenotypes in postnatal animal models of ASD³².

Recently, there are reports showing that application of genome editing tools postnatally in specific brain regions of ASD mouse models, including prefrontal cortex, anterior cingulate cortex and hippocampus, could successfully rescue autistic-like defects, indicating that modulation of synaptic activity via genetic manipulations may also provide a reliable way to regulate animal behaviors during pathological status⁴⁷⁻⁴⁹. However, for therapeutic purposes, viral injections into specific regions of the brain of ASD patients may not be ideal approaches so far.

To overcome these obstacles, we have developed an easier way of genome editing targeting neurons in the brain via a BBB-crossing AAV system. We showed that through a newly designed CBE system and intein-mediated split strategy, CRISPR-based CBE could be successfully delivered into the brain via dual AAVs and performed base editing effectively. Though the AAV-PHP.eB vector usually preferentially infected neurons in cortical and hippocampal regions, we observed that the protein level of MEF2C in the specific brain regions and autistic-like behaviors of *Mef2c* L35P^{+/-} mice, including abnormal social interaction and hyperactivity, were both largely rescued. These results establish the framework of using genome base editing tools *in vivo* in the brain to correct genetic mutations and provide potential therapeutic approaches for people with ASD. Because we were able to reverse phenotypes even postnatally, it gives hope for the development of therapeutic approaches for adolescents and adults living with ASD.

Declarations

Report summary

Further information is available in the Nature Research Reporting Summary linked to this article.

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Author contributions

Conceptualization, TLC, ZQ. Base editing plasmids construction, JLC. Biochemical experiments, SJZ, JCW. Mouse experiments: WKL, YTY, ZYX, SFS, ZFC. Laser dissections, WKL, BQX, SQZ, CZ. Next generation sequencing preparation, WKL, JLC, WJH. NGS data analysis, BY. Animal data analysis: WKL, JCW. Viral injection and analysis, WLP, WKL. Writing: WKL, TLC, ZQ.

Competing interests

Authors declare no competing interests.

Methods

Discovery of *MEF2C* rare *de novo* SNV and clinical information on the patient harboring *MEF2C* Leu35Pro.

Previously, we collected and sequenced blood genomes acquired from one aged two years and half boy with ASD related phenotypes as well as his parents with Xinhua hospital affiliated to Shanghai Jiaotong University School of Medicine and the patient clinically diagnosed with Rett-like syndrome was based on the definition of Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition. Additionally, on the aspects of bioinformatics analysis of the *MEF2C* variants discovered and identified in this article, the longest of *MEF2C* transcript isoform was used as a reference sequence (NM_001193347.1, NP_001180276.1). According to the clinical diagnosis of Xinhua hospital, the patient with autosome *MEF2C* rare *de novo* SNV, Leu35Pro exhibited neuronal developmental retardation, poor eye contact, motor abnormality and involuntary hand movement.

Engineer and construction of plasmids.

Human *MEF2C* WT transcript 3 (NM_001193347.1) CDS sequence, which was synthesized in Genescript corporation, was built into empty FUGW-EGFP driven by UbC promoter (Addgene plasmid 81018; using for HEK293 cell transfection) and pCAGGS driven by CAG promoter (modified by our lab; using for neuronal cell transfection) vector. On the aspect of the construction of *MEF2C* L35P (L35F, L35I, L35A) vectors, we adopted KOD-Plus- Mutagenesis Kit (TOYOBO Bio-Technology, CO., LTD.) to reproduce T to C (corresponding replacement) conversion on the position of *MEF2C* WT CDS, which was an inverse PCR (iPCR)-based site-directed mutagenesis kit using KOD-plus DNA polymerase (No. KOD-201) as a high-fidelity PCR enzyme. To be mentioned, IRES sequence was inserted into the location between human *MEF2C* WT CDS and EGFP, and additionally FLAG tag was inserted into the plasmid following *MEF2C* WT CDS. In addition, two different shRNA were inserted into FUGW-H1 vector (Addgene plasmid 25870) for knock-down assays. Besides, the empty FUGW-EGFP vector was co-transfected into cultured primary mouse cortical neurons for enhancing the intensity of GFP fluorescence. What needed to be mentioned was that these shRNA sequence we designed was located at the non-homologous region of mouse *Mef2c* transcript 3 through aligning to human *MEF2C* transcript 3, suggesting that these two designed shRNA were not available to target to *MEF2C* mRNA to achieve the purpose of knock-down. All FUGW, FUGW-H1, pCAGGS plasmids were packaged into lentivirus for improving transfection efficiency in cultured mouse neurons.

For base editing efficiency of APOBEC3A-CBE system *in vitro*, *Mef2c* sgRNA was cloned into pU6-sgRNA-EF1 α -UGI-T2A-mCherry plasmid linearized with BsaI, and paired oligonucleotides were synthesized, annealed, and inserted for sgRNA expression vectors construction. Besides, pCBH-SpG-1249-APOBEC3A^{Y130F}-T2A-EGFP plasmid was engineered with APOBEC3A inserted into SpG on the position of 1249 amino acid (PX461, Addgene plasmid 48140) ¹. AncBE4-SpG was generated as described

previously⁵⁰, in which nCas9 was replaced with SpG (D10A) variant. N-A3A (Y130F)-SpG was generated by replacing deaminase in AncBE4-SpG with APOBEC3A (Y130F).

For *Mef2c* gene correction in *Mef2c* L35P^{+/-} mice by means of delivery of APOBEC3A-CBE system, AAV-hSyn-1-1248-pU6-sgRNA plasmid was linearized with BsmBI, and paired oligonucleotides were inserted for sgRNA expression. AAV-hSyn-1249-APOBEC3A^{Y130F}-1368-UGI plasmid was engineered of AAV-hSyn-1249-SpG plasmid linearized with SpeI and BsrGI, and APOBEC3A^{Y130F}-UGI component with same enzymes, and inserted for vector construction (Fig. 5a). Subsequently, above two vectors were packaged into AAV-PHP.eb virus by Shanghai Obio corporation in order to efficiently infect with neuronal cells in the whole brain via crossing the BBB. The corresponding sequences used in the plasmid construction were listed as followed.

Human *MEF2C* L35P point mutation sequence:

forward: 5'-CGAGCGTGCTGTGTGACTGTGAGATTGC-3'.

reverse: 5'-GCTCATAAGCCTTCTTCATCAACCCAAATTCCTC-3'.

Human *MEF2C* L35F point mutation sequence:

forward: 5'-TTCAGCGTGCTGTGTGACTGTGAGATT-3'.

reverse: 5'-GCTCATAAGCCTTCTTCATCAACCCAAATTCCTC-3'.

Human *MEF2C* L35I point mutation sequence:

forward: 5'-ATAAGCGTGCTGTGTGACTGTGAGATT-3'.

reverse: 5'-GCTCATAAGCCTTCTTCATCAACCCAAATTCCTC-3'.

Human *MEF2C* L35A point mutation sequence:

forward: 5'-GCCAGCGTGCTGTGTGACTGTGAGATT-3'.

reverse: 5'-GCTCATAAGCCTTCTTCATCAACCCAAATTCCTC-3'.

Mouse *Mef2c* shRNA-1 sequence: 5'-GCCATCAGTGTCTGAGGATGT-3'.

Mouse *Mef2c* shRNA-2 sequence: 5'-GTCTGAGGATGTGGATCTGCT-3'.

The DsRed shRNA sequence was used as balnk control: 5'-AGTTCCAGTACGGCTCCAA-3'.

Mef2c sgC8 sequence: 5'-TATGAGCCGAGCGTGCTGTG-3'.

Mef2c sgC15 sequence: 5'-AAGGCTTATGAGCCGAGCG-3'.

Genetic modified mice

The C57BL/6 *Mus musculus* was used as the experimental model in this article. All procedures were approved by the Animal Care and Use Committee of the Center for Excellence in Brain Science & Intelligence Technology, Chinese Academy of Sciences, Shanghai, China. The use and care of animals were in accordance with the guidelines of this committee. According to animal welfare requirements, all experimental mice were bred in a pathogen free (PF) unit under constant temperature (approximately 22 °C), humidity (approximately 55%RH), ventilation and automatic circadian rhythm on the condition of a 12h light/dark cycle (light from 7 a.m. to 7 p.m., dark from 7pm to 7 am) with food and water provided ad libitum. *Mef2c* L35P knock-in mice were designed and constructed by CRISPR/Cas9 mediated homologous recombination strategy on the genetic background of C57BL/6 mouse by Biocytogen Co.,Ltd. The sequences of small guide RNA (sgRNA) for *Mef2c* L35P knock-in mice and the targeting donor for *Mef2c* L35P knock-in mice were shown as followed. The genotype of *Mef2c* L35P knock-in mice, used in experiments was identified by sanger sequencing and tail genome PCR primers were shown as followed.

Mef2c L35P knock-in sgRNA sequence:

5'-ATATGTTCACTTACTGCGAC TGG-3'.

5'-ATGAAGGACTATATAGTCCA AGG-3'.

Mef2c L35P knock-in mice sequence:

forward: 5'-GAATGTGTTAGCACCCAAGACTCTG-3'.

reverse: 5'-GCATGTTGCAGCCATAGATGGGGTA-3'.

Fluorescence-activated cell sorting.

Neuronal cells transfected with sgRNA-mCherry and CBE-EGFP plasmids were digested into a single-cell suspension and collected before acquisition on a flow cytometer at DIV4. Cells were sorted and counted on a BD FACScan flow cytometer (FACS Aria II) (BD Biosciences). In addition, we used the 561- to 614-nm channel to detect mCherry signal and the 488- to 513-nm channel for EGFP. Data were acquired with BD CellQuest Pro software (BD Biosciences) and analyzed with FlowJo flow cytometry analysis software (Tree Star).

Stereotactic and tail intravenous injection of AAV virus.

For sparse labelling to investigate neuronal spine development *in vivo*, the mixture of AAV-hSyn-Cre (0.05μl of $1\sim1.25\times10^{13}$ vg ml⁻¹, generated by Shanghai Taitool corporation) and AAV-hSyn-DIO-tdTomato (0.05μl of 2.9×10^{12} vg mL⁻¹, generated by Guangzhou PackGene corporation) was directly injected stereotactically into bilateral medial prefrontal cortex (mPFC) of continuously anaesthetized mice after

glass pipette penetration. The stereotactic coordinates targeting to the mPFC region were shown as followed: anteroposterior (AP), +2.45 mm; mediolateral (ML), 0.3 mm; dorsoventral (DV), -1.0 mm. The mice were euthanized and perfused one month later after viral stereotactic injection.

For investigating the effect of amelioration of aberrant phenotype in *Mef2c* L35P^{+/-} mice after delivery of APOBEC3A-CBE system, AAV-PHP.eB-hSyn-EGFP, AAV-PHP.eB-hSyn-1-793-U6-sgC8 and AAV-PHP.eB-794-APOBEC3A^{Y130F}-1368 were packaged by Shanghai Obio corporation and titered through qPCR.

Anaesthetized 1-month-old *Mef2c* L35P^{+/-} mice were injected through tail vein with the mixture of AAV-PHP.eB-hSyn-1-793-U6-sgC8 (150µl of 4.9×10¹² vg ml⁻¹) and AAV-PHP.eB-793-APOBEC3A^{Y130F}-1368 (150µl of 4.4×10¹² vg ml⁻¹), while AAV-PHP.eB-hSyn-EGFP (300µl of 4.5×10¹² vg ml⁻¹) was injected into *Mef2c* WT and L35^{+/-} mice with the same manipulation as negative control. We euthanized the mice at 10-12 weeks of age to examine ASD-related neurodevelopmental pathological phenotypes.

Genome DNA on-target and off-target activities with DNA deep-sequencing.

We extracted genome DNA from hippocampus tissues of *Mef2c* L35P^{+/-} mice injected with either EGFP or CBE and then amplified and mixed the target sequences with the addition of index sequence by PCR. Illumina TruSeq Nano DNA LT Library Prep Kit (FC-121-4001, Illumina, USA) was used with 20 ng of mixed DNA samples for the construction of sequencing libraries with standard Illumina protocols. DNA-seq was conducted using Illumina Novaseq 6000 platform in Personal Biotechnology Co., Ltd, Shanghai, China.

Potential off-target sites were predicted as described previously with online software (benchling.com)⁵¹. All potential off-target sites with off-target score >1 (11 sites) were selected for subsequent analysis. PCR products for all 11 sites were successfully obtained and sequencing. Amplicons were aligned using CRISPResso2⁸ to evaluate the editing frequencies (C to T or G to A) at off-target sites.

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Figures

Figure 1

Figure 1

A *de novo* mutation in the *MEF2C* gene associated with ASD causes rapid degradation of MEF2C protein.

(a) Schematic illustration of the *de novo* *MEF2C* variant identified in a Chinese ASD simplex family (Squares and circles represent males and females; blank represent non-carrier; Half blank and half black represent carrying the *de novo* variant in the *MEF2C* gene). **(b)** Validation of the *de novo* variant of *MEF2C* by sanger sequencing (Red box indicates the variant). **(c)** The location of the *de novo* variant

(L35P) in the MEF2C protein. Green dots indicate 13 SNVs on *MEF2C* exons reported previously; MADS: MCM1, Agamous, Deficiens, Serum response factor; MEF2, myocyte enhancer factor 2; TAD, transcriptional activation domain; NLS, nuclear location signal. **(d)** Western blotting of Flag-tagged MEF2C WT and L35P expressed in cultured primary cortical neurons. Quantitative analysis of mRNA level ($n = 3$) **(e)** and protein level ($n = 4$) **(f)** of Flag-tagged MEF2C WT and L35P expressed cultured primary cortical neurons normalized to GAPDH. **(g)** Western blotting of Flag-tagged MEF2C WT and L35P expressed in HEK293 cells. Quantitative analysis of mRNA level ($n = 6$) **(h)** and protein level ($n = 4$) **(i)** of Flag-tagged MEF2C WT and L35P expressed in HEK293 cells. **(j)** Western blotting of *MEF2C* WT and L35P expressed in HEK293 cells, with cycloheximide (CHX) treatment for indicated time before immunoblotting analysis. **(k)** Quantitative analysis of relative protein expression of *MEF2C* WT and L35P in **(l)**. ($n = 4$, two-way ANOVA). **(l)** Western blotting of MEF2C WT and L35P expressed in HEK293 cells, with bortezomib (BTZ) or CHX treatment for 12 hr before immunoblotting analysis. **(m)** Quantification of relative MEF2C protein levels normalized to GAPDH in **(l)** ($n = 3$, one-way ANOVA). **(n)** Western blotting of MEF2C-WT or MEF2C-L35P co-transfected with either empty vector or L35P in HEK293 cells. **(o)** Quantitative analysis of Western blotting in **(n)** ($n = 4$, one-way ANOVA). Each dot in western blotting represents one independent experiment. n is biological repeat numbers of independent experiments. Statistical values represent the mean $\pm$ s.e.m.* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ **** $p < 0.0001$.

Figure 2

Figure 2

Reduced MEF2C protein levels and autistic-like behaviors in *Mef2c* L35P knock-in mice.

(a) Western blotting of MEF2C protein in various brain regions extracted from *Mef2c* WT and L35P^{+/-} mice. Quantitative analysis of relative Mef2c expression of PFC (b) and hippocampus (c) in *Mef2c* WT and L35P^{+/-} mice ($n = 3$). (d) Immunohistochemical staining for Mef2c (green) and DAPI (nuclei marker,

blue) in the cerebral cortex, hippocampus and amygdala of *Mef2c* WT and L35P^{+/-} mice (S1BF, somatosensory cortex 1 barrel field; DG, dentate gyrus; LA, lateral amygdala; BLA, basolateral amygdala; BMA, basomedial amygdala; white dotted box indicates corresponding brain region). Scale bars, 1000μm (left); 300μm (middle); 400μm (right). Quantitative analysis of average fluorescent intensity of *Mef2c* in **(d)**: somatosensory cortex **(e)**, dentate gyrus **(f)** and lateral amygdala **(g)** of *Mef2c* WT ($n=9$ brain slices from 3 mice) and L35P^{+/-} mice ($n=10$ brain slices from 3 mice). **(h)** Representative heat maps for locomotion of *Mef2c* WT and L35P^{+/-} mice on the social approach and social novelty session in the three-chamber test. Quantification of cumulative duration of interacting with mouse and empty cage of *Mef2c* WT **(i)** and L35P^{+/-} mice **(j)** ($n = 14$ for each group). **(k)** The preference index of social approach test for *Mef2c* WT and L35P^{+/-} mice. Quantification of cumulative duration of interacting with novel stranger mouse and familiar mouse of *Mef2c* WT **(l)** and L35P^{+/-} **(m)** mice ($n = 14$ for each group). **(n)** The preference index of social novelty test for *Mef2c* WT and L35P^{+/-} mice. **(o)** Representative locomotion track traces of *Mef2c* WT and L35P^{+/-} mice in the open field test. Quantitative analysis of total distance **(p)**, mean velocity **(q)**, and duration in the center zone **(r)** of *Mef2c* WT and L35P^{+/-} mice in the open field test ($n = 14$ for each group). **(b, c, e, f, g, k, n, p, q, r)** unpaired Student's *t* test. **(i, g, l, m)** paired Student's *t* test. Statistical values represent the mean $\pm$ s.e.m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ **** $p < 0.0001$.

Figure 3

Figure 3

Design and validation of the AeCBE gene editing system

(a) Schematic diagram of CRISPR/Cas9-based CBE gene editing system, including SpG, sgRNA, UGI and APOBEC3A^{Y130F} (red circle indicates the mutation site, green circles indicate correct base pair). (b) Design of sgRNAs targeting to the mutation site in the mouse *Mef2c* gene. (c) Schematic flow for validating the

efficiency of the AeCBE system in vitro in mouse cortical neurons. Cultured mouse cortical neurons were co-electroporated with sgRNA-mCherry and AeCBE-EGFP plasmids followed by sorting with both mCherry and GFP signals. **(d)** Representative sanger sequencing results of primary cortical neurons from *Mef2c* L35P^{+/-} mice after AeCBE gene editing (red arrowhead indicate gene loci without gene editing (upper panels) and with SgC8 (lower left panel), SgC15 (lower right panel). The percentage of T (c.104T) is obtained by calculating T base ratio ($T / (C+T) * 100\%$). **(e)** Illustration of AeCBE, APOBEC3A-SpG, and AncBE4 constructs. Sanger sequencing data **(f)** and Next-generation sequencing analysis **(g)** of *Mef2c* c.104C/T with three base editing systems in mouse neurons. Editing rate = $(T(\text{after}) - T(\text{before})) / C(\text{before})$ %. * $p < 0.05$, one-way ANOVA test.

Figure 4

Restoration of Mef2c protein level and abnormal inhibitory interneuron development in *Mef2c* L35P^{+/+} mice after the AeCBE gene editing *in vivo*.

(a) Schematic illustration of dual AAV plasmids engineered by intein-dependent protein split strategy. (b) Representative immunofluorescent staining for EGFP (green) and DAPI (blue) to determine the AAV

infection efficiency in the brain through intravenous injection in the tail vein (RSG, retrosplenial granular cortex; RSA, retrosplenial agranular cortex; MPtA, medial parietal association cortex; LPtA, lateral parietal association cortex; V2L, secondary visual cortex, lateral area; AuV, secondary auditory cortex, ventral area). Scale bars, 1000µm (upper left and bottom); 500µm (upper right). **(c)** Representative immunofluorescent staining for spCas9 (red) and DAPI (blue) to examine AeCBE delivery efficiency in the brain (S1Tr, primary somatosensory cortex, trunk region; S2, secondary somatosensory cortex; white dotted box indicates corresponding brain region). Scale bars, 1000µm (left); 500µm (middle); 100µm (zoom magnification, right). Representative western blotting of prefrontal cortex **(d)** and hippocampus **(f)** extracted from *Mef2c* WT mice treated with AAV-EGFP, and *Mef2c* L35P^{+/-} mice injected with either EGFP or AeCBE. **(e, g)** Quantification for western blotting in **(d)** and **(e)**, respectively ($n = 4$ mice per group). **(h)** Immunohistochemical staining for Mef2c (green) and DAPI (blue) in the cerebral cortex outer layer, hippocampus and amygdala of *Mef2c* WT mice injection of AAV-EGFP, and *Mef2c* L35P^{+/-} mice injected with either EGFP or AeCBE. Scale bars, 1000µm (left); 300µm (middle); 400µm (right). Quantification of average fluorescent intensity of Mef2c in the somatosensory cortex **(i)**, dentate gyrus **(j)** and lateral amygdala **(k)** of *Mef2c* WT EGFP ($n=11$ brain slices from 4 mice) L35P^{+/-} EGFP ($n=12$ brain slices from 4 mice) and L35P^{+/-} AeCBE mice, in **(h)** ($n=12$ brain slices from 3 mice). **(l)** Editing efficiency of AeCBE in prefrontal cortex examined by next-generation sequencing. Editing rate = (T(after)-T(before))/C (before) %. (GFP: $n = 6$, AeCBE: $n = 5$, n is mice number). **(m)** Immunohistochemical staining for PV (red), Mef2c (green) and DAPI (blue) in the retrosplenial cortex and dentate gyrus of *Mef2c* WT mice injection of AAV-EGFP, and *Mef2c* L35P^{+/-} mice injected with either EGFP or AeCBE. Scale bars, 200µm., Quantification of PV-positive cell density in the retrosplenial cortex **(n)** ($n=10$ brain slices from 4 mice per group). Statistical values represent the mean $\pm$ s.e.m. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, unpaired two-tailed Student's *t*-test.

Figure 5

Figure 5

Amelioration of autistic-like behaviors in *Mef2c* L35P^{+/-} mice with CBE gene editing *in vivo*.

(a) Representative locomotion heat maps of *Mef2c* WT mice treated with AAV-EGFP, and *Mef2c* L35P^{+/-} mice injected with either EGFP or CBE in the three-chamber test. Cumulative duration of the social approach session of *Mef2c* WT mice treated with AAV-EGFP (b), and *Mef2c* L35P^{+/-} mice injected with

either EGFP (**c**) or CBE (**d**) ($n=13$). (**e**) the preference index of social approach for three groups. Cumulative duration of the social novelty episode of *Mef2c* WT mice injected of AAV-EGFP (**f**), and *Mef2c* L35P^{+/-} mice injected with either EGFP (**g**) or CBE (**h**) ($n=13$). (**i**) the preference index of social novelty of three groups. One-way ANOVA test was applied in (**e, i**). (**j**) Cumulative sniffing time in the first trial of the social intruder test for *Mef2c* WT mice treated with AAV-EGFP ($n=14$), *Mef2c* L35P^{+/-} mice injected with either EGFP ($n=12$) or CBE ($n=15$). (**k**) Cumulative sniffing time in five respective trials of *Mef2c* WT mice treated with AAV-EGFP ($n=14$), and *Mef2c* L35P^{+/-} mice injected with either EGFP ($n=12$) or CBE ($n=15$) (two-way ANOVA). (**l**) The social recognition index of the social intruder test for three groups (unpaired two-tailed Student's *t*-test). Mean cumulative sniffing time (black curve) and data for individual mice (gray curve) in the social intruder test for *Mef2c* WT mice treated with AAV-EGFP (**m**, $n=14$), *Mef2c* L35P^{+/-} mice injected with either EGFP (**n**, $n=12$) or CBE (**o**, $n=15$, one-way ANOVA). (**p**) Representative locomotion track traces of *Mef2c* WT mice treated with AAV-EGFP and *Mef2c* L35P^{+/-} mice injected with either EGFP or CBE in the open field test. Quantification of total moving distance (**q**) and mean velocity (**r**) of three groups ($n=13$; unpaired two-tailed Student's *t*-test). (**s**) Quantitative analysis of cumulative time spent in self-grooming and scratching of *Mef2c* WT mice treated with AAV-EGFP and *Mef2c* L35P^{+/-} mice injected with either EGFP or CBE ($n=13$; unpaired two-tailed Student's *t*-test). *n* represents mice number. Statistical values represent the mean $\pm$ s.e.m. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

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