



Generation of tryptophan hydroxylase 2 gene knockout pigs by CRISPR/Cas9-mediated gene targeting

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Abstract

Unbalanced brain serotonin (5-HT) levels have implications in various behavioral abnormalities and neuropsychiatric disorders. The biosynthesis of neuronal 5-HT is regulated by the rate-limiting enzyme, tryptophan hydroxylase-2 (TPH2). In the present study, the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) system was used to target the *Tph2* gene in Bama mini pig fetal fibroblasts. It was found that CRISPR/Cas9 targeting efficiency could be as high as 61.5%, and the biallelic mutation efficiency reached at 38.5%. The biallelic modified colonies were used as donors for somatic cell nuclear transfer (SCNT) and 10 *Tph2* targeted piglets were successfully generated. These *Tph2* KO piglets were viable and appeared normal at the birth. However, their central 5-HT levels were dramatically reduced, and their survival and growth rates were impaired before weaning. These *Tph2* KO pigs are valuable large-animal models for studies of 5-HT deficiency induced behavior abnormality.

Keywords: CRISPR/Cas9, tryptophan hydroxylase-2 gene, serotonin, Bama mini pigs

Introduction

The neurotransmitter serotonin (5-hydroxytryptamine, 5-HT), an important extracellular signaling molecule, has been implicated in a broad range of physiological functions in the central and peripheral nervous systems. In vertebrates, 2 tryptophan hydro-

xylase (TPH) isoforms, TPH1 and TPH2 encoded by 2 distinct genes, are responsible for the rate-limiting step of the biosynthesis of 5-HT^[1–2]. It has been well documented that TPH1 and TPH2 differ in terms of their spatial distribution. TPH1 is extremely abundant in the gut and pineal gland, accounting for the main source of circulating 5-HT, whereas TPH2 is specifically

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expressed in serotonergic neurons of the raphe nuclei of the brainstem, responsible for the synthesis of central 5-HT^[3–11]. Because genetic variation and functional mutations in the human *Tph2* gene have been shown to be associated with cognition and emotional regulation as well as neuropsychiatric disease and behavioral abnormalities^[12–16], *Tph2* has drawn increasing attention in scientific community.

During the past decade, the ensuing genetically modified *Tph2* mouse models in which the biosynthesis of neuronal 5-HT is selectively ablated in the brain have been generated, and these have provided considerable insight into understanding various neurobehavioral processes regulated by serotonergic neurotransmission^[11,17–21]. However, these rodent models cannot fully recapitulate human 5-HT deficiency properties because their physiology and gene expression differ from those of humans, and studies using *Tph2*-deficient large animal models have not been reported^[10,22–23]. Currently, pigs are extensively used as a large-animal model for biomedical research because they share many similarities with humans in terms of anatomy, physiology, immunology, compared to rodents^[24–26]. Pigs are less expensive to procure and involve fewer ethical issues than other large animals, such as non-human primates. Pigs also have advantages over rodent models for cognition and behavior studies in that they have high-level of emotional and cognitive abilities, and their anatomical brain structures are similar to those of humans^[27–29].

Recently, the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) system has significantly improved gene targeting efficiency and has been extensively used to generate genetically modified animal models in various species, including mice^[30], rabbits^[31], sheep^[32], and pigs^[33–34]. In this study, *Tph2*^{-/-} piglets were successfully generated by using the CRISPR/Cas9 system combined with somatic cell nuclear transfer (SCNT) technology, which could be utilized to elucidate the physiological function of *Tph2* in a further study.

Materials and methods

Ethics statement

This study was conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Nanjing Medical University. All pigs were housed in Nanjing Medical University animal facilities, and standard animal husbandry protocols were followed.

Targeting vector construction

Targeting sgRNA 1 and sgRNA 2 for the *Tph2* coding region were designed using online tools (<http://crispr.mit.edu/>) and synthesized by Genscript Co., Ltd. (Nanjing, China). Oligos of sgRNAs were annealed and sub-cloned into BbsI-digested pX330 (addgene #423230) plasmid, respectively. The resulting targeting vectors for *Tph2* were confirmed by Sanger sequencing.

Generation of *Tph2* targeted cell lines

Primary fibroblasts (PFFs) derived from 34 days old Bama mini-pig fetuses were cultured in medium consisting of high glucose DMEM (Gibco, Grand Island, NY, USA), 15% FBS (Gibco, Grand Island, NY, USA) and 1 × Penn/Strep solution (Gibco, Grand Island, NY, USA). 1 µg *Tph2* targeting vector was introduced into PFFs followed the methods described previously^[35]. The electroporated cells were maintained with G418 at 800 µg/mL thereafter. Single colonies were picked and cultured in 48-well plates. Confluent wells were treated with 0.05% trypsin and re-suspended using 300 µL culture medium. Aliquot cells were plated in a 12-well plate, and the remaining cells were lysed for *Tph2* genotyping. The primers were: 5'-gattgggccgt-caaatcctg-3' and 5'-ctacctgtcagtgagcctc-3'. PCR conditions were 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and finally 72°C for 7 minutes. The PCR products of each colony were purified using a NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany) and then cloned into pMD18-T vector (Takara Clontech, Tokyo, Japan) for sequencing.

Production of mutant piglets

SCNT procedure was described by Chen *et al.*^[35]. Briefly, oocytes were collected and cultured for 42–44 hours. The cumulus cells and nuclei of in vitro matured oocytes were then removed. The mutant cell lines were pooled as nuclear donor cell to be injected into the perivitelline space of the oocytes. Then, the electrically activated reconstructed embryos were cultured in embryo culture medium at 38°C for 3 days. About 260 blastocysts were transplanted into the uteruses of the surrogate pigs. The genomic DNA isolated from the ear biopsy of the neonates was used to assess mutagenesis at the targeted *Tph2* site by sequencing.

T7E1 cleavage assay

Fibroblasts transfected with Cas9 plasmids of different sgRNAs (as described above) were cultured

for 48 hours. Genomic DNA was isolated and PCR was performed to amplify the genomic region spanning the CRISPR target sites for *Tph2*. The T7E1 cleavage assay was performed using an EnGen® Mutation Detection Kit (NEB, Beverly, MA, USA) as previously described^[32].

Histology and immunofluorescence analysis

The tissues of brainstem from *Tph2*^{-/-} pigs and wild-type pigs were fixed with 4% buffered paraformaldehyde for 48 hours. The entire tissue was then dissected, embedded in paraffin wax, and sectioned at 5 µm. Sections deparaffinized in xylene and rehydrated were stained with hematoxylin and eosin or immune-stained overnight at 4°C with anti-TPH2 polyclonal antibody (Abcam, Cambridge, MA, USA). Images were obtained using a Digital Sight DS-Ri1 camera on a Nikon Eclipse 80i microscope.

Western blot analysis

Brainstems from euthanized piglets were prepared by homogenization in the presence of protease inhibitors (ThermoFisher Scientific, Waltham, MA, USA), and centrifuged to remove residual debris. The samples were heat-denatured at 95°C for 5 minutes with 5× SDS-PAGE loading buffer and fractionated on 10%

SDS-PAGE gels (Bio-Rad, Hercules, CA, USA). GAPDH (HRP-60004, Proteintech, Manchester, UK) was used as a standard control protein. Following electrophoresis, proteins were transferred to a nitrocellulose membrane and blocked with 5% skim milk powder. Primary antibodies used Anti-TPH2 antibody (ab121013, Abcam, Cambridge, MA, USA), which use a concentration of 2 µg/mL.

Results

CRISPR/Cas9-mediated *Tph2* gene targeting in PFFs

To knock out the *Tph2* gene in pigs, 2 sgRNAs targeted the exon 7 of *Tph2* were designed following previously described procedures^[32]. The precise target sites are shown in **Fig. 1A**. To test their cleavage efficacy, these 2 sgRNAs were cloned into pX330 vector and transfected into PFFs derived from 35-day-old Bama mini fetuses. Genomic DNA was harvested from transfected PFFs after 48 h. PCR amplicons spanning the *Tph2* target region were subjected to T7 Endonuclease I assay followed by gel electrophoresis analysis. The results showed that both sgRNAs could induce indels in the *Tph2* target region, while sgRNA1 shows slightly higher cleavage efficiency (19.2%) than

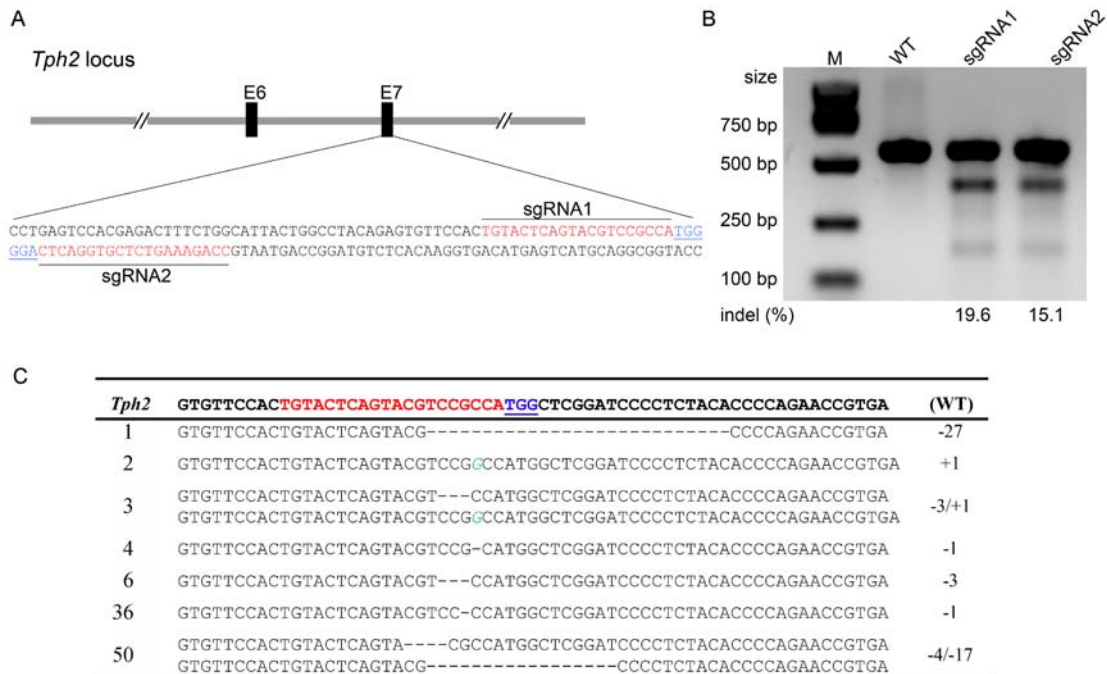


Fig. 1 CRISPR/Cas9 mediates *Tph2* gene targeting in PFFs. **A**: Schematic of Cas9/sgrNAs targeting sites of pig *Tph2* locus. The sgRNA targeting sequences are highlighted in red, and the protospacer-adjacent motif (PAM) sequences are highlighted in blue and underlined. **B**: T7E1 assay of PCR products spanning the *Tph2* targeting region. M: DNA marker; WT: PCR product of wild-type; sgRNA1 and sgRNA2: PCR products of PFFs transfected with sgRNA1 and sgRNA2, respectively. **C**: Genotypes of *Tph2* biallelic modified colonies. "-": deletion; "+": insertion; italic green letters denote inserted base pairs.

sgRNA2 (15.1%) (**Fig. 1B**). In this way, sgRNA1 was chosen for subsequent *Tph2* gene targeting.

To establish *Tph2* mutation cell lines, the Cas9-sgRNA1 targeting vector and a TD-tomato plasmid containing a neomycin resistance (*Neo*) gene were co-transfected into an early passage of primary PFFs. Cells were maintained with G418 thereafter until single resistant colonies were formed. Genotyping analysis of each colony was performed using TA-cloning and Sanger sequencing. The results indicated that 12 colonies had monoallelic modifications in the *Tph2* targeting region and 20 had biallelic modifications (**Table 1**). About 38.5% of the cell colonies had biallelic mutations (**Table 1**). Genotypes of *Tph2* biallelic modified colonies are shown in **Fig. 1C**.

Production of *Tph2*^{-/-} pigs by SCNT

To produce *Tph2* KO piglets, PFFs colonies with biallelic mutations were mixed as donor cells for SCNT. Totally, 1,800 reconstructed embryos were transferred into 7 recipient gilts. Each recipient was monitored by B-ultrasonic examination about one month after operation, and 2 pregnant gilts were discovered to be expecting, showing a pregnancy rate of 28.6% (**Table 2**). The 2 recipients were maintained to term and gave birth to 12 live-born piglets without displaying any obvious developmental defects or differences from wild-type neonates (**Fig. 2A**). Genotyping results showed all piglets except No. 11 to be biallelic *Tph2* mutants. Piglet No. 11 was determined to be a *Tph2* wild type, probably due to contamination of colonies

with a few wild type PFFs. Eight piglets (No. 1, No. 3, No. 5, No. 6, No. 7, No. 8, No. 9, and No. 10) carried a-4/-17 bp deletion derived from No. 50 donor cells, 2 piglets (No. 2 and No. 4) born a + 1 bp insertion originate from No. 2 donor cells, and No. 13 piglet contained a-1 bp deletion derived from No. 4 colony, respectively (**Fig. 1C** and **Fig. 2B**).

To determine the TPH2 protein expression level of the gene targeted piglets, Western blot analysis was carried out using the whole protein extracted from brain stem of *Tph2* targeted piglets and age-matched wild type controls. As shown in **Fig. 2C**, unlike in the WT control, the expression of TPH2 protein was undetectable in *Tph2* targeted piglets (**Fig. 2C**). To further characterize TPH2 expression, immunohistochemistry was conducted on brainstem sections. Consistent with the Western blot results, *Tph2* signal was observed only in WT and not in the *Tph2* KO piglets (**Fig. 2D**). These results confirmed that the *Tph2* targeted piglets generated in this study were authentic *Tph2* KO animals.

Survival and body weight of *Tph2*^{-/-} piglets were reduced during lactation

To assess the developmental consequence of *Tph2* KO in piglets, brainstem sections were stained using the hematoxylin/eosin (H&E) method. The results showed no obvious difference in the structures of neuronal cells between *Tph2* KO and WT pigs, indicating that *Tph2* KO may have little influence on formation of the organizational structure of brainstem (**Fig. 3A**). How-

Table 1 Summary of mutant colonies of CRISPR/Cas9-mediated *Tph2* gene targeting

Experiment	Monoallelic mutation	Biallelic mutation	Indel-positive
1	5/27	9/27	14/27
2	7/25	11/25	18/25
Total	12/52 (23.1%)	20/52 (38.5%)	32/52 (61.5%)

Note: Mutant colonies/total colonies were tested in 2 independent tests.

Table 2 Somatic cell nuclear transfer results for the generation of knockout cloned pigs

Recipient	Donor cells	Transferred embryos	Pregnancy	Newborn piglets
1	31, 2	280	NO	-
2	2, 50	255	YES	10
3	41, 4	260	NO	-
4	46, 2, 41	265	NO	-
5	12, 36	255	YES	2
6	2, 31	245	NO	-
7	4, 12	260	NO	-
Total		1,820	28.6%	12

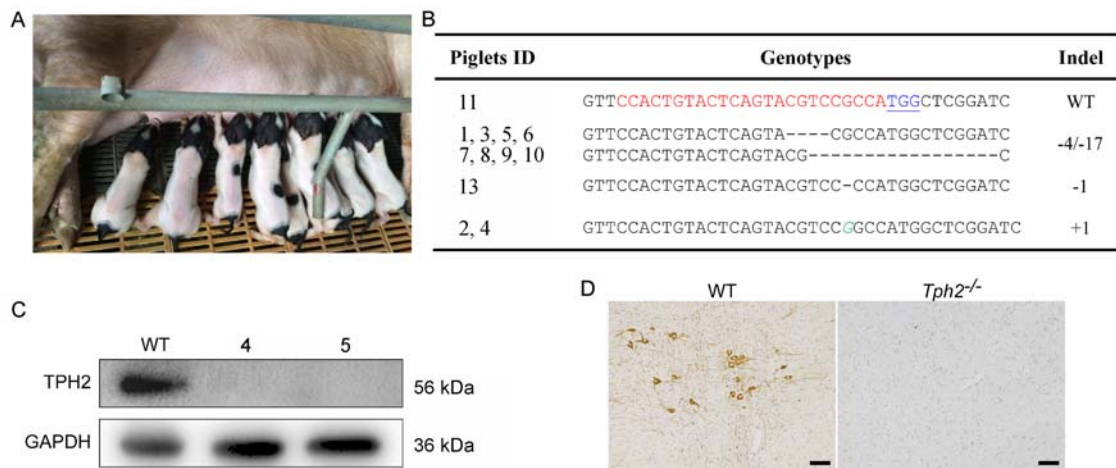


Fig. 2 Generation of *Tph2* gene targeted piglets via somatic cell nuclear transfer (SCNT). A: Photograph of newborn cloned piglets carrying *Tph2* gene mutation. B: Genotypes of cloned piglets. "-": deletion; "+": insertion; italic green letters denote inserted base pairs. C: Representative Western blot of TPH2 protein of cloned piglets and wild type control. D: Representative images of TPH2 immunohistochemistry on brainstem sections. Scale bar is 50 μ m.

ever, *Tph2* KO piglets showed a decrease in survival rate (36.4%) compared with WT piglets from birth to weaning (**Fig. 3B**). *Tph2* KO piglets were also shown significantly less body weight than age-matched WT

controls before weaning (**Fig. 3C**). These data indicated that lack of central 5-HT may have a detrimental effect on piglet breast feeding behavior which resulted in a much less body weight and lower survival rate of *Tph2*

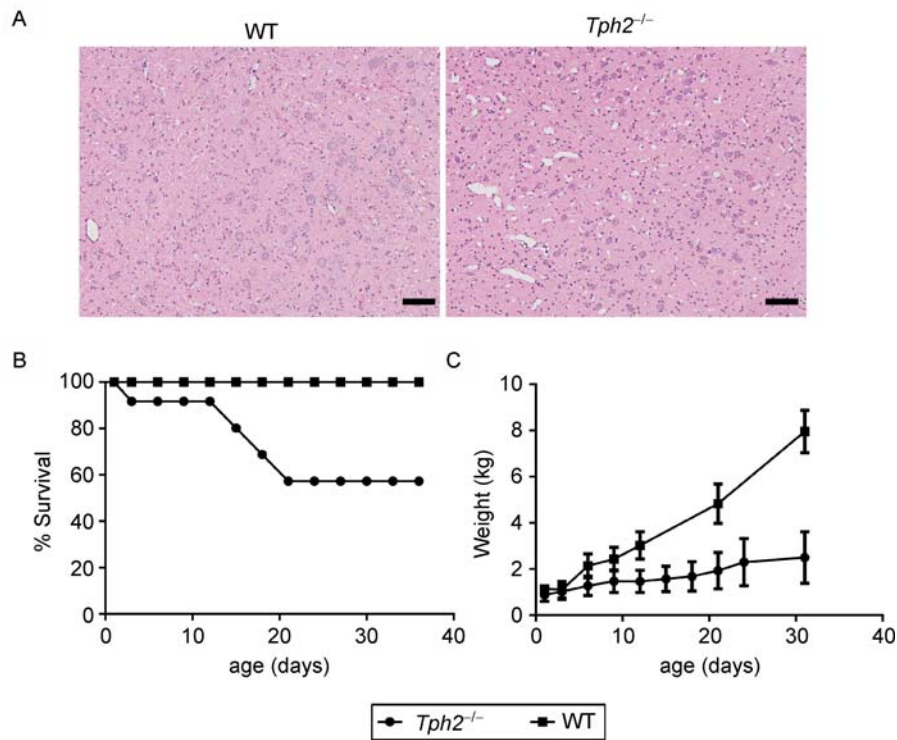


Fig. 3 Survival and body weight of the *Tph2*^{-/-} piglets were reduced before weaning. A: Representative H&E images of organizational structure of neuronal cells of the *Tph2*^{-/-} piglets and WT control. Scale bar is 50 μ m. B: The survival curve of *Tph2*^{-/-} and WT piglets. *Tph2*^{-/-} pigs showed a higher mortality before weaning than in WT. C: Body weight of *Tph2*^{-/-} and WT piglets. Data are presented as the mean \pm SEM. WT, $n = 4$; *Tph2*^{-/-}, $n = 10$ at birth, $n = 6$ at the endpoint.

KO piglets before weaning. Extensive well-controlled behavior studies will be conducted once more *Tph2* KO piglets are produced.

Off-target analysis

Although the CRISPR/Cas9 system has the advantage of high gene targeting efficiency in pigs, the undesired off-target effects are still a major concern^[34,36]. To determine whether off-target mutagenesis occurred in the *Tph2* KO piglets, 20 potential off-target sites (OTSs) for sgRNA1 were founded by using the CRISPR design tool (<http://crispr.mit.edu/>). These sequences are listed in **Supplementary Table 1**, available online. Five OTSs with high score were chosen for PCR amplification using genomic DNA isolated from *Tph2* KO piglets and WT controls. The primers used to amplify the OTSs are listed in **Table 3**. Sanger sequencing of the PCR amplicons showed that none of the sequencing reads exhibited mutations, indicating that there had been no off-target effects at these OTSs in *Tph2* KO piglets.

Discussion

In the present study, *Tph2* targeted pigs were successfully generated by combination of the CRISPR/Cas9 system and SCNT technology in which the TPH2 was totally depleted in the brain. The gene targeting efficiency of the sgRNA/Cas9 in PFFs reached as high as 61.5% (**Table 1**), which was much higher than that of the homologous-recombination-based *Tph2* targeting in mice^[17,21]. The *Tph2* targeting efficiency in PFFs in this study is 61.5% with 38.5% biallelic mutation, which is higher than that of *IgM* reported by Chen *et al.*^[35] (53.3% targeting efficiency with 13.3% biallelic mutation). This is probably due to sgRNAs specifically for *IgM* and *Tph2* are of different cleavage

efficiency. In addition, 10 homozygous *Tph2*^{-/-} pigs were generated by just 1 round of SCNT. In this way, the high efficiency and ease of the Cas9/sgRNA system can greatly facilitate the rapid production of KO pig models.

Although off-target effects are a major concern of the CRISPR/Cas9 gene targeting system^[36-38], no off-target event was detected in *Tph2*^{-/-} piglets produced in the present study. However, off-target mutagenesis may occur in the cloned *Tph2*^{-/-} piglets because the *Sus scrofa* genome sequence is incomplete, which has hampered comprehensive searches for off-target events. To produce the *Tph2*-targeted piglets, the several knockout PFF colonies were mixed for SCNT procedure and the reconstructed embryos were transferred into a single recipient. Interestingly, results showed that the number of piglets developed from a particular *Tph2* KO cell line cell (No. 50) was markedly higher than that of the other cell colonies (**Table 4**). These results indicated that either the No. 50 cell colony had higher competency or other cell colonies might contain undesired off-target mutations that could impair the integrity of the cell genome and impair its development during embryo manipulation. For this reason, transferring pooled embryos derived from multiple cell lines of different clonability into a single surrogate could be a practical strategy to increase the chance to achieve cloned gene-targeted pigs.

Consistent with *Tph2* KO mice models^[17], the raphe serotonergic neurons in *Tph2*-disrupted piglets are completely devoid of 5-HT immunoreactivity, but no gross brain morphological abnormalities occur. These results provide evidence for the conclusion that TPH2 is solely responsible for raphe neuron 5-HT synthesis and 5-HT may not be indispensable to the development and maintenance of raphe serotonergic neurons. Moreover, reduction of the body weight and the postnatal survival shown in the *Tph2*^{-/-} piglets before weaning are consistent with observations made in 5-HT depleted

Table 3 Primers for amplifying off-target sites

Primer	Sequence (5'- 3')	Amplicon (bp)
OTS1 for	CCTTTGATTGAAGCTGTGTGAA	307
OTS1 rev	CAATGTCCTCTGACCGACTG	
OTS2 for	AATCACTCAGAACAGAGCCAAG	303
OTS2 rev	ATCCTCCTTTACGCCAGGTC	
OTS3 for	GAAGATGGACATTGCCAGGAC	314
OTS3 rev	CGCCAACAAAGTAAACCAAACT	
OTS4 for	TGTGCTGGACATATACTTGAGA	306
OTS4 rev	GGAGGATTGAGAAGAAGGTGAT	
OTS5 for	ACACCTCCTAACCCAAGTGAG	338
OTS5 rev	CACAAGTAACCAGAGCCACAG	

Table 4 Summary of Tph2^{-/-} pigs

Piglets ID	Birth length (cm)	Birth weight (g)	Health status	Donor cell
1	19	563	Alive	50
2	22.5	916	Dead on day 18	2
3	20	604	Dead on day 21	50
4	22.5	897	Dead on day 3	2
5	23	983	Alive	50
6	24	1,018	Alive	50
7	21.5	938	Alive	50
8	23.5	964	Alive	50
9	23.5	1,020	Alive	50
10	22	893	Dead on day 15	50
11	23	1,140	Alive	WT
12			Mummy fetus	
13	20	1,420	Alive	36

Tph2 knockout mice^[19,21], indicating 5-HT plays a similar role in neonatal viability and growth rate across species. Dysregulation of 5-HT has long been thought to induce various mental conditions including depression, anxiety disorders, bipolar disorder, autism, and schizophrenia^[12–16]. Behavioral abnormalities were not evaluated in these *Tph2*^{-/-} pigs because of the scope of this study and the long time required for such experiments. However, amplification of the *Tph2*^{-/-} pigs by cross-breeding for these experiments are currently ongoing in our laboratory.

In summary, these *Tph2*^{-/-} piglets are a valuable model that will be useful for investigation of the developmental and functional consequences of altered brain-specific 5-HT level and its role in cognition, emotional regulation, and neuropsychiatric disease risk.

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