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Research Paper

Characterizing a novel *vGlut3-P2A-iCreER* knockin mouse strain in cochlea

Chao Li [a, 1], Yilai Shu [c, d, 1], Guangqin Wang [a], He Zhang [a, b], Ying Lu [a], Xiang Li [a], Gen Li [e, f, g], Lei Song [e, f, g], Zhiyong Liu [a, *]

[a] *Institute of Neuroscience, CAS Center for Excellence in Brain Science and Intelligence Technology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, 200031, China*
[b] *University of Chinese Academy of Sciences, Beijing, 100049, China*
[c] *ENT Institute and Otorhinolaryngology Department, Affiliated Eye and ENT Hospital, State Key Laboratory of Medical Neurobiology, Fudan University, Shanghai, China*
[d] *Key Laboratory of Hearing Medicine of National Health and Family Planning Commission (NHFPC), Shanghai, China*
[e] *Department of Otolaryngology-Head and Neck Surgery, Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China*
[f] *Ear Institute, Shanghai Jiao Tong University School of Medicine, Shanghai, China*
[g] *Shanghai Key Laboratory of Translational Medicine on Ear and Nose Diseases, Shanghai, China*

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ABSTRACT

Precise mouse genetic studies rely on specific tools that can label specific cell types. In mouse cochlea, previous studies suggest that vesicular glutamate transporter 3 (*vGlut3*), also known as *Slc17a8*, is specifically expressed in inner hair cells (IHCs) and loss of *vGlut3* causes deafness. To take advantage of its unique expression pattern, here we generate a novel *vGlut3-P2A-iCreER* knockin mouse strain. The P2A-iCreER cassette is precisely inserted before stop codon of *vGlut3,* by which the endogenous *vGlut3* is intact and paired with iCreER as well. Approximately, 10.7%, 85.6% and 41.8% of IHCs are tdtomato + when tamoxifen is given to *vGlut3-P2A-iCreER/+; Rosa26-LSL-tdtomato/+* reporter strain at P2/P3, P10/P11 and P30/P31, respectively. Tdtomato + OHCs are never observed. Interestingly, besides IHCs, glia cells, but not spiral ganglion neurons (SGNs), are tdtomato+, which is further evidenced by the presence of Sox10+/tdtomato+ and tdtomato+/Prox1(Gata3 or Tuj1)-negative cells in SGN region. We further independently validate *vGlut3* expression in SGN region by *vGlut3 in situ* hybridization and antibody staining. Moreover, total number of tdtomato + glia cells decreased gradually when tamoxifen is given from P2/P3 to P30/P31. Taken together, *vGlut3-P2A-iCreER* is an efficient genetic tool to specifically target IHCs for gene manipulation, which is complimentary to *Prestin-CreER* strain exclusively labelling cochlear outer hair cells (OHCs).

1. Introduction

Our auditory organ cochlea resides in the inner ear, and its key part for detecting sound is named as Organ of Corti. The Organ of Corti contains several cell types: Inner Hair Cells (IHCs), Outer Hair Cells (OHCs) and neighboring supporting cells (SCs). OHCs are sound amplifier and IHCs transform sound stimuli into electrophysiological signal that is further transmitted into cochlear spiral ganglion neurons (SGNs) for next information processing before ending at auditory cortex. The excitatory neurotransmitter, glutamate, is critical for communication between IHCs and SGNs. vGlut3 (vesicular glutamate transporter 3) encoded by gene *Slc17a8* transports glutamate from IHCs and loss of *vGlut3* causes deafness (Moser and Starr, 2016; Obholzer et al., 2008; Ruel et al., 2008; Seal et al., 2008). Prior to embryonic day 15 (E15), no vGlut3 protein is expressed in cochlea (Seal et al., 2008). It is turned on primarily in IHCs around perinatal ages and maintained afterwards (Akil et al., 2012; Flores et al., 2015; Seal et al., 2008). In contrast, Prestin encoded by *Slc26a5* is a motor protein and expressed in OHCs but not IHCs (Liberman et al., 2002; Zheng et al., 2000), which is further confirmed by lineage tracing analysis with *Prestin-CreER* knockin mouse (Fang et al., 2012). *Prestin-CreER* has become a great tool for

* Corresponding author.
E-mail address: Zhiyongliu@ion.ac.cn (Z. Liu).
[1] The authors contribute equally to this work.

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labelling and gene manipulation in cochlear OHCs at all ages after P2/P3. However, such a tool for cochlear IHCs is not available yet.

We aim to provide a tamoxifen-inducible iCreER mouse strain which can both temporally and spatially target cochlear IHCs. During cochlear IHC development, besides *vGlut3*, *Fgf8* is also IHC specific and not expressed in OHCs, which recently is confirmed again by *Fgf8-P2A-FLPo* mediated fate mapping approach (Quadros et al., 2017). *Fgf8-CreER* mouse strain in which *Fgf8* coding region is replaced by CreER is also available (Hoch et al., 2015). However, because *Fgf8* is only transiently expressed in differentiating IHCs and become undetectable in adult IHCs (Jacques et al., 2007; Liu et al., 2014; Zhang et al., 2017), *Fgf8-CreER* cannot label IHCs at adult ages. Instead, *vGlut3* is turned on in IHCs at perinatal ages and continuously expressed at all postnatal ages (Seal et al., 2008), therefore it is a good candidate gene to drive an inducible Cre for targeting IHCs specifically. A BAC transgenic *vGlut3-Cre* strain is available (Grimes et al., 2011). But, its Cre activity is turned on as soon as *vGlut3* is transcribed. Here, we aim to produce a *vGlut3* endogenous promoter/enhancer driven, tamoxifen inducible Cre strain in which temporal control of Cre activity is allowed.

Before generating a *vGlut3-P2A-iCreER* knockin mouse strain, we first systematically characterized and validated *vGlut3* mRNA and protein expression patterns. Our data demonstrate that *vGlut3* is first turned on in cochlear IHCs in a basal to apical gradient, with basal and middle IHCs at embryonic day 16.5 (E16.5), and apical at E18-E19. In addition, we find that cochlear OHCs weakly and transiently express *vGlut3* between E19 and Postnatal day 10 (P10), and both its turn on and off also follows basal to apical gradient.

The *vGlut3-P2A-iCreER* knock-in mouse strain was generated by homologous recombination approach using Crispr/Cas9 system. We precisely inserted P2A-iCreER cassette immediately before the stop codon (TAA) of *vGlut3* whose endogenous expression was kept intact. In this manuscript, we focused on cochlear part and only used vestibular organ (either sacculus or utricle) as internal controls when necessary. Fate mapping *vGlut3-P2A-iCreER/+; Rosa26-LSL-tdtomato/+* strain showed that within organ of Corti, only IHCs were tdtomato + when tamoxifen was given at all three different postnatal ages. Tdtomato + OHCs were never observed. Interestingly, in cochlear spiral ganglion neuron (SGN) region, glia cells but not SGNs also expressed tdtomato. Taken together, *vGlut3-P2A-iCreER,* in our knowledge, was the first mouse genetic tool strain that could specifically target IHCs, but not OHCs, at all postnatal ages. Therefore, it can be used to label cochlear IHCs, ablate/damage IHC, perform genetic manipulation and so forth, while keeping OHCs intact at the same time.

2. Materials and methods

2.1. Generation of the vGlut3-P2A-iCreER knockin mouse strain

We used Crispr/Cas9 approach to generate *vGlut3-P2A-iCreER* knockin mouse. The *vGlut3* single-guide gRNA (sgRNA): *5′-CTGAGAAGTCTCCTTCGGCC-3′* with PAM sequence *TGG* was injected into the *C57BL/6* mouse zygotes together with Cas9 and donor DNA (target vector in Fig. 4B). Founder 0 (F0) mice were mosaic, and mouse tail junction PCR was used to screen potential F0 mice with correct gene targeting, which were further bred with wild type C57BL/6 mice and F1 mice were further screened by southern blot and tail junction PCR to guarantee no random insertion as well as correct gene targeting.

For mouse tail genotyping, three primers (F1, F2 and R) were used simultaneously. The nucleotide sequence of primer F1: *5′-GCTGGTACACTACAGCGGAGTCATC-3′;* nucleotide sequence of primer F2: *5′-TGCAAGAACGTGGTGCCCCTCTATG-3′*; nucleotide sequence of primer R: 5′-TTAATCTCCCCCTTTCCCACGATTTGG-3'. As shown in Fig. 4F, pair of F1 and R produced a 407 bp band in wild type (WT) allele. Pair of F2 and R generated a 310 bp band in knockin (KI) allele. The PCR condition was below: 95 °C -3min; 95 °C −30sec, 62 °C −30sec, 72 °C −30sec with 35 cycles; 72 °C −10min. Note that with this PCR parameter, primer pair of F1 and R cannot produce a band in KI allele due to their much longer distance.

2.2. Southern blot of mouse tail DNA

Genomic DNA extracted from mouse tails (3–4 weeks old of age) was digested with PstI (3′ probe) or NcoI (iCre probe) (cat# R3140M and R3193M, New England BioLabs). High quality of DNA was further separated on a 1% agarose gel and transferred to GE Healthcare Life Sciences Amersham Hybond -N + Membrane (cat# 45-000-927, Fisher Scientific). The membrane was hybridized at 42 °C overnight using DIG Easy Hyb Granules (cat# 11796895001, Roche) containing a PCR-generated probe with the PCR DIG Probe Synthesis kit (cat# 11636090910, Roche). For probe labeling, 3′-probe and internally iCre DIG-labeled probes were prepared by PCR using Taq DNA polymerase and incorporating DIG-11-dUTP according to the manufacturer's instructions. Hybridization signals were detected using the DIG Luminescent Detection kit (cat# 11363514910, Roche). For 3′-probe, wild type (WT) and knockin (KI) DNA cut with PstI generates a 10.2k bp and 8.9k bp band, respectively (Fig. 4D). For iCre internal probe, only KI allele DNA cut with NcoI gives a 3.2k bp band (Fig. 4E).

2.3. In situ hybridization of vGlut3 probe

We first constructed vector containing *vGlut3* anti-sense CDS sequence (500 bp, +1226 to +1725 of NM_182959.3) drived by *T7* promoter. The vector is linearized by NotI (cat# R3189s, New England BioLabs) and transcribed by T7 RNA polymerase (cat# P2075, Promega). Riboprobe was labeled with a digoxigenin (DIG)-labeling kit (cat# 11277073910, Roche).

Mouse inner ear tissues were fixed in 4% paraformaldehyde (PFA) overnight at 4 °C. Before embedded in OCT and sectioned with 18 μm thickness, they were in 30% sucrose overnight. Upon *in situ* hybridization, slides were dried first at 50 °C for 1 h, further fixed in DEPC-4%PFA at room temperature (RT) for 20 min. Then, slides were treated with Proteinase K (cat# 25530049, Thermo fisher) at room temperature (RT) for 5 min, followed by second fixation in DEPC-4%PFA at RT for 10min, 0.1M RNAse-free Triethanolamine (cat# V900257, Sigma) at RT for 10 min. Slides were hybridized with DIG-labeled *vGlut3* anti-sense RNA probe overnight at 65 °C, followed by incubation in anti-DIG-AP antibody (cat# 11093274910, Roche) at RT for 2 h, or at 4 °C overnight. Colorimetric reaction was carried out using NBT/BCIP Kit (cat# 11681451001, Roche).

2.4. Sample process, histology and immunofluorescence

The following primary antibodies were used: anti-Myosin-VI (rabbit, 1:200, 25–6791, Proteus Bioscience), anti-Prox1 (rabbit, 1:500, AB5475, Millipore), anti-Prestin (goat, 1:200, sc-22692, Santa Cruz Biotechnology), anti-Sox2 (goat, 1:1000, sc-17320, Santa Cruz Biotechnology), anti-vGlut3 (rabbit, 1:500, 135203, Synaptic System), anti-Parvalbumin (mouse, 1:1000, P3088, Sigma), anti-Gata3 (goat, 1:200, AF2605, R&D systems), anti-Sox10 (goat, 1:200, sc-17342, Santa Cruz Biotechnology) and anti-Tuj1 (mouse, 1:500, 801201, BioLegend). All the secondary antibodies that were compatible with different combinational use of first antibodies are purchased from Thermo Scientific company (Molecular Probes).

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