Chapter 15

Optogenetic Interrogation of ChR2-Expressing GABAergic Interneurons After Transplantation into the Mouse Brain

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Abstract

This paper describes research methods to investigate the development of synaptic connections between transplanted GABAergic interneurons and endogenous neurons in the adult mouse hippocampus. Our protocol highlights methods for retroviral labeling adult-born GCs, one of the few cell types in the adult brain to be continuously renewed throughout life. By precise targeting of the retrovirus, labeling of adult-born GCs can be combined with optogenetic stimulation of the transplanted cells and electrophysiology in brain slices, to test whether the GABAergic interneurons integrate and establish inhibitory synaptic connections with host brain neurons. Modifications to adult neurogenesis are an important contributing factor in the development and severity of TLE and seizures. When combined with retroviral labeling, the approaches we describe in this chapter can be used to determine whether transplantation modifies the process of adult neurogenesis or other properties of the hippocampus. These approaches are helping to define parameters for potential cell replacement therapies to be used in patients with intractable seizure disorders.

Key words Transplantation, Stem cells, Optogenetics, Channelrhodopsin2, Electrophysiology, Retrovirus, Hippocampus, Neurogenesis

1 Introduction

Optogenetics provides a powerful approach for investigating the functional connectivity of transplanted cells within intact brain circuits and in brain slices. Here, we describe a detailed protocol for harvesting, transplanting, and studying the functional connectivity of Channelrhodopsin2 (ChR2) expressing fetal cells derived from embryonic mouse forebrain medial ganglionic eminence (MGE) and transplanted in the adult mouse hippocampus. We also describe procedures for monitoring seizures after transplantation into the hippocampus in mice with pilocarpine-induced temporal lobe epilepsy (TLE). This extended protocol is based on our prior publications describing the effects of transplanting MGE-derived GABAergic progenitors [1, 2].

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GABAergic interneurons that populate the basal ganglia, cerebral cortex, and hippocampus in the mammalian central nervous system (CNS) are born during embryonic life in proliferative regions located in the anterior neural tube. These five proliferative regions include the medial, caudal, and lateral ganglionic eminences (MGE, CGE, and LGE, respectively), the preoptic area (POA), and the septal region. The MGE, CGE, LGE, and POA give rise to populations of neurons that synthesize the neurotransmitter gamma-aminobutyric acid (GABA) [3]. During the embryonic proliferation of cells in the GEs, a distinctive crease defines the boundary between the MGE and the LGE. This morphological boundary facilitates precise dissection of the MGE from the more lateral LGE and CGE. Parvalbumin-expressing (PV+) and somatostatin-expressing (SST+) GABAergic interneuron progenitors dissected from the MGE have been extensively studied using transplantation into mouse models of neurological disorders, including amblyopia (vision loss caused by monocular deprivation), epilepsy, and neuropathic pain.

Based on extensive research investigating their developmental origins and migratory patterns, the five proliferative regions in the basal forebrain are now recognized as the embryonic sources of all forebrain GABAergic neurons in the cerebral cortex, piriform cortex, hippocampus, and amygdala, as well as the striatum [4]. The primary regions supplying cortical and hippocampal GABAergic neurons are the MGE (60%), CGE (30%), and POA (10%) [4]. These cells normally migrate from their proliferative zone in the GEs toward the cerebral cortex and hippocampus following stereotyped routes using a mode of migration that is termed "tangential" (to distinguish it from the main radial mode of cortical neuron migration). The property of tangential migration enables MGE-derived GABAergic progenitors to disperse throughout the developing cerebrum and hippocampus, and after transplantation.

The GABAergic interneurons generated in the GEs and POA are morphologically, electrophysiologically, and molecularly diverse. Efforts to classify these populations of interneurons reached a consensus in 2009 in a document that describes the features that can distinguish among cortical interneuron subtypes [5]. In the hippocampus CA1 region, further classification was based on the locations of the cell bodies and their dendritic and axonal projections. Morphological and electrophysiological analyses of interneurons distinguished by the expression of the calcium-binding protein parvalbumin (PV), the neuropeptide somatostatin (SST), and the ionotropic serotonin receptor (5HT3aR) showed that these three classes define distinct interneuron populations in the cerebral cortex and hippocampus. Within these major subtypes, additional diversification is possible based on transcriptome data, but these three major subclasses allow a general hierarchical classification Scheme [6].

Recently, studies have utilized genetic or viral tools to express Channelrhodopsin2 (ChR2), a modified algal protein from *Chlamydomonas reinhardtii* a light-activated cation channel, in these GABAergic interneuron subclasses to study their functions in different forebrain circuits. With the development of cre-expressing transgenic lines of mice that have promoter/enhancer directed expression of cre or FLP recombinase in functionally distinct classes of GABAergic interneuron subclasses, it is now possible to selectively express genes of interest such as ChR2 to study their functions within intact forebrain circuits or after transplantation. Optogenetic studies in the intact brain require positioning of an optical fiber within the regions of interest to allow activation of ChR2-expressing GABAergic interneurons, whereas investigations in brain slices allow the use of focused beams of blue light onto selected regions within the brain slice. The latter technique facilitates whole-cell patch-clamp recordings on neighboring cells to determine the functional effects of exciting GABAergic interneurons within the structure of interest.

Our approach relies on a line of mice that were created with a bacterial artificial chromosome (BAC) transgene that regulates ChR2 expression under the control of the vesicular GABA transporter (VGAT, *Slc32A1*). The VGAT-ChR2-YFP BAC transgene was designed in the laboratory of Dr. Guoping Feng at Massachusetts of Institute of Technology. The transgene contains a mammalian codon optimized *Chlamydomonas reinhardtii*-derived ChR2 gene (*COP4*) modified to contain a gain-of-function H134R substitution (mhChR2; also called hChR2-H134R) fused in-frame with an enhanced yellow fluorescent protein (EYFP). In comparison to ChR2, the mhChR2 causes greater stationary photocurrents. This mhChR2::YFP fusion protein sequence was inserted into the coding region of VGAT locus on a mouse BAC by homologous recombination, and the resulting 196 kbp VGAT-mhChR2-YFP BAC transgene was injected into fertilized oocytes to create transgenic mouse founders. The founder mice were bred to C57BL/6J mice to establish a colony (line 8) and backcrossed multiple generations before this transgenic line of mice was deposited at the Jackson Laboratory (Stock No. 000664). Mice in this colony have approximately twenty copies of the transgene. Blue light (450–490 nm) drives opening of this cation channel and allows rapid and reversible photo stimulation of action potentials in ChR2-expressing GABAergic interneurons.

In this protocol, we describe our methods for dissecting mhChR2::YFP-expressing MGE-derived GABAergic progenitors from embryonic day 13.5 (E13.5) mouse embryos. We describe procedures for dissociating and resuspending the progenitor cells in transplantation media containing growth factors and survival factors and our method for precision stereotaxic transplantation of the dissociated cells into the dentate gyrus of the hippocampus in the

adult mouse brain. After transplantation into the adult mouse hippocampus, MGE-derived cells typically migrate away from the site of transplantation and integrate into hippocampal circuits over the course of about 4–6 weeks. Additionally, within several weeks, the transplanted cells develop distinct neurochemical phenotypes, based on the expression of SST+, PV+, and other neuropeptides [2].

In slices prepared from mice that received transplants of MGE cells into the DG, we established that blue light activation of the ChR2-YPF expressing cells induced strong hyperpolarizing (inhibitory) currents in neighboring cells in the hippocampus; moreover, the transplanted cells showed spontaneous excitatory and inhibitory currents [1, 2]. These findings indicate that after transplantation, MGE-derived progenitors receive excitatory and inhibitory synaptic inputs and form putative synaptic contacts onto neighboring neurons, including granule cells (GCs) in the DG. In vivo electroencephalographic (EEG) monitoring of seizure activity showed that several weeks following transplantation of MGE-derived GABAergic interneurons (from non-ChR2-expressing donor mice), spontaneous recurrent seizures were suppressed in mice with temporal lobe epilepsy induced by pilocarpine (TLE) [1, 7]. Additionally, MGE-derived GABAergic interneurons appear to follow the same developmental lineage patterns and acquire firing properties comparable to endogenous MGE-derived GABAergic interneurons after transplantation [8]. These findings suggest that upon transplantation, developing GABAergic progenitor cells from the MGE integrate into the adult mouse hippocampus and develop molecular and electrophysiological properties similar to endogenous hippocampal interneurons.

We have extended our investigations of the effects of transplanting MGE progenitors into the hippocampus of mice with TLE to investigate which types of neurons in the host brain receive inhibitory synaptic input from the transplants. The rationale for these studies is that the dentate gyrus of the adult hippocampus is one of the few brain regions that shows continued production of new neurons throughout life – chiefly granule cells (GCs) in the DG. Adult hippocampus neurogenesis occurs in birds [9], rodents [10], and primates [11, 12]. Neurogenesis can be increased by manipulations such as providing enriched environment [13], exercise [14], and seizures [15]. Considerable evidence links the development of spontaneous recurrent seizures to a disturbance in the normal patterns of adult neurogenesis in the hippocampus [15–19]. In addition to increasing the number of new, adult-born GCs in the mouse hippocampus in TLE, many of the newborn GCs migrate abnormally, and their somas are oriented improperly (ectopic GCs). Many GCs develop abnormal dendritic arbors that include branches projecting into the hilus of the dentate gyrus instead of the molecular layer (hilar-projecting dendrites).

Additionally, many GCs become hyperexcitable, due partly to the formation of recurrent axon collaterals through a process known as mossy fiber sprouting. These changes contribute to hyperexcitability and the emergence of spontaneous recurrent seizures (SRS) in rodent models of TLE. An additional change in the pilocarpine model is that many of the endogenous GABAergic interneurons in the hippocampus degenerate or become dysfunctional, and mossy cells [20], which provide feed-forward excitation to GABAergic interneurons [20, 21], may also die.

The process of adult neurogenesis has been studied with different techniques including DNA uptake of injected thymidine analogues [22], immunohistochemical detection of endogenous cytoskeletal markers (e.g., Doublecortin for young migrating neurons) [23], and in vivo retroviral labeling [24]. To use thymidine analogues and doublecortin immunoreactivity, it is necessary to process the tissue for immunocytochemical staining. However, retroviral labeling can provide fluorescent expression that can be visualized in a fluorescent microscope in living brain slices. The MMLV-based replication defective retroviruses that express fluorescent proteins are capable of stably integrating a DNA of interest into host genome of dividing cells during mitosis. Additionally, retroviruses can be designed and produced with CRISPR/Cas9 system and a fluorescent protein to stably transduce dividing cells in model organisms [25]. In this protocol, we inject a high-titer retrovirus by stereotaxis into the subgranular zone of the adult mouse dentate gyrus in the hippocampus to label newborn GCs. This procedure can be done prior to, or following, transplantation of ChR2-expressing MGE progenitors. Several days after transduction, the retrovirally labeled adult-born neurons express fluorescent protein in processes and soma. With more extended periods of time, the fluorescent protein fills the axons and dendrites. It is therefore a superb technique for studying structural and functional properties of adult-born neurons and their connectivity with the ChR2-expressing transplants.

2 Materials (See Table 1)

2.1 MGE Dissection and Dissociation

1. Hank's balanced salt solution (HBSS w/o Ca^{+2} or Mg^{+2}) for dissection: Add 9.5 g of HBSS powder and 0.35 g $NaHCO_3$ to 750 mL ddH_2O in a 1 L graduated cylinder, and bring pH to 7.4. Make volume to 1 L with ddH_2O. Sterilize by filtering through 0.2 μm filter and store on ice.
2. Make transplantation media: 2 mL L-15 media, 15 μL FGF, 3 μL caspase inhibitor ZVAD-FMK, 20 μL B27, 1 μL murine EGF, and store it at 37 °C in water bath.

Table 1
Equipment and reagents

Name	Company	Catalog number	Comments
Animals			
C57BL/6NHsd	Envigo	#047	
B6.Cg-Tg(Slc32a1-COP4∗H134R/EYFP)8Gfng/J	The Jackson Laboratory	014548	Also referred to as VGAT-ChR2-YFP line 8
MGE dissection			
L-15 medium	Gibco	11415-064	
Fibroblast growth factor	Sigma	F0291	
Epidermal growth factor	Invitrogen	53303-018	
Caspase inhibitor	Promega	G7231	
B27	Gibco	17504-044	
HBSS powder	Sigma	F0291	
2.5% trypsin (10X)	Gibco	15090-046	
Trypsin inhibitor	Gibco	007-100	
150 × 15 mm	Fisher Scientific	FB0875714	
Microsurgical knife	F.S.T	10316-14	
Dissecting microscope	Leica	M165FC	
FHS/F-01 headlamp	Biological Laboratory Equipment Maintenance & Service Ltd.		FHS/EF-2G2 emission filters
Benchtop centrifuge	Eppendorf	5424	
Centrifuge	Beckman	GS-6R	
Dumont #5SF forceps	Fine Science Tools	11252-00	
Sharp fine scissors	Fine Science Tools	14028-10	Straight blunt tips
Vannas Spring scissors	Fine Science Tools	15000-08	Straight tip, 2.5 mm cutting edge
Cell culture			
293GP cell line	Bryan Luikart	http://www.synapticplasticity.com/	For retrovirus
293-R cell line	Bryan Luikart	bluikart@yahoo.com	For retrovirus titer

(continued)

Table 1
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Name	Company	Catalog number	Comments
Iscove's modification of DMEM (IMDM)	Corning	10-016-CV	Complete IMDM with 10% FBS, 1% NEAA, 1% L-Gln, and 1% P/S
Fetal bovine serum (FBS)	Corning	35-011-CV	
MEM nonessential amino acids (NEAA)	Corning	25-025-CI	
L-Glutamine solution, 100X (L-Gln)	Corning	25-005-CI	
Penicillin/ Streptomycin solution, 100X (P/S)	Corning	30-002-CI	
Polystyrene 10 cm plate	USA Scientific	CC7682-3394	
Trypsin EDTA 1X	Corning	25-053-CI	
Caffeine	Sigma-Aldrich	C0750-500G	
Transfection			
5 mL polystyrene tubes	Fisher Scientific	352054	
VSV-g plasmid	Bryan Luikart Lab		
MMLV-red Rubi plasmid	Bryan Luikart Lab		
Calcium chloride dihydrate ($CaCl_2$)	Fisher Scientific	C69-500	Make a 2.5 M solution in ddH_2O
Sodium chloride (NaCl)	Fisher Scientific	S271-3	
HEPES	Fisher Scientific	BP2939-100	
Na_2HPO_4	Sigma	S9390-1 kg	
KH_2PO_4	Fisher Scientific	P285-500	
KCl	Fisher Scientific	3040-01	
2X HEPES-buffered saline (HBS)			500 mL: 8.2 g NaCl, 5.95 g HEPES, 0.106 g Na_2HPO_4, pH 7.01

(continued)

Table 1
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Name	Company	Catalog number	Comments
Testing efficacy of virus and immunostaining			
0.22 μM filter unit	Cell-treat	229706	
0.45 μM syringe filter unit	Millex-HV	SLHV033RS	
30 cc L/L syringe	BD	302832	
50 mL conical tube	Cell-treat	229420	
15 mL conical tube	Cell-treat	229410	
polyethylene glycol 6000 (PEG 6000)	EMD Millipore	528877-1 kg	
Phosphate-buffered saline (PBS) (10×)			NaCl (80 g), KCl (2.0 g), Na_2HPO_4 (14.4 g), KH_2PO_4) (2.4 g) pH 7.4
0.5 mL microcentrifuge tubes	USA Scientific	1605-0000	
Matrigel	Corning	356234	
6-well plate	Fisher Scientific	353046	
Paraformaldehyde	Electron Microscopy Sciences	15174-S	
Normal goat serum	Vector	S-1000	
Triton X-100	Sigma-Aldrich	×100–500 mL	
10 mL serological pipette	Fisher Scientific	357551	
Chicken anti-GFP	Aves	GFP-1020	
Rabbit anti-mCherry	Thermo Scientific	PA5-34974	
Goat anti-chicken-FITC	AVES	F-1005	
Goat anti-rabbit 568	Molecular Probes	A11011	
Stereotaxic surgery			
Isothesia (isoflurane)	Henry Schein	SKU 029405	
2% topical lidocaine	Hi Tech	00603-1393-64	
Stainless steel scalpel blades, #21, 100-pk	Surgical Design		
Puralube Vet Ophthalmic Ointment	Dechra	17033-211-38	

(continued)

Table 1
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Name	Company	Catalog number	Comments
Glass syringe (5 μL, removable needle)	Hamilton	7634-01	
Needle 26GA, 0.5″ 30	Hamilton	7804-03	
Needle 30GA, 0.5″ 45	Hamilton	7803-07	
Glass syringe (10 μL, removable needle)	Hamilton	7635-01	
Automated stereotaxic injection system	Stoelting Quintessential	53311	
Charcoal filter, VetEquip	Harvard Apparatus		
Cotton tipped applicators	Quality Medical Products		
Neo-Predef with tetracaine powder 15 g	Pharmacia & Upjohn	1471000	
VetBond	Tissue adhesive, 3 M Corp		
Electrophysiology			
Ketamine hydrochloride	Ketaset, Zoetis		
Xylazine	Anased, Lloyd Laboratories		
Vibratome	Leica	VT1000S	
Microscope with U-MWIB fluorescent	Olympus	BX51	
Uniblitz shutter			To gate incoming excitation light
Master-8 device	A.M.P.I instruments		
Axopatch 200B amplifier	Molecular Devices		
ITC-18 A/D converter	InstruTech		
Texas Red Avidin D solution	Vector Labs		

(continued)

Table 1
(continued)

Name	Company	Catalog number	Comments
Software			
IMARIS	Bitplane		3D neuron reconstruction
IGOR	Wavemetrics		Electrophysiology analysis software programming language

3. Prepare medium-bore and fine-bore fire-polished Pasteur pipettes and autoclave.
4. Sterilize tools for the dissection of embryos: 2 Dumont #5SF forceps, 2 pairs of sharp fine scissors (straight blunt tips), 2 pairs of fine sharp scissors (sharp tips), 2 pairs of Vannas Spring scissors (straight tip, 2.5 mm cutting edge), and 1 microsurgical knife.
5. 0.125% trypsin in HBSS.
6. 0.07% trypsin in HBSS.

2.2 Packaging of Retrovirus

1. Complete media: IMDM with 10% FBS, 1% NEAA, 1% L-Gln, and 1% Penicillin/Streptomycin solution (50 mL FBS, 5 mL NEAA, 5 mL L-Gln, 5 mL Penicillin/Streptomycin, make up volume to 500 mL with IMDM).
2. Collection media: IMDM with 2% FBS and 40 mg/100 mL caffeine (2 mL FBS, 40 mg/100 mL caffeine, make up volume to 100 mL with IMDM).
3. 2.5 M $CaCl_2$: add 36.75 g $CaCl_2$ powder to ddH_2O, and raise volume to 100 mL.
4. 2× HEPES: add 8.2 g NaCl, 5.95 g HEPES, 0.106 g Na_2HPO_4 to a graduated cylinder with 450 mL ddH_2O, bring pH to 7.01, and make to a final volume of 500 mL.
5. 40% PEG 6000 in 1.5 M NaCl: add 40 g PEG and 8.7 g NaCl to ddH_2O in a 100 mL cylinder, and make to a final volume of 100 mL.
6. 10× PBS: 1.36 M NaCl, 26.83 mM KCl, 101.96 mM Na_2HPO_4, 17.64 mM KH_2PO_4, and raise volume to 1 L with ddH_2O. pH 7.4.

2.3 Electrophysiology

1. Anesthesia: ketamine hydrochloride (120 mg/kg, i.p.; Ketaset) and xylazine (10 mg/kg, i.p).
2. High sucrose artificial cerebral spinal fluid (ACSF) for brain slicing: 27.07 mM $NaHCO_3$, 1.5 mM NaH_2PO_4, 1 mM $CaCl_2$, 3 mM $MgSO_4$, 2.5 mM KCl, and 222.14 mM sucrose.

3. Incubation ACSF: 125 mM NaCl, 1 mM $CaCl_2$, 3 mM $MgSO_4$, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM KCl, 25 mM glucose, 3 mM myo-inositol, 2 mM Na-pyruvate, and 0.4 mM ascorbic acid.
4. Recording ACSF: 125 mM NaCl, 1.5 mM $CaCl_2$, 1.0 mM MgSO4, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 3.5 mM KCl, 25 mM glucose, 3 mM myo-inositol, 2 mM sodium pyruvate, and 0.4 mM ascorbic acid.
5. Intracellular solution: 135 mM gluconic acid, 135 mM CsOH, 1 mM EGTA, 8 mM MgCl, 0.1 mM $CaCl_2$, 10 mM HEPES, 2 mM Mg-ATP, and 0.3 mM Na-GTP, 11 mM biocytin (*see* **Note 1**).
6. Leica VT1000S vibratome for brain slicing.
7. Olympus BX51 microscope with U-MWIB fluorescent cube set, producing excitation wavelengths 460–490 nm.
8. Uniblitz shutter to gate incoming excitation light with msec precision.
9. Master-8 device (A.M.P.I.) to program the timing of light pulses and coordinate with the computer acquisition of electrophysiological recordings.
10. Axopatch 200B amplifier for voltage-clamp recording.
11. ITC-18 (InstruTech) A/D converter for digitizing the electrophysiology.
12. IGOR software (wavemetrics) for recording electrophysiology on a computer, attached to the InstruTech A/D converter (any reputable software platform that can be triggered by a 5 V TTL pulse from the Master-8 to the software's respective A/D converter should work).

2.4 Biocytin Morphological Reconstructions

1. 0.24 M phosphate buffer (0.24 M PB): 33.76 g sodium phosphate monobasic into 750 mL ddH_2O, 7.72 g sodium hydroxide (NaOH) into 150 mL ddH_2O. Mix these two solutions together, and add more ddH_2O to make 1 L. Check pH (7.2–7.4).
2. 30% sucrose: 30 g sucrose, 50 mL 0.24 M PB, and raise volume to 100 mL with boiling ddH_2O.
3. 4% PFA: 10 mL 32% paraformaldehyde and raise volume to 80 mL with 70 mL 0.12 M PB (from Electron Microscopy Sciences)
4. 0.12 M phosphate buffer (PB): 500 mL ddH_2O, 500 mL 0.24 M PB.
5. 0.5 M potassium phosphate buffer monobasic solution: 68.05 g monobasic KH_2PO_4 and bring volume to 1 L with ddH_2O.

6. 0.5 M KPBS dibasic: 114.1 g dibasic monohydrous $K_2HPO_4 \times H_2O$ or 87.09 g anhydrous K_2HPO_4 and raise volume to 1 L with ddH_2O
7. 0.02 M KPBS: 7 mL 0.5 M KPBS monobasic, 33 mL KPBS 0.5 M dibasic, 9 g NaCl, and increase volume to 1 liter with ddH_2O. pH 7.4
8. Triton-X stock solution: 3 mL 0.02 M KPBS and 9 μL Triton-X.
9. Texas Red Avidin D solution: 12 microliters Texas Red and 3 mL Triton-X stock solution.

3 Methods

3.1 Dissection of Medial Ganglionic Eminence (MGE), Dissociation, and Transplantation into Adult Mouse Hippocampus

Set up timed breeding between a male VGAT-ChR2-YFP adult mice (stock number: 014548, The Jackson Labs; [26]) and two wild-type females (C57Bl/6NHsd, Envigo) in the evening (Day 0) and leave the mice together overnight. The following morning (E0.5) confirm mice with vaginal plugs and separate them into new, clean cages. The embryos should be dissected from the pregnant female(s) 13 days after the day of mating, on gestation day E13.5 to obtain MGE cells (*see* **Note 2**).

3.1.1 MGE Dissection

1. Euthanize pregnant female by cervical dislocation.
2. Soak abdomen with spray bottle containing 70% EtOH.
3. Lift the skin just below the midline of the stomach, and make a fine cut through the skin without opening the peritoneum. Using blunt-ended fine scissors, separate the overlying skin from the peritoneum, and make lateral cuts in the skin and fold back. Holding the peritoneum with forceps, make a large cut through the peritoneum to expose the abdominal cavity and the uterus.
4. Holding the uterus with forceps, lift gently while cutting away connective tissue until the entire uterus is free and transfer into a sterile 150 mm Petri dish containing ice-cold HBSS. Place the Petri dish in a sterile laminar flow hood (*see* **Note 3**).
5. Dissect each embryo from the uterus and transfer into a second sterile 150 mm Petri dish containing sufficient ice-cold HBSS to cover the embryos.
6. Place the 150 mm Petri dish on a chilled aluminum plate on the stage of a stereo microscope for fluorescent imaging and dissection.
7. Identify and separate the transgenic embryos using the dissecting microscope while viewing the embryos under the GFP filter. The transgenic embryos can also be identified by using

specialized goggles (FHS/F-01 headlamp equipped with FHS/EF-2G2 emission filters, Biological Laboratory Equipment Maintenance and Service Ltd.).

8. Remove the head from one embryo (leaving the rest of the embryos intact in the dish in ice-cold HBSS).
9. Orient the head by holding the nose with Dumont forceps. Cut through the skin overlying the skull to expose the skull. Make a cut along the midline of the skull and along the posterior border of the cerebral hemispheres to expose the brain. Open the skull by extending these cuts until the brain is exposed.
10. Keeping the scissors horizontal cut under the cerebral hemispheres to remove them from the brain.
11. Place each cerebral hemisphere dorsal side down and gently cut two slits in the lateral edge of the cortices to expose the ganglionic eminence (GE).
12. The GE should be visible just inside the fold of the cortex on the medial edge. It is the most prominent structure in the forebrain right now, and the crease is its' characteristic feature. The MGE is the medial portion of the GE.
13. Make straight cuts with the micro surgical knife (stab knife) to separate the CGE and LGE from MGE. Turn the MGE on its side and trim off the bottom.
14. Transfer the two pieces of MGE to a 50 mL conical tube containing ice-cold HBSS by drawing the tissue pieces into the tip of a sterile fire-polished sterile glass Pasteur pipette with a latex bulb.
15. Repeat the dissection for each of the transgenic embryos.

3.1.2 Dissociation of Embryonic MGEs

1. Prepare a 5 mL solution of 0.125% trypsin in HBSS in a 15 mL conical tube.
2. Using a sterilized fire-polished sterile glass Pasteur pipette, transfer the MGE in the trypsin solution. Incubate the conical tube for 12 min at 37 °C in the water bath.
3. While the trypsin is working, prepare 5 mL of 0.07% trypsin inhibitor in HBSS.
4. Once the trypsin is done and the cells have settled on the bottom of the tube, use a glass pipette to suction up the tissue and release them into the trypsin inhibitor. Incubate for 10 min at 37 °C in the water bath.
5. Triturate rapidly using a sterile large-bored fire-polished glass Pasteur pipette 10 times. Take care not to create bubbles. Repeat trituration with a medium tip fire polished glass pipette for 10 times. At this point, most of the tissue should be dissociated. Do not over-triturate as this will damage the cells.

6. Spin the tube in the benchtop centrifuge (IEC) on speed 1 for 45 s to remove debris. Decant the supernatant into a new tube, leaving behind the debris. (We want the supernatant because the debris is just excess tissue from the dissection.)
7. Spin supernatant with cells in centrifuge (e.g., Beckman GS-6R) at 200 × *g* at 23 °C for 10 min. After spinning, you should see a pale white pellet at the bottom of the tube (hard to see).
8. Decant the trypsin inhibitor, leaving behind the cell pellet, and replace with 1 mL of transplantation medium. Triturate 3–4 times with a sterile small-bore fire-polished glass Pasteur pipette to dissociate the pellet and create a single cell suspension.
9. Take 5 μL of the cell suspension with a sterile tipped Eppendorf pipette and use in a hemocytometer to determine the density of the cells.
10. Transfer cell suspension to a 1.5 mL sterile Eppendorf tube.
11. Spin Eppendorf tube on benchtop centrifuge (Eppendorf 5424) at 586 × *g* for 2 min.
12. Transfer pellet to a small PCR tube and resuspend cells to the final concentration (100,000 cells/μL) in transplantation medium.
13. Keep the cells on ice and use immediately for transplantation.

3.2 Retroviral Labeling of Adult-Born Granule Cells in the Mouse Hippocampus to Study Connectivity of ChR2-Expressing MGE Transplants

3.2.1 Packaging of Retrovirus

Day 1: Starting cultures from frozen stocks

1. Rapidly thaw a vial of frozen 293 GP (gag/pol) cells at 37 °C in a water bath.
2. Pipette and transfer suspension of cells to a 15 mL conical tube.
3. Add 5 mL of complete IMDM media, and mix gently and centrifuge cells at 500 × *g* for 3 min.
4. Remove supernatant, and resuspend the cell pellet in 10 mL of complete IMDM media.
5. Incubate cells overnight at 37 °C with 5% CO_2.

Day 2: Aspirate the existing media and add 10 mL of pre-warmed complete IMDM media.

Day 4: Once the cells become confluent, split to 2.5–3.0 × 10^6 cells per plate (10 cm plate).

Split using the following procedure:

1. Remove media and wash cells once with PBS at RT.
2. Treat with 2 mL of trypsin for 3 min at 37 °C.
3. Add 3 mL of complete IMDM media to inhibit trypsinization.

4. Resuspend cells gently by pipetting and transfer to a 15 mL tube.
5. Centrifuge at 500 × *g* for 3 min. Resuspend the cells in 1 mL of media. Dilute 10 μL of cells in 90 μL of media. Count the cells using a hemocytometer. Replate cells with complete medium.

Day 5: Calcium phosphate-mediated transfection and viral particle collection

1. Change 75% of medium 2 h prior to transfection (10 mL total).
2. Prepare transfection reagents for two 10 cm plates using two 10 mL polystyrene tubes. Label the first tube as "DNA," and label the second tube "2× HBS."
3. Adjust the concentration of plasmid DNA to 1 μg/μL in TE pH 7.4.
4. Add following mix to tube "DNA" (add slowly while tapping the tube to mix): 15 μL VSV-g, 20 μL MMLV-redRubi, 865 μL molecular biology grade H_2O, 100 μL 2.5 M $CaCl_2$.
5. Add 1000 μL of 2× HBS to the second tube.
6. Take the 1 mL contents of the first tube, and slowly add one drop at a time into the 2× HBS tube while constantly tapping the tube.
7. Leave the tube in dark for 30 min.
8. Add 1 mL in slow droplets to each plate and leave overnight.

Day 6: Replace media with 8 mL of collection media.

Day 7: First harvest: Aspirate the media containing viral particles using a pipette with the plate tilted, and dispense into a 50 mL conical tube. Store the 50 mL conical tube at 4 °C. Replace with 8 mL of collection media.

Day 8: Second harvest: Again, aspirate the media, and add it to the same 50 mL conical tube used on the previous day.

3.2.2 Purification of Retroviral Particles

1. Prepare 5× PEG 6000 solution: 40% PEG 6000 and 1.5 M NaCl (autoclave and slowly mix the solution on a shaker while cooling.
2. Centrifuge viral particles containing media at 2000 × *g* for 10 min to pellet down insoluble material.
3. Carefully remove and filter (0.45 μm low protein binding) media containing viral particles (do not use a 0.2 μm filter, as it can shear the envelope from the virus making it noninfectious).
4. Add 5× PEG 6000 solution to 1× (final concentration should be 8% PEG 6000 and 0.3 M NaCl).
5. Mix by upending several times (do not shake).

6. Incubate at 4 °C for 12 h or more, remixing occasionally.
7. Spin viral containing PEG solution at 3900 × *g* for 45 min.
8. Remove supernatant and spin again for 2 min at 4700 × *g*. Discard supernatant.
9. Resuspend pellet by adding 1/10th to 1/100th original volume of PBS and put on rocker for 30 min.

3.2.3 Determining the Titer of the Retrovirus

Preparation of plates for determining titer. Coat the plates with Matrigel.

1. Dilute 1 mL of Matrigel with 9 mL of IMDM.
2. Put 0.5 mL of diluted Matrigel in each well of the 6-well plate and leave at 37 °C for 30 min.
3. Remove the Matrigel mixture and plate the cells.

Day 1: Plate 293R cells in a 6-well plate (for a 10 cm plate, use 1 mL of trypsin and 3 mL of media to stop it). Plate at a density of 5×10^4 in 1 mL media per 1 well of the 6-well plate.

50% confluence is good for determining titer and is usually reached 24 h after plating.

Day 2: For titer, add 10 μL of virus into first well. Take 100 μL from the first well, and add to second well and so on. Incubate for 24 hours.

Day 3: Add 1 mL media to each of the wells.

Day 5: Add 2 mL of warm 4% PFA (warm by putting in the water bath at 37 °C) to each well. Leave for 5 min.

1. Remove the PFA and add 2 mL of 4% PFA. Leave for 5 min.
2. Rinse twice (15 min each) with PBS-T (0.4%) pH 7.4.
3. Block non-specific binding by incubating in 10% NGS in PBS-T for 1 h at RT.
4. Remove blocking solution and replace with rabbit anti-mCherry primary antibody (1:1000) diluted in 2.5% NGS in PBS-T at RT overnight.

Day 6: Rinse 3 × 15 min with PBS-T.

1. Add Goat anti-rabbit-568 secondary antibody (1:1000) in 2.5% NGS in PBS-T at RT for 1 h.
2. Rinse 3 × 15 min with PBS-T.
3. Mount with Prolong with DAPI.

Determination of titer: mCherry-expressing cells should be visible after 72 h and can be seen without staining. If no fluorescently labeled cells can be seen in well 5 and 6, it's a very low-titer virus (start the retrovirus production again). If you can see the mCherry-expressing cells, proceed with staining. Only wells 4, 5, and 6 need to be stained. Count the number of retrovirally labeled

cells in well 5 and well 6, and multiply by their respective dilution factors. Take an average of the count in well 5 and 6 to determine the titer. The 10^8 transfection units per milliliter (TU/mL) of retroviral particles is a good titer to use with stereotaxic surgeries.

3.3 Stereotaxic Injection of MGE Cells and Retrovirus

Maintain sterile condition in the surgery suite. This is achieved by using autoclaved surgery tools, spraying counter tops with 70% ethanol, using sterile gloves, and applying antibiotic powder to the incision site after it is closed.

3.3.1 Preparation for Surgery

1. Autoclave surgery tools. Prepare recovery chamber by turning on heating pad to maintain body temperature for recovery.
2. Isoflurane is the most commonly used halogenated anesthetic in animal research. Researchers can be exposed, via inhalation, to waste anesthetic gases, including isoflurane, if care is not taken to scavenge or prevent release into the laboratory environment. Induction of anesthesia and surgical procedures should be performed using a well-designed and well-maintained gas scavenging system, such as a biosafety cabinet ducted to the outside or a rodent surgical ventilation system equipped with charcoal gas scavenging filters. Turn on the ventilation system and check for proper air filtering. Use adequate precautions to ensure that waste isoflurane gas is removed from the surgical area (recommended number is 6 or more changes/hour; check with your environmental health and safety office).

3.3.2 Beginning Stereotaxic Surgery

1. Prepare mouse for surgery by anesthetizing with isoflurane in an induction chamber. Confirm animal is properly anesthetized by pinching foot. Inject 0.03 mL of meloxicam subcutaneously to control inflammation and pain.
2. Place mouse (C57Bl/6NHsd, Envigo) in stereotaxic apparatus. Line up teeth with the hole in apparatus, and quickly fix the nose cone.
3. Adjust ear bars, making sure that the ear bars are fully immobilizing the mouse's skull laterally and are equally pushed in on either side.
4. Apply ophthalmic ointment to the mouse's eyes.
5. Sterilize the skin by applying povidone-iodine and lidocaine to reduce pain.
6. Make a longitudinal incision along the midline axis of the skull with the scalpel blade, exposing bregma and lambda.
7. Apply H_2O_2 to the skull with a cotton swab to aid in bregma and lambda visualization through dissecting microscope.

8. Place drill bit at bregma looking through dissecting microscope and zero the digital stereotaxic coordinates on the *x*, *y*, and *z* planes.
9. To level bregma and lambda, zero *z*-axis at bregma and check the dorsal ventral (*z*) plane by moving to lambda so that the *z*-coordinate is roughly equal at both bregma and lambda. Adjust if there is greater than or equal to 0.04 mm difference.
10. Move drill to desired coordinates. For the MGE-transplant use (M/L = ± 2.1 mm, A/P = −2.5 mm) and for retrovirus use (M/L ± 1.1, A/P –1.9 mm; M/L ± 2.1, A/P –2.5), then slowly and carefully drill through the skull.
11. After drilling holes, switch drill with the syringe and attach it to the Quintessential Stereotaxic Injector.
12. Withdraw enough cells/virus to inject in both hemispheres. Move needle to first drill site. Lower the tip of the bevel just beneath the skull and zero the *z*-coordinate when half of the bevel has disappeared. For transplanting MGE cells (50,000 cells per site), slowly lower the needle to the deepest *z*-coordinate. The *z*-depths are −1.8 and −2.2 mm. The injection rate is 0.2 μL/min using a stereotaxic injector. After the injection is finished, wait for 2 min, and then raise needle to inject again to the dorsal coordinate. Wait for 5 min before removing syringe (*see* **Note 4**).
13. For stereotaxic injections of retrovirus, use the following stereotaxic coordinates for making two injection sites in the adult male mouse: DV −2.5 and −2.3; DV −2.2 and −1.8. The injection rate is 0.25 μL/min. Use 0.5 μL virus per site. Make the first injection into the more ventral location, and leave the needle in place for 2 min before a second injection is made into the more dorsal location. Wait for 5 min before withdrawing needle.
14. Repeat the injections for other drill holes.
15. After injections, wet skin and skull with sterile saline. Use blunt forceps to hold skin together and apply surgical adhesive (e.g., Vetbond or similar product) to adhere skin together. Apply lidocaine and antibiotic powder to the wound.
16. Remove mouse from the stereotaxic apparatus and place into heated recovery chamber. Monitor the mouse continuously until the mouse regains consciousness and begins to move around. Return the mouse to the home cage. House individually until the wounds have healed.
17. In the days following the surgery, provide mice with soft diet and treats. Monitor the mouse's weight and wound healing.

For representative results, see Fig. 1.

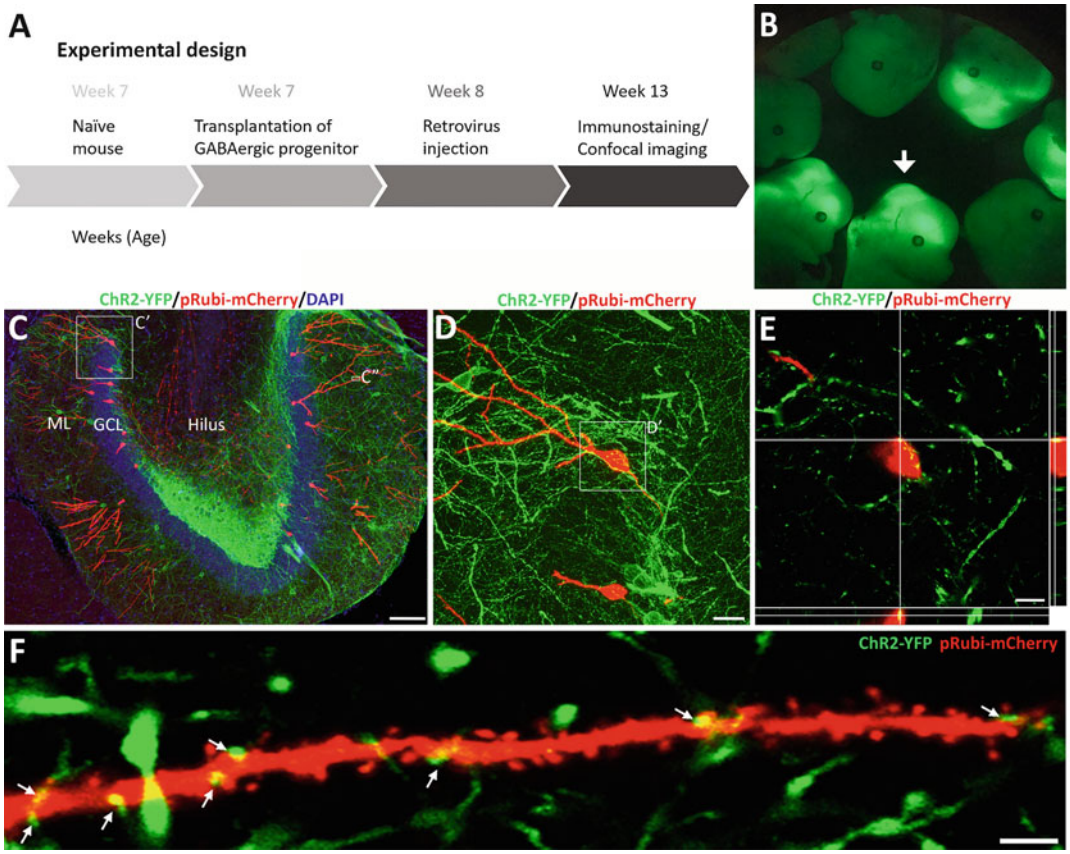

Fig. 1 Transplanted MGE-derived ChR2-YFP-expressing GABAergic interneuron progenitors differentiate into mature interneurons and form extensive axonal networks throughout the host dentate gyrus. Some of the cells localize to the hilus of DG, while others migrate into the granule cell layer and molecular layer. The axons contact host brain adult-born GCs that are labeled with the pRubi retrovirus. Retrovirally expressed mCherry completely fills the somas, dendrites axons of these GCs. (**a**) An experimental timeline for the procedure described in this protocol. MGE-derived GABAergic progenitors were transplanted into the hilus of dentate gyrus of 7-week-old mice. One week later, retrovirus injections were made, and 5 weeks post RV injection mice were perfused for analysis. (**b**) Transgenic embryos express GFP in the brain and spinal cord. These transgenic embryos glow green in color under GFP filter as indicated by an arrow. (**c**) Low-magnification image of the DG in a naive mouse that received injections of RV at 1-week post-transplantation. (**d**) High magnification image of boxed region (C') showing RV-labeled GCs (red) and transplanted MGE-derived GABAergic interneurons expressing ChR2-EYFP (green). (**e**) Optical slice of boxed region (D') showing sites of putative synaptic contacts between the retrovirally labeled GC and the transplanted GABAergic interneurons. (**f**) Higher magnification view of boxed region (A") showing putative sites of synaptic contacts along GC dendrite. (**a**) Scale equals 100 μm, (**b**) scale equals 20 μm, (**c)** scale equals 10 μm, (**d**) scale equals 2.5 μm

3.4 Electrophysiological and Optogenetic Analyses of Functional Synaptic Connections Between Transplanted ChR2-Expressing GABAergic Interneurons and Retrovirally Labeled Adult-Born GCs in the Mouse Hippocampus

3.4.1 Brain Slice Preparation and Electrophysiology

1. Anesthetized the mouse with the ketamine solution. After checking to make sure the mouse is deeply anesthetized, quickly removed the brain (1 min. or less), transferring it to oxygenated, ice-cold high sucrose ACSF.
2. Mount the brain, dorsal surface down, onto the ice-water chilled stage of a vibratome (Leica VT1000S). Slices are cut (350 μm thick) in the horizontal plane from ventral to dorsal.
3. Transfer the slices to a warmed (37 °C) incubation ACSF. Following the last collected slice, the beaker containing the slices is set on the table at room temperature and allowed to incubate for one hour (in the same ACSF).
4. Transfer an individual slice into the electrophysiological recording chamber (RC-17 heated chamber, Warner Instruments) containing a steady flow (approx. 4 mL/min) of warmed (34 °C) recording ACSF.
5. Identify a neuron of interest for whole-cell patch-clamping (WCPC), using standard WCPC technique. The neuron is voltage-clamped at +10 mV to help identify GABAergic inhibitory postsynaptic potentials (IPSCs).

3.4.2 Optogenetic Stimulation

The transplanted MGE-derived interneurons expressing ChR2 were stimulated with a blue light that was produced with a mercury lamp housed in a standard BX51 Olympus microscope. The light was filtered with a U-MWIB cube set, producing excitation wavelengths 460–490 nm. This light stimulus was gated via a Uniblitz shutter. Both the Uniblitz shutter and the computer were coordinated in time with a Master-8 stimulator (AMPI instruments): a button press on the Master-8 immediately started the electrophysiological recording, and after 3 s of this spontaneous activity (baseline recording), a set of 5 millisecond (ms) pulses, each separated by 200 ms, were sent to the shutter (Fig. 2). The electrophysiological recording ended after 3 s following the last 5 ms pulse. The light intensity at the level of the hippocampal slices was approximately 2 mW and the stimulus consisted of a circular area of illumination measuring 0.17 mm^2. The light stimuli consisted of five pulses of 5 ms duration with an interstimulus interval of 200 ms. The pulses were triggered using a Master-8 stimulator (A.M.P.I.).

3.4.3 Biocytin Morphological Reconstructions

1. Fill a 12 mL bottle about 2/3 with 4% PFA (PFA should be fresh—no more than 1 week old).
2. Using a high velocity setting, pull the electrode away from the neuron.
3. Transfer the slice from recording to the PFA bottle, being careful to include only the minimal amount of ACSF from the recording chamber. This can be achieved by letting the slice fall toward the tip of the transfer pipette and then just touching the

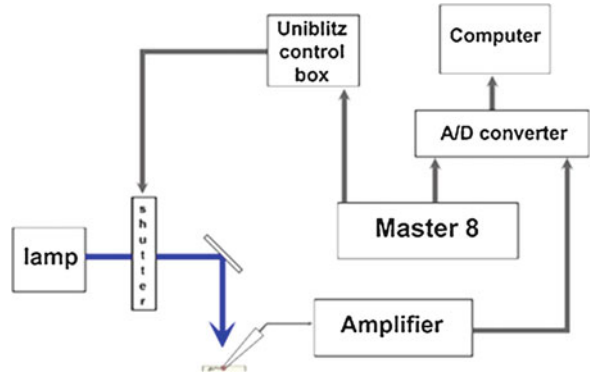

Fig. 2 Schematic illustration of the setup for optogenetic stimulation of neurons in a brain slice while recording a single neuron. Gray arrows indicate direction of electronic signals, either providing a TTL pulse (a simple "go" or trigger signal) or, in the case of the electrode, amplifier A/D converter, and computer pathway, the recorded activity of the neuron

transfer pipette to the surface of the 4% PFA solution. Allow the slice to remain in 4% PFA for a minimum of 30 min and no more than 12 h (*see* **Note 5**).

4. Slices from PFA to sucrose (wash): Remove PFA from the slice bottle (with a disposable transfer pipette), and dispose of PFA into a hazardous waste container. Repeat 3×.
   - Pour 0.12 M PB into bottle to about 1/3 full.
   - Place bottle on shaker for 5 min.
   - Remove 0.12 M PB.
   - Pour approximately 5 mL of 30% sucrose (made with 0.12 M PB) into small 12 mL bottle container. Place the 30% sucrose bottle with slice in 4 °C refrigerator overnight.

3.4.4 Texas Red Staining Protocol

Day 1: All steps under agitation at room temp, including final incubation.

1. Rinse in 0.12 M PB for 15 min.
2. Rinse in 0.02 M KPBS twice, each time for 15 min.
3. Incubate overnight in 3 mL Texas Red Avidin D solution.

Day 2: All steps at room temperature.

1. Rinse in 0.02 M KPBS three times, each time for 20 min under agitation.
2. Mount on a glass slide and coverslip, using an anti-fade mounting medium for fluorescence (e.g., VectaShield, Electron Microscopy Sciences), with the slice containing the patched cell mounted toward the coverslip. Be careful not to create bubbles and/or compress tissue when placing the coverslip on top of the slice. Seal edges of coverslip with nail polish.

Individual neurons that are retrovirally labeled and filled during electrophysiological recordings with biocytin can be further studied to determine morphological properties by performing 3D single neuron reconstructions and Sholl analyses, using commercially available software (IMARIS, Bitplane). An example of neuronal morphology studied with IMARIS cell reconstruction software is shown in Fig. 3.

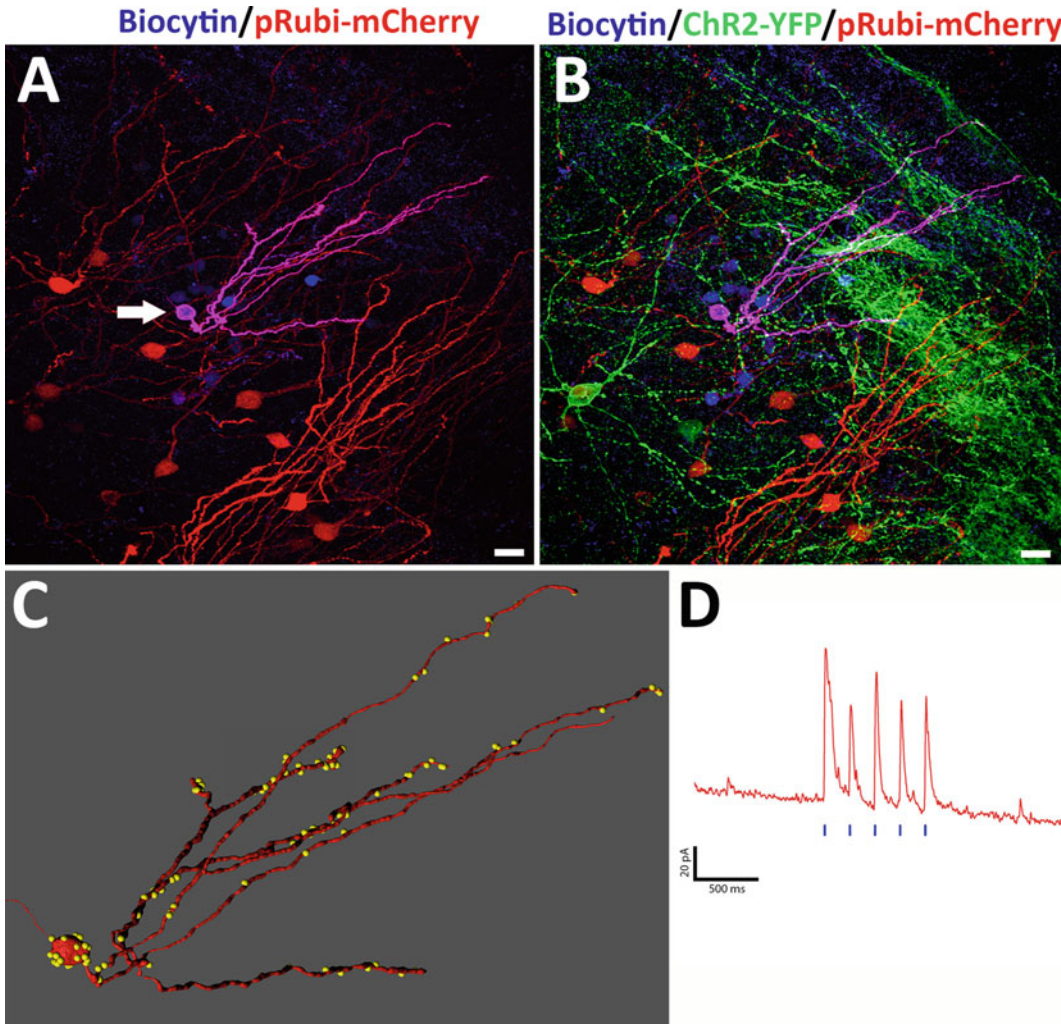

Fig. 3 Optogenetic demonstration of functional inhibition of adult-born GC by transplanted ChR2-expressing MGE-derived GABAergic interneurons. Patch-clamp recordings were performed from adult-born RV-labeled GCs, while the transplanted interneurons were depolarized optogenetically by illuminating hippocampal slices with blue light. (**a**) The electrophysiologically characterized, biocytin-filled GC is indicated by a white arrow in the group of adult-born GCs (red). (**b**) RV-labeled adult-born GCs are surrounded by dense plexus of MGE-derived ChR2-EYFP-expressing GABAergic axons (green). (**c**) 3D rendering of the same biocytin-filled GC by means of IMARIS software. The yellow dots indicate the locations of putative inhibitory synaptic contacts from the transplants. (**d**) Optogenetic stimulation of transplanted interneurons induced IPSCs in this GC following blue light stimulation. The vertical blue bars indicate blue light pulses of 5 ms duration and 200 ms interpulse interval

4 Notes

1. All ACSF solutions are constantly aerated with 95% CO_2, 5% O_2.
2. Introduce the females into the male mouse's cage. Make sure that the bedding in the male mouse's cage is not changed for several days before the day of mating. This time point marks the first day of gestation for any females that become pregnant. We briefly introduce the females into the male mouse's cage for 5 min for several days before the day of mating, and then leave them together in the male's cage for overnight breeding, as this seems to facilitate breeding. Breeding multiple females to the male mouse at one time generally increases the likelihood of obtaining timed-pregnant females. The VGAT-ChR2-YFP transgenic mouse line is maintained and bred in the hemizygous state. Thus, not all the embryos will contain VGAT-ChR2-YFP gene; however YFP expression is evident in transgenic embryos when they are viewed under blue light wavelengths in a dissecting microscope. In addition to observing vaginal plugs, we routinely check whether the timed-pregnant females gain weight during gestation. Weigh the females the day of mating and again after 10 days. Pregnant females should gain approximately 2 g by this stage of gestation.
3. Take care not to contaminate embryos by preventing the uterus from contacting non-sterile surfaces.
4. Injecting cells in multiple sites within the hippocampus increases the dispersion of GABAergic progenitor cells throughout the hippocampus.
5. It's important to know which side of the slice your neuron was recorded from, as you'll want that side to be "face up" when you mount the slice on a glass slide.

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Ethics Statement

All protocols were reviewed and approved by the Wesleyan University Institutional Animal Care and Use Committee (IACUC).

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