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A 'tool box' for deciphering neuronal circuits in the developing chick spinal cord

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ABSTRACT

The genetic dissection of spinal circuits is an essential new means for understanding the neural basis of mammalian behavior. Molecular targeting of specific neuronal populations, a key instrument in the genetic dissection of neuronal circuits in the mouse model, is a complex and time-demanding process. Here we present a circuit-deciphering 'tool box' for fast, reliable and cheap genetic targeting of neuronal circuits in the developing spinal cord of the chick. We demonstrate targeting of motoneurons and spinal interneurons, mapping of axonal trajectories and synaptic targeting in both single and populations of spinal interneurons, and viral vector-mediated labeling of pre-motoneurons. We also demonstrate fluorescent imaging of the activity pattern of defined spinal neurons during rhythmic motor behavior, and assess the role of channel rhodopsin-targeted population of interneurons in rhythmic behavior using specific photoactivation.

INTRODUCTION

The vertebrates' spinal cord consists of millions of neurons, connected through abundant synapses, which form functional neuronal circuits. Determining the organizing principles of these complex circuits is necessary for understanding how they process information, and support sensorimotor integration and motor behavior. The genetic dissecting of spinal neuronal circuits is fast becoming one of the most successful approaches to open the black boxes of neuronal networks (1). This approach requires: (i) systematic targeting of specific neuronal cell types; (ii) mapping the connectomes of specific populations of neurons; (iii) characterization of the activity patterns of circuit constituents and (iv) determination of the effects of loss- or gain-of-function of specific neuronal populations on circuit output and motor behavior.

The rate-limiting step in genetic dissection of spinal circuits is the molecular targeting of specific populations of spinal neurons. Germline targeting can be complicated by shared expression in several populations of neurons. Overcoming these problems in a mouse model can be achieved through intersected expression of site-specific recombinases (2), a time-consuming and costly process.

The accessibility of the embryonic chick spinal cord and the ability to surgically manipulate the cord, limbs and dorsal root ganglia (DRG), together with the evolutionary conservation of many sensorimotor circuits, have long suggested the chick embryo as an ideal model system for deciphering sensorimotor connectomes. In recent years, this idea has been brought a step closer, through new techniques demonstrating integration of exogenous DNA (3,4), germline transgenesis (5,6) and RNAi knockdown of gene expression (7–10). The ability to directly target the spinal cord, through electroporation, provides the opportunity to genetically, spatially and temporally direct expression to specific populations of spinal neurons. Such studies have enabled mapping of axonal routes of spinal interneurons (11–13), and clarification of the roles of guidance cues in spinal interneuron axonal projections (14–16).

Here we extend our studies (11–13,17–18), to describe a circuit-deciphering 'tool box'. This tool box includes (i) an assortment of enhancer elements and details of an enhancer-intersection methodology, to genetically target specific spinal neuronal subsets, and restrict gene expression to specific spinal interneurons; (ii) mutated alleles of Cre and a modified Chickbow strategy to allow axon tracking at single neuronal resolution; added to these, we have designed a second layer of cellular and activity reporters, specifically, transsynaptic viruses and modifier genes; (iii) genetically-targeted synaptic markers to identify synaptic contacts with target neurons labeled by fluorophores encoded by pseu-

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dorabies viruses (PRVs); (iv) genetically-encoded calcium sensors allowing calcium imaging of neuronal activity patterns, with concurrent electrophysiological recording of motor output; (v) photoactivation of specific populations of neurons, targeted with ChR2 to monitor their contribution to the motor output produced during stereotypic behavior.

Collectively, this set of molecular tools provides an acute, quick and efficient means to characterize the connectome, mechanisms of action and specific involvement of defined interneurons (INs) in sensorimotor integration and control of movements in the developing chick.

MATERIALS AND METHODS

In ovo electroporations

Fertilized White Leghorn chicken eggs were incubated at 38.5–39°C. A DNA solution of 5 mg/ml was injected into the lumen of the neural tube at HH stage 17–18 (E2.75-E3). Electroporation was performed using 3 × 50 ms pulses at 25 V, applied across the embryo using a 0.5-mm tungsten wire and a BTX electroporator (ECM 830). Embryos were incubated for 3–17 days prior to further treatment or analysis. For long-range incubations (longer than E12) following electroporation or viral injection, 150–300 μl of antibiotic solution, containing 100 unit/ml penicillin in Hank's Balanced Salt Solution (Biological Industry, Beit-Haemek), was added on top of the embryos.

Immunohistochemistry

Embryos were fixed overnight at 4°C in 4% paraformaldehyde/0.1 M phosphate buffer, washed twice with phosphate buffered saline (PBS), incubated in 30% sucrose/PBS for 24 h, and embedded in Optimal Cutting Temperature (O.C.T.). Cryostat sections (E5–6—14 or 20 μm) were collected on Superfrost Plus slides and kept at −20°C. For 100-μm sections, spinal cords (SC) were isolated from the fixed embryos and subsequently embedded in warm 5% agar (in PBS), and 100-μm sections (E12–E17) were cut with a Vibratome. Sections were collected in wells (free-floating technique) and processed for immunolabeling.

The following primary antibodies were used—rabbit polyclonal GFP antibody (Molecular Probes, Eugene, Oregon, USA), mouse anti-GFP, rabbit anti-RFP and goat ChAT antibody (Cemicon, Temecula, CA, USA), and Lhx1/5 (4F2) (provided by T. Jessell, Columbia University, New York, NY, USA). Mouse anti-synaptotagmin antibody (ASV30) and mouse anti myc (9E10) (hybridoma bank, University of Iowa, Iowa City, USA). rabbit anti-Pax2 antibody (ABcam) antibody. Cy2, RRX and Cy5 were used as fluorochromes conjugated to the secondary antibodies (Jackson). Images were taken under a microscope (Axioscope 2; Zeiss) with a digital camera (DP70; Olympus) or confocal microscope (FV1000; Olympus).

DNA

The EdI1, Foxd3 (169), Ngn1 and TAG1/Axonin1 enhancer elements were described previously (11–12,19). The CAGG::PBase was obtained from the Sanger Institute (Cambridge). PB-CAGG-pLox-Luc-pLox-GFP-PB plasmids were provided by Xiaozhong Wang (3). This plasmid was modified accordingly: the *Luc* gene was replaced by a STOP cassette. The *GFP* gene was replaced with either of the following genes: *Gap43GFP, Gap43mCherry, Gap43myc, Taumyc, SV2-GFP, SynB-GFP, GCaMP3* and *ChR2-cherry*.

PRV injection

We used three isogenic recombinants of an attenuated pseudorabies virus strain Bartha (PRV Bartha) that express enhanced green fluorescent protein (PRV152), monomeric red fluorescent protein (PRV614) or the Brainbow proteins (PRV263). The viruses were harvested from vero cell cultures at titers 4×10^8, 7×10^8 and 1×10^9 plaque forming units (pfu/ml), respectively. Viral stocks were stored at −80°C. Injections of 3 μl of PRV152, PRV614 or PRV263 were made in multiple sites of the shank muscles of E13 or E14 chick embryos, using Hamilton syringe (Hamilton; Reno, NV, USA) equipped with a 33-gauge needle. The embryos were incubated for 24–48 h and sacrificed for analysis.

Spinal cord isolation and stimulation

Spinal cords of E11–13 chick embryos were isolated in a dissection chamber superfused with oxygenated Tyrode's solution at 16–18°C (20). Then, preparations were transferred to a motorized recording chamber continuously superfused with, warmed (27°C) and oxygenated Tyrode's solution, pH 7.3, and mounted with ventral or lateral face up. The motor rhythm was induced by a single or double pulse stimulus of the sacral segments of the cord or of one of the lumbosacral dorsal root ganglia, and recorded using suction electrodes attached to right or left sartorius and femorotibial nerves.

Light stimulation

The ChR2-expressing neurons were stimulated using blue light emitting diode (LED) at 480 nm through a 100-μm optic fiber probe pointed to illuminate the ventral surface of the spinal cord.

Calcium imaging

Spinal cords of E11–13 chick embryo were isolated (see above) and transferred to a motorized recording chamber (see above). The preparation was mounted with its ventral or lateral face up. The GCaMP3-expressing neurons were visualized using a water immersion X20 objective (n/a 0.5) on an upright epifluorescent microscope (BX51WI Olympus, Japan) equipped with a digital CCD (Aqua, QImaging, UK). Changes in fluorescence emission at 530 nm, produced by excitation at 488 nm, were monitored at 25–30 frames/s. To minimize bleaching, the fluorescence excitation (Lumen 200; Prior Scientific, UK) was set at the lowest possible level and the exposure time was shortened to 15–20 s. A Transistor-Transistor Logic (TTL) pulse was used to open the CCD shutter, initiate the stimulus train and close the shutter. The TTL pulse was fed into the A/D converter used for electrophysiological data acquisition for subsequent offline synchronization of the optical and concurrent electrophysiological recordings. The motor rhythm was

induced by stimulation of the sacral segments of the cord or one of the dorsal roots and recorded using suction electrodes attached to the sartorius and femorotibial nerves.

Signal processing of the imaging and electrophysiological recordings were done using 'SpinalCore' (21). The fluorescent emission was measured before (F) and during stimulation. Calcium transients were recorded as changes in the mean pixel intensity (ΔF) in the soma of the imaged cell and in 10 regions of interest (ROIs) encircling the cell (surrounding ROIs) before and during the stimulation and expressed as relative change in fluorescence ($\Delta F/F$). The surrounding ROIs were used to measure the out-of-focus fluorescence and its $\Delta F/F$ was subtracted from the $\Delta F/F$ of the soma to obtain the specific $\Delta F/F$ of the neuron. The optical data were interpolated with the concurrently recorded motor output from the sartorius and femorotibial nerves and analyzed using Wavelet (WT) transformation algorithms. The phase between the optical and electrophysiological data was calculated from the spectral density plots of the coherent power obtained after omitting non-coherent regions from the cross-WT spectrum following a Monte Carlo-based WT coherent analysis (21,22).

RESULTS

Increasing the targeting specificity: enhancer element intersection

We have previously generated a library of enhancer elements that direct expression to defined spinal neuronal populations (11–12,19,23–24,25–30) (Table 1). To restrict expression to a single neuronal population**,** we employed enhancer-intersection strategy. Two enhancer elements that drive the expression of two different recombinases (Cre, FLPo or PhiC31o) were coupled to a dual-conditional reporter containing two recombinase-specific STOP cassettes (Figure 1A). Thus, the reporter gene is activated only in cells that co-express the two recombinases.

The efficiency of removing the double STOP cassette was tested by electroporation of two recombinases along with the appropriate double conditional target plasmid and ubiquitously expressed mCherry plasmids. The combination of Cre+FLPo yielded the highest efficiency expression of GFP, while the combination of FLPo and PhiC31o resulted in lower activation of the target gene (Figure 1A). A similar decrease in labeling efficiency was observed when FLPo and PhiC31o were expressed in dI1 neurons under the control of EdI1 enhancer element (Supplementary Figure S1D).

Previously we exploited Cre/Gal4 enhancer intersection to label dI2 neurons (12). In order to use Cre and FLPo recombinases for enhancer intersection in dI2 neurons, FLPo was expressed under the control of FoxD3-169 enhancer and Cre under the Ngn1 enhancer. Ninety-five percent of GFP-expressing cells were $Lhx1^{+}/Pax2^{-}$ dI2-neurons (Figure 1B). For sustained long-term expression of reporter gene, we used the PiggyBac system. The double conditional GFP cassette was flanked with PiggyBac arms (BParms). Ubiquitously expressed PiggyBac transposase (PBase) was supplemented to the plasmid-targeting mixture. Expression of GFP was detected in numerous dI2 neurons with crossed-projections 12 days post electroporation, at E15 (Figure 1C).

In summary, the employment of spatially-restricted electroporation to one side of the spinal cord, coupled with the refinement of expression domains via enhancer intersection and PiggyBac-mediated transgene transposition, provides a rapid and reproducible means for mapping axonal trajectories of genetically- and topographically-defined neuronal populations.

Targeting at single neuron resolution

The robust expression of a reporter gene downstream from an IN-specific enhancer, yields a widespread expression in somata and axons (12,31) (Figure 2C and F; Supplementary Figure S1A). Further understanding of the connectivity of genetically-targeted spinal neurons requires a precise determination of the trajectories of single axons. We used two different approaches to obtain single-neuron single-axon resolution.

(i) Mutated Cre alleles: *In vitro* studies and experiments in yeasts demonstrated that substitution of amino acids in the active site of Cre-recombinases reduces the recombination activity of Cre (32). Three-point mutations that convert E168 to S, Q or V were tested for activation of Cre-dependent GFP in the chick spinal cord. The efficiency of E168S, E168V (Supplementary Figure S1B and C) and E168Q (Figure 2) Cre mutations, in labeling dI1 neurons, was examined. The mutated Cre forms were cloned downstream of the EdI1 enhancer element and electroporated along with a Cre-conditional GFP plasmid ($CAG::STOP^{LoxP}$-EGFP). The number of neurons in a cross-section was scored (Figure 2B). The use of mutated Cre resulted in labeling of 0–3 neurons in 90% of the sections. In many of the cross-sections, a single dI1 neuron and its axon were visualized (Figure 2D and E; Supplementary Figure S1B and C). In contrast, the wild-type Cre labeled more than three neurons in 55% of the sections (Figure 2B).
To assess the use of mutated CreQ for tracking axonal trajectories, EdI1::CreE176Q and EdI1::FLPo were simultaneously electroporated along with the reporter cassettes: Cre-dependent mCherry and FLPo-dependent GFP, respectively (Figure 2A, C and F). dI1 neurons reveal crossed and uncrossed projections and the majority of dI1 axons project rostrally (Figure 2C–F) (12). The trajectories of distinct dI1 neurons, labeled with the mutated CreQ, on the background of the entire dI1 population, labeled with FLPo, were analyzed in the cross-sections (Figure 2C) and 'open book' preparations of E6 spinal cords (Figure 2F). An EdI1::CreE176Q-based tracing of three thoracic neurons reveals rostral crossed projections of one, and caudal uncrossed projections of the other two neurons (Figure 2F").

(ii) Neuronal specific expression of Chickbow reporters: Brainbow is a powerful genetic tool for labeling cells with multiple colors by expressing a random combination of three/four fluorescent genes (33–35). Based on the observation that a random number of plas-

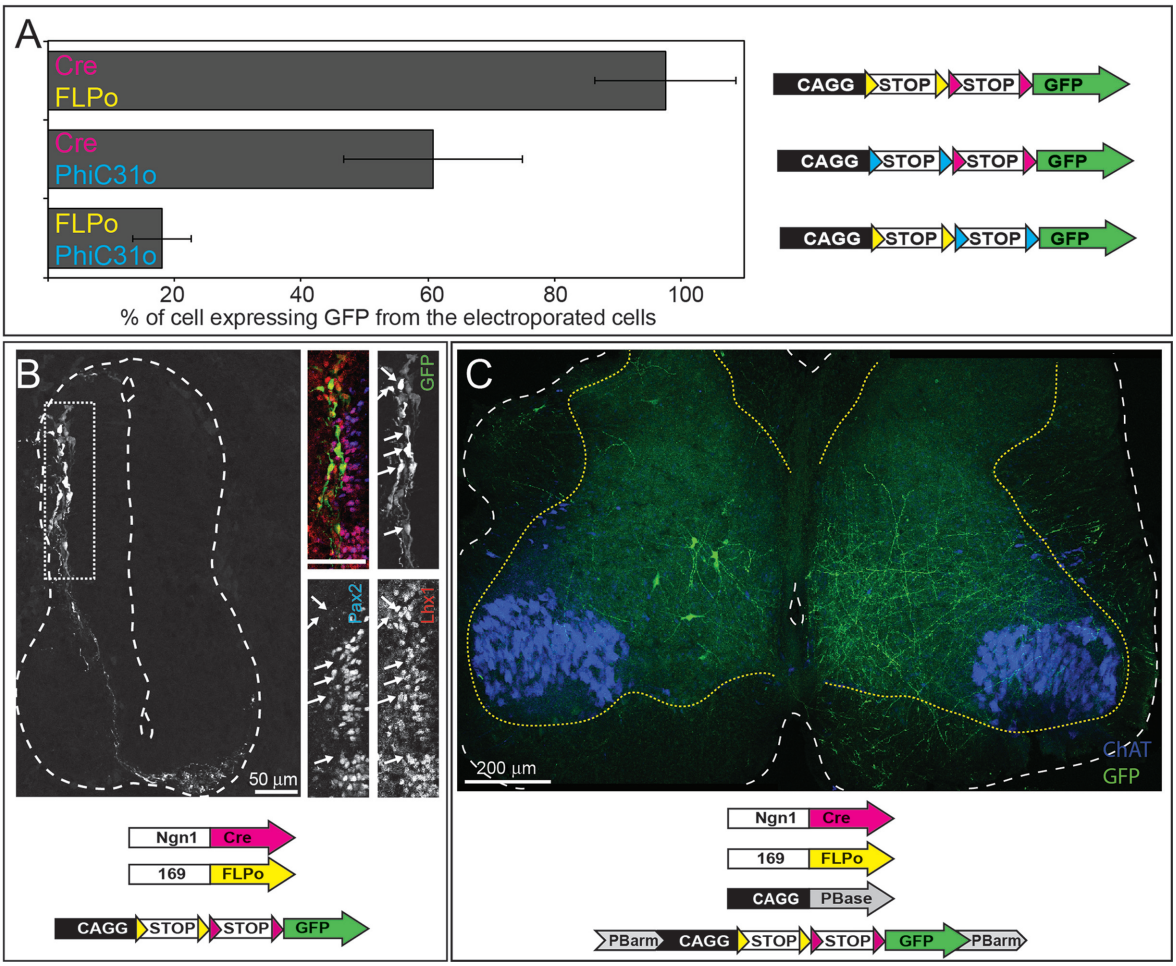

Figure 1. Genetic targeting of spinal neurons by enhancer element intersection. (**A**) The efficiency of removal of the double STOP cassette by two recombinases was tested by co-electroporation of two recombinases (Cre+FLPo, Cre+PhiC31o and FLPo + PhiC31o) along with the appropriate conditional target plasmid and a control ubiquitously expressed mCherry plasmid. The percentage of cells co-expressing GFP and mCherry from the total number of mCherry-expressing cells was scored. (**B**) For labeling dI2 neurons, FLPo under the control of FoxD3-169 enhancer and Cre under the Ngn1 enhancer were co-electroporated along with the FLPo-Cre double conditional GFP plasmid. The neuronal fate of reporter-expressing cells was validated at E5 by co-labeling with antibodies to Pax2 (dI4, dI6, V0 and V1) and Lhx1/5 (dI2, dI4, dI6, V0, V1) (right hand panels). Confocal image of a 20-µm cross-section thoracic level of E5 spinal cord. (**C**) For prolonged expression, the dual conditional cassette was inserted between PiggyBac arms, and the PiggyBac transposase (PBase) was added. Confocal image of a 100-µm cross-section through LS4 segment of E15 chick embryo demonstrating expression of GFP in commissurally projecting dI2 neurons.

mids are incorporated into the cells following electroporation of multiple (>4) plasmids (Supplementary Figure S2B), we modified the Brainbow strategy for multicolor labeling of chick spinal neurons. Three plasmids, with different Cre-dependent reporter genes—mGFP, mCherry and Taumyc (or myristylated myc—*m*-myc)—were electroporated into E3 chick hemi-neural tubes along with Cre-expressing plasmids (Figure 3A). Secondary antibodies conjugated to Cy2, Cy3 and Cy5, respectively, were used to intensify the fluorescence signal. Expression of Cre was targeted to dI1 by the EdI1 enhancer element (Figure 3A). The modified Brainbow method was sufficient to resolve the trajectories of a single caudally turning dI1 axon (Figure 3E, for further details see Figure 3E legend), and axonal bifurcation (Figure 3D, arrows) that could not be detected using monochromatic reporters. For multicolor labeling of dI2, we combined the enhancer-intersection and Chickbow methods (Figure 3B). Cell bodies and axons of dI2, labeled with color combinations are evidenced (Figure 3F).

To obtain stable and cell-specific expression of the Chickbow, we used the Piggybac system. The conditional mGFP, mCherry and Taumyc cassettes were flanked with PBarms and ubiquitously-expressed PBase was added to the Chickbow plasmids (Supplementary Figure S2A). Multicolored dI1 neurons can be seen in a spinal cross-section at E15 (Supplementary Figure S2C and D).

Thus, the modified Chickbow method was found to be a powerful tool to track trajectories of defined INs, and together with the mutated-Cre and the enhancer targeting, reveal the diversity of axonal trajectories within a genetically-defined neuronal population.

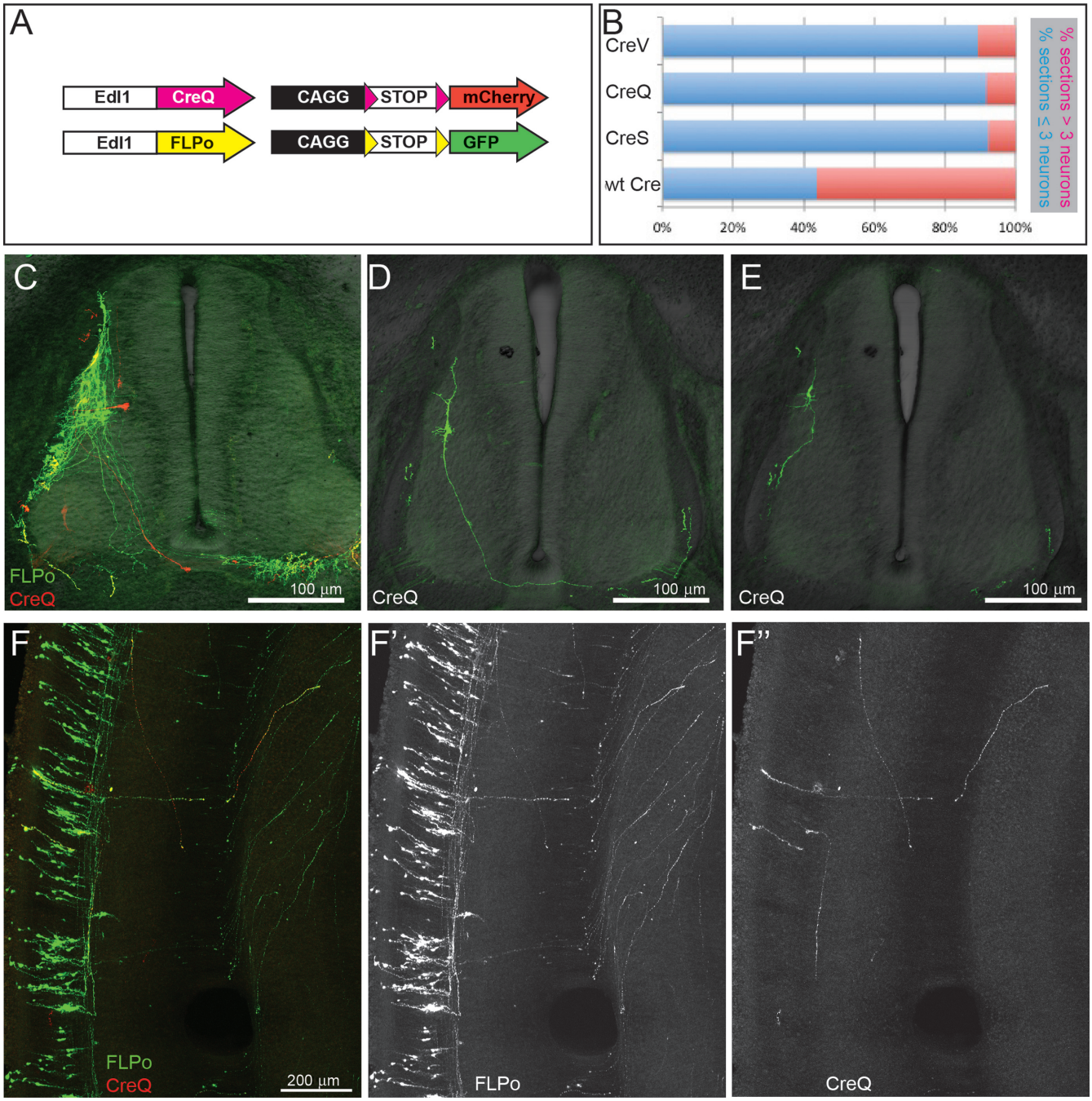

Figure 2. Employing mutated recombinase for single-axon resolution. (**A**) Schematic representation of the plasmids used for multiple (EdI1::FLPo) and single (EdI1::CreQ) labeling of dI1 neurons and axons. (**B**) Quantitative analyses showing the efficiency of the different Cre alleles. The number of dI1 neurons in 14-μm cross-sections was scored. The percentage of cross-sections harboring three or less neurons (blue) and more than three neurons (red) are presented. The numbers of cross-sections that were analyzed are: CreS—466, CreQ—160, CreV—326 and Cre—110. (**C**–**E**) Confocal image of 50-μm cross-sections of chick E6 lumbar spinal cord. Expression of GFP driven by EdI1::FLPo, yields a widespread expression in soma and axons. By contrast, expression of *m*Cherry (C) or GFP (D,E) driven by the mutated Cre isoform, EdI1::E168Q, yields far fewer reporter-positive soma/axons. Note, dI1 neurons that project their axons either commissurally (D) or ipsilaterally (E) are shown. (**F**) For assessing the use of the mutated Cre for tracking longitudinal trajectories of axons, EdI1::CreE176Q and EdI1::FLPo were simultaneously electroporated along with the corresponding reporter cassettes: Cre-dependent *m*Cherry and FLPo-dependent GFP, respectively (A). The trajectories of distinct dI1 neurons, labeled with the mutated Cre (**F″**), on the background of the entire dI1 population (**F′**), labeled with FLP, were analyzed in open book preparation of E6 spinal cord.

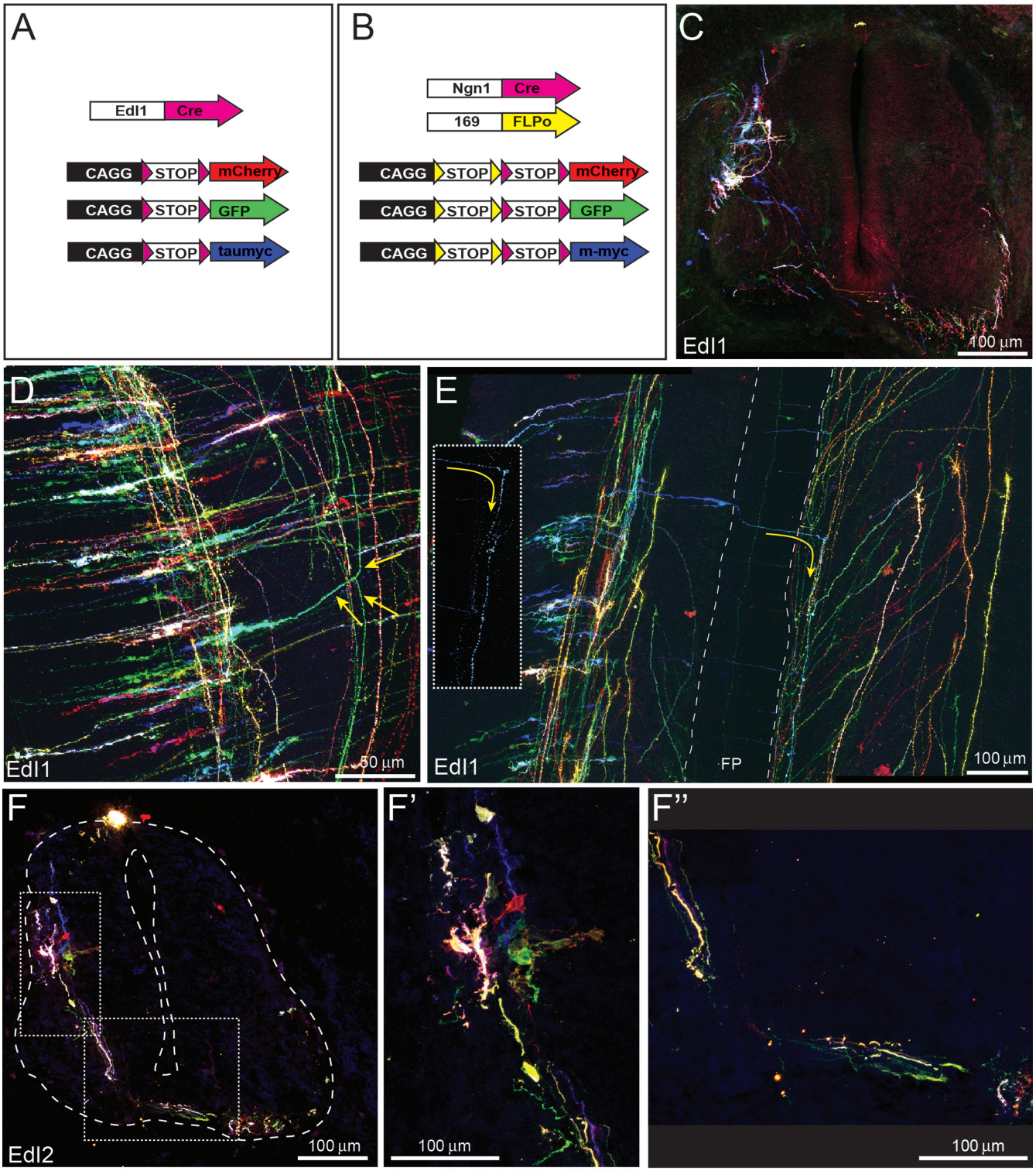

Figure 3. Neuronal specific expression of Chickbow reporters in the spinal cord. (**A**,**B**) Schematic representation of the plasmids used for labeling dI1 (A) and dI2 (B) neurons. *m*-myc = myristylated *myc*. (**C-E**) Chickbow labeling of dI1 neurons. Confocal images of a 20-µm cross-section through the lumbar region of E6 chick embryo spinal cord (**C**), and through open book preparations (**D**,**E**) of E6 spinal cord. Note that dI1 neurons and axons are labeled with multiple colors. The yellow arrows in (D) point to a dI1 neuron with a bifurcating axon, turning rostrally and caudally at the ipsilateral side. A single caudally projecting axon (E; light blue, denoted by a yellow arrow) can be clearly resolved (E, inset, pointed by a yellow arrow) and further enhanced, after extracting the light blue axons from the original ROI. The white broken lines in (E) demarcate the floor plate (FP) boundary. (**F**) Chickbow labeling of dI2 neurons. Confocal image of 20-µm cross-section of E6 lumbar spinal cord is demonstrated. Enlarged (20×) images of the boxed area in (F) are shown in (**F′**) (dI2 soma) and (**F″**) (dI2 axons at the FP). dI2 cell bodies and axons are labeled with multiple colors.

Table 1. Genetic targeting of spinal neurons by enhancer element intersection

	Atoh1	284	Ngn1	169	168	215	242	Ptf1a	Nato3	mTAG1	cTAG1
DRG					✓	✓				✓	✓
dI1	✓	✓	✓				✓			✓	
dI2			✓	✓	✓		✓				
dI3						✓	✓				
dI4								✓			
dI5											
dI6											
V0											
V1				✓	✓						
V2											
MN						✓				✓	✓
V3											
FP									✓		
Ref	(12,54)				(24)	(11)		(53)	(23)	(19)	

Summary of expression patterns of enhancer elements in the chick neural tube. Direct expression to defined spinal neuronal populations was attained using three approaches. First, we generated a library of enhancer elements by searching the VISTA enhancer browser (25–27) for central nervous system regulatory elements (11,12). Second, we obtained additional enhancer elements by screening evolutionally-conserved non-coding elements residing in the vicinity of IN-expressed genes (19,23). Third, we screened elements known from published data (28–30).

Identification of synaptic targets of spinal neurons

Mapping the synaptic connections between constituents of a given neuronal network is crucial for understanding its mechanism of action. Here we describe synaptic-specific reporter tools and retrograde *trans*-synaptic PRV viruses that together, could be used to label pre-motoneurons and their synaptic connections.

(i) Labeling with synaptic reporters: to label synapses, we used two chimeric proteins in which a synaptic protein is fused to GFP. Synaptobrevin-GFP (SynB-GFP) and synaptic vesicle protein 2, SV2-GFP (36,37) were cloned into a Cre-dependent PiggyBac target vector (3). Each plasmid was electroporated into E3 chick spinal cords along with ubiquitously-expressed PBase and Cre plasmids (Figure 4A, and Supplementary Figure S3). Embryos were harvested at E15 or E17. Cross-sections of the lumbar spinal cord were labeled with anti-choline acetyltransferase (ChAT, a known motoneuron marker) and anti-synaptotagmin (a synaptic marker). Figure 4B–E, and Supplementary Figure S4B, show synaptic boutons, co-expressing either SynB-GFP or SV2-GFP, and synaptotagmin on ChAT$^+$ cells at the ventrolateral spinal cord, contralateral to the injection, thus demonstrating putative synaptic contacts between commissural axons and motoneurons. Noticeably, all the large SV2-GFP$^+$ boutons contacting the Chat$^+$ motoneurons are synaptotagmin positive (Figure 4B–E). Small SV2-GFP$^+$/synptotagmin$^-$ dots, that do not contact motoneurons, are most likely SV2-GFP protein particles that are not assembled to vesicles.

(ii) Tracking pre-motor interneurons by PRV injected to limb muscles: *trans*-synaptically propagating neurotropic viruses are powerful mapping tools of neural pathways (38,39). The genetically-targetable retrograde and anterograde *trans*-synaptic herpes virus tracers (herpes simplex virus type 1 and PRV) are available to map the input and output of specific neuronal subpopulations (38,40–41). PRV-Bartha strain spreads mainly retrogradely through axons (42). PRV-Bartha has been used to trace neuronal circuits in hamster (43), rats (44) and mice (41,45). Here we used PRV-Bartha-encoded fluorescent proteins to trace pre-motoneurons in the chick lumbar spinal cord. PRV-

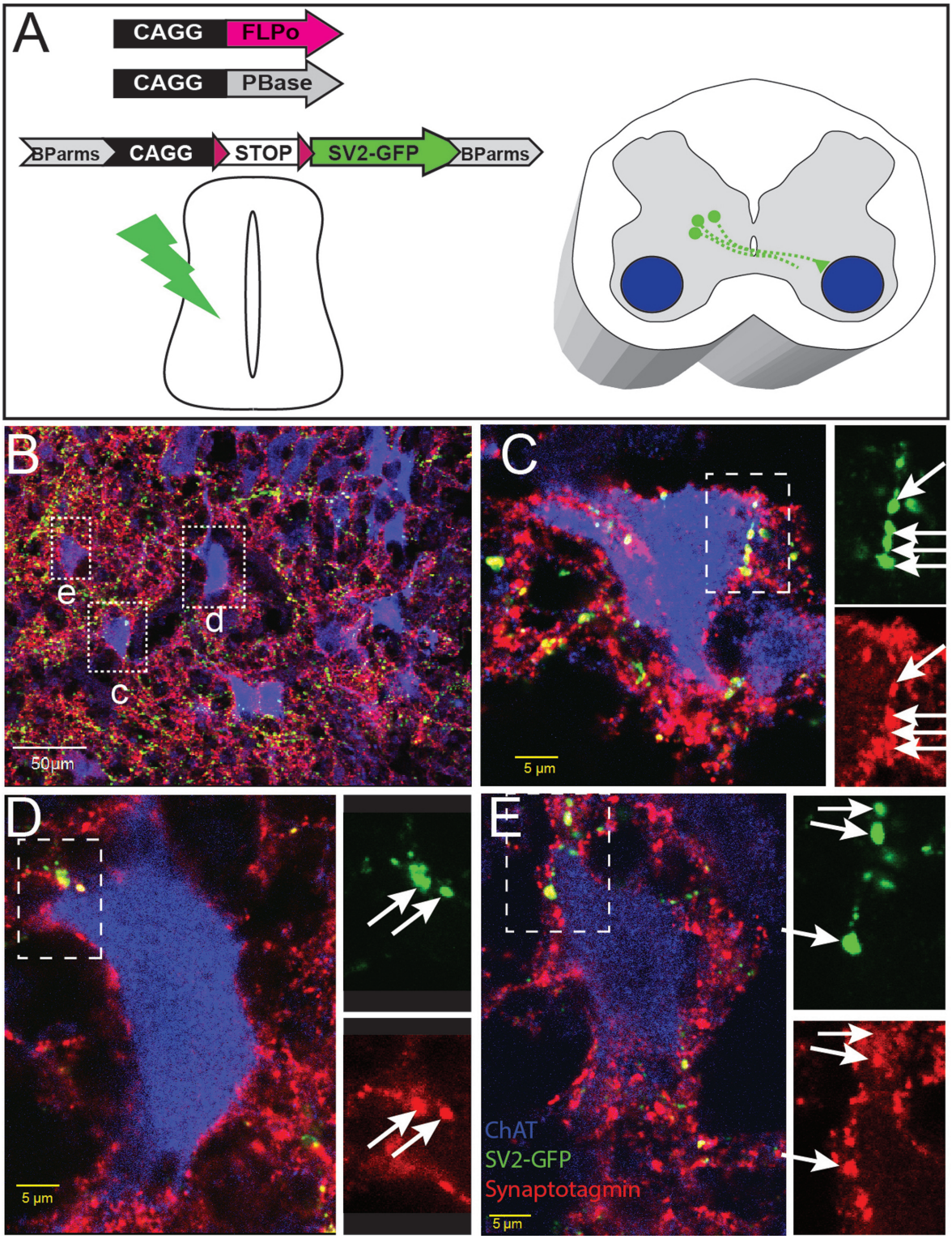

Figure 4. Using synaptic reporters to label synapses. (**A**) SV2-GFP was cloned into Cre-dependent PiggyBac target vector and was electroporated into E3 chick spinal cord along with ubiquitously-expressed PBase and Cre plasmids. (**B**–**E**) Confocal image of a 20-µm cross-section through LS6 segment in E17 chick embryo spinal cord, labeled to detect GFP (green), synaptotagmin (red) and ChAT (blue) (B). Optical, high-power (60×) sections of the boxed areas in (B) are presented as enlargements in (C–E). White arrows point to synaptic boutons co-expressing SV2-GFP and synaptotagmin.

152 (a GFP-encoding virus) was injected unilaterally into E13 chick shank muscles (Figure 5A). GFP-labeled spinal motoneurons were revealed 24–30 h post injection (Figure 5B and D, Supplementary Figure S4A). Ipsi- and contralaterally-labeled interneurons were detected along 10 lumbosacral spinal segments after 36 h (Figure 5C and E), and throughout the entire spinal segments after 48 h (Figure 5B, Supplementary Figure S4B–E). Similar kinetics of GFP-coupled PRV labeling was reported in the mouse spinal cord (46). Some of the early GFP-labeled interneurons co-expressed parvalbumin and calbindin (Supplementary Figure S4F and G), well known markers of first order motoneurons in rodents (46).

Conditional infectious or replicating viruses, as well as viruses harboring a conditionally-expressed reporter, are employed to restrict infection and progression of viral spread (42,47). For revealing the connectivity of genetically-defined pre-motoneuron, we combined enhancer-mediated targeting of spinal neurons with the use of a conditional reporter-expressing PRV strain. As proof-of-concept, we tested Cre-mediated activation of a reporter gene in commissural pre-motor neurons. Cre targeting to commissural neurons was achieved at E3, using unilateral electroporation of a plasmid mixture that enables FLPo-mediated activation of conditional Cre (Figure 5F). At E14, we injected PRV-263, a Brainbow PRV (42), into the limb muscles contralateral to the Cre-expressing side. The viral genome of PRV-263 carries a Brainbow cassette expressing a red reporter in infected cells that is excised in the presence of Cre and thereby allows expression of yellow or cyan reporters. Hence, it is expected that the RFP labeling in infected Cre-expressing commissural neurons, will be converted to either YFP or CFP. Figure 5G shows that the expression of RFP and YFP+Cyan (detected with anti-GFP antibody, referred as GFP variants) could be readily detected 35 h after viral injection. While RFP-expressing neurons can be visualized in both injected and contralateral sides, the expression of the GFP variants is restricted to contralateral neurons. Thus, the GFP variants expressing interneurons described above are suggested to be commissural pre-motoneurons.

Imaging the activity of spinal neurons targeted with genetically-encoded calcium sensors

The execution of motor tasks involves an integrated activity of diverse sets of neural circuits in the spinal cord. Understanding the contribution of identified spinal interneurons to specific behaviors is hampered by difficulties to characterize the properties and patterns of activity of the numerous neurons that make up the complex spinal neuronal networks in vertebrates. The ability to determine the activity patterns of genetically-defined neurons has recently been made possible by optical imaging of neurons targeted with the genetically-encoded calcium indicator GCaMP3 (48). Here we demonstrate the use of GCaMP3 to monitor the activity of targeted motoneurons in isolated chick spinal cord preparations together with recording from the respective flexor and extensor spinal nerves, during rhythmic activity produced by electrical stimulation of the sacral spinal segments.

The calcium indicator GCaMP3 was cloned into a Cre-dependent PiggyBac vector. Targeting to motoneurons was attained using electroporation of the conditional GCaMP3 plasmid (Figure 6A) into E3 chick neural tube along with ubiquitously-expressed PBase and Cre under the control of the chick motoneuron-specific enhancer of the *TAG1/Axonin1* gene (19) (Table 1, Figure 6B). Electroporated embryos were sacrificed at E12; the spinal cords were isolated and mounted in an *in vitro* chamber equipped for combined optical imaging of the targeted neurons and electrophysiological recording of the motor output. Fluorophores were excited at the appropriate wavelength, and images were acquired from the ventral or lateral surface of the cord using epifluorescence microscopy and a cooled 14bit CCD camera, before and during motor rhythm produced by electrical stimulation (Figure 6C). Figure 6C shows that the stimulation elicited an alternating flexor-extensor rhythm recorded from the satrorius and femorotibialis nerves, respectively, and that the simultaneous calcium imaging and electrophysiological recordings could be used to resolve the activity patterns of flexor and extensor motoneurons during the motor rhythm (Figure 6C and Supplementary Movie 1).

Photoactivation of spinal networks expressing channel rhodopsin2

Targeted expression of optogenetic tools can be employed to activate or inactivate neuronal populations and evaluate their contribution to specific motor tasks (49,50). Here we demonstrate that photoactivation of microbial opsins is capable of triggering an alternating flexor-extensor rhythm in the embryonic chick spinal cord. To activate spinal neurons, we used a light responsive sodium channel ChR2 fused to mCherry cloned into a PiggyBac vector under the control of the constitutively active CAG enhancer (Figure 7A). The plasmid was electroporated bilaterally to the neural tube of E3 chick embryo with ubiquitously-expressed PBase expressing plasmid. Embryos were sacrificed at E13 and their spinal cords isolated and mounted in an *in vitro* chamber equipped for electrophysiological recordings. Strong expression of ChR2-mCherry in INs was observed at both sides of the spinal cord (Figure 7B). TTL-triggered fiber-coupled LED was used for specific photoactivation of the isolated E13 spinal cords at 480 nm. The light stimulus produced an alternating flexor-extensor rhythm recorded from the sartorius and femorotibialis nerves, respectively (Figure 7C). Similar approach can be applied to other genetically-identified spinal INs.

DISCUSSION

In the present study, we demonstrate the use of a 'tool box' we designed for a rapid decoding the wiring, connectome, activity and function of genetically-targeted spinal interneurons in the embryonic chick spinal cord.

The ability to target specific subtypes of neurons relies on availability of cell-type-specific enhancer elements, or the ability to knock-in a transgene into the locus of

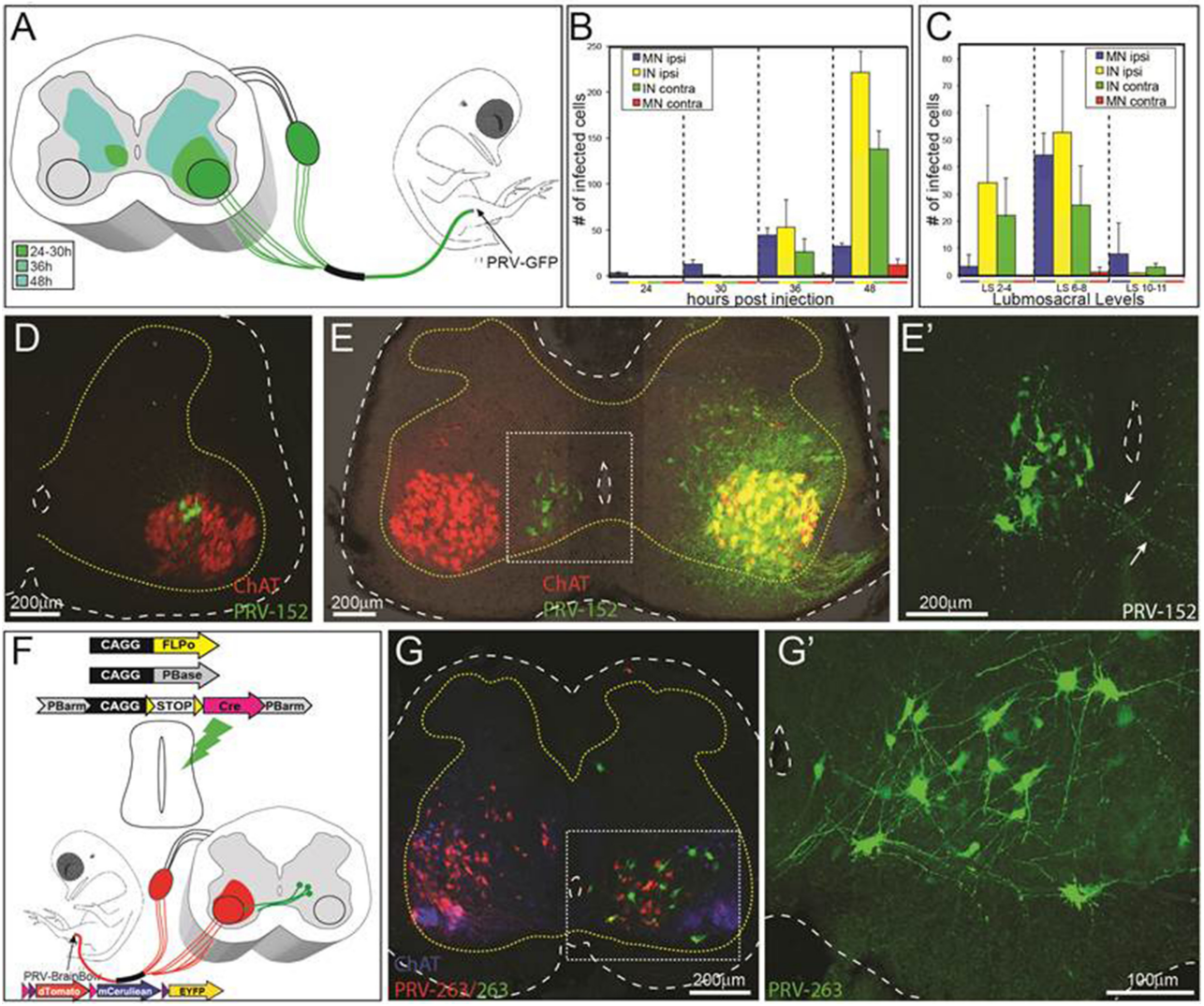

Figure 5. Tracking pre-motor interneurons by PRV injection into the limb muscles. (**A**) Schematic, showing injection of PRV-152 (GFP-encoding virus) into E13 shank muscles. (**B**) Quantification of the number of GFP-expressing motoneurons and interneurons in the injected and contralateral sides in lumbusacral levels 6–8, at 24, 30, 36 and 48 h post viral injection. (**C**) Quantification of the spatial distribution of infected motoneurons and interneurons, 36 h post injection, in the lumbosacral levels. (**D**–**E**) Confocal images of 100-µm cross-sections through LS6 segment in E14 chick embryo spinal cord, 30 (D) and 36 h (E) post viral infection. (**E′**) Enlarged (20×) confocal image of the boxed area in (E). White arrows are pointed at crossing commissural axons. (**F**) Schematic, showing a FLPo conditional Cre cassette, cloned into PiggyBac vector and electroporated into the chick spinal cord at E3 along with ubiquitously-expressed Cre and PBase plasmids. At E13, PRV-263 is injected into the shank muscles. (**G**) Confocal image of a 100-µm cross-section through LS2 segment in E14 chick embryo spinal cord, 36 h post infection with PRV-263 into the contralateral hindlimb shank muscles. dTomato-expressing ipsilateral motoneuron, ipsilateral INs and contralateral INs are visible. In addition, GFP variants expressing INs are evident on the contralateral side. The boxed area in (G) is enlarged (20×) in (**G′**).

a tissue-specific gene. However, in many cases specificity is determined by a repertoire of genes, rather than individual genes. Intersection between enhancers or modalities can restrict targeting to a specific cell population. We have previously demonstrated confinement of expression to the spinal INs: dI2 and dI3 via enhancer intersection of Cre and Gal4 drivers and UAS/STOP^{loxP} reporter (11,12). To extend and stabilize expression, we used two recombinases and a double removable-STOP signal. The joined use of enhancer intersection and chick hemitube electroporation poses a unique advantage over other model systems. A mixture of three to four plasmids can be electroporated into a specific rostrocaudal (R/C) level (lumbar, thoracic, brachial and hindbrain) and the axonal projections can be followed in both sides of the spinal cord. The laterality (ipsi- or contralateral) and longitudinal direction of the projection (rostral or caudal) can only be determined by spatially restricted expression of reporters. Similar neuronal-targeting task in mice requires crossing of three mouse lines. The spatial and cell-type restriction of expression to specific neurons, side (left/right) and R/C level of the spinal cord via germline targeting, is not feasible. *In utero* electroporation into the mouse E11.5–12.5 spinal cord of ubiquitously-expressed transgenes is being used for ectopic expression. However, the use of tissue-specific enhancers in mouse spinal cord electroporations for tracking

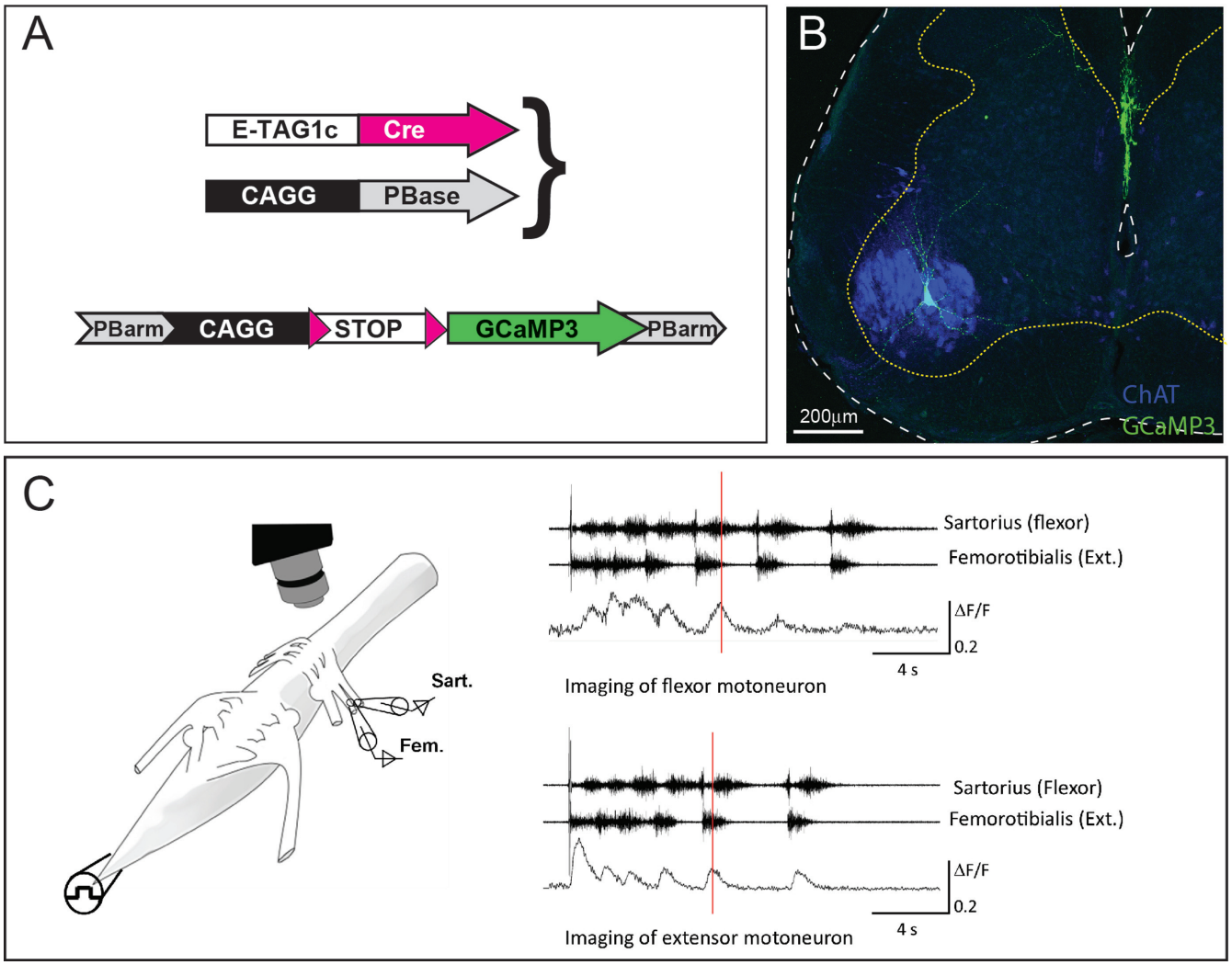

Figure 6. Imaging of flexor and extensor motoneurons targeted with GCaMP3 during rhythmic activity produced by electrical stimulation of the sacral segments. (**A**) GCaMP3 was cloned into a Cre-dependent PiggyBac vector. This plasmid was electroporated into E3 chick neural tube along with ubiquitously-expressed PBase and Cre under the control of the chick TAG1/Axonin1 that drives expression in motor neurons. The embryos were incubated for 9 days. At E12, the embryos were sacrificed and their spinal cords were isolated. (**B**) Confocal image of a 100-μm cross-section through the LS6 segment in E13 chick embryo spinal cord. The section is stained for GFP (green) and ChAT (blue). The GFP-expressing cell is localized (cyan) within ChAT-positive motoneuron pool at the ventrolateral aspect of the cord. (**C**) The isolated spinal cord preparation is illustrated mounted with ventral side up in an *in vitro* chamber. The most caudal part of the spinal cord is inserted into a stimulation suction electrode. Suction electrode recordings of the motor output are obtained from the sartorius and femorotibialis nerves and the activity of GCaMP3-targeted motoneurons is viewed using water immersion objective on an upright epifluorescent microscope equipped with a digital CCD. Combined calcium imaging of the activity of motoneurons targeted with GCaMP3 and the motor output produced by stimulation of the caudal segments of the spinal cord. Stimulation of the sacral part of the cord (3 pulse 10 Hz train at 50 μA) elicited an alternating flexor and extensor rhythm, recorded from the satrorius and femorotibialis nerves, respectively. The activity pattern of two different motoneurons targeted with GCaMP3 was imaged from the ventral surface of the LS1 segment (see methods) concurrently with the recorded motor output. The fluorescence changes (ΔF/F) of one of these neurons were in phase with the rhythmic bursting recorded from the sartorius nerve (imaging of flexor motoneuron), while the ΔF/F of the other motoneuron was in phase with femorotibialis bursting (imaging of extensor motoneuron).

axonal pathways has not been demonstrated. The availability of genetically and topographically targeting tools can be used for labeling simultaneously two neuronal populations (11,12), for studying the specific connectivity between two different neuronal populations that reside at the same R/C level, or at different R/C levels.

Genetic methods for labeling and manipulating single neurons, MARCM and MADM, have been introduced in fly and mouse, respectively (51). We present alternative single-cell targeting methods that are efficient, swift and economical: mutated Cre isoforms and cell-specific Chickbow. Mutated Cre isoforms can be used with any of the reporters we described: nuclear, axonal, synaptic, Chickbow and GCaMP- target plasmids and PiggyBac-transposase. In addition, single cell manipulations can be attained by co-electroporation of Cre-dependent ectopic genes, dominant-negative forms and RNA-knockdown cassettes. Hence, the mutated Cre alleles are part of a modulatory collection of plasmids that can be used for single or alternatively broad expression of a variety of target genes. Diverged biological questions can be addressed by using different set of plasmids from the existing collection. The modified Chickbow

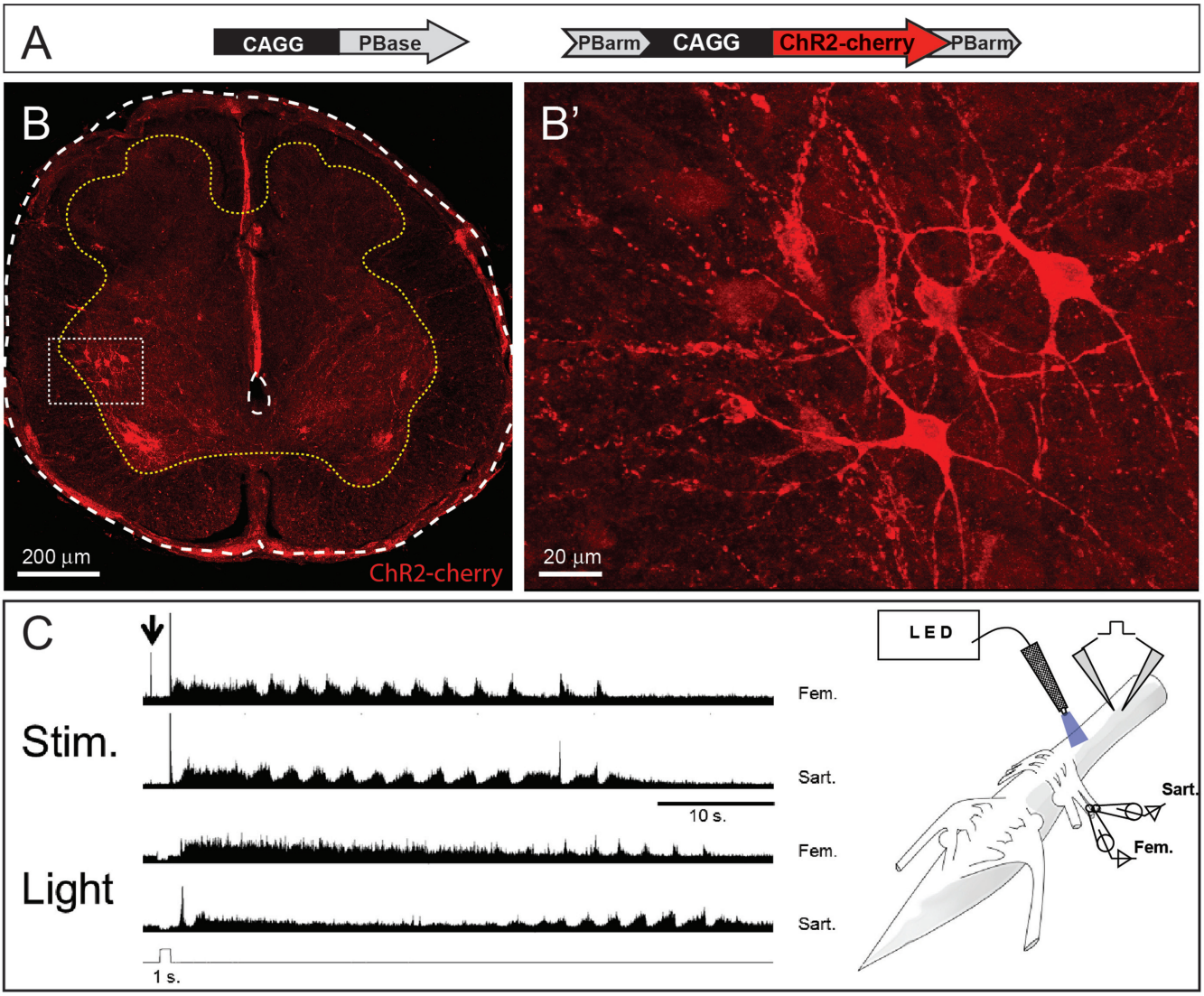

Figure 7. Stimulus and light induced alternating flexor-extensor bursting in isolated chick spinal cord expressing ChR2. (**A**) ChR2-mCherry was cloned to PiggyBac vector under the control of the constitutively active CAG enhancer. This plasmid was electroporated to E3 chick NT along with ubiquitously-expressed PBase expressing plasmid. Embryos were further incubated for 10 days. At E13, the embryos were sacrificed and their spinal cords were isolated. (**B**) Confocal micrograph of a 100-µm cross-section through the T7 spinal segment showing expression of ChR2-mCherry in spinal neurons. Sections were stained for mCherry using anti-RFP antibodies. (**C**) Flexor-extensor alternating rhythm produced by photoactivation of ChR2-expressing neurons in the isolated chick spinal cord. The rhythm produced by bipolar stimulation of the ventral funiculus and recorded from the sartorius and femorotibialis nerves (2 top records, stimulus), is superimposed with the rhythm produced by a 1-s, 480-nm light stimulation of the ventral surface of the spinal cord. The illustration on the right shows the isolated preparation, the bipolar stimulation electrodes and the light spot emitted through a fiber optic guide attached to a TTL-activated LED.

system we used is also modulatory. Additional colors can be added to the three reporters we used, just by adding extra plasmid that contains a Cre-dependent reporter. Double conditional Cre+FLPo reporters were used successfully for multicolor labeling of dI2 via enhancer intersection and double conditional reporters, demonstrating the validity of this method to numerous cell types.

Identification of the post- and presynaptic neurons of a given cell is crucial for mapping neuronal networks. We have used targeted expression of anterogradely-transported synaptic reporters and retrograde *trans*-synaptic PRV virus for labeling pre-motor INs. Neurotropic rabies and herpes viruses, which can propagate *trans*-synaptically, have been employed for mapping sensory-motor circuits in rodents (41,52–54). The mutual use of muscle-injected PRV coupled with targeting tools, as we currently demonstrate (Figure 5F), can facilitate the genetic characterization of the cellular component of sensorimotor circuits. We have demonstrated that targeted expression of Cre contralateral to the virus-injection limb, allows identification of pre-motor commissural neurons.

Genetic identification of neurons that contact spinal motoneurons (MNs), requires cell-type-specific markers. However, the expression of most of the cell-fate markers is confined to early stages of development. Targeting of Cre to specific neuronal type coupled with the use of PRV-

Brainbow, as we have demonstrated, is one option. An alternative approach is to target expression of reporter (GFP) to specific IN using the targeting tools (Table 1) and infect the limb with PRV expressing another reporter (RFP). Co-labeled INs at 34–36HPI, are likely to be genetically-defined pre-MNs.

Understanding of the mechanism of action of neural circuits in vertebrates and their relation to motor behaviors depends on identification of constituents of the network, the connectivity between them and on evaluation of their contribution to the studied behavior. The neuronal specific expression of genetically-encoded neural-activity sensors and modifiers, opens the way for characterization of the firing and pharmacological properties of the studied populations of neurons, the relation between the firing pattern and the motor output produced during different motor behaviors, and for clarification of the specific function of defined constituents of spinal circuits in the studied behavior. The reduced expression of reporters of neuronal activity in a genetically-identified spinal neuron in the en-bloc spinal cord preparation that preserves the entire spinal networks, enabled us to image the activity pattern of genetically-identified spinal neurons with single cell resolution, from the ventral, lateral and dorsal aspects of the spinal cord during motor activity produced by afferent stimulation, spontaneous motor rhythms and white matter pathways stimulation. Further studies of the circuitry and connectomes of networks involved in rhythmic motor actions will be carried out using the techniques we describe together with improved resolution and depth of recordings from the *in vitro* en-bloc preparation and in-ovo embryo using multiphoton microscopy imaging combined with electrophysiological recordings.

In summary, the use of a library of enhancers, enhancer intersection for delineating expression pattern, PiggyBac-mediated transgene transposition, wide collection of reporters, coupled with the ability to spatially and quantitatively restrict expression, provide an accurate and efficient experimental means for mapping neuronal trajectory of genetically-defined neurons. Targeted expression of synaptic reporters and directed injection of PRV virus can assist in mapping the connectomes of INs within the spinal cord. Finally, neuronal specific expression of genetically-encoded neural-activity sensors and neuronal activity modifiers can clarify the function of defined constituents of spinal circuits during known motor behaviors in the developing chick spinal cord.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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