ANESTHESIOLOGY

Synaptic Dynamics of the Feed-forward Inhibitory Circuitry Gating Mechanical Allodynia in Mice

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Mechanical allodynia characteristic of neuropathic pain is caused by neuroplastic changes in the spinal cord dorsal horn
- Aβ primary afferent nerve fibers provide the required nociceptive input for allodynia

What This Article Tells Us That Is New

- Using the clustered regularly interspaced short palindromic repeats/clustered regularly interspaced short palindromic repeats-associated nuclease 9 technique, a novel mouse strain was created allowing study of a key set of spinal interneurons
- The data suggest hyperexcitability of spinal protein kinase C γ expressing interneurons facilitates allodynia after nerve injury

ABSTRACT

Background: The authors' previous studies have found that spinal protein kinase C γ expressing neurons are involved in the feed-forward inhibitory circuit gating mechanical allodynia in the superficial dorsal horn. The authors hypothesize that nerve injury enhances the excitability of spinal protein kinase C γ expressing interneurons due to disinhibition of the feed-forward inhibitory circuit, and enables Aβ primary inputs to activate spinal protein kinase C γ expressing interneurons.

Methods: *Prkcg-P2A-tdTomato* mice were constructed using the clustered regularly interspaced short palindromic repeats and clustered regularly interspaced short palindromic repeats-associated nuclease 9 technology, and were used to analyze the electrophysiologic properties of spinal protein kinase C γ expressing neurons in both normal conditions and pathologic conditions induced by chronic constriction injury of the sciatic nerve. Patch-clamp whole cell recordings were used to identify the nature of the dynamic synaptic drive to protein kinase C γ expressing neurons.

Results: Aβ fiber stimulation evoked a biphasic synaptic response in 42% (31 of 73) of protein kinase C γ expressing neurons. The inhibitory components of the biphasic synaptic response were blocked by both strychnine and bicuculline in 57% (16 of 28) of neurons. Toll-like receptor 5 immunoreactive fibers made close contact with protein kinase C γ expressing neurons. After nerve injury, the percentage of neurons double-labeled for c-fos and *Prkcg-P2A-tdTomato* in animals walking on a rotarod was significantly higher than that in the nerve injury animals (4.1% *vs.* 9.9%, 22 of 539 *vs.* 54 of 548, $P < 0.001$). Aβ fiber stimulation evoked burst action potentials in 25.8% (8 of 31) of protein kinase C γ expressing neurons in control animals, while the proportion increased to 51.1% (23 of 45) in nerve injury animals ($P = 0.027$).

Conclusions: The *Prkcg-P2A-tdTomato* mice the authors constructed provide a useful tool for further analysis on how the spinal allodynia gate works. The current study indicated that nerve injury enhanced the excitability of spinal protein kinase C γ expressing interneurons due to disinhibition of the feed-forward inhibitory circuit, and enabled Aβ primary inputs to activate spinal protein kinase C γ expressing interneurons.

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Neuropathic pain, such as postherpetic neuralgia or trigeminal neuralgia, is considered to be a chronic disease, and is difficult to cure. Mechanical allodynia, also known as touch-evoked pain, refers to the pain caused by innocuous stimuli in pathologic conditions such as nerve injury. It is a typical symptom of neuropathic pain, and can be evoked by dressing and washing. The spinal dorsal horn is a primary center for the integration and sensitization of pain information. Changes of the synaptic plasticity of the neural circuits in the spinal dorsal horn, which are composed of excitatory and inhibitory interneurons and projection neurons, exert a significant role in the formation of allodynia.

Protein kinase C γ expressing neurons belong to interneurons of the spinal superficial dorsal horn, mainly distributed in the lamina IIi, which mainly accept inputs from low-threshold mechanoreceptive afferents. In an article published in *Science* in 1997, it was first proposed that deletion of the *PKCγ* gene significantly alleviated nerve injury–induced mechanical allodynia without affecting acute pain responses,

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indicating that protein kinase C γ may play an important role in the development of mechanical allodynia after nerve injury.[1] Our recent study indicated that spinal protein kinase C γ expressing interneurons receive a feed-forward inhibitory control that prevents the low-threshold mechanoreceptive information transmission to the nociceptive circuits in the spinal dorsal horn. Dysfunction of the feed-forward inhibitory circuit elicits mechanical allodynia.[2] Recently, more reports dissecting the spinal allodynia gate have supported the notion that spinal feed-forward inhibitory circuits play a crucial role in mediating innocuous information that goes through the nociceptive pathway to elicit allodynia.[3–11] Although these studies indicated that the spinal protein kinase C γ expressing interneuron is the centerpiece of the gate circuits, a dynamic or electrophysiologic analysis of the synaptic drive on protein kinase C γ expressing interneurons from primary afferent fibers and spinal inhibitory interneurons is largely absent from previous work, due to the lack of genetic tools to label this population of spinal neurons. In addition, the plasticity of the feed-forward inhibitory circuit controlling protein kinase C γ expressing interneurons in pathologic conditions has not been fully revealed. Therefore, the current study was aimed to analyze the synaptic dynamics between spinal protein kinase C γ expressing interneurons and inhibitory interneurons in both physiologic and pathologic conditions using a genetic labeled animal model.

In this study, we first developed *Prkcg-P2A-tdTomato* knock-in mice based on clustered regularly interspaced short palindromic repeats and clustered regularly interspaced short palindromic repeats–associated nuclease 9 technology, in which the protein kinase C γ expressing interneurons were genetically labeled by tdTomato fluorescent protein. Then, we analyzed the dynamic synaptic drive on protein kinase C γ expressing neurons by patch-clamp whole cell recordings in both physiologic and pathologic conditions using the genetically fluorescent-labeled mice.

Materials and Methods

In response to peer review, several experiments were added, including chronic constriction injury of the sciatic nerve and rotarod assay.

Animals

C57BL/6 mice and Kunming mice (mean ± SD; 25 ± 5 g, 6 to 8 weeks old) for constructing the *Prkcg-P2A-tdTomato* mice were provided from the Beijing Vital River Laboratory Animal Technologic Company (China). Male *Prkcg-P2A-tdTomato* mice were used for behavioral experiments (6 to 8 weeks old) and electrophysiologic experiments (3 to 5 weeks old). All mice were reared in a specific pathogen free environment with 12 h light/dark circle (light from 7:00 AM to 7:00 PM) and temperature maintained at 22 to 24°C. Animal use was carried out in accordance with the requirements of the Fourth Military Medical University Ethics Committee (Xian, China). All experimental procedures were approved by the Ethics Committee. *Prkcg-P2A-tdTomato* mice were randomly distributed to different groups according to the experimental strategy. Researchers performing the behavioral and electrophysiologic experiments were blind to the group assignment. During the experimental operation, efforts were made to use minimal numbers of animals. All animals were killed by transcardiac perfusion under deep anesthesia (pentobarbital sodium). All experiments were conducted between 8:00 AM to 6:00 PM. The primary outcome of the experiments was the specific synaptic responses recorded from protein kinase C γ expressing neurons.

Prkcg-P2A-tdTomato Mice Generation

The Prkcg-P2A-tdTomato mouse line was generated using the clustered regularly interspace short palindromic repeats and clustered regularly interspaced short palindromic repeats–associated nuclease 9 technology.[12,13] The clustered regularly interspaced short palindromic repeats/clustered regularly interspaced short palindromic repeats–associated nuclease 9 system provides immunity by integrating the fragments of invading phage and plasmid DNA into clustered regularly interspaced short palindromic repeats, and using the corresponding clustered regularly interspaced short palindromic repeats RNAs to guide the degradation of homologous sequences. Briefly, to derive the knock-in mice that tdTomato could be expressed under control of the Prkcg gene, we designed the small guide RNA containing the T7 promoter sequence targeting the site before the stop codon of Prkcg gene and constructed the targeting vector containing the P2A-tdTomato sequence. Clustered regularly interspaced short palindromic repeats–associated nuclease 9 messenger RNA, small guide RNA, and corresponding donor vector were injected into the fertilized eggs of C57BL/6 females. After injection, the live fertilized egg was transplanted to the pseudopregnant Kunming females. The mutant offspring were genotyped by Southern blot and polymerase chain reaction.

Design of Clustered Regularly Interspaced Short Palindromic Repeats Small Guide RNAs

According to the design, the P2A-tdTomato cassette was inserted before the stop codon of the *Prkcg* gene (Supplemental Digital Content, fig. S1A, http://links.lww.com/ALN/C266). Clustered regularly interspaced short palindromic repeats small guide RNA6, which is the highest small guide RNA, were screened for on-target activity using the UCA kit (Beijing Biocytogen, China).

Construction of Donor Plasmid

The targeting vector containing the P2A-tdTomato sequence was inserted before the stop codon of *Prkcg* gene; therein the tdTomato sequence was sided with 1.4 kb and 1.4 kb homolog arms. The targeting vector was constructed by using endotoxin-free plasmid DNA kit.

In Vitro Transcription

The T7 promoter sequence was separately put into the small guide RNA (small guide RNA target sequence: 5'-TGAGATTACATGACAGGCAC-3') and clustered regularly interspaced short palindromic repeats-associated nuclease 9 coding sequence upstream by the polymerase chain reaction method, produced by using the T7 *in vitro* transcription kit (Ambion, USA). Both were purified and eluted with the MEGA clear kit and ribonuclease-free water.

Microinjection

We chose C57BL/6 female mice as embryo donors and took Kunming mouse strains as pseudopregnant foster mothers. The mixed clustered regularly interspaced short palindromic repeats–associated nuclease 9 messenger RNA, small guide RNA, and donor vectors were injected into the fertilized eggs of C57BL/6 females. After injection, the live fertilized egg was transplanted to the pseudopregnant Kunming females, and the Founder 0 mouse was born. The Founder 1 generation was obtained by mating positive Founder 0 generation mice with wild-type mice.

Genotyping

Genomic DNA was extracted from the tails or toes of the 7-day-old mice (Founder 0 generation) or 3- to 5-week-old mice using the alkaline lysis method. To identify the genotypes of the offspring of the mice, we designed three pairs of primers (Takara Bio Inc., USA): the primers EGE-LC-035-5'Mut-F and EGE-LC-035-3'Mut-R were in the wild gene sequences, and the primers EGE-LC-035-5'Mut-R and EGE-LC-035-3'Mut-F were in the foreign gene sequences. EGE-LC-035-5'Mut-F/EGE-LC-035-3'Mut-R was mainly used to identify the existence of wild alleles. At the same time, it can also identify specific genotypes of mice, conditional on taking the polymerase chain reaction results of two pairs of primers (EGE-LC-035-5'Mut-F/EGE-LC-035-5'Mut-R and EGE-LC-035-3'Mut-F/EGE-LC-035-3'Mut-R) into account.

Southern Blot Analysis

According to the Southern blot design strategy, the extracted mice genomic DNA was digested by the EcoNI or NcoI (New England BioLabs, USA). They were then subject to electrophoresing in 1% agarose gel and transferred to a nylon membrane (Hybond N+; Amersham International plc, United Kingdom). The extracted mice genomic DNA was then hybridized overnight using a digoxigenin-labeled probe (Roche, USA) at 42°C.

Immunohistochemistry

Young adult male *Prkcg-P2A-tdTomato* mice (3 to 5 weeks old) were deeply anesthetized (intraperitoneal) by pentobarbital sodium followed by transcardiac perfusion of physiologic saline and 40 ml ice-cold 4% paraformaldehyde. After perfusion, the tissues were dehydrated with 20% and 30% sucrose at 4°C, respectively. Then the tissues, embedded in Tissue-Tek O. C. T. Compound (USA), were cut into 25-μm sections by Cryostat Microtome (Leica, Germany). First, these sections were washed three times in 0.1 × phosphate buffer saline at room temperature. Second, the sections were incubated in rabbit anti–protein kinase C γ antibody (diluted 1:800; Santa Cruz-211, USA) or mouse anti–Toll-like receptor 5 (1:100; Santa Cruz-517439, USA) monoclonal antibody for 12 to 18 h at 4°C, and then treated with secondary donkey anti-rabbit IgG conjugated with Alexa Fluor 488 (diluted 1:500, Molecular Probes-A21206, USA) or donkey anti-mouse IgG conjugated with Alexa Fluor 488 (1:500; Molecular Probes-A32766, USA) respectively for 2 to 3 h at room temperature. Finally, sections were mounted on glass slides after the same washing procedure, and were dehydrated and covered with antifluorescence quenching glass.

In accordance with our previous study,[2] after the electrophysiologic experiment was completed, the spinal slices were fixed with 4% paraformaldehyde overnight and gradiently dehydrated with 20% and 30% sucrose. They were washed three times with Tris-Triton (Tris-Base, Sigma-PH0713, USA; TritonX-100, Sigma-T9284, USA) buffer and blocked with 4% normal goat serum in Tris-Triton buffer for 1 h. They were then incubated in rabbit anti–protein kinase C γ (1:400; Santa Cruz-211, USA) or mouse anti–Toll-like receptor 5 (1:100; Santa Cruz-517439, USA) monoclonal antibody for 72 h at 4°C. On the fourth day, they were washed in Tris-Triton buffer, and were incubated for 24 h with donkey anti-rabbit IgG conjugated with Alexa Fluor 647 (1:500; Molecular Probes-A32795, USA) or donkey anti-mouse IgG conjugated with Alexa Fluor 647 (1:500; Molecular Probes-A32787, USA), respectively. Both were incubated with streptavidin conjugated with Fluorescein green (1:500; Vector Laboratories-SA-5001; USA). All images in the section were obtained by confocal microscopy (Olympus FV1200, Japan).

In Situ Hybridization

In situ hybridization associated procedures and probes (vesicular glutamate transporter 2, glutamic acid decarboxylase 67, and glycine transporter 2) have been described previously.[14,15] We obtained the fluorescent and *in situ* hybridization signals by a fluorescent microscope (Olympus FV1200, Japan). The Prkcg-tdTomato fluorescent signals of male mice were collected first, and then we obtained the *in situ* hybridization signals (for vesicular glutamate transporter 2, glutamic acid decarboxylase 67, and glycine transporter 2) with pseudo fluorescent color, which were merged onto the tdTomato images using Photoshop software (Adobe Systems Incorporated, USA).

Preparation of Lumbar Spinal Cord Slice with a Dorsal Root Attached

Parasagittal 400- to 500-μm-thick lumbar spinal cord slices with a dorsal root (5 to 10 mm long) attached were prepared

as described previously (Supplemental Digital Content, fig. S2A, http://links.lww.com/ALN/C267).[16] The suction electrode and recording electrodes were used to stimulate the dorsal root to evoke synaptic responses and to record cells in the slices. Briefly, *Prkcg-P2A*-td*Tomato* mice (male, 3 to 5 weeks old) were deeply narcotized with pentobarbital sodium, transcardially perfused with ice-cold sucrose artificial cerebrospinal fluid. Then the lumbosacral spinal cord with dorsal root was removed and placed on the agar-made table. Parasagittal 400- to 500-μm-thick spinal cord slices with dorsal root attached were cut by a vibrating microtome filled with ice-cold sucrose artificial cerebrospinal fluid. Then the spinal cord slices were placed in the normal artificial cerebrospinal fluid equilibrated with a mixture of O_2 and CO_2 (19:1) to recover at room temperature for 1 h.

Dorsal Root Simulation and Whole Cell Recordings

In line with our previous electrophysiology protocol, the resistance of patch pipettes filled with biocytin was maintained at 5 to 10 MΩ.[16,17] The tight whole cell recordings were made from protein kinase C γ expressing neurons and protein kinase C γ^- neurons, located in lamina IIi of parasagittal spinal cord slices and distinguished by the expression of fluorescent protein (Supplemental Digital Content, fig. S2, A and B, http://links.lww.com/ALN/C267). In membrane tests, the membrane resistance, membrane capacitance, series resistance, and leak current were recorded. Resting membrane potentials were measured at the I=0 mode. Rheobase, firing threshold, and amplitude were measured at I-Clamp mode. Rheobase was the current intensity of 40ms duration resulting in the first action potential. The action potential firing threshold was defined as the amplitude in one third of the slope of action potential. Action potential amplitude refers to the voltage difference between the starting and highest point of action potential.[18] The action potential firing pattern was determined by depolarizing pulses of 1-s duration. Neurons with series resistance of more than 20 MΩ were excluded from further analysis.

Primary afferent-evoked postsynaptic potential was evoked by electrical stimulation of the dorsal root. Conduction velocities of this synaptic transmission were calculated by the latency of the evoked reaction and the length of dorsal root. Judgment of whether responses were from Aβ, Aδ, or C fibers was made based on both response threshold and conduction velocities (Supplemental Digital Content, fig. S2, http://links.lww.com/ALN/C267). Data were acquired, digitized, and analyzed by the Axopatch 200B amplifiers (Axon Instruments, USA), the Digitizer 1440A, and pCLAMP10.7 software (Axon Instruments), respectively.

Determination of Morphological Characteristics

The morphology of spinal neurons was decided mainly by the lamina distribution, dendrite length, and orientation of the dorsal-ventral and rostral-caudal dendrites.[16,17] Briefly, islet cells confined to lamina II mainly extended their dendritic tree in the rostrocaudal direction, even reaching 600 μm, and had a limited extension in the dorso-ventral plane. Central cells also spread their dendrites in the rostro-caudal and the dorso-ventral direction. The length of their dendritic tree was less than those of islet cells (less than 200 μm). Vertical cells were predominantly located within lamina IIo and the laminae IIo/IIi border, extending dendrites mainly in dorso-ventral directions and showing a fan-like appearance. The radial cells exhibited their arbor in relatively more directions, with relatively shorter dendrite length. Their cell bodies were usually located within laminae IIi.

Imaging

In order to calculate the coincidence rate between tdTomato and protein kinase C γ, 1-μm-thick optical sections were acquired and photographed on an Olympus FV1200 confocal microscope in the z stack model. Confocal settings were consistent for scans of the same staining. Quantification of overlay was performed on 10 to 14 optical sections of three mice in ImageJ (National Institutes of Health, USA) using the Cell Counter Plugin, in which three fields were randomly selected in each section.

Chronic Constriction Injury of the Sciatic Nerve

Nerve injury models were completed by using a modified procedure of Bennett and Xie.[19] Briefly, the mouse was anesthetized by 3% isoflurane in oxygen, and after ensuring the state of anesthesia, the isoflurane was reduced to 1.5%. The left common sciatic nerve was exposed by pincette at mid-thigh level, proximal to its trifurcation. Five millimeters of the sciatic nerve was freed of adhering tissue by detacher. Then three ligatures of 5–0 suture were tied loosely around the sciatic nerve, 1 mm apart. After that, the incision was sutured layer by layer. Finally, the mouse was put into a warm box. The animals showed excessive licking and limping of ipsilateral hind paw. The mouse with motor dysfunction of the operative hind limb was excluded.

Rotarod Assay

To give mice nonnoxious stimulation and induce c-fos expression, the *Prkcg-P2A-tdTomato* mice were trained on the rotarod (ZS-YLS-4C; ZS Dichuang; China) at 30 rpm for 1 h before c-fos staining.[10] The mice were randomly divided into four groups: control group, control + rotarod group, nerve injury group, and nerve injury + rotarod group. The latter two groups received nerve injury. On the seventh day after nerve injury, mice from the four groups continuously ran for 1 h at 30 rpm on the rotarod. Then, all the mice were deeply anesthetized (intraperitoneal) by pentobarbital sodium followed by transcardiac perfusion of physiologic saline and 40 ml ice-cold 4% paraformaldehyde. According to the method mentioned in the Immunohistochemistry section, the immunostaining of c-fos (1:500; SYSY-226003; USA)

was performed. Finally, the percentage of neurons double-labeled for c-fos and Prkcg-P2A-Tdtomato was calculated.

Data Analysis

Continuous and categorical variables are expressed as mean ± SD and frequency (%), respectively. The normal distribution assumptions of continuous variables have been evaluated by P-P plots and Q-Q plots. Two animals did not survive during nerve injury operation and were excluded from the analysis. There were no missing data. No statistical power calculation was conducted, and the sample size was based on our previous experience with this design. Although multiple neurons were selected from the same animal, each neuron was assumed independent for analysis. For categorical variables, differences in proportions between two groups were tested with the chi-square test. *P* values less than 0.05 are considered significant. Outliers, if any, were not excluded from statistical analyses. Graph plotting was performed using Prism GraphPad7.0 software (GraphPad Software Inc., USA). Data analysis was conducted using SPSS22.0 software (IBM, USA).

Results

Prkcg-P2A-tdTomato Marks Almost All Protein Kinase C γ Expressing Neurons in the Spinal Dorsal Horn

We first identified the distribution of protein kinase C γ-tdTomato neurons in the spinal dorsal horn. As illustrated in

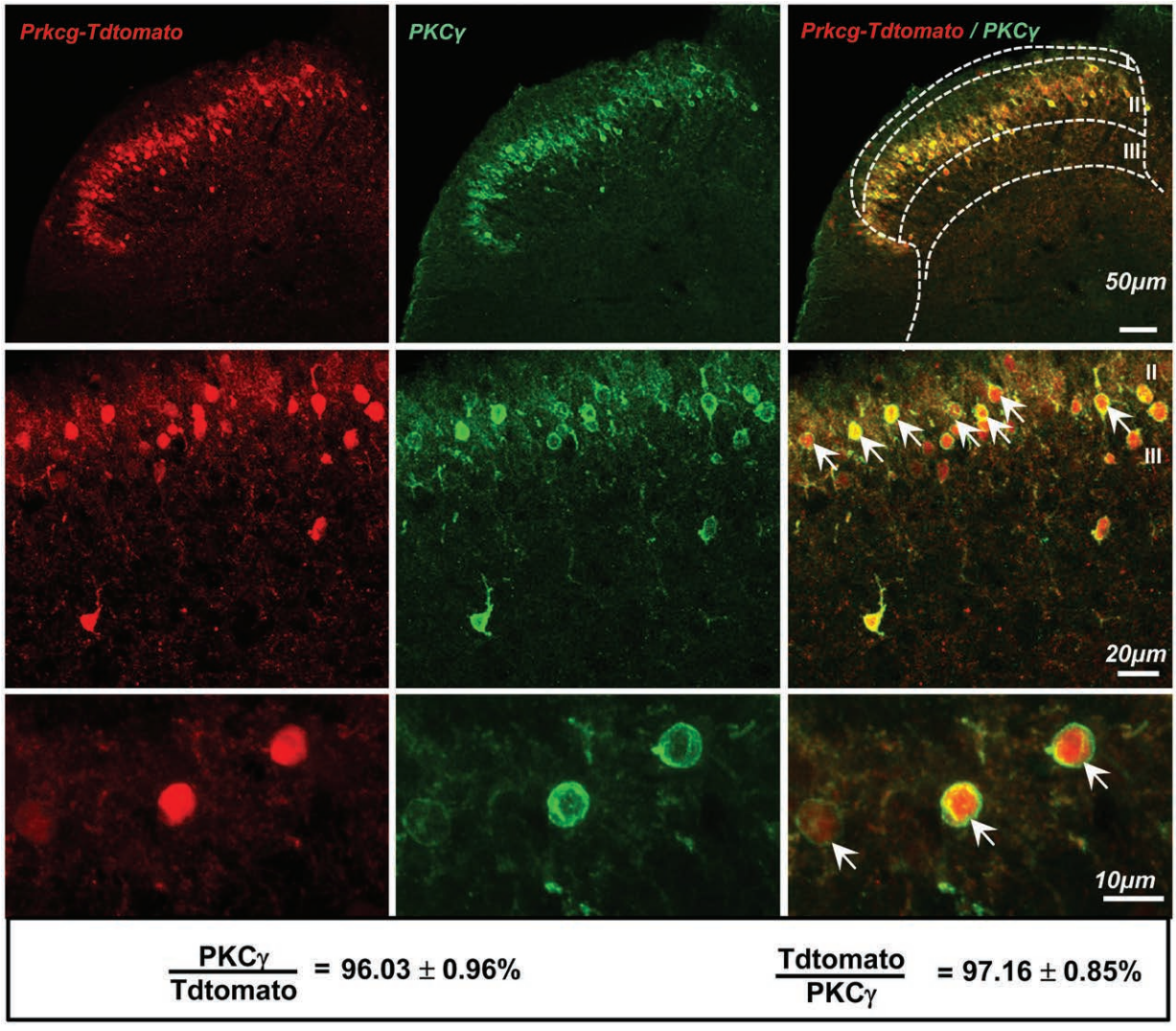

Fig. 1. *Prkcg-P2A-tdTomato* labeled most protein kinase C γ (PKCγ) expressing neurons in the spinal dorsal horn. Double staining of tdTomato with PKCγ antibody on transverse lumbar spinal cord slices of 3-week-old *Prkcg-P2A-tdTomato* mice. *Arrows* indicate the overlay neurons. n = 12 sections from three mice.

figure 1, the colocalization of protein kinase C γ-tdTomato and protein kinase C γ immunofluorescence was mainly observed in lamina IIi of the spinal dorsal horn. Scattered protein kinase C γ neurons were also expressed weakly in lamina I, lamina IIo, and lamine III to V. The distribution pattern of protein kinase C γ expressing neurons in the spinal dorsal horn corresponded to previous reports.[10,20] Double staining showed that 96 ± 0.9% (n = 12 sections from three mice) of tdTomato$^+$ neurons exhibited protein kinase C γ immunoreactivity, while 97.2 ± 0.9% (n = 12 sections from three mice) of protein kinase C γ immunostaining neurons expressed tdTomato (fig. 1), indicating that the *Prkcg-P2A-tdTomato* faithfully marked almost all protein kinase C γ expressing neurons in the spinal dorsal horn. Compared with the traditional gene knock-in technology, the clustered regularly interspaced short palindromic repeats/clustered regularly interspaced short palindromic repeats–associated nuclease 9 system has significant advantages.[12,13] Additionally, the fluorescence of tdTomato lasted for a long time (until several weeks after the electrophysiology experiment was finished). What is more noteworthy is that protein kinase C γ antibody and tdTomato achieved higher coexpression. Thus, the *Prkcg-P2A-tdTomato mice* we developed are suitable for electrophysiologic recordings.

We next conducted double *in situ* hybridization of protein kinase C γ-tdTomato with several excitatory and inhibitory neuron markers (Supplemental Digital Content, fig. S4, http://links.lww.com/ALN/C269). We discovered that 87.6 ± 6.8% (n = 13 sections from three mice) of protein kinase C γ expressing neurons expressed the vesicular glutamate transporter 2 (Supplemental Digital Content, fig. S4A, http://links.lww.com/ALN/C269), a marker of glutamatergic excitatory neurons.[8,21] In addition, 14.1 ± 6.2% (n = 14 sections from three mice) of protein kinase C γ expressing neurons expressed glutamic acid decarboxylase 1 (the γ-aminobutyric acid–mediated [GABAergic] inhibitory neuron marker; Supplemental Digital Content, fig. S4B, http://links.lww.com/ALN/C269), and 4.6 ± 0.3% (n = 10 sections from three mice) of protein kinase C γ expressing neurons expressed glycine transporter 2 (the glycinergic inhibitory neuron marker; Supplemental Digital Content, fig. S4C, http://links.lww.com/ALN/C269), indicating that protein kinase C γ expressing neurons in the lamina IIi of spinal dorsal horn are mainly excitatory interneurons, which is consistent with previous studies, and implies that these protein kinase C γ expressing neurons undertake the task of information transmission or information cascade amplification.

Aβ Afferent Driven Feed-forward Inhibitory Circuit Innervating Protein Kinase C γ Expressing Neurons

In order to address the type of inputs innervating protein kinase C γ expressing neurons, we patched neurons expressing tdTomato in parasagittal spinal slices from *Prkcg-P2A-tdTomato* mice (3 to 5 weeks old). Fluorescent light images of tdTomato-marked neurons from these slices are shown in Supplemental Digital Content, figure S2B (http://links.lww.com/ALN/C267). In the patch-clamp experiment, we recorded 73 PKCγ$^+$ neurons expressing tdTomato and 36 protein kinase C γ$^-$ neurons without fluorescent protein. In a separate experiment, the dorsal root compound action potential recordings were used to determine the intensity range for activation of Aβ, Aδ, and C fibers (Supplemental Digital Content, fig. S2, http://links.lww.com/ALN/C267). The electric strengths for activation of Aβ, Aδ, and C fibers in our setup were 0.1 to 0.3 V, 0.4 to 1 V, and 1.4 to 6 V, respectively. The corresponding conduction velocities for Aβ, Aδ, and C fibers were 3.3 to 4.5 m/s, 1.4 to 1.8 m/s, and 0.5 to 1.0 m/s.

As illustrated in Supplemental Digital Content, figure S2A (http://links.lww.com/ALN/C267), synaptic responses were evoked by dorsal root stimulation through a suction electrode in recorded protein kinase C γ expressing neurons. Recordings were made from 73 fluorescent protein kinase C γ expressing neurons located in lamina IIi under the I-clamp mode. In general, protein kinase C γ expressing neurons are more receptive to inhibitory afferents at resting membrane potentials. Aβ fiber stimulation evoked a biphasic synaptic response in 42% (31 of 73) of protein kinase C γ expressing neurons at resting membrane potentials (fig. 2B). The biphasic synaptic response was characterized as an evoked monosynaptic excitatory postsynaptic potential followed by a polysynaptic inhibitory postsynaptic potential, because the evoked monosynaptic excitatory postsynaptic potentials disappeared at holding potential = 0 mV, while the polysynaptic inhibitory postsynaptic potential disappeared at holding potential = 70 mV (fig. 2B). The excitatory inputs are from primary Aβ fibers, while the inhibitory inputs are from inhibitory interneurons, which received primary Aβ fiber input (fig. 2A). These results are consistent with our previous findings in rats.[2] The Aβ fiber-mediated feed-forward inhibitory circuit to protein kinase C γ expressing neurons acts as a gate that prevents Aβ input from activating protein kinase C γ expressing neurons. This gate response might be a useful model for analysis of the mechanisms underlying mechanical allodynia.[2]

Further, 1% of protein kinase C γ expressing neurons (1 of 73) directly generated action potential without polysynaptic inhibitory postsynaptic potential with dorsal root stimulation at Aβ strength (Supplemental Digital Content, fig. S5, Aa and D, http://links.lww.com/ALN/C270). When increasing the intensity of stimulation, 7% (5 of 73) of protein kinase C γ expressing neurons exhibited evoked inhibitory postsynaptic potential-excitatory postsynaptic potential, inhibitory postsynaptic potential, and excitatory postsynaptic potential with action potentials mediated by Aδ fibers (Supplemental Digital Content, fig. S5, Ab–d and D, http://links.lww.com/ALN/C270). We also found that a few protein kinase C γ expressing neurons received collaborative inputs from A and C fibers (4%, 3 of 73) and C

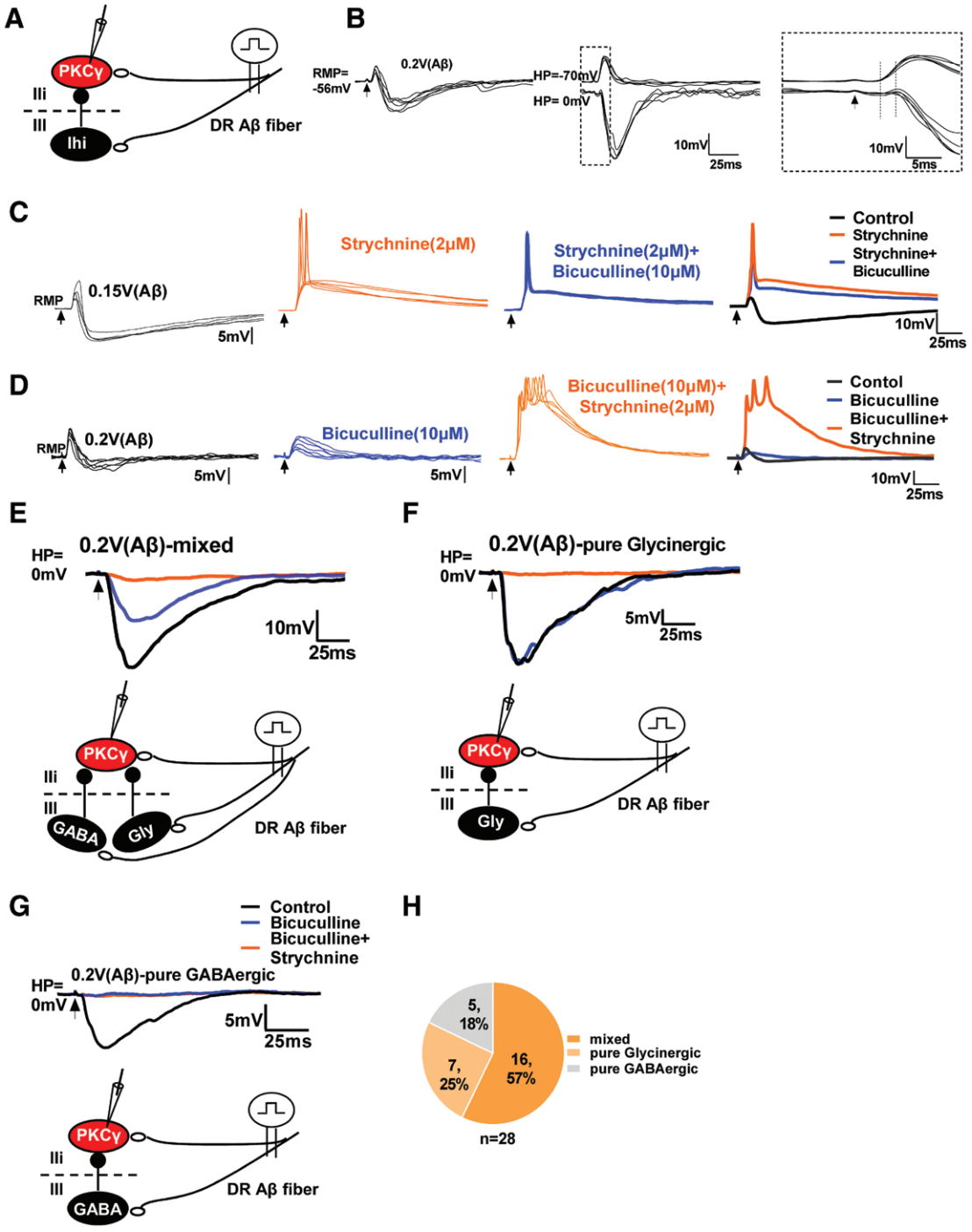

Fig. 2. Aβ-mediated biphasic eEPSP-eIPSP evoked in PKCγ-tdTomato neurons. (*A*) Schematic showing PKCγ-tdTomato$^+$ neurons in lamina IIi that receive Aβ input and feed-forward inhibition driven by Aβ input. (*B*) A biphasic synaptic response was evoked by dorsal root stimulation at Aβ intensity. The inset shows the latency gap between the eEPSP and eIPSP when the membrane potential was held at -70 mV or 0 mV. (*C*) Strychnine blocks the dorsal root evoked IPSP and generates APs in PKCγ$^+$ neurons at resting membrane potentials. Successive application of bicuculline does not change the amplitude of AP. (*D*) Application of bicuculline blocks eIPSP and strychnine generates long-lasting APs in PKCγ$^+$ neurons at resting membrane potentials. (*E*) An example of a PKCγ-tdTomato neuron receiving mixed GABAergic and glycinergic inputs. (*F*) An example of a PKCγ-tdTomato neuron receiving pure glycinergic input. (*G*) An example of a PKCγ-tdTomato neuron receiving pure GABAergic input. (*H*) Pie chart analysis of numbers of PKCγ$^+$ neurons receiving mixed afferent inputs, pure glycinergic, and pure GABAergic afferent inputs. AP, action potential; eEPSP, evoked monosynaptic excitatory postsynaptic potential; eIPSP, polysynaptic inhibitory postsynaptic potential; GABA, γ-aminobutyric acid; GABAergic, γ-aminobutyric acid–mediated; HP, holding potential; PKCγ, protein kinase C γ; PKCγ$^+$, protein kinase C γ expressing.

fibers alone (8%, 6 of 73; Supplemental Digital Content, fig. S5, Ba–d, C, and D, http://links.lww.com/ALN/C270).

For the purpose of comparison, we also recorded 36 protein kinase C γ⁻ neurons around protein kinase C γ expressing neurons in lamina IIi to study the primary afferent fibers that drove them. In general, the protein kinase C γ⁻ neurons in lamina IIi mainly accepted excitatory inputs (Supplemental Digital Content, fig. S6, http://links.lww.com/ALN/C271). The protein kinase C γ⁻ neurons were mainly innervated by Aδ fibers (48%, 17 of 36), which generated excitatory postsynaptic potential and excitatory postsynaptic potential-inhibitory postsynaptic potential synaptic responses (Supplemental Digital Content, fig. S6, C, D, and J, http://links.lww.com/ALN/C271). Aβ-fiber stimulation induced excitatory postsynaptic potentials and excitatory postsynaptic potential-inhibitory postsynaptic potential synaptic responses in 14% (5 of 36) and 8% (3 of 36) of recorded protein kinase C γ⁻ neurons, respectively (Supplemental Digital Content, fig. S6, A and B, http://links.lww.com/ALN/C271). Moreover, a few protein kinase C γ⁻ neurons received joint input of A and C fibers or C-fiber–mediated synaptic inputs only (Supplemental Digital Content, fig. S6E–H, http://links.lww.com/ALN/C271).

These results indicated that protein kinase C γ expressing neurons are different from protein kinase C γ- neurons in terms of synaptic inputs: Protein kinase C γ expressing neurons mainly received the Aβ-fiber mediated inhibitory inputs, while protein kinase C γ- neurons in the lamina IIi mainly accepted the excitatory inputs from Aδ fibers.

Toll-like Receptor 5–Positive Aβ Afferent Innervating Protein Kinase C γ Expressing Neurons

Recent studies show that Toll-like receptor 5 is coexpressed with neurofilament-200 in Aβ fibers in the dorsal horn.[11] It is important to know if protein kinase C γ expressing neurons also receive Toll-like receptor 5 Aβ inputs. Neurofilament protein 200 is the definite marker of Aβ- low-threshold mechanoreceptors, and neurofilament protein 200 and Toll-like receptor 5 are almost completely colocalized in the spinal dorsal horn.[11,22] In response to peer review, we performed immunochemical staining to explore the connections between Toll-like receptor 5–positive fibers and protein kinase C γ expressing neurons. Illustrated in figure 3A, Toll-like receptor 5–positive fibers were distributed widely in the spinal cord dorsal horn and overlapped with genetically labeled protein kinase C γ expressing neurons. Toll-like receptor 5–positive fibers made close contact with the cell body (fig. 3B) and dendrites (fig. 3C) of protein kinase C γ expressing neurons labeled by biocytin. These results suggested that Toll-like receptor 5–positive Aβ afferent directly innervates protein kinase C γ expressing neurons.

Both Glycinergic and GABAergic Interneurons Contribute to the Feed-forward Inhibition of Protein Kinase C γ Expressing Neurons with Predominant Glycinergic Contribution

In order to further identify the inhibitory interneurons making feed-forward inhibition on protein kinase C γ expressing neurons, the polysynaptic inhibitory postsynaptic potentials recorded from protein kinase C γ expressing neurons were challenged by application of strychnine (2 μM) and/or bicuculline (10 μM) to block the glycinergic and/or GABAergic transmission (fig. 2, C and D). In seven recordings at resting membrane potentials from protein kinase C γ expressing neurons showing biphasic synaptic responses, application of strychnine completely reversed the inhibitory postsynaptic potentials and generated action potentials. Subsequent application of bicuculline hardly affected the remaining excitatory postsynaptic potentials and action potentials (fig. 2C). Conversely, when bicuculline was applied first to block the GABAergic transmission in another five recordings at resting membrane potentials, the inhibitory postsynaptic potentials were partially reversed, but did not recruit action potentials. Subsequent application of strychnine further reversed the inhibitory postsynaptic potentials and recruited action potentials (fig. 2D). These results suggest that both glycinergic and GABAergic interneurons contributed to the feed-forward inhibition of protein kinase C γ expressing neurons with predominant glycinergic contribution.

Additionally, we found that another 37% (27 of 73) of protein kinase C γ expressing neurons merely received Aβ-fiber–mediated polysynaptic inhibitory postsynaptic potential inputs (fig. 4). Equally, both glycinergic and GABAergic transmissions constituted the inhibitory component. In order to dissect the pure inhibitory component, the holding potentials were set at 0 mV to exclude the influence of excitatory postsynaptic potentials. During the steady recording period, bicuculline and strychnine were applied consecutively. We found that feed-forward inhibition of 57% (16 of 28) of protein kinase C γ expressing neurons was sensitive to both strychnine and bicuculline, while 25% (7 of 28) of protein kinase C γ expressing neurons only had the strychnine-sensitive component, and 18% (5 of 28) of protein kinase C γ expressing neurons only had the bicuculline-sensitive component (fig. 2E–H).

Spinal Protein Kinase C γ Expressing Neurons Possess Diverse Morphological Features

After electrophysiologic recordings, 56 protein kinase C γ expressing neurons (figs. 5 and 6) and 12 protein kinase C γ⁻ neurons were successfully labeled by injecting biocytin into the cells. The neurons marked by biocytin were stained with protein kinase C γ antibody to ascertain that the neurons we patched were really protein kinase C γ expressing neurons, as shown in figure 5. The protein kinase C

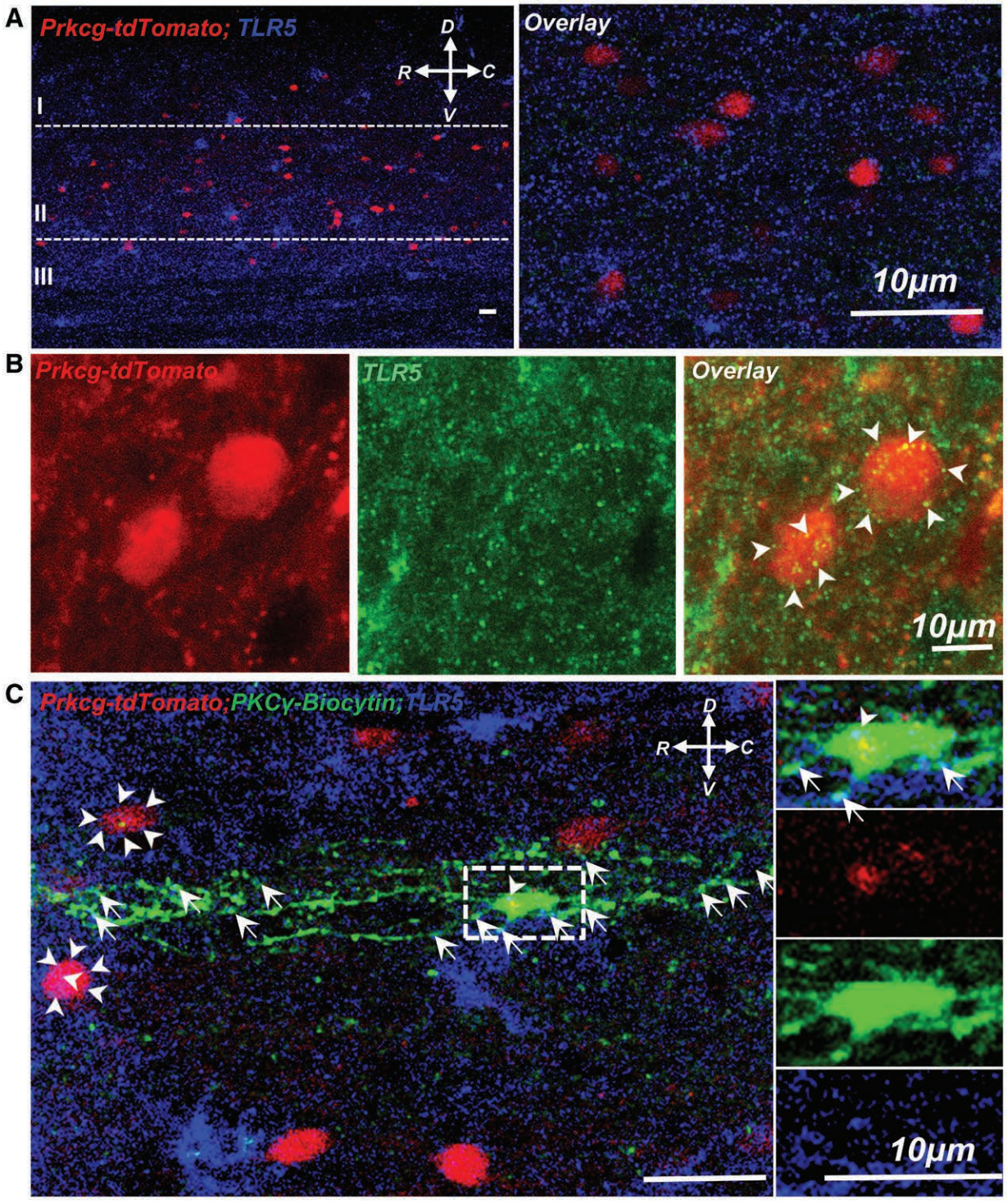

Fig. 3. Toll-like receptor 5 (TLR5)–positive Aβ afferent innervating protein kinase C γ expressing (PKCγ$^+$) neurons. (*A*) Double staining of tdTomato with TLR5 antibody on sagittal lumbar spinal cord slices of 3-week-old *Prkcg-P2A-tdTomato* mice. (*B*) TLR5-positive fibers made close contact with the cell body of genetically labeled PKCγ$^+$ neurons. (*C*) TLR5-positive fibers made close contact with the cell body and dendrites of PKCγ$^+$ neurons labeled by intracellular biocytin. *Arrowheads* indicate the contact points on the cell body. *Arrows* indicate the contact points on the dendrites.

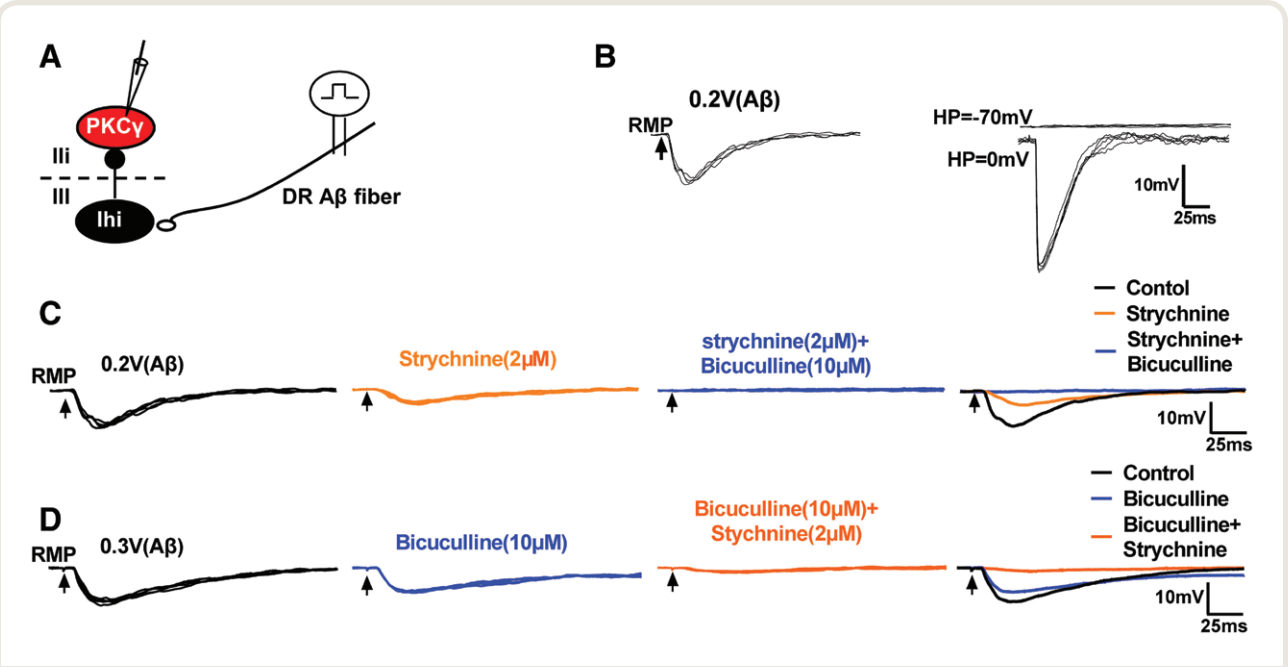

Fig. 4. Aβ-mediated eIPSP evoked in PKCγ-tdTomato neurons. (*A*) Schematic showing PKCγ$^+$ neurons in lamina IIi that only receive the feed-forward inhibition. (*B*) Dorsal root (DR) stimulation at Aβ intensity evoked unitary IPSP synaptic response. Amplitude of IPSP was increased or disappeared when the holding potential was held at 0 mV or -70 mV. (*C*) Strychnine and bicuculline together blocked the DR-evoked IPSP at resting membrane potentials. (*D*) Application of bicuculline followed by strychnine blocked the DR-evoked IPSP at resting membrane potentials. eIPSP, polysynaptic inhibitory postsynaptic potential; IPSP, inhibitory postsynaptic potential; PKCγ, protein kinase C γ; PKCγ$^+$, protein kinase C γ expressing; RMP, resting membrane potential.

γ expressing neurons were classified mainly based on the length and orientation of the dorsal-ventral and rostral-caudal dendrites. In agreement with classic morphology classification schemes,[23–26] we found that central cells were the dominant morphologic type of protein kinase C γ expressing neurons, accounting for 48% (27 of 56) and displaying an average spread in the rostro-caudal direction of 324.4 ± 78.8 μm and a dorso-ventral spread of 92.8 ± 39.8 μm (fig. 5A–D, and fig. 6, A and F). Additionally, 13% (7 of 56) of protein kinase C γ expressing neurons were classified as islet cells, mainly extended their dendritic tree in the rostrocaudal direction (582.1 ± 84.1 μm), and had a limited extension in the dorsoventral plane (103.6 ± 41.3 μm, fig. 6, D and F). Previous studies have proposed that 4.8% of protein kinase C γ expressing neurons are islet cells. However, it has been considered that islet cells belong to inhibitory neurons,[3,27] suggesting that although most protein kinase C γ expressing neurons are excitatory interneurons, the possibility of inhibitory neurons cannot be ruled out,[28] which is consistent with the results of *in situ* hybridization. Although it has been reported that vertical cells are predominantly located within lamina IIo and the laminae IIo/IIi border, we found that 10 of 56 protein kinase C γ expressing neurons exhibited vertical morphology in our study, extending dendrites mainly in dorsoventral directions and showing a fan-like appearance (fig. 6, B and F). Additionally, 9 of 56 protein kinase C γ expressing neurons exhibited their arbor in relatively more directions, and were defined as radial neurons (fig. 6, C and F). Moreover, we could not identify 3 of 56 protein kinase C γ expressing neurons, which were defined as unclassified type (fig. 6, E and F). The specific proportion of protein kinase C γ- neurons was not counted because of the small sample size. However, protein kinase C γ- neurons also had central, islet, vertical, radial, and unclassified morphology types (data not shown). Consequently, we cannot differentiate protein kinase C γ expressing and protein kinase C γ- neurons simply on the basis of morphologic classification.

Spinal Protein Kinase C γ Expressing Neurons Have Diverse Electrophysiologic Properties

The firing patterns of protein kinase C γ expressing neurons were divided into three categories, determined by 1-s depolarizing current injections at rheobase: irregular firing, phasic firing, and tonic firing (fig. 7A). Phasic-firing neurons showed two or more bursts of action potentials separated by silent periods of at least 100 ms throughout current injection.[29] Irregular-firing neurons manifested repeated action potentials occurring at irregular intervals.[30] Tonic-firing neurons showed regular firing during the current injection and even manifested adaptation.[31] In total, 42% (31 of 73) of the recorded protein kinase C γ expressing neurons showed

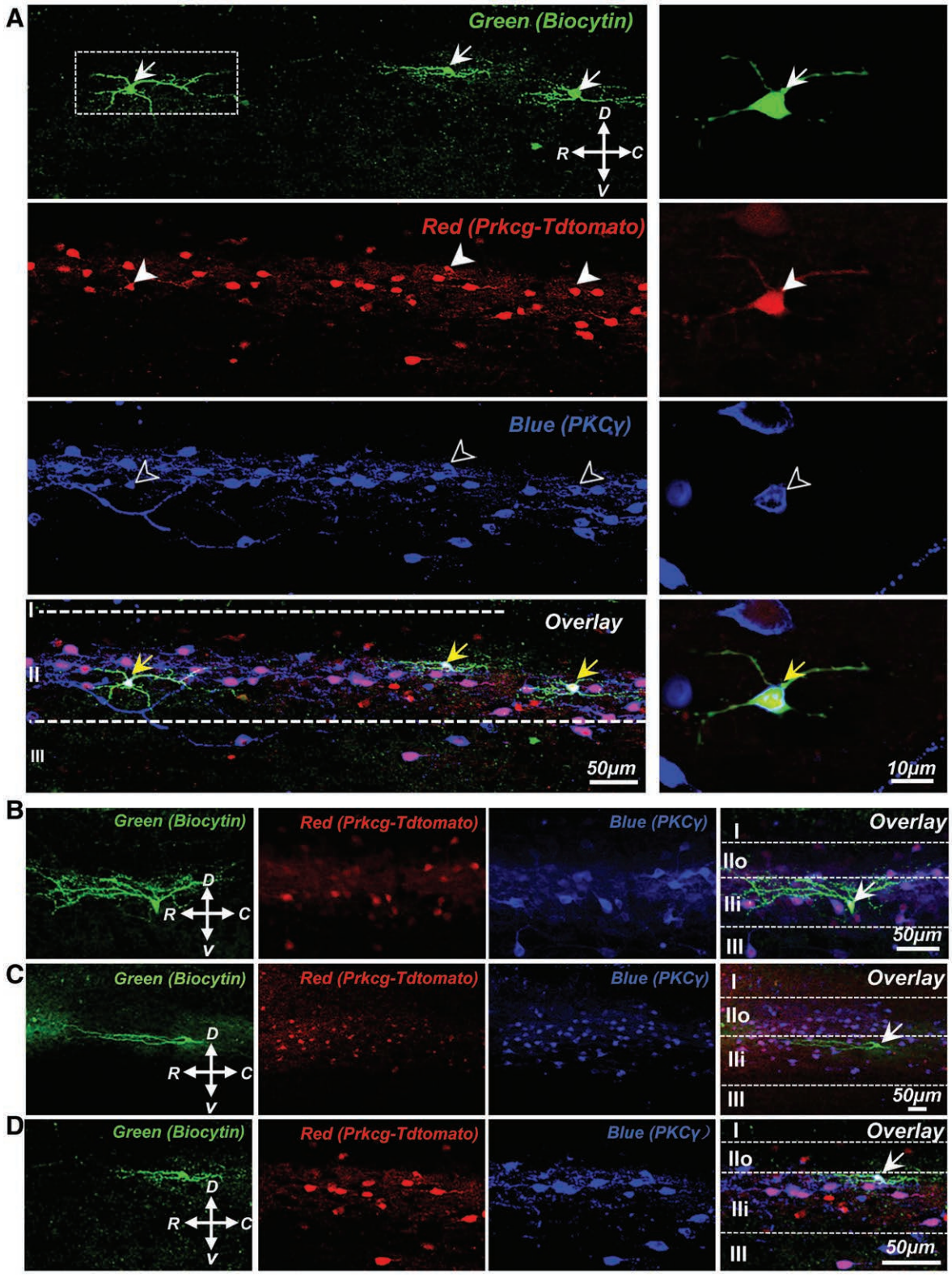

Fig. 5. Confocal images of typical PKCγ⁺ neurons. (*A*) Examples of three recorded neurons. *Arrows* indicate recorded neurons labeled with intracellular biocytin. *Solid arrowheads* indicate the corresponding tdTomato-expressing neurons. *Hollow arrowheads* refer to the recorded neurons stained with PKCγ antibody. *Yellow arrows* indicate the overlay neurons. (*B–D*) Images of the recorded PKCγ⁺ neurons in figure 3D, figure 4D, and figure 5C, respectively. C, caudal; D, dorsal; PKCγ, protein kinase C γ; PKCγ⁺, protein kinase C γ expressing; R, rostral; V, ventral.

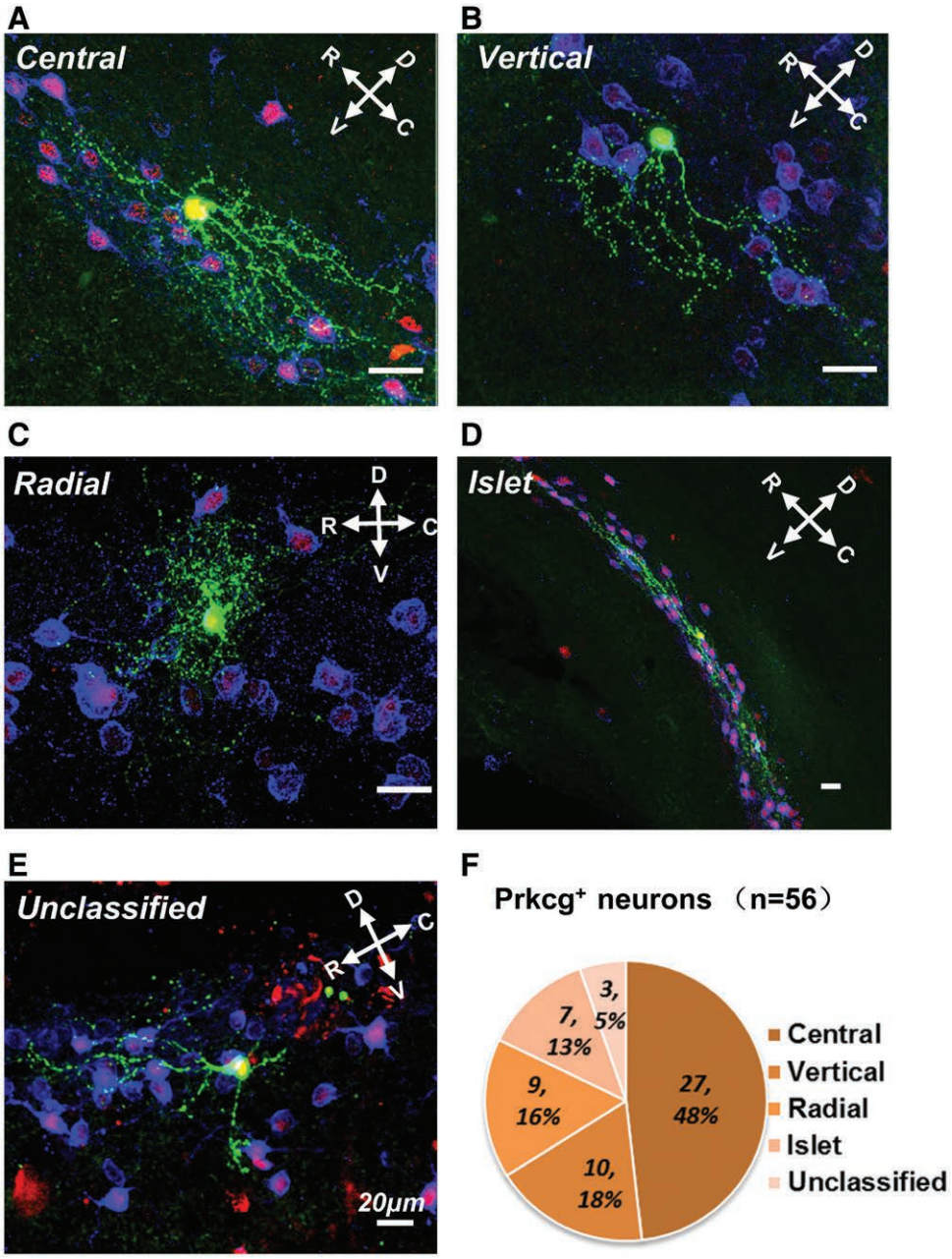

Fig. 6. Spinal PKCγ⁺ neurons possess diverse morphological features. Examples of central (*A*), vertical (*B*), radial (*C*), islet (*D*) and unclassified (*E*) biocytin-filled Prkcg-tdTomato neurons with triple immunofluorescence staining. (*F*) Pie chart analysis of numbers of each morphological type. C, caudal; D, dorsal; R, rostral; V, ventral.

an irregular firing pattern, 32% (23 of 73) showed a phasic firing pattern, and 26% (19 of 73) displayed a tonic firing pattern (fig. 7A). Among 36 protein kinase C γ⁻ neurons, the most prevalent action potential firing pattern was tonic (15 of 36, 42%), followed by delayed (13 of 36, 36%), phasic bursting (4 of 36, 11%), and regular firing patterns (4 of 36, 11%; Supplemental Digital Content, fig. S6, I and K, http://links.lww.com/ALN/C271). Delayed-firing neurons showed a certain (greater than 100 ms) delay followed by action potential. Thus, irregular firing patterns predominated in the protein kinase C γ expressing neurons, while the tonic firing pattern predominated in protein kinase C γ⁻ neurons. There is no doubt that the discharge pattern, as a classification standard of neurons, can classify neurons, but there is overlap between protein kinase C γ expressing and protein kinase C γ⁻ neurons in terms of their firing patterns. It is thus incomplete to judge protein kinase C γ expressing neurons merely according to their discharge patterns.

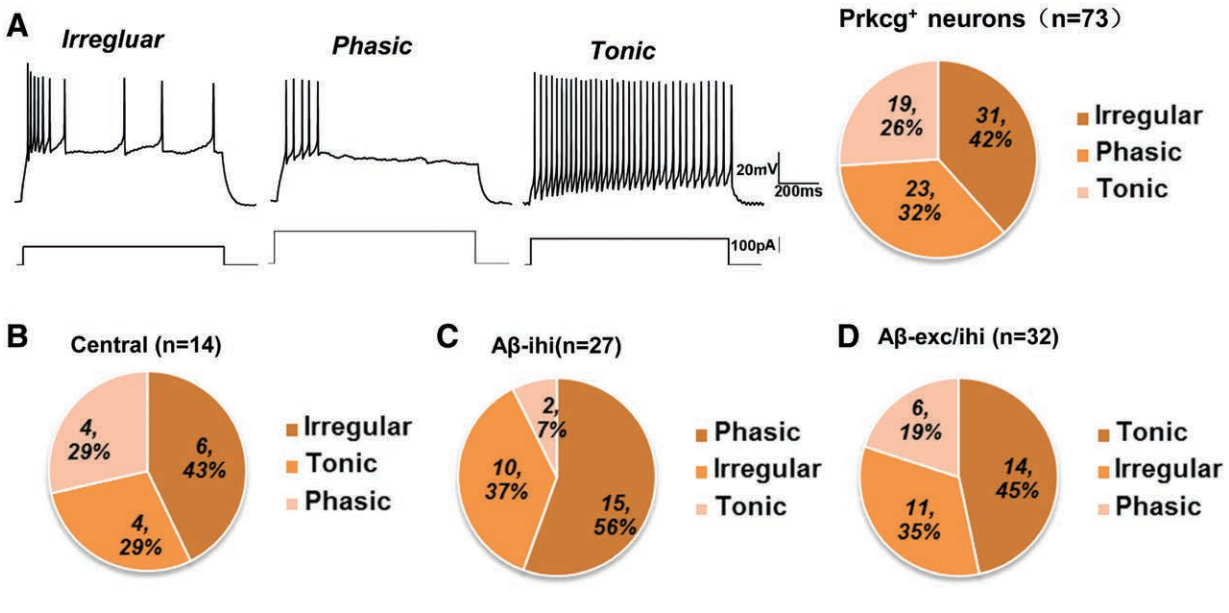

Fig. 7. Spinal PKCγ+ neurons have diverse firing patterns. (*A*) Examples and pie chart analysis of three main firing patterns of protein kinase C γ expressing neurons during 1 s depolarizing rheobase current. (*B*) The proportion of three main firing patterns in central type of protein kinase C γ expressing neurons. (*C*) The proportion of three main firing patterns in protein kinase C γ expressing neurons receiving Aβ-mediated inhibitory input. (*D*) The proportion of three main firing patterns in protein kinase C γ expressing neurons receiving Aβ-mediated excitatory and inhibitory inputs. Aβ-ihi, Aβ-mediated inhibitory input; Aβ-exc/ihi, Aβ-mediated excitatory and inhibitory inputs; PKCγ+, protein kinase C γ expressing.

Spinal Protein Kinase C γ Expressing Neurons Were Activated by Innocuous Stimulation after Peripheral Nerve Injury

In response to peer review, additional experiments were conducted to explore if the excitability of the protein kinase C γ expressing interneuron is enhanced in nerve injury induced pathologic conditions. The c-fos expression can be used to monitor the activity of neurons and has been shown to be induced in spinal protein kinase C γ neurons after innocuous stimulation.[10] We first compared the percentage of double-labeled neurons for c-fos and *Prkcg-P2A-tdTomato* after 60 min walking on a rotating rod (n = 3) to that observed in control animals (n = 3). We found that although the numbers of c-fos expression neurons increased in the walking group, the percentage of neurons double-labeled for c-fos and *Prkcg-P2A-tdTomato* did not differ significantly (2.6% *vs.* 2.8%,4 of 154 *vs.* 5 of 177 $P > 0.999$) in the two groups (fig. 8, A, B, and E). However, in pathologic conditions after nerve injury, the percentage of double-labeled neurons in the walking group was significantly higher than that in the nerve injury group (fig. 8C–E, 4.1% *vs.* 9.9%, 22 of 539 *vs.* 54 of 548, $P < 0.001$).

The Excitability of Spinal Protein Kinase C γ Expressing Neurons Was Enhanced after Nerve Injury

In response to peer review, additional patch clamp recordings were used to compare the Aβ fiber evoked action potentials of Prkcg-P2A-tdTomato protein kinase C γ expressing neurons in control and nerve injury conditions. The Prkcg-P2A-tdTomato fluorescent labeled mice were used. The sagittal spinal cord slices were made from control (4 to 5 weeks old) and nerve injury animals (1 to 2 weeks after nerve injury). Dorsal root stimulation was used to evoke the synaptic responses in protein kinase C γ expressing neurons. Dorsal root stimulation at Aβ fiber strength evoked EPSPs followed by burst action potentials in 25.8% (8 of 31) of protein kinase C γ expressing neurons in control animals, while the proportion increased to 51.1% (23 of 45) in nerve injury animals (fig. 8, F and G), indicating that the proportion of protein kinase C γ expressing neurons activated by Aβ fiber input significantly increased after nerve injury compared to normal conditions (25.8% *vs.* 51.1%, P = 0.027). These results indicated that nerve injury enhanced the excitability of spinal protein kinase C γ expressing interneuron due to disinhibition of the feed-forward inhibitory circuit, and enabled Aβ primary inputs to activate spinal protein kinase C γ expressing interneurons.

Discussion

In the current study, we constructed *Prkcg-P2A-tdTomato* knock-in mice based on clustered regularly interspaced short palindromic repeats/clustered regularly interspaced short palindromic repeats–associated nuclease 9 technique, which allows recording protein kinase C γ expressing neurons

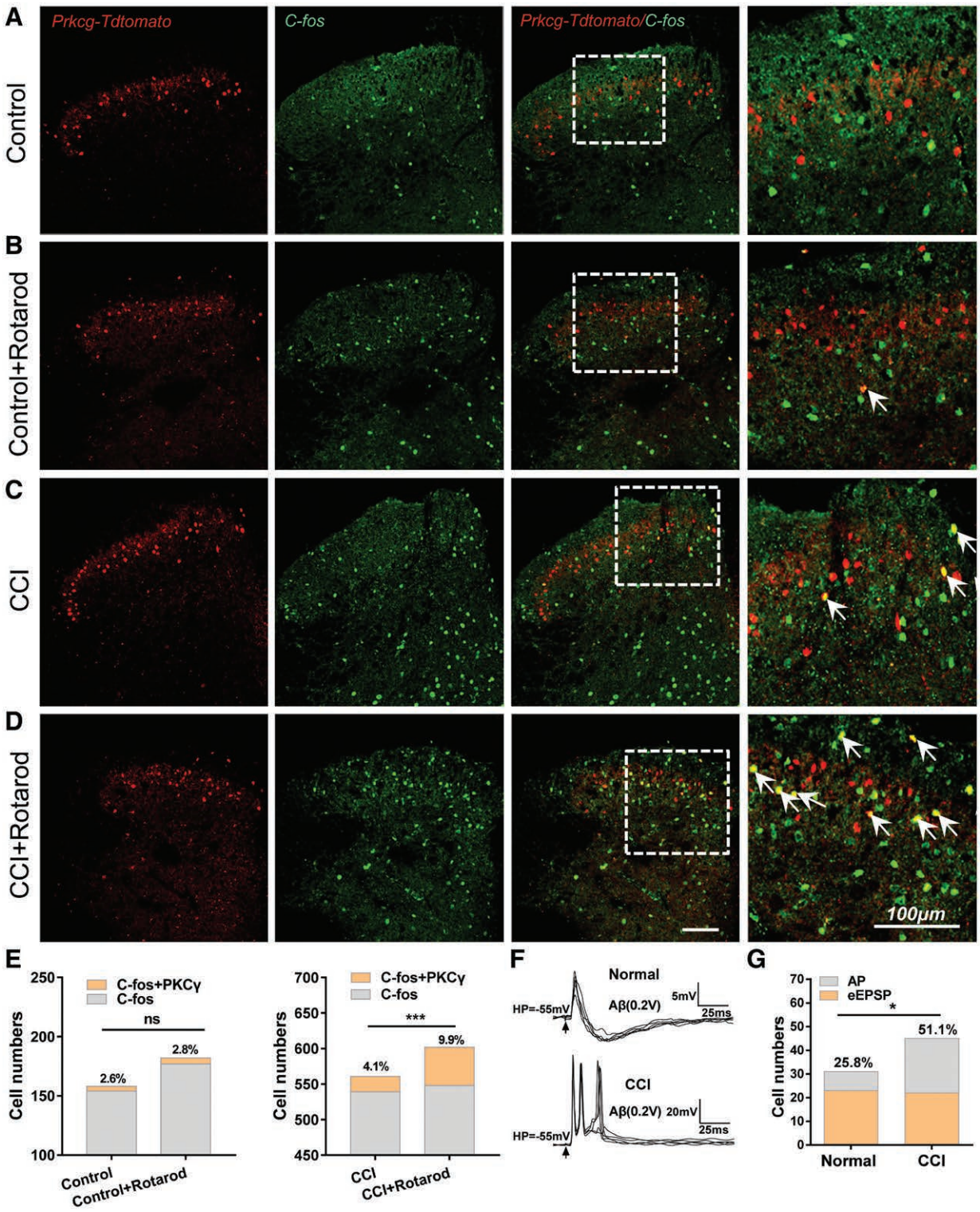

Fig. 8. (*Continued*)

Fig. 8. The excitability of spinal PKCγ⁺ neurons was enhanced after nerve injury (*A* and *B*) Immunostaining spinal sections show c-fos expression and Prkcg-tdTomato neurons in control animals and animals after 60 min walking on a rotating rod. (*C* and *D*) Immunostaining spinal sections show c-fos expression and Prkcg-tdTomato neurons in nerve injury animals and nerve injury animals after 60 min walking on a rotating rod. (*E*) The percentage of neurons double labeled for c-fos and *Prkcg-P2A-tdTomato* did not differ significantly (2.6% *vs.* 2.8%, 4 of 154 *vs.* 5 of 177, $P > 0.999$) in groups A and B; in pathologic conditions after nerve injury, the percentage of double labeled neurons in the walking group was significantly higher than that in the nerve injury group (4.1% *vs.* 9.9%,22 of 539 *vs.* 54 of 548, $P < 0.001$, chi-square test). (*F*) Dorsal root stimulation at Aβ fiber strength evoked typical biphasic EPSPs-IPSPs in PKCγ⁺ neurons of control animals, and EPSPs followed by burst APs in PKCγ⁺ neurons of nerve injury animals. (*G*) The proportion of PKCγ⁺ neurons firing APs increased from 25.8% (8 of 31) to 51.1% (23 of 45) after nerve injury ($P = 0.027$, chi-square test). *, $P < 0.05$;***, $P < 0.001$. AP, action potential; CCI, chronic constriction injury; eEPSP, evoked monosynaptic excitatory postsynaptic potential; HP, holding potential; ns, no significance; PKCγ, protein kinase C γ; PKCγ⁺, protein kinase C γ expressing.

precisely in visual identification and greatly improves the experimental efficiency. In addition, the fluorescence of genetically labeled neurons could last for several weeks after patch clamp recordings, which is suitable for the morphological identification of recorded neurons. Previous studies have indicated that the spinal protein kinase C γ expressing interneuron is the centerpiece of the allodynia gate circuits.[3–11] The *Prkcg-P2A-tdTomato* mice provide a useful tool for further study of the working mechanism of spinal allodynia gate.

Clustered regularly interspaced short palindromic repeats/clustered regularly interspaced short palindromic repeats–associated nuclease 9, as a gene editing technology, has many advantages compared with traditional methods such as zinc finger nucleases and transcription activator-like effector nucleases. It has been also used for gene knockout, gene replacement, gene activation, disease modeling, and even gene therapy.[12,13] This technology may facilitate the creation of a new generation of animal models for various diseases.

Previous studies have usually classified neurons according to their basic electrophysiological characteristics such as discharge patterns and morphological characteristics, but rarely involve the distinction of primary afferent fibers.[25,26,29,31–35] In this study, we found that protein kinase C γ expressing neurons have a variety of discharge patterns and morphological characteristics, although central morphology and an irregular firing pattern were dominant, implying that it is not reliable to judge the type of spinal neurons only by discharge patterns and morphological characteristics. Therefore, we propose that the classification of spinal neurons should be based not only on their electrophysiologic and morphological characteristics, but also on the types of primary afferent fibers accepted by the neurons. The types of primary afferent fibers accepted by the neurons may be more reliable as a means to represent their functions.

Myelinated Aβ, part of low-threshold Aδ and C fibers transmitting innocuous information mainly target the ventral part of lamina IIi to lamina V of the spinal dorsal horn. On the other hand, C and Aδ fibers transmitting nociceptive information mainly target the lamina I to the dorsal part of lamina IIo.[36,37] The current study confirmed that the protein kinase C γ expressing neuron was innervated by intricate low-threshold Aβ, Aδ, and high-threshold C fiber inputs, with Aβ-fiber–mediated biphasic evoked excitatory postsynaptic potentials-inhibitory postsynaptic potentials predominating among the synaptic responses. We have previously found that innervation from both inhibitory interneurons and primary Aβ-fibers to protein kinase C γ expressing neurons in the spinal dorsal horn form a feed-forward inhibitory circuit.[2] Toll-like receptors are mainly involved in immune and glial cells.[38] Blockade of Toll-like receptor 5 dose-dependently suppressed nerve injury–induced mechanical allodynia.[11] These findings provide a potential target for the treatment of mechanical allodynia.[22] Our results suggest that Toll-like receptor 5–positive Aβ afferent directly innervates protein kinase C γ expressing neurons. How the Toll-like receptor 5–positive Aβ afferent influences the function of protein kinase C γ expressing neurons needs further investigation. Our results are consistent with previous reports that c-fos expression can be induced in protein kinase C γ expressing neurons after innocuous stimulation.[10] We further found that a large amount of spinal protein kinase C γ expressing neurons are activated by innocuous inputs after nerve injury.

Nevertheless, there are some limitations to this study. Notably, we merely recorded the protein kinase C γ expressing neurons in lamina IIi, ignoring a small portion of neurons distributed in lamina I, IIo, and III. We also did not observe the behavioral changes of mice by regulating protein kinase C γ expressing neurons *in vivo*. In our further research, we will examine neurons in these other locations in order to determine whether they are consistent with the characteristics of neurons in lamina IIi. Furthermore, our ongoing studies aim to explore how nerve injury impairs the functions of inhibitory interneurons and then allows the nonnoxious input to activate protein kinase C γ expressing neurons.

In summary, the *Prkcg-P2A-tdTomato* mice we constructed in this study provide a useful tool for the study of the protein kinase C γ expressing neurons involved in spinal cord circuits gating mechanical allodynia. By using this animal model, we have confirmed our previous findings that spinal protein kinase C γ expressing neurons mainly receive low-threshold Aβ primary afferent input, and the feed-forward inhibitory input driven by the low-threshold Aβ primary afferent. The convergent inputs control the excitability of protein kinase C γ expressing neurons

and gate the Aβ inputs passing through the spinal nociceptive pathway. We further found that both glycinergic and GABAergic spinal interneurons contribute to the feed-forward inhibition of protein kinase C γ expressing neurons with predominant glycinergic contribution. In addition, we propose that spinal protein kinase C γ expressing neurons possess diverse morphological and electrophysiologic features; it is therefore incomplete to judge protein kinase C γ expressing neurons merely according to their dendritic arbor and discharge patterns. The current study supports our previous conclusion that drugs targeted at reducing excitability of spinal protein kinase C γ expressing neurons could be used to reduce mechanical allodynia after nerve injury.

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Competing Interests

The authors declare no competing interests.

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