Thorough GABAergic innervation of the entire axon initial segment revealed by an optogenetic ‘laserspritzer’

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Abstract GABAergic terminals of chandelier cells exclusively innervate the axon initial segment (AIS) of excitatory neurons. Although the anatomy of these synapses has been well-studied in several brain areas, relatively little is known about their physiological properties. Using vesicular γ-aminobutyric acid transporter–channelrhodopsin 2–enhanced yellow fluorescence protein (VGAT-ChR2-YFP)-expressing mice and a novel fibreoptic ‘laserspritzer’ approach that we developed, we investigated the physiological properties of axo-axonic synapses (AASs) in brain slices from the piriform cortex (PC) of mice. AASs were in close proximity to voltage-gated Na⁺ (Naᵥ) channels located at the AIS. AASs were selectively activated by a 5 μm laserspritzer placed in close proximity to the AIS. Under a minimal laser stimulation condition and using whole-cell somatic voltage-clamp recordings, the amplitudes and kinetics of IPSCs mediated by AASs were similar to those mediated by perisomatic inhibitions. Results were further validated with channelrhodopsin 2-assisted circuit mapping (CRACM) of the entire inhibitory inputs map. For the first time, we revealed that the laserspritzer-induced AAS-IPSCs persisted in the presence of TTX and TEA but not 4-AP. Next, using gramicidin-based perforated patch recordings, we found that the GABA reversal potential (E_GABA) was −73.6 ± 1.2 mV when induced at the AIS and −72.8 ± 1.1 mV when induced at the perisomatic site. Our anatomical and physiological results lead to the novel conclusions that: (1) AASs innervate the entire length of the AIS, as opposed to forming a highly concentrated cartridge, (2) AAS inhibition suppresses action potentials and epileptiform activity more robustly than perisomatic inhibitions, and (3) AAS activation alone can be sufficient to inhibit action potential generation and epileptiform activities in vitro.
Introduction

Axo-axonic cells (AACs) are known for the unique location of their synapses, which are formed on the axon initial segment (AIS) of pyramidal neurons (Szentagothai & Arbib, 1974; Jones, 1975; Fairen & Valverde, 1980; Somogyi et al. 1983) and are hence termed axo-axonic synapses (AASs). AACs are hypothesized to perform crucial roles in governing the initiation and propagation of action potentials (APs) from the AIS to the distal axon (Miles et al. 1996; DeFelipe, 1999; Howard et al. 2005; Inda et al. 2006). While several studies have shown that AACs exert a large and fast inhibitory effect on postsynaptic neurons (Pawelzik et al. 1999; Maccaferri et al. 2000; Tamas & Szabadi, 2004; Glickfeld et al. 2009), other studies have demonstrated that AACs may be excitatory/depolarizing (Szabadi et al. 2006) or bi-directional, depending on the brain state (Khirug et al. 2008; Woodruff et al. 2009, 2011). Thus, the physiological function of AACs is a scientifically unresolved question.

The controversies regarding the physiological properties of AASs may be attributable to the heterogeneity of AACs (DeFelipe et al. 1985; Inda et al. 2009) and the techniques employed. The majority of studies used the dual-recording approach with one electrode in the presynaptic AAC and a second electrode in the soma of the postsynaptic neuron to document unitary IPSCs. Two major drawbacks to this approach are; (1) it is challenging to obtain a large number of cell pairs due to the scarcity of AACs, and (2) the presynaptic AAC is highly prone to injury.

The optogenetic approach has become the method of choice to selectively activate specific neurons or synapses (Nagel et al. 2003; Boyden et al. 2005; Arenkiel et al. 2007; Wang et al. 2007; Sohal et al. 2009; Zhao et al. 2011). However, the optogenetic approach has not been applied to AAS studies. Recently, we discovered that virtually every pyramidal neuron was innervated by AASs within the piriform cortex (PC; Wang & Sun, 2012). The current study takes advantage of this high innervation ratio and seeks to understand further details of AAS physiology by using two innovative optogenetic approaches: (1) an innovative laser delivery electrode that we developed and named ‘laserspritzer’, analogous to the term ‘picospritzer’ used by electrophysiology labs (Sun et al. 2014), and (2) channelrhodopsin-assisted circuit mapping approach (CRACM) which has been successfully used to study glutamatergic innervation maps (Petreanu et al. 2007, 2009). We applied these two innovative approaches to the vesicular GABA transporter–channelrhodopsin 2 (VGAT-ChR2-YFP) mice (Zhao et al. 2011). We could selectively activate GABAergic innervations formed on either the soma or the AIS and investigate somatically recorded IPSCs using both gramicidin-based perforated patches and cell-attached recordings in normal and ‘epileptic’ postsynaptic excitatory cells. Our results demonstrate that AASs innervate the entire AIS. AASs can be selectively laser activated, and play a strong inhibitory role in spike-induction, regardless of the resting membrane potentials. AASs exert stronger inhibitory effects on spike induction than perisomatic inhibitions. Due to the unique location of AASs, the functions of AASs are likely to be very important in vivo. The unique functions of these synapses in a circuit are likely to be attributable to the mechanism by which chandelier cells are integrated within a circuit.

Methods

Animals

The animals used in this study were vesicular γ-aminobutyric acid transporter–channelrhodopsin 2 (H134R)-enhanced yellow fluorescence protein (YFP) mice (hereafter called VGAT-ChR2-YFP mice). In this line of mice, ChR2-YFP fusion protein is selectively expressed under the control of the VGAT promoter (Zhao et al. 2011). In a subset of experiments, we also used calmodulin II–green fluorescent protein (CamKII-GFP) mice in which GFP is expressed in majority of glutamatergic neurons of PC (Wang et al. 2013). Adult animals from postnatal weeks 6–8 were used. All animals were housed at the University of Wyoming Animal Facility in a temperature-controlled environment with a 12 h light–12 h dark cycle. The use of mice adhered to protocols approved by the IACUC of the University of Wyoming.
Brain slice preparation, light fixation and immunohistochemistry

Mice were deeply anaesthetized using isoflurane and decapitated. A light fixation approach was used in this study as described by Schneider Gasser (Schneider Gasser et al. 2006). Briefly, brain slices (500 μm thick) were cut using a vibratome (TPI, St Louis, MO, USA) as described previously (Young & Sun, 2009). They were incubated in fixative (4% paraformaldehyde, PFA) for 60 min and rinsed with 0.01 M phosphate-buffered saline (PBS; pH 7.4) three times for 20 min each time. After being stored for 2 days in the cryoprotectant (30% sucrose solution in 0.1 M phosphate buffer) at 4°C, brain slices were further cut into 20 μm sections using a cryostat at −25°C. Immunostaining was carried out as described previously (Wang & Sun, 2012): after being rinsed twice with PBS, sections were incubated for 30 min in PBS with 0.5% H2O2, then PBS rinsed twice for 10 min each, incubated in PBS with 0.3% Triton X-100, 0.05% Tween20, and 4% normal goat serum for 2 h at room temperature, then incubated in PBS with 0.2% Triton X-100 and primary antibodies overnight at 4°C. The next day, sections were PBS rinsed twice and incubated for 3 h at room temperature in secondary antibodies (in PBS with 0.3% Triton X-100). Lastly, the sections were PBS rinsed, mounted, and coverslipped using Vectashield mounting medium. The primary antibodies used were monoclonal mouse anti-ankyrin-G (1:250, Santa Cruz, Dallas, TX, USA, SC-15700), polyclonal rabbit anti-GAT-1 (1:500, Millipore, MA, USA, AB1570; Wang & Sun, 2012), and monoclonal mouse anti-NaN.1.2 (1:500, NeuroMab, Davis, USA, K69/3; Merrick et al. 2010). The secondary antibodies used in this study were Alexa Fluor 488 and 594, goat anti-mouse or goat anti-rabbit IgG (heavy and light chains; 1:1000; A-11001 and A-11008, Invitrogen, Carlsbad, CA, USA). The confocal microscopy images were obtained by using a Zeiss 710 microscope under a ×100 oil immersion objective. Intrinsic YFP fluorescence was used as the marker for the fusion protein ChR2-YFP. Co-localization analysis was performed using Image J (NIH) as described previously (Bolte & Cordelieres, 2006). Quantitative analysis of presynaptic boutons was performed using z-stack confocal images of Alexa 594 or neurobiotin-filled neurons as described (Wang & Sun, 2012).

Brain slice electrophysiology

Mice were deeply anaesthetized using isoflurane and decapitated. The brains were quickly removed and transferred into cold (~4°C) oxygenated slicing medium containing the following (in mM): 2.5 KCl, 1.25 NaH2PO4, 10.0 MgCl2, 0.5 CaCl2, 26.0 NaHCO3, 11.0 glucose, and 234.0 sucrose. Both coronal and horizontal slices were used in this study. Horizontal slices were prepared as described (Markopoulos et al. 2008). Brain slices (250–300 μm) were made using a vibratome and incubated in a holding chamber in artificial cerebral spinal fluid (aCSF) at 35 °C for at least 1 h. The aCSF consisted of the following (in mM): 126.0 NaCl, 2.5 KCl, 1.25 NaH2PO4, 1.0 MgCl2, 2.0 CaCl2, 26.0 NaHCO3, and 10.0 glucose. Solutions were gassed with 95% O2–5% CO2 to a final pH of 7.4 at a temperature of 35 ± 1°C. Individual slices were transferred to the recording chamber, fixed to a modified microscope stage, and allowed at least 30 min to equilibrate before recording. While recording, slices were minimally submerged and superfused continuously with oxygenated aCSF (4.0 ml min−1). A low-power objective (× 2.5) was used to identify layer II of the PC. A high-power water-immersion objective (× 63) with Nomarski optics and infrared video was utilized to visualize individual neurons for recording. Recording pipettes were fabricated from capillary glass (World Precision Instruments, M1B150F-4; Sarasota, FL, USA) using a Sutter Instrument P80 puller (Novato, CA, USA) and exhibited tip resistances of 3 ± 2 MΩ when filled with the intracellular solutions below. A Multi-clamp 700B amplifier (Molecular Devices, Foster City, CA, USA) was used for voltage-clamp and current-clamp recordings. Current- and voltage-clamp protocols were generated using pClamp 9.2 software (Molecular Devices). For whole-cell recordings of synaptic currents in pyramidal neurons under voltage clamp, the patch pipette saline was composed of (in mM): 100 caesium gluconate, 10.0 phosphocreatine-Tris, 3.0 MgCl2, 0.07 CaCl2, 4.0 EGTA, 10.0 Hepes, 4.0 Na2-ATP, and 1.0 Na-GTP (pH adjusted to 7.4 and osmolarity adjusted to 280 mosmol l−1). For whole-cell recordings of ChR2-mediated direct current in interneurons under voltage clamp, the patch pipette saline was composed of (in mM): 100 potassium gluconate, 10.0 phosphocreatine–Tris, 3.0 MgCl2, 0.07 CaCl2, 4.0 EGTA, 10.0 Hepes, 4.0 Na2-ATP, and 1.0 Na-GTP (pH adjusted to 7.4 and osmolarity adjusted to 280 mosmol l−1). Either neurobiotin (0.5%; Vector Laboratories) or Alexa 594 (0.2 mg ml−1; Invitrogen, A10438; Kim et al. 2012) was added into the recording pipette solution. Data were accepted for analysis when access resistance in whole-cell recordings ranged from 6 ± 2 MΩ and remained stable (<25% change) throughout the recording. For both direct and synaptic currents mediated by ChR2, responses were recorded at a holding potential of 0 mV. Direct ChR2 currents were induced in the presence of a cocktail containing NBQX (5 μM), picrotoxin (50 μM) and AP-5 (50 μM) and tetrodotoxin (TTX, 100 nM, Sigma-Aldrich). A single laser stimulation (8 ms) was triggered every 20 s to prevent the desensitization of ChR2. After each brain slice was recorded, it was transferred immediately into a fixative (4% paraformaldehyde, PFA) for 40 min to overnight, and then rinsed in PBS (0.01 M) twice for 20 min each time. Slices with Alexa 594
were directly mounted using Vectashield mounting medium with or without 4,6-diamidino-2-phenylindole (DAPI). Slices with neurobiotin were processed for neurobiotin-3,3′-diaminobenzidine tetrahydrochloride (DAB, D5905, Sigma-Aldrich, St. Louis, MO, USA) histochemistry.

Characterization of ChR2-IPSCs from AASs using a ‘laserspritzer’ approach

Layer IIa semilunar neurons in the PC, which do not have basal dendrites, were used for electrophysiological recordings to study ChR2-mediated inhibitory postsynaptic currents (ChR2-IPSCs). The purpose was to minimize possible contamination from inhibitory inputs of basal dendrites (Haberly, 1983; Valverde & Santacana, 1994; Suzuki & Bekkers, 2006). We previously showed that the AISs of these neurons run, without exception, in the direction of layer III (vertically to layer II), and virtually all of the glutamatergic cells were innervated by AASs (Wang & Sun, 2012). Therefore, we defined an axon location (for the purpose of laser stimulation) 40 μm away from soma, in the direction of layer III. ChR2-IPSCs were recorded under voltage clamp at 0 mV. A laserspritzer probe (Sun et al. 2014) was fabricated from a conventional multi-mode optic fibre with a core diameter of 250 μm, and pulled to retain a final diameter of <5 μm (Fig. 1A). The fine optic fibre was inserted into a conventional patch pipette which could be moved by a micromanipulator to achieve subcellular stimulation. The capillary pipette protects the soft fibre and limits the bending. The spatial resolution of the laserspritzer was tested using ChR2-expressing interneurons; details are given in the Results section. A fibre with desired spot excitation (≈<10 μm spot size with 0.02–0.04 mW mm⁻² laser power) was collected for experimentation. The minimal laser intensity was determined for each cell at the soma, then tested at the axon (40 μm away) to induce AAS-mediated currents (Fig. 1B). Neurons were filled with Alexa 594 and examined for axon integrity immediately after the recording (Fig. 1C). The order of stimulation for the two locations (soma and axon) was randomized (Fig. 1B). Normalized axonic vs. somatic ChR2-IPSCs amplitudes were evaluated for statistical significance using the non-parametric Wilcoxon signed-rank test. The laser power of the laserspritzer was measured by placing the laserspritzer on a handheld laser meter (Edmund Optics, Barrington, NJ, USA) with 1.57 mm² aperture. Laser intensities used in this study ranged from 0.01 to 0.1 mW mm⁻².

Gramicidin-based perforated recording

Gramicidin-based perforated patch recordings were carried out as described (Owens et al. 1996). Briefly, patch pipette saline consisted of the following (in mM): 100 potassium gluconate, 10.0 phosphocreatine–Tris, 3.0 MgCl₂, 0.07 CaCl₂, 4 EGTA, 10.0 Hepes, 4.0 Na₃-ATP, and 1.0 Na-GTP (pH adjusted to 7.4 and osmolarity adjusted to 280 mosmol l⁻¹). Gramicidin (Sigma-Aldrich) was prepared as a stock solution of 5 mg ml⁻¹ immediately prior to each experiment and kept on ice until diluted into a pipette solution (20–25 μg ml⁻¹) every 2 h. The same pipette solution without gramicidin was used to tip-fill the pipettes. After seal formation, perforation was evaluated by presenting 5 mV test pulses to the cell under voltage clamp. Recordings were started when access resistance reached below 50 MΩ. Cells with a membrane potential lower than −50 mV were used for further recordings. GABAₐ reversal potential (E_{GABA}) was measured under voltage clamp in perforated patches. In pyramidal neurons, ChR2-IPSCs were recorded at different membrane potentials from −50 to −90 mV, in 10 mV increments. All measurements of E_{GABA} were carried out in the soma, regardless of laser stimulation location (axon or soma). Current responses corresponding to each voltage level were plotted, and data points were fitted with a linear equation. The GABA-mediated reversal potential was defined as the x-intercept value of the fit.

ChR2-assisted circuit mapping (CRACM) of inhibitory inputs

CRACM is a powerful tool for functional mapping of neural circuits and long-range connections using ChR2 photo-stimulation (Petreanu et al. 2007, 2009). Here, we used this approach to measure subcellular location-specific inhibitory inputs. Photo-activation of ChR2 was performed by shattering the beam of a blue laser (470 nm) in the specimen plane via a × 5 objective. The movement of the laser beam was precisely controlled with mirror galvanometers, triggered by scanning and data acquisition software Ephus (http://www.ephus.org). A user-defined mapping grid with row and column spacing of 50 μm was applied for recordings. Inhibitory maps were recorded at a holding potential of −60 mV, in the presence of AMPA, the NMDA antagonist NBQX (2 μM) and CPP (5 μM), using a CsCl-based pipette solution.

Epileptiform activity in vitro

To examine the effect of AAS activation on epileptiform activity in vitro, brain slices were exposed to aCSF with 0 mM Mg²⁺ and 500 μM 4-AP. The 0-Mg²⁺ aCSF consisted of the following (in mM): 126.0 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 2.0 CaCl₂, 26.0 NaHCO₃, and 10.0 glucose. A submerged recording chamber was used. A bipolar electrode was placed in layer III of the PC to
induce epileptiform after-discharges. After the gigaohm seal was achieved, cell-attached recordings were made. Upon completion of cell-attached recordings, whole-cell current-clamp recordings were made in the same cells to obtain additional data. Laser stimulation was applied at appropriate latencies (1–100 ms) after the electric stimulation.

**Data and statistical analyses**

Electrophysiology data analysis was carried out using Clampfit (Version 10.2, Molecular Devices). ChR2-IPSC events were detected using a template, the kinetics of which resembled that of a typical GABA-mediated IPSC. To quantitatively analyse the configuration of ChR2-IPSCs amplitudes, we used a multi-peak Gaussian distribution. One-way analysis of variance (ANOVA) was used to examine statistical significance between groups. The non-parametric Wilcoxon signed-rank test was used to compare ChR2-IPSCs amplitudes and onset jitter of soma vs. axon location. The standard for significant difference was defined as $P < 0.05$. All graphic representations of data illustrations are given as means ± SEM. The number of observations ($n$) contributing to each mean is reported in the Results section, or indicated in the graphs.

**Results**

**Morphological features of ChR2-containing AASs**

We first examined the localization of ChR2-YFP in GABAergic terminals. The YFP positive (YFP+) synaptic varicosities were seen surrounding soma, dendrite, and AIS regions of individual Alexa 594-labelled pyramidal and semilunar cells (Fig. 1Cb). To verify the GABAergic synaptic nature of the strong YFP varicosities, we performed immunohistochemical staining against common GABAergic synaptic markers. Co-localization analysis (Mander’s coefficient) of YFP and GABA transporter-1 (GAT-1) immunoreactivities (Fig. 2Aa) in confocal images showed a 98.2 ± 1.5% overlap ($n = 6$ mice), indicating that ChR2-YFP was expressed in nearly all GABAergic terminals. We next examined the subcellular location of ChR2-YFP within layer II of the PC. Protein markers GAT-1 and ankyrin-G, both enriched at the AIS (Wang & Sun 2012), were used to examine GABAergic terminals to see if they were formed near the AIS. Confocal images of YFP and ankyrin-G immunoreactivities showed that YFP+ terminals innervated both the soma and AIS of excitatory neurons (Fig. 2Ab). Within both the AIS and soma, YFP+ terminals were found to be co-localized (95.6 ± 4.5%, $n = 30$) with voltage-gated sodium channels 1.2 (Na,1,2, Fig. 2B). In addition, AASs were found on the AIS where Na,1,2 clustered (Fig. 2B, white arrowheads). The spacial proximity between AASs and Na,1,2 suggests a critical role of AASs in the regulation of action potential initiation. In addition, ChR2-YFP varicosities in both the AIS and soma were confirmed in every recorded semilunar cell with an intact AIS. Therefore, we concluded that ChR2-YFP was located at all GABAergic terminals innervating both the AIS and soma of layer II semilunar cells in the PC, paving the way for further optogenetic investigations in these cells.

**The spatial resolution of the fibreoptic laserspritzer electrodes**

Next, we characterized the spatial resolution of laserspritzer electrodes in brain slices by examining ChR2-mediated inward currents in layer I YFP+ interneurons (i.e. ChR2-expressing cells; Fig. 3Aa). The majority of recorded ChR2-containing interneurons were neuroglia-form interneurons with regular-spike firing patterns and very limited dendritic arbors. Synaptic currents were blocked by a cocktail containing NBQX, picrotoxin and AP-5. We first examined the relationship between the location of the laserspritzer along the radial axis (perpendicular to the recording electrode and pia surface of PC) and ChR2-mediated currents under the same laser intensity (Fig. 3B). On average, the amplitudes of ChR2-mediated currents recorded on the soma dropped to less than 10% of somatically induced values when the laserspritzer electrode was placed at 21.5 ± 6.3 μm away from soma in either apical or basal directions, i.e. only 10% of the ChR2 current was activated at this distance ($n = 7$ cells, Fig. 3Bb). The FWHM (full width at half-maximum) at this laser intensity (0.04 mW mm$^{-2}$) was 40 μm ($n = 6$, Fig. 3Bb). Next, we examined the relationship between the location of the laserspritzer along the horizontal axis (i.e. in line with the direction of the recording electrodes) and ChR2-mediated currents under the same laser intensity. On average, the amplitudes of ChR2-mediated currents recorded on the soma dropped to less than 10% of somatically induced values when the laserspritzer electrode was placed at 9.5 ± 4.3 μm away from soma, i.e. only 10% of the ChR2 current were activated at this distance ($n = 7$ cells, Fig. 3Bc). The FWHM at this laser intensity (0.04 mW mm$^{-2}$) is 10 μm ($n = 6$, Fig. 3Bc). Next, we measured the relationship between action potential (AP) probabilities and laserspritzer location. Supra-threshold laser intensities, defined as 1.5 × laser intensities at which action potentials were induced at the soma, were used in this experiment. On average, when the laserspritzer was placed at > 36.1 ± 14.6 μm away from the soma in either apical or basal directions, the probability of AP induction was reduced to less than 10% of...
somatically induced values \( (n = 7 \text{ cells, Fig. 3C}) \). Together, these data suggests that the laserspritzer has an effective spatial resolution of 10 (minimal intensity) to 40 \( \mu \text{m} \) (supra-threshold). Therefore, when the laserspritzer was placed at \( >40 \) \( \mu \text{m} \) away from soma, it had negligible effects on ChR2 currents in the recorded soma (Sun et al. 2014). We conclude that the spatial resolution of the laserspritzer was less than 40 \( \mu \text{m} \) (Sun et al. 2014), a resolution similar to or better than that achieved with the CRACM (Petreanu et al. 2007, 2009).

**Comparison of AIS and somatically induced ChR2-IPSCs**

Subsequently, we examined the properties of ChR2-IPSCs induced by either the AIS or somatic stimulation in layer IIa semilunar cells, respectively. Semilunar cells have no basal dendrites which makes them ideal for avoiding basal dendrite contamination during AIS stimulations (Haberly, 1983; Valverde & Santacana, 1994; Suzuki & Bekkers, 2006). Each recorded cell and its axons were

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**Figure 1. Strategy for subcellular optical stimulation: the laserspritzer approach**

A, fabrication of laserspritzer. Microscopic images of a fibreoptic cable at different stages of fabrication. Blue dotted line highlights the size of laser illumination in air. B, photomicrograph of recorded neurons and laserspritzer in soma vs. axon location in a slice, visualized under differential infrared contrast (DIC). Scale bars = 10 \( \mu \text{m} \). Ca, schematic diagram (left) and photo of a semilunar cell with axon (right) showing the subcellular optical activation at the soma and axon location. Scale bar = 10 \( \mu \text{m} \). Cb and c, confocal images of an axon-intact (b) and an axon-truncated (c) Alexa 594-filled semilunar neuron (magenta), surrounded by the VGAT-ChR2-YFP (green). Scale bars = 10 \( \mu \text{m} \).
visualized via intracellular loading of Alexa 594 (Fig. 4Aa). First, we placed the laserspritzer near the soma and induced perisomatic ChR2-IPSCs with minimal laser intensity. Next, we moved the laserspritzer 40–50 μm away from the soma, towards layer III of the PC (i.e. AIS region) and induced axonic ChR2-IPSCs using the same intensity (Fig. 4Aa and Ba). Prior to recording, we determined if the axon of recorded cells was intact (i.e. > 20 μm in length), and subsequently grouped all cells into axon-intact and axon-truncated cell types (Fig. 4Ab vs Aa). For both cell types, ChR2-IPSCs recorded at the two locations (i.e. soma and AIS) were examined. We found that the IPSCs induced at either location embodied similar amplitudes (Fig. 4Ca and Da) and kinetics in cells with intact axons (Figs. 4 and 5). Conversely, in cells with truncated axons, events were induced only at the soma but not the AIS (Fig. 4Bb). The results suggest that in neurons with intact axons, ChR2-IPSCs induced at the AIS were likely to be mediated by AASs. We also examined the jitter of response onsets for the soma- and AIS-induced responses in intact neurons (Fig. 5Aa), and found that all events had a narrow window of jitter (jitter = 1.61 ± 1 ms, n = 12 at soma and 1.15 ± 0.8 ms, n = 12 at AIS). This suggests that the ChR2-IPSCs induced at both locations were most probably monosynaptic events that did not originate from mixed sources (e.g. disynaptic or polysynaptic). To further characterize the properties of the ChR2-IPSCs, we first analysed the amplitude of ChR2-IPSCs at the two locations (i.e. soma vs. AIS). In intact neurons, multi-peak Gaussian curves fitted for amplitudes at the AIS versus soma location showed similar distributions (centre, width and height; Fig. 4Ca, n = 16 cells). There were no significant differences between the normalized amplitudes recorded at the two locations tested with one-way ANOVA (P = 0.29) and non-parametric Wilcoxon row-ranking test (P = 0.97, shown in Fig. 5Ab). Cumulative fraction plot for the amplitude histogram largely overlapped (Fig. 4Da). In contrast, in axon-truncated neurons, normalized amplitudes recorded showed significant differences (one-way ANOVA, P < 0.001) between the two locations. A cumulative fraction plot for the amplitude histogram of the axon location showed a significant shift to the left (Fig. 4Db). There were no significant differences in amplitudes of ChR2-IPSCs between the soma- versus axon-induced synaptic responses in neurons with intact axons. There were no significant differences in the soma-induced synaptic responses between axon-intact vs. axon-truncated neurons (Fig. 5B). We therefore concluded that the amplitudes of ChR2-IPSCs mediated by AASs and perisomatic inhibitions were similar when they were both recorded at the soma. The averaged time to peak (ms) and decay time constant (τ, ms) of all events in each cell was analysed (Fig. 5C). No significant differences were found between soma- and AIS-induced responses in axon-intact neurons and soma-induced responses in axon-truncated neurons. Hence, we concluded that the strength and kinetics of AAS-IPSCs were similar to those mediated by perisomatic ChR2-IPSCs evoked and recorded in the somata of the same neurons. However, given the location of the AIS (~40 μm from the soma, the recording site), it is likely that local IPSCs at the AIS are more potent than perisomatic IPSCs (see Discussion).

**Effects of microsurgical removal of axons**

To help validate the axonal origin of ChR2-IPSCs induced at the AIS and estimate the relative weight
of AAS vs. non-AAS inhibitions, we first performed acute micro-axotomy experiments. We initially placed an extracellular stimulating electrode near the recorded semilunar cell and recorded evoked IPSCs. Next, we moved a patch pipette with negative pressure (suction) across the main axons to achieve acute microsurgical axotomy (Fig. 6Aa). We found that the amplitude of evoked IPSCs (eIPSCs, $V_{\text{Hold}} = 0$ mV) but not evoked EPSCs (eEPSCs, $V_{\text{Hold}} = -50$ mV, the reversal potential of GABA$_{A}$-mediated IPSCs), was reduced by 51±8% (Fig. 6Ab, $n = 8$ cells). Because the microsurgery only affected a small area local to the AIS specifically (Fig. 6Aa, right), the results suggest that AASs contribute to nearly 50% of total eIPSCs. Furthermore, we designed a novel slice cutting method using CamKII-GFP mice to visualize layer II glutamatergic cells. A modified tangential slice was made perpendicular to the direction of descending axons of layer II semilunar cells (Fig. 6B). The thickness of the slice was 200 $\mu$m, designed to preserve the somata but not axons of layer II semilunar cells (Figs. 6Bf and 7). We found that the amplitude of eIPSCs but not eEPSCs in these cells was significantly smaller than the amplitude recorded in a 200 $\mu$m slice (Fig. 6Bh). In summary, our local and slice axon truncation experiments were supportive of the idea that AASs make significant contributions to the eIPSCs recorded in the somata of layer II semilunar cells.

Optogenetically induced AAS-IPSCs persist under a blockage of action potentials with TTX

Long-range monosynaptic glutamatergic EPSCs can be evoked by photostimulation of ChR2 in the presence of TTX. However, the ChR2-mediated current alone is usually not sufficient to trigger glutamate release. Therefore, 4-AP (1 mM) has typically been applied with TTX to induce monosynaptic EPSCs from these terminals, presumably by augmenting the depolarization at the terminals (e.g. Fig. 7B; Petreanu et al. 2007, 2009; Cruikshank et al. 2010). It is unclear if a similar pharmacological ‘cocktail’ recipe would work for ChR2 located in GABAergic terminals. Firstly, we made whole-cell recordings from semilunar cells, and applied laserspritzer stimulation in the soma and AIS, respectively,
to induced perisomatic and AAS-IPSCs (Fig. 7). As anticipated, bath application of TTX (1 mM) abolished the ChR2-IPSCs in both somatic and axonal locations (Fig. 7C). To our surprise, adding 4-AP (1–10 mM) did not restore the ChR2-IPSCs (Fig. 7D, n = 20 cells). In contrast, the combination of TTX (1 mM) and TEA (7.5 mM) restored ChR2-IPSCs to levels similar to control aCSF levels in both somatically and axonically induced IPSCs (Fig. 7E and F). Thus, our data show for the first time that at GABAergic terminals (basket and chandelier), TEA-sensitive channels play a more prominent role in the regulation of nerve terminal depolarization.

**ChR2-assisted circuit mapping (CRACM) of inhibitory inputs**

To provide comprehensive information on the strength of inhibitions in different subcellular locations of PC semilunar cells versus in neocortical pyramidal neurons of S1, we used the CRACM method (with similar spatial resolution to our laserspritzer, Petreanu et al. 2007, 2009) in VGAT-ChR2-YFP mice. The CRACM methods have traditionally been used to provide information on glutamatergic inputs. Glutamatergic antagonists NBQX (2 μM) and CPP (5 μM) were applied to eliminate potential contamination from spontaneous glutamatergic EPSCs. As shown in Fig. 8B, laser that was applied near the AIS region induced IPSCs with the largest amplitudes in PC semilunar cells (n = 5). In contrast, the perisomatic stimulation induced IPSCs with smaller amplitudes. Neocortical pyramidal neurons in layer 5B of S1 showed small amplitude IPSCs associated with axon stimulation (Fig. 8Ab and c, white arrow), which was consistent with previous data in these neurons using paired recordings (Jiang et al. 2013). As a comparison, the inhibitory map of a single PC semilunar cell shows a characteristic ‘tail’ that highly corresponds with the location of the AIS (Fig. 8Bc, white arrow). These results were consistent with our laserspritzer experiments, suggesting that PC semilunar cells were innervated by robust AASs. Next, we changed the laser intensities and examined the map changes within PC semilunar cells. In 3 out of 4 cells tested, AASs were reduced by 50% with lower laser intensities (e.g. Fig. 8Ca). We further examined this with single axon location laserspritzer stimulation by incrementally increasing the laser intensities. Our data show that in 50% of the cells, one stimulation site (~10 μm) was innervated by only one AAS, because the amplitude of AAS-IPSCs did not change (Fig. 8Cb). However, in about 40% of cells, AAS-IPSC amplitudes increased by one- to twofold (Fig. 8Cb). Due to the spatial limitations of the laserspritzer stimulation, we concluded that a single axonal stimulation site (~10 μm length with minimal intensity laser stimulation) is innervated by one to three independent axons. However, the distribution of AASs is spread out among the entire AIS (~50 μm, see Fig. 9 and next section below).

**AASs innervate the entire length of the AIS**

To further examine spatial distribution of AASs along the AIS, we first examined the distribution of AAS terminals

![Figure 4. ChR2-mediated inhibitory postsynaptic currents (ChR2-IPSCs) at the AIS vs. soma location](image-url)
(visualized by GAT-1 puncta) along the AIS (visualized by ankyrin-IR). AAS innervation is defined with a clear overlap between GAT-1 immuno-positive puncta on the ankyrin-IR-positive AIS within at least two dimensions using z-stack confocal imaging (Fig. 9A, panels 3–5). The densities of GAT-1-positive puncta were plotted against the distance from the soma along the AIS. Our data indicate that GAT-1-positive puncta were distributed uniformly along the entire length of the AIS (45±6 μm, Fig. 9B), suggesting that the entire length was innervated by AASs. To further examine if these innervations were functional, we made whole-cell recordings from semilunar cell pairs (n = 7), located within a 10 μm range (e.g. Fig. 9Ca) of TTX (1 μM) and TEA (7.5 mM). In each cell, the laserspritzer was first placed at the soma, then moved in 10 μm increments along the main axis of the AIS toward layer III (Fig. 9Ca). Our data show that axonal laser stimulation induced robust IPSCs throughout the entire length of axons in cells with intact axons. In contrast, in adjacent (<10 μm) axon-truncated cells, the same axon stimulations did not induce significant IPSCs at any axonal sites (Fig. 9Cb). Since the spatial resolution of the laserspritzer was larger than the distance between the two recorded adjacent cells which were surrounded by the same axon layers, therefore, it was highly unlikely that the light stimulation-induced IPSCs were due to passing basket axons near AIS regions. Furthermore, transmission along passing axons requires action potential propagation, which was eliminated by TTX, especially on the distal stimulation site (>30 μm). Therefore our data support a novel hypothesis that AASs innervate the entire length of the AIS with equal strength.

Figure 5. Properties of ChR2-IPSCs
Aa, ChR2-IPSC onset jitter (ms). Left, representative traces of soma and axon ChR2-IPSCs showing the onset portion; right, jitter of IPSC onsets from soma vs. axon location in the same cell were plotted. Continuous line, y = x; dotted line, linear fitting of the data points (n = 12 cells, P = 0.36, R² = 0.59). Ab, the amplitudes of somatic ChR2-IPSCs are plotted against axonic ChR2-IPSCs in the same cell. Continuous line, y = x; dotted line, linear fitting of the data points (n = 15 cells, P = 0.97, R² = 0.81). B and C, ChR2-IPSCs amplitude (Ba), CV (Bb), time to peak (Ca) and decay time constant (τ, Cb) in all cells groups. In all box charts, the values represented are the minimum, 25th percentile, median, 75th percentile, and the maximum. **P < 0.01.
Both AAS and somatic ChR2-IPSCs are hyperpolarizing and suppress spikes across a wide range of membrane potentials

In order to maintain intact intracellular Cl− concentration, we performed gramicidin-based perforated patch recordings (see Methods). The GABA reversal potentials ($E_{\text{GABA}}$) for ChR2-IPSCs recorded from and induced at the soma and AIS were very similar (soma location, $n = 8$ cells, $E_{\text{GABA}} = -72.8 \pm 1.1$ mV; AIS location, $n = 22$ cells, $E_{\text{GABA}} = -73.6 \pm 1.2$ mV, $P = 0.71$, Fig. 10A). Next, the effect of ChR2-IPSCs on postsynaptic excitability was investigated under a range of membrane potentials under current clamp. The mean series resistance was $50.3 \pm 2.8 \text{ MΩ}$. After obtaining perforation, spiking in the recorded cells was initiated by supra-threshold current

![Figure 6. Axon lesion experiment](image)
injections achieved by a current steps (80 ms) of increasing intensities (200 ± 40 pA) until spikes were induced consistently. To examine the effect of ChR2-IPSCs on postsynaptic spiking probabilities under different ‘resting’ membrane potentials, the recorded neurons were held at variable membrane potentials, ranging from −50 to −90 mV, in 10 mV increments. Our results showed that laserspritzer stimulation (6 ms) at either the soma or AIS location had inhibitory effects on spike induction (Fig. 10C). This effect was consistent over the range of membrane potentials (−50 mV to −90 mV, in 10 mV increments). In all cells that were tested for paired

Figure 7. Monosynaptic nature of ChR2-IPSCs
A, the laserspritzer is placed at the soma and AIS location of a semilunar neuron (SN) to stimulate axonal terminals provided by chandelier cells (ChC) and basket cells (BC) respectively. B, schematic diagram showing the terminal of a GABAergic synapse and hypothetical locations of ion channels. C, representative traces of ChR2-IPSCs with (grey trace) and without TTX (black trace) application. D, representative traces of ChR2-IPSCs with no drugs (black), 1 μM TTX and 0.3 mM 4-AP (blue), 1 μM TTX and 7.5 mM TEA (red), and 7.5 mM TEA alone (green). Blue bars represent time of the laser stimulation. E, time series showing the ChR2-IPSC amplitudes over the entire course of the experiments. Open and filled circles represent the IPSC amplitudes with the application of TEA+TTX and 4-AP+TTX, respectively. Open and filled bars indicate the timing of drug application. F, normalized amplitudes of ChR2-IPSCs before and after TEA+TTX application induced at soma location (top) or axon location (bottom).
stimulations (i.e. somatic vs. axonic), the overall results were that AAS inhibitions provide a statistically stronger suppression of spike induction (Fig. 10Cb, n = 20 cells, \( P < 0.05 \) tested with \( t \) test and one-way ANOVA). We therefore concluded that both AAS and perisomatic inhibition provided robust inhibition and independently abolished firings induced by somatic depolarization; however, AASs were more powerful in inhibiting spikes induced by somatic current injections.

AAS activation significantly reduces epileptiform discharges \textit{in vitro}

Lastly, we investigated the effect of AAS activation by laserspritzer stimulation on the stimulus-induced epileptiform discharges. Electric stimulation at layer III of the PC in the presence of 0 mM Mg\(^{2+}\) aCSF and 4-AP solution (see Methods) induced epileptiform after-discharges (Fig. 11B). In order to maintain intracellular Cl\(^-\) concentration, cell-attached voltage-clamp

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**Figure 8. CRACM of inhibitory inputs in neocortical and PC cells**

Aa and Ba, example CRACM map superimposed on a brightfield image of S1 (Aa) and PC (Ba) brain slice. Responses were induced by a 1 mW, 1 ms blue (473 nm) laser flash, with the laser scanned in a grid pattern (50 \( \mu \)m spacing, 12 \( \times \) 26). Ab and Bb, example traces showing inward IPSCs recorded at \( V_{\text{hold}} = -60 \) mV in the presence of glutamate antagonist NBQX (2 \( \mu \)M) and CPP (5 \( \mu \)M). Ac and Bc, example of inhibitory input maps superimposed on the reconstructed neurobiotin-labelled and computer-reconstructed S1 pyramidal (Ac) and PC semilunar (Bc) neurons. Triangles indicate the location of the soma in the recorded cell. The white arrow indicates that strong inhibition coincides with the AIS of the recorded cell. Ca, effects of reducing laser power on the inhibitory map in the same cell as in B. White arrows, AIS location; white square, map of somatic inputs. Cb, AASs were induced by the laserspritzer with varying laser intensities. Amplitudes of AAS-IPSCs were normalized to those induced by minimal laser intensities. A histogram plot shows the distribution of normalized AAS-IPSCs. Dashed lines, one- or twofold normalized amplitude.

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recordings (patch pipette solution had a reversal potential for \( \text{GABA}_A = -40 \text{ mV} \) when recorded at whole-cell mode) were carried out after gigaohm seal formation. Whole-cell current-clamp recordings were also repeated in the same cells. The pipette solution contained Alexa 594 to validate the presence or absence of AIS (e.g. Fig. 11A). The burst after-discharges were largely eliminated by laserspritzer stimulation (6 ms) at the axon location. The epileptiform activities returned to control level after the laser stimulation was switched off (Fig. 11B). Single laserspritzer stimulation at the axon location 1–100 ms after electric stimulation reduced epileptiform after-discharges by 64 ± 11% under cell-attached mode \((n = 13, P = 0.006, \text{Fig. 11Cc})\). Laserspritzer stimulation at the soma location (6 ms) had a significantly smaller effect (reduced by 45±11%, \(n = 10\)). There were significant differences in the effects on spike number induced by laserspritzer stimulation between the AIS or soma locations (Fig. 11Cc \(P < 0.05\)). Therefore, we concluded that AIS and somatic stimulation alone can both effectively suppress epileptiform activities, however, the AASs were more effective at suppressing spike inductions under the same epileptic conditions.

**Discussion**

This study took advantage of the optogenetic approach to study, for the first time, the properties and function of AASs *in vitro*. To activate and study AASs, we developed a novel fibreoptic electrode laserspritzer with a spatial resolution of less than 40 \(\mu m\) (Sun *et al.* 2014). The laserspritzer is inexpensive and can be adopted with ease in an electrophysiological setup. The size, light weight and flexible nature of the laserspritzer enables rapid and convenient micromanipulation near recorded neurons (Sun *et al.* 2014). The laserspritzer can be inserted into deep slices near the axonal or dendritic branches of recorded neurons, which provides selective targeting of subcellular compartments (e.g. apical or proximal dendrites). This study provides an example of how this novel approach can be employed to examine inputs formed on different subcellular compartments at

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**Figure 9. AASs were distributed along the entire AIS**

A, representative confocal image shows GAT-1 puncta location along the ankyrin-IR positive AIS. Single (1, 3 4 and 5; scale bar = 1 \(\mu m\)) and 3D flattened confocal photos (2, scale bar = 5 \(\mu m\)). B, distribution of GAT-1 puncta along the entire ankyrin-G positive AIS (45 \(\mu m\)) was determined by using the high resolution z-stack images as shown in A3–5. C, whole-cell recordings were made from a pair of Alexa 594-filled semilunar cells (magenta, one intact (top) and one with axon truncation at AIS (bottom)) located less than 10 \(\mu m\) apart (Ca). ChR2-YFP is shown in green. IPSCs were induced by somatic or axonic stimulation in 10 \(\mu m\) increments along the AIS. Scale bar = 10 \(\mu m\). Cb, the amplitudes of IPSCs induced at different axonal locations were plotted against the amplitudes of somatically induced IPSCs in axon intact cells (black squares) vs. axon truncated cells (black triangles, \(n = 7\) pairs).
a resolution similar to or better than the widely used CRACM approach. Different sizes of laser illumination areas can be achieved by adjusting the laser intensity. The PC was chosen as the brain region of interest because it has the highest AAS density across all brain regions (Wang & Sun, 2012). We selected semilunar cells in all of our recordings to characterize ChR2-mediated synaptic currents. They are a group of excitatory neurons located exclusively in layer IIa of the PC, and lack basal dendrites (Haberly, 1983; Valverde & Santacana, 1994; Suzuki & Bekkers, 2006). By making recordings in PC semilunar cells, we avoided the activation of ChR2-IPSCs from basal dendrites near the AIS location. In axon-truncated neurons, no ChR2-mediated inhibitory synaptic events were detected when the laserspritzer was placed at the AIS location, suggesting that the ChR2-IPSCs induced at this location were mediated by AASs. It is also unlikely that more perisomatic GABAergic axons were severed in axon-truncated cells, because somatically induced ChR2-IPSCs remained the same (Fig. 5Ba). We further reasoned that the ChR2-IPSCs induced at the axon location were unlikely to be the result of direct activation of interneuron soma due to the scarcity of interneurons within layer II of the PC, compared to pyramidal neurons. Moreover, ChR2-IPSCs induced by the laserspritzer had a very narrow onset window (jitter less than 1.6 ms; Fig. 5Aa) which was consistent with the properties of monosynaptic synaptic events. The laser intensities applied at the AIS location to induce ChR2-IPSCs were much lower than the intensities required for inducing action potentials in the soma of interneurons (e.g. Fig. 3Ca). This may occur because of the small volume and large resistance of the ChR2-containing synaptic terminals. Finally and most importantly, the laserspritzer-induced IPSCs persisted in the presence of TTX and TEA (Fig. 7) and were removed by the microsurgical lesion of axons, suggesting that release sites were located at stimulated terminals. For these reasons, we believe that monosynaptic inhibitory synaptic events were activated by the laserspritzer at the AIS.

**AACs are inhibitory in the PC**

The physiological function of AACs has been a controversial issue. Early studies of AAC function revealed an inhibitory function using paired recordings between a presynaptic AAC and a postsynaptic pyramidal neuron

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**Figure 10. The effects of ChR2-IPSCs on neuronal spiking probabilities recorded using a gramicidin-based perforated patch**

Aa, representative gramicidin-based perforated patch recordings of averaged traces of ChR2-IPSCs at axon location induced under different membrane potentials. Ab, current–membrane potential relationship of ChR2-IPSCs induced at axon (red) and soma (black), respectively (axon location, n = 22 cells; soma location, n = 10 cells). Ac, GABA reversal potentials (mV) at axon (red diamonds) vs. soma (black squares) in axon-intact neurons only. B, current injection protocol and the resulting representative current clamp traces in a PC neuron recorded using gramicidin-based perforated patch. Blue bar represents the timing of the laser stimulation. Ca, normalized spiking probabilities with the laserspritzer at axon vs. soma location, respectively (axon location, n = 33 cells; soma location, n = 18 cells). x-axis, timing of spike train with laser stimulation applied at time 0. Cb, effect of laserspritzer stimulation at axon vs. soma location on spiking probabilities in the same cells (n = 20 cells with 10 cells in each group, *P < 0.05).
In contrast, several more recent studies using perforated-patch recordings in pyramidal neurons reported that there was a depolarizing effect on AACs and that $E_{GABA}$ at the AIS was depolarized by $\sim 20$ mV more than the resting potential (Szabadics et al. 2006; Khirug et al. 2008; Woodruff et al. 2009). Using a less invasive approach (local unitary field potential recording), Glickfeld and colleagues demonstrated that AACs primarily played an inhibitory role in the hippocampus (Glickfeld et al. 2009). In the current study, we showed that the inhibition provided by AASs had a reversal potential of $-73.6 \pm 1.2$ mV, and showed consistent inhibitory effects of AASs on spike induction across a wide range of membrane potentials tested (Fig. 10C). The reasons for the controversies over AAC function among these studies may include the heterogeneity of AACs, varying animal ages, and some technical differences. In our study, a presumed homogeneous population of AACs innervating layer IIa of the PC was studied. All GABA reversal potential measurement and the evaluation of the effects of AAC inputs on postsynaptic spiking were performed using gramicidin-based perforated patches for the maintenance of intact intracellular Cl$^{-}$ concentration (Abe et al. 1994; Ebihara et al. 1995; Kyrozis & Reichling, 1995). Though no information is available on the maturation of AASs in mice, several studies showed a protracted maturation of AASs in monkey prefrontal cortexes throughout the juvenile stage (Cruz et al. 2003; Fish et al. 2013), suggesting that the age of animals for studying the function of AACs is critical. The ages of the animals in the current study were between postnatal weeks 6 and 8, which ensured the developmental maturation of AASs. Furthermore, anatomical and physiological studies have reported that each AIS is innervated by more than one AAC in the neocortex and the PC (Fairen & Valverde, 1980; De Carlos et al. 1987; Tamas & Szabadics, 2004; Larriva-Sahd, 2010; Inan et al. 2013). Although it is impossible to determine the exact number of AASs/AACs activated by laser stimulation, the location-specific activation approach in the current study

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Figure 11. The effects of ChR2-IPSCs on stimulus-induced epileptiform after-discharges recorded using cell-attached recordings.  
A, an Alexa 594-filled cell with intact axon. B, representative traces of a cell-attached recording under voltage-clamp mode, showing the effect of AAS and perisomatic innervation activations on stimulus-induced epileptiform after-discharges induced by electric stimulation of layer III in a modified aCSF containing 0-Mg$^{2+}$ and 500 μM 4-AP. Vertical black bars represent the time for single electric stimulations and blue bars represent the time for laser stimulations. Ca, representative traces for a cell-attached recording under voltage-clamp mode. Top and bottom traces were collected with and without laser stimulation at the axon location, respectively. Cb, spike numbers of individual neurons with and without laser stimulation at axon and soma location, respectively.Cc, normalized spike numbers obtained from cell-attached recordings in all groups (**P < 0.001, *P < 0.05).
provided, for the first time, the capability of activating all axo-axonic innervations onto a single AIS from multiple AACs (Fig. 9C). This is physiologically relevant because several studies have reported electric coupling between AACs, but the function and power of the coupling is largely unknown (Woodruff et al. 2011; Tamas & Szabadics, 2004).

**Function of AACs within the PC and in antiepileptic treatment**

The PC circuit is a highly unique model circuit for the study of cortical sensory processing and associative memory (Haberly & Bower, 1989; Johnson et al. 2000; Haberly, 2001; Franks & Isaacson, 2005; Barnes et al. 2008; Bathellier et al. 2009; Johenning et al. 2009; Stettler & Axel, 2009; Poo & Isaacson, 2009, 2011; Davison & Ehlers, 2011; Franks et al. 2011; Wiegand et al. 2011). Inhibitory interneurons in the PC have long been appreciated for their role in the regulation of the temporal patterning of neural activity, olfactory coding and circuit oscillations (Wilson & Bower, 1992; Haberly, 2001; Luna & Schoppa, 2008; Kay et al. 2009; Poo & Isaacson, 2009, 2011; Kay & Beshel, 2010; Zhan & Luo, 2010; Zelano et al. 2011; Suzuki & Bekkers, 2012). Among the diverse interneuron populations in the PC (Young & Sun, 2009; Suzuki & Bekkers, 2010a,b), the physiology and function of AACs has never been characterized. A few recent studies found that AACs are located in layers II and III (Larriva-Sahd, 2010; Wang & Sun, 2012) with their axonal terminals encasing the AIS of neighbouring pyramidal neurons and their dendrites extending to layer Ib and layer II. This suggests that AACs are recruited by glutamatergic inputs from intracortical or non-piriform association fibres. Our results demonstrated a strong inhibitory power of AACs, suggesting their crucial role in the cortical feedback regulation of spiking timing in glutamatergic neurons. Substantial evidence has implicated the loss or reorganization of AASs in the pathology of epilepsy. Close examination of surgically removed cortices from human epileptic patients demonstrated a selective yet significant loss of AASs at the epileptic foci (Ribak, 1985; Marco & DeFelipe, 1997), and apparent reorganization in the surrounding areas (Arellano et al. 2004). One of the proposed models for epileptogenesis is that the selective loss of AASs at the epileptic foci causes the auto-associative fibre system to be especially vulnerable to epileptiform activity initiation (Williams et al. 1977; Ribak, 1985; Freund & Buzsaki, 1988; Marco & DeFelipe, 1997; Arellano et al. 2004). The PC has been shown to be the region most prone to seizure activities, as well as chronic epileptic genesis within the limbic system (Racine et al. 1988; Loscher & Ebert, 1996). In the barrel cortex of mice, chandelier cells were found to respond more robustly than any other type of cortical neuron to overall excessive cortical excitation (Zhu et al. 2004). Our results indicate that AACs exert powerful and transient inhibitions in the PC, suggesting that they may also be critical for dampening excessive cortical excitation, making them a possible therapeutic target for epilepsy treatment. However, the capacity of AASs in controlling excessive network activities, such as epileptiform discharges has not been investigated directly. In the current study, an in vitro epilepsy model (stimulus-induced epileptiform activity in the presence of 0-Mg²⁺ and 4-AP) was used to study the effect of AAS activation by laser stimulation. With both cell-attached and whole-cell mode recordings, we demonstrated that AAS activation was sufficient to reduce the stimulus-induced epileptiform after-discharges by 64 ± 11%, significantly higher than that produced by perisomatic inhibitions (45 ± 11%, Fig. 11C). This suggests that chandelier neurons play a critical role in controlling excessive innervations induced by recurrent network activities in the PC.

**Conclusions**

Using a novel optogenetic approach that we developed (Sun et al. 2014), we have provided evidence of the inhibitory role of AASs. Our anatomical and physiological results led to the following novel conclusions. (1) AASs innervate the entire length of the AIS, as opposed to forming a highly concentrated cartridge. (2) AAS inhibition suppressed action potentials and epileptiform activity more robustly than perisomatic inhibitions. Due to the unique laminar location of dendrites of chandelier cells (IB and II; Larriva-Sahd, 2010; Wang & Sun, 2012) and powerful innervation within the AIS region of PC principal neurons, AACs may play a unique role in both the regulation of neural oscillations and the controlling of excess network excitations within the PC. (3) AAS activation alone can be sufficient to put a brake on action potential generation and epileptiform activities in vitro.

**References**


Additional information

Competing interests

The authors declare no conflicting interests.

Author contributions

X.W. and Q.Q.S. conceived and designed the experiments. X.W. and Q.Q.S. performed the experiments. X.W., B.M.H. and Q.Q.S. analysed the data and wrote the paper. All authors read and approved the final version of the paper.

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