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Characterization of Axo-Axonic Synapses in the Piriform Cortex of *Mus musculus*

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Abstract

Previous anatomical and physiological studies have established major glutamatergic and GABAergic neuronal subtypes within the piriform cortical circuits. However, quantitative information regarding axo-axonic inhibitory synapses mediated by chandelier cells across major cortical subdivisions of piriform cortex is lacking. Therefore, we examined the properties of these synapses across the entire piriform cortex. Our results show the following. 1) γ -Aminobutyric acid membrane transporter 1-positive varicosities, whose appearance resembles chandelier cartridges, are found around the initial segments of axons of glutamatergic cells across layers II and III. 2) Both the density of axo-axonic cartridges and the degree of γ -aminobutyric acid membrane transporter 1 innervation in each axo-axonic synapse are significantly higher in the piriform cortex than in the neocortex. 3) Glutamate decarboxylase 67, vesicular GABA transporter, and parvalbumin, but not calbindin, are colocalized with the presynaptic varicosities, whereas gephyrin, Na-K-2Cl cotransporter 1, and GABA_A receptor $\alpha 1$ subunit, but not K-Cl cotransporter 2, are colocalized at the presumed postsynaptic sites. 4) The axo-axonic cartridges innervate the majority of excitatory neurons and are distributed more frequently in putative centrifugal cells and posterior piriform cortex. We further describe the morphology of chandelier cells by using parvalbumin-immunoreactivity and single-cell labeling. In summary, our results demonstrate that a small population of chandelier cells mediates abundant axo-axonic synapses across the entire piriform cortex. Because of the critical location of these inhibitory synapses in relation to action potential regulation, our results highlight a critical role of axo-axonic synapses in regulating information flow and olfactory-related oscillations within the piriform cortex in vivo.

INDEXING TERMS

olfaction; interneuron; axo-axonic synapses; GABA; inhibitory network; olfactory cortex

The identification, categorization, and discrimination of olfactory stimuli rely on the neural processing in the piriform cortex (PC; Wilson et al., 2006; Gottfried, 2010). Network oscillation between the olfactory bulb and the cortex is an important feature of the olfactory system during olfactory processing (Freeman, 1978; Kay and Freeman, 1998). Excitatory pathways linking these two circuits occur through mitral cell axons projecting to the PC (Haberly and Price, 1977; Luskin and Price, 1983). However, the exact mechanism of the oscillations in the PC remains undefined. In vitro models of oscillations, such as gamma (30–80 Hz) and theta (5–12 Hz) rhythms, demonstrate an absolute requirement for phasic inhibitory synaptic transmission (Whittington and Traub, 2003b; Buzsaki and Draguhn,

2004; Buzsaki et al., 2004). Distinct interneurons with different firing patterns and axonal targets (dendritic vs. somatic) may play distinct roles in the formation of different forms of oscillations in vivo. A complete understanding of mechanisms underlying odorant induced oscillations in the cortex requires a thorough knowledge of interneuronal subtypes and their synaptic targets in the olfactory networks. This knowledge of fine structural organization of inhibitory and excitatory microcircuits is a prerequisite for thorough understanding of the central mechanism of olfactory processing (Restrepo, 2009; Isaacson, 2010).

Several studies have been conducted to characterize the inhibitory neurons in the PC, with the focus on basket cells (Haberly and Feig, 1983; Haberly, 1983; Ekstrand et al., 2001). Recently, rapid progress has been made in understanding the diversity of interneurons in the PC (Zhang et al., 2006; Young and Sun, 2009; Gavrilovici et al., 2010; Suzuki and Bekkers, 2010; Stokes and Isaacson, 2010). Among the diverse groups of interneurons identified, distinct interneurons providing inhibition onto different layers (Young and Sun, 2009; Suzuki and Bekkers, 2010) or different cellular compartment (e.g., dendrites and soma of pyramidal cells) have been identified (Stokes and Isaacson, 2010). However, chandelier cells have not been characterized physiologically or morphologically in the aforementioned studies. Axo-axonic inhibitory synapses (AAIS) innervating pyramidal neurons provided by chandelier cells in the PC have been described by several groups (DeFelipe, 1999; Inda et al., 2009; Larriva-Sahd, 2010). The function of AAIS is important for the following reasons. 1) AAIS form a high density of synapses onto the initial segment of pyramidal neurons, in close opposition to voltage-gated sodium channels (DeFelipe, 1999; Howard et al., 2005). 2) Inhibition from AAIS can effectively shut down the outputs of the pyramidal cells (Zhu et al., 2004; Szabadics et al., 2006). Despite the importance of AAIS, quantitative anatomical and histological information on AAIS in the PC is still lacking. For example, the AAIS distribution in different subdivisions of PC is currently unknown (anterior piriform cortex [APC] vs. posterior piriform cortex [PPC], different layers; Johnson et al., 2000; Illig and Haberly, 2003). The detailed properties of AAIS in the PC, e.g., molecular composition and density of innervation per AAIS, are unclear. Morphological and physiological properties of chandelier cells supplying the AAIS are also still uncharacterized. The goal of the present study is to examine comprehensively the detailed anatomical properties of AAIS in APC and PPC, with the goal of providing necessary information for future physiological study or pharmacological and molecular targeting of these synapses.

MATERIALS AND METHODS

Animals

Six adult male CD-1 mice, two additional male adult GAD67-GFP-positive mice on a CD-1 background strain (provided by Dr. Yuchio Yanagawa), and two additional male adult CamKII-GFP mice on a CD-1 background strain (provided by Dr. Gábor Szábo) were used in this study. All mice were housed at the University of Wyoming animal facility on the fifth floor of the Biological Sciences Building in a temperature-controlled environment with a 12-hour light/12-hour dark cycle. The use of mice was based on protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Wyoming.

Brain slice preparation, fixation, and immunohistochemistry

For tissue fixation, we used a light fixation approach as described by Schneider Gasser et al. (2006). Briefly, brain slices (300–450 μ m thickness) were made using a Vibratome. Sections were based on brain slice cutting methods we have described in electrophysiological recordings (Young and Sun, 2009). Slices were then transferred to scintillation vial containing 3 ml fixative (4% paraformaldehyde [PFA]) for 20 minutes. We next removed fixative and rinsed the slices with 0.01 M phosphate-buffered saline (PBS; pH 7.4) several

times before they were transferred to 3 ml cryoprotectant (30% sucrose solution in 0.1 M phosphate buffer) and stored for at least 2 days at 4°C. Brain slices were resectioned using a cryostat at -25°C into 10- or 40-µm sections. Free-floating sections were then stained for antibodies as follows: sections were rinsed in PBS twice; incubated for 30 minutes in PBS with 0.5% H₂O₂; washed twice for 10 minutes in PBS; incubated for 2 hours at room temperature in PBS with 0.3% Triton X-100, 0.05% Tween, and 4% normal goat serum; and incubated overnight at 4°C in PBS containing 0.2% Triton X-100 and primary antibodies listed in Table 1. Sections were then rinsed twice in PBS and incubated for 3 hours at room temperature in secondary diluents (PBS with 0.3% Triton X-100). The sections were then rinsed, mounted, and coverslipped using Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI) or without DAPI.

Antibody characterization

We examined the specificity of main antibodies by using Western blotting. Briefly, freshly dissected cortex containing piriform cortex and adjacent cortical areas from P30 CD-1 mice was immediately homogenized in ice-cold RIPA buffer (Thermo Scientific, Rockford, IL) and processed for Western blotting based on methods described earlier. The Western blotting results are shown in Figure 1.

Ankyrin-G—The antibody recognized two bands, at 480 kDa and 270 kDa, which are thought to correspond to the two forms of ankyrin on Western blots of Caco 2 (human colonic carcinoma cell line) whole-cell lysates (manufacturer's data sheet). It stained a specific pattern of cellular morphology and distribution in this study as described previously for monkey (Cruz et al., 2009), rat (Rasmussen et al., 2007; Van Wart et al., 2007), and mouse (Boiko et al., 2003; Van Wart et al., 2007) brain.

NKCC (H-110)—Mouse NKCC₁ and NKCC₂ share 100% identity. Human and mouse NKCC₁ share 94% identity. The antibody recognizes both NKCC₁ and NKCC₂ in mouse tissue (manufacturer's technical support). NKCC₂ is kidney specific and is absent in the brain across species (Lytle et al., 1995; Vanden Heuvel et al., 2006). The antibody recognized a single band of 135 kDa on Western blots of WI 38 (human lung fibroblast cell line) whole-cell lysates (manufacturer's data sheet; Xu et al., 1994). The antibody also detected a protein doublet (145–155 kDa) on Western blots of rat brain, and the signal was not seen when the antibody was preadsorbed with NKCC₁-MBP (maltose-binding protein) fusion protein (Plotkin et al., 1997). In mouse brain, it stained a pattern of cellular morphology and distribution in this study as previously described for mice brain (Delpire et al., 1999).

GAD67—GAD67 antibody recognized a single band of 67 kDa on Western blots of mouse brain lysates (Fig. 1B). Specific cellular morphology and distribution were similar to previous immunohistochemistry staining results with this antibody in rat (Rotllant et al., 2010), mouse (Jinno and Kosaka, 2010; Schubert et al., 2010), and human (Karolewicz et al., 2010) brain.

Calbindin—Calbindin antibody recognized a single band of 28 kDa on Western blots from rat brain homogenate, and the staining is specifically inhibited by the calbindin-D28K immunizing peptide (manufacturer's technical information). The pattern of cellular morphology and distribution in stained sections in this study was consistent with that shown previously in earlier studies (Gargini et al., 2007; Jakovcevski et al., 2009).

PV—The antibody reacted with PV from brain and muscle (Gomez-Nieto and Rubio, 2011; Currier et al., 2011). According to immunoblot of mouse brain lysates by the manufacturer,

it recognized a single band of 12 kDa. The antibody recognized another weak band at about 50 kDa in our Western blots of mouse brain lysates (data not shown). In this study, the antibody stained a pattern similar to that in previous reports on mouse brain (Zeng et al., 2009; Siembab et al., 2010).

KCC₂—The antibody recognized a single band of 140 kDa on Western blots of rat brain lysates. PreadSORption with 1 µg of control peptide per milliliter of diluted Ab abolishes the signal (Williams et al., 1999). An unexpected weak band at about 250 kDa was detected on our Western blots of mouse brain lysates (Fig. 1C). In mouse brain, it stained a specific pattern of cellular morphology and distribution as previously described (Blaesse et al., 2006; Delpy et al., 2008; Pradhan et al., 2011).

VGAT—The immunogen peptide shows no significant homology with other known proteins. Western blots of mice brain lysates detected only one expected band at about 51 kDa as shown in Figure 1D and as described previously (Tabuchi et al., 2007). In mouse brain, the antibody recognized a cellular morphology and distribution in this study similar to that previously reported (Tabuchi et al., 2007).

GABA_{Aα1}—On Western blots of mouse brain lysates, the antibody recognized a single band at about 51 kDa (Fig. 1A). Immunostaining of mouse brain with this antibody detected a significant reduction of signal in GABA_{Aα1} knockout mice compared with wild type (Lappe-Siefke et al., 2009). On Western blots of mouse brain lysates, the antibody recognized a single band at about 51 kDa (Tabuchi et al., 2007; Belichenko et al., 2009; Fig. 1A).

GAT-1—As described earlier (Garcia-Marin et al., 2010), a single band of 67 kDa was detected on Western blots of mouse brain homogenates, and the signal was abolished by preadsorption with a GAT-1 cognate peptide, GAT-1588–1599 (QAGSSASKEAYI; Guo et al., 2009). It also stained a specific pattern of cellular morphology and distribution as previously described for rat, mouse, monkey, and human brain (Ribak et al., 1996; Blazquez-Llorca et al., 2010; Galvan et al., 2010; Guo et al., 2009).

Gephyrin—Mouse and human gephyrin share 100% identity. Specificity of the antibody was validated with recombinant rat gephyrin expressed in *Escherichia coli* as a positive control and fibroblasts of human patients with gephyrin deletion as a negative control (Reiss et al., 2001). It stained the same pattern of cellular morphology and regional distribution in mouse brain in this study as in previous reports (Kralic et al., 2006).

Image processing

The immunofluorescent specimens were examined with an epifluorescent microscope (Carl Zeiss) equipped with AxioCam digital color camera. Double- or triple-immunofluorescent images were analyzed with the AxioVision LE imaging suite (Carl Zeiss). Confocal microscopy images were sampled with a Zeiss 710 microscope. Immunopositive cells were counted using the Automeasure function of AxioVision LE imaging suite, combined with manual corrections. At least three defined areas in each anatomical subdivision and layer were measured, and the number of immunopositive cells was counted within the areas through visualized quantification. Each count represented the average of at least five individually stained sections from three separate brains. To count the number of inhibitory boutons (GAT-1-IR), a Zeiss 710 confocal microscope equipped with a ×100 oil immersion plan achromatic objective and a ×20 objective lens was used. With the ×20 objective lens, we first identified subdivisions of the PC and then switched to the ×100 objective lens. The number of presynaptic inhibitory (i.e., GAT-1-positive) varicosities was counted in defined

regions in individual sections. Perisomatic inhibitory varicosities were defined as small (0.5–1 μm) GFP-positive puncta located within 1 μm of a GFP-negative cell body. After counting, all density data were corrected with a factor related to objects being measured and section thickness, based on the method of Abercrombie (1946), summarized by Guillery (2002). Briefly, Abercrombie's formula for the ratio of the "real" number to the observed number is $T/T + h$, where T = section thickness and h = mean diameter of the cell body (or diameter of ankyrin-G cartridges) along the axis perpendicular to the plane of the section, the z axis. We obtained precise distances between PV^+ cell body and ankyrin-G cartridges in the Z -axis by tracing the objects with Neurolucida.

Fluoro-Gold (hydroxystilbamidine) dye injection and IHC

The method for in vivo Fluoro-Gold (FG; Fluorochrome LLC) injection was approved by the local IACUC. Briefly, mice were deeply anesthetized with an IP injection of ketamine (80 mg/kg) and xylazine (9 mg/kg). Next, the head of the mouse was shaved, and the animal was placed in a stereotaxic apparatus and restrained using a snout clamp. The area of the head to be incised was disinfected with 75% ethanol. A small amount of lidocaine was administered to the area to be incised, and a single incision was made in the scalp along the midline of the skull. With forceps, the skin on the head was held open, and a small hole was made in the skull over one olfactory bulb using a dental drill. After the drilling, a small amount of FG was administered through the hole into the olfactory bulb using a needle. Once the dye had been applied, a small amount of antibiotic ointment (Neosporin) was applied to the skull, and the incision was closed using a single suture coupled with a small dab of ethyl cyanoacrylate (Krazy Glue) to ensure the closure of the wound. Mice were kept warm until recovery of motion was observed and were then placed in a clean cage with food and water. The length of the entire process was usually 0.5–2 hours. After recovery, mice were housed in individual cages and monitored daily for the presence/development of surgical complications such as skin or brain infection or swelling, altered food intake, pain, distress, etc. Mice that developed surgical complications were killed. After 5–7 days, mice were anesthetized with 55 mg/kg of Na pentobarbital and sacrificed for further examination as described above. In subsequent IHC experiments, FG^+ cells were directly visualized under a UV lamp and a DAPI filter.

Data analysis

Data were analyzed in either Excel (Microsoft). Parameters were compared across groups using single-factor analysis of variance (ANOVA) tests, followed by Tukey's honestly significant difference (HSD) test in order to determine intergroup significance. Data are presented as mean $\pm$ SEM, unless noted otherwise. Figures were created in CorelDraw X4 (Ottawa, Ontario, Canada). Photomicrographs were imported but not modified in any way during the figure production process.

RESULTS

Distribution of chandelier cell cartridges in PC vs. neocortex

Coronal APC and PPC sections (10 μm in thickness), as defined earlier (Johnson et al., 2000; Illig and Haberly, 2003), were processed with ankyrin-G and GAT-1 IHC and examined under a Zeiss 710 confocal microscope. In layers II and III of APC and PPC, ankyrin-G-IR and GAT-1-IR greatly overlapped with each other: ankyrin-G-IR appeared as intensely labeled, mostly vertically (some horizontally, particularly in layer III) oriented candlestick-like structures whose diameter tapered down to one end, with the wide end near the unlabeled soma of presumed glutamatergic neurons (Fig. 2), and GAT-1-IR appeared as a plexus of puncta wrapped around ankyrin-G-IR (Fig. 2). These observations agree with the morphology of axon initial segments (AIS) accompanied by chandelier-mediated AAIS

previously described for other brain regions (Inda et al., 2007, 2009; Cruz et al., 2009). Therefore, these structures were identified as AISs and AAIS, respectively. In layer I of APC, a dense plexus of GAT-1-IRs and ankyrin-G-IRs was also found in the lateral olfactory tract (LOT; Fig. 3); however, these structures lacked the distinctive feature of AIS (e.g., they lacked of vertical oriented cartridges and were thin and tortuous) and were not systematically analyzed in this study. Within the PC, the density of ankyrin-G-IR cartridges was highest in layer II of PPC ($0.003 \pm 0.001/\mu\text{m}^2$), followed by layer II of APC ($0.0022 \pm 0.001/\mu\text{m}^2$), layer III of PPC ($0.0013 \pm 0.001/\mu\text{m}^2$), and layer III of APC ($0.001 \pm 0.0005/\mu\text{m}^2$; Fig. 3). These values were several-fold higher than those of layer II/III of motor area of neocortex ($0.0007 \pm 0.0001/\mu\text{m}^2$, $n = 8$, $P < 0.01$; e.g., Fig. 4). Because the cell densities in these areas were intrinsically different, we next compared percentage of cells expressing the ankyrin-G cartridges among the total cell population. In the ankyrin-G- and GAT-1-immunostaining sections, cell bodies appeared as dark hollows (Fig. 2), which were utilized to count the total cell population in each section. The density of ankyrin-G-positive AISs was then divided by the total cell population in each area to obtain a percentage value. Consistent with differences in the absolute density of ankyrin-G cartridges, a much higher percentage value was found across all PC subdivisions (73% in layer II of APC to 93% in layer III of PPC, averaged from four mice) vs. neocortex (44%, averaged from four mice; Fig. 4).

We next examined the morphological details of the presumed AAIS cartridges in the PC and compared them with those of the neocortex in high-magnification confocal images. As shown in Figures 2–4, a very prominent feature of the AAIS cartridges in the PC was that the ankyrin-G-IR cartridges were surrounded by a higher concentration of GAT-1-IRs compared with neocortex. Quantitatively, the degree of innervation of ankyrin-G-IR cartridges by GAT-1-positive boutons was further examined by using the Z-stack feature of confocal images. As shown in Figure 5A, GAT-1-positive presynaptic boutons were defined as GAT-1-positive puncta that overlapped with ankyrin-G-IR in at least two dimensions in the orthogonal view of the Z-stack images. The density of GAT-1-positive presynaptic boutons along AIS was defined as the number of GAT-1-positive boutons per $1 \mu\text{m}$ of AIS on confocal images. GAT-1 bouton density was significantly higher in PC than in motor cortex ($1.0 \pm 0.1/\mu\text{m}$ in PC vs. $0.6 \pm 0.1/\mu\text{m}$ in neocortex, $P < 0.001$; Fig. 5B). Within the PC, GAT-1 bouton density was significantly higher in PPC vs. APC ($1.2 \pm 0.1/\mu\text{m}$ in PPC vs. $0.8 \pm 0.1/\mu\text{m}$ in APC, $P < 0.05$; Fig. 5). On the other hand, the length of AIS was significantly higher in neocortex (motor area) than in PC (Fig. 5). These results suggest that AAIS may provide stronger axon-axonic inhibition per cell in the PC vs. neocortex.

Colocalization studies

We next examined a number of presynaptic and postsynaptic GABAergic markers for their colocalization with the GAT-1-positive boutons at the AIS (Table 1). We tested antibodies for their specificity in mouse brain lysates and found that all antibodies that we used were selective for the targeted proteins (Fig. 1). We found that the presynaptic GABAergic markers (Table 1) VGAT and GAD67 were coexpressed in AAIS of PC (e.g., Figs. 6, 7). Similarly to GAT-1-IR, a high density of VGAT⁺ puncta was found next to ankyrin-G-expressing cartridges (Fig. 6). GAD67-GFP- and GAD67-IR-positive boutons were found to be highly colocalized with GAT-1-IR and to form close contact with ankyrin-G cartridges (Fig. 7). In contrast, PV only partially colocalized with ankyrin-G-IR cartridges (discussed further below). Postsynaptic GABAergic markers gephyrin, NKCC₁, and GABA_A α ₁, but not KCC₂, were highly expressed in the AAIS as well (Fig. 6). Gephyrin-IR, NKCC-IR, and GABA_A α ₁ all appeared as concentrated puncta located on the surface of the ankyrin-G-IR (Fig. 6), suggesting that multiple, segregated postsynaptic GABAergic sites were formed at each chandelier synapse. The calcium-binding protein calbindin was also detected in AIS.

However, calbindin appeared to be associated with the glutamatergic neurons only (data not shown). These results suggest that the molecular composition of the AAIS in PC is similar to that of AAIS in neocortex at adult stages as described earlier (DeFelipe, 1999; Howard et al., 2005; Lewis, 2011).

AAIS innervation of specific glutamatergic neuron subtypes

Glutamatergic cells of PC can be further divided into several subgroups based on their cellular morphology (e.g., semilunar vs. pyramidal), intrinsic properties, and laminar locations (Haberly, 1983; Suzuki and Bekkers, 2006). Here we examined whether AAIS was differentially expressed in PC centrifugal cells vs. noncentrifugal cells (Kiselycznyk et al., 2006; Restrepo et al., 2009). In this experiment, we used GAD67-GFP mice to distinguish GABAergic from non-GABAergic cells (Tamamaki et al., 2003). We injected the retrograde dye FG into the MOB in vivo, which led to well-labeled cells within the PC. All of the FG-labeled cells in the PC were found within layer II/III and could be anatomically identified as pyramidal cells or semilunar cells based on the morphology of their soma and initial dendritic segments as well as their lack of GAD67-GFP expression (Fig. 7). From high-magnification confocal images, we counted the percentage of centrifugal cells (i.e., FG⁺ cells) expressing ankyrin-G-IR cartridges located right next to the FG-labeled soma (Fig. 7D–H). This datum was compared with the percentage of AAIS in noncentrifugal glutamatergic cells (i.e., FG-negative and GAD67-GFP-negative soma; e.g., Fig. 7). To our surprise, we found that AAIS were more highly expressed in centrifugal cells ($92 \pm 4\%$, $n = 4$ mice) than in noncentrifugal glutamatergic cells ($71 \pm 3\%$, $n = 4$, $P < 0.05$; e.g., Fig. 7). When we examined the density of ankyrin-G-IR cartridges, we found that the density ($0.0023 \pm 0.001/\mu\text{m}^2$, $n = 4$ mice) was similar to the density of total excitatory neurons ($0.003 \pm 0.001/\mu\text{m}^2$) from a previous study in our laboratory. These results indicated that chandelier synapses were widely expressed in the majority of excitatory neurons of PC and more abundantly in the centrifugal cells.

Morphological properties of PV-expressing interneurons and their relation to AAIS

In neocortex, chandelier cells are a subpopulation of PV-expressing interneurons (DeFelipe, 1999; Lewis, 2011). We therefore used coronal PC sections (40 μm in thickness) processed with PV-IHC to survey PV⁺ cell morphology in an attempt to identify chandelier cells. As shown in Figure 8, PV⁺ somata were seen in layer II and III, with slightly higher density in layer II (0.00022 cells/ μm^2) than in layer III (0.00018 cells/ μm^2). Most PV⁺ cells in layer II had predominantly vertically oriented dendritic trees. Some of the dendrites expanded across all layers into layer Ia; others stopped largely within layer II (Figs. 8, 9). Dendrites of layer III PV cells had varied projection orientations: 30% of PV cells had widespread horizontal expansions, whereas 30% cells had vertical orientations, and the rest of the cells projected locally with no preference (Fig. 8A–C). Somata morphology of PV cells also differed, with a higher percentage of multiangular cell bodies in layer III (48% vs. 28% in layer II, $n = 4$ mice) and a more oval or pear-like appearance in layer II (45% vs. 30% in layer III, $n = 4$ mice). Detailed distributions of PV cell types with different dendritic patterns are shown in Figure 8F. Within cell dendritic subtypes, bitufted interneurons (i.e., “type a” cells) as well as other cell types have been shown to be associated with chandelier cells in neocortex and in PC (Zhu and Connors, 1999; DeFelipe, 1999; Woodruff et al., 2010; Larriva-Sahd, 2010; Lewis, 2011). With confocal images, we found that PV⁺ terminals were most commonly seen in a perisomatic location (i.e., basket synapses) in layer II of PC (Fig. 8). In fact, in most of the AIS measured, only $52\% \pm 5\%$ ($n = 8$) were found to have positive innervation by PV⁺ chandelier terminals (e.g., Fig. 8D). Moreover, in low-magnification photos (Fig. 8B), dense plexi of basket networks were found in layer II, whereas in layer III PV⁺ cartridges were not apparent. We further examined possible innervations of glutamatergic cells by PV cells in CAMKII-GFP mice, in which GFP was expressed only in glutamatergic

cells. As shown in Figure 8C, in the border of layer II/III, where CamKII-GFP cells AIS were prominently visible, PV⁺ cartridges were found near the AIS region of these cells (arrowheads in Fig. 8C). In layer III, PV⁺ cartridges were also seen in the presumed AIS region of many CamKII-GFP cells (arrowheads in Fig. 8C). These results support the idea that subsets of PV cells provide AAIS synapses in the PC.

To examine further the morphology of single chandelier cells, we intracellularly labeled individual GAD67-GFP cells with neurobiotin during patch-clamp studies. In a very low percentage (4%) of cells recorded (n = 100), four bitufted interneurons whose axonal terminals showed the chandelier-type cartridges were found (e.g., Fig. 9). All four cells exhibited a firing pattern with peak firing frequency >120 Hz and a linear frequency–current relationship (Fig. 9), which is consistent with fast-spiking firing types. In agreement with the idea that one chandelier cell provides AAIS inhibition to many pyramidal neurons, these four neurons showed elaborate axonal arbors that expanded to large areas (~2,000 μm^2 ; e.g., Fig. 9).

DISCUSSION

Features of AAIS in PC and neocortex

AAIS innervating the AIS of pyramidal cells formed by chandelier cells have been reported for different brain regions (including PC) of several mammalian species (Inda et al., 2007, 2009; Cruz et al., 2009). This type of synapses has been examined with several postsynaptic molecular markers: ankyrin-G, βIV spectrin, gephyrin, Caspr2, voltage-gated sodium channels, and KV1.2 channels. These proteins are thought to be involved in regulating postsynaptic structure and receptor localization at AIS (Howard et al., 2005; Inda et al., 2006; Cruz et al., 2009). Several presynaptic markers, particularly the high-affinity plasma membrane transporter GAT-1 (Inda et al., 2006, 2007, 2009) and PV, have been shown to be expressed in the presynaptic AAIS varicosities (Howard et al., 2005; Inda et al., 2006; Cruz et al., 2009). In human neocortex, AAIS terminal densities have been shown to be much higher in the association cortex (frontal orbitary cortices) vs. primary sensory cortices (e.g., S1) and their levels are usually higher in supragranular layers of neocortex (Inda et al., 2007). For mice, Inda et al. (2007) reported greater abundance of GAT-1-positive AAC terminals in the PC compared with other neocortical regions (Inda et al., 2009). Our results regarding the density of ankyrin-G-IR cartridges in PC are consistent with this result. In addition, we found that GAT-1-IR-positive puncta are highly colocalized at AIS labeled with ankyrin-G in PC (Figs. 2–4). Overall, the mean AIS coverage rate by GAT-1-IR is greater than 70% in both APC and PPC across all layers (Fig. 3), suggesting abundance of GABAergic innervation in the AIS region of PC. Indeed, the high coverage rate is due to a larger number of GAT-1-positive presumed boutons (Fig. 5). Thus, the inhibitory coverage rate per AAIS was higher than in neocortex of mice, as seen in human and monkey frontal cortex (Inda et al., 2006; Cruz et al., 2009). In addition to the higher inhibitory coverage in AIS of PC, the density of AAIS was found to be close to the density of total excitatory cell populations but was even higher in putative centrifugal cells (Fig. 7). Moreover, a much higher percentage of cells in PC was associated with both GAT-1 and ankyrin-G cartridges than was present in neocortex motor area (Figs. 3, 4). Together, these results suggest that AAIS-mediated inhibition could play a very important role in regulating the spike outputs from the majority of pyramidal cells of PC, particularly in cells that provide centrifugal modulation of OB.

Earlier studies have shown a great diversity of properties of AAIS (e.g., morphology, distribution, density, and molecular compositions) across different species (Lewis, 1998; DeFelipe, 1999; Howard et al., 2005). Some of these features also exhibit developmental changes (Cruz et al., 2003, 2009). Even in the same species (e.g., humans or mice), large

differences in the density and distribution of AAIS terminals containing the high-affinity plasma membrane transporter GAT-1 between cortical areas and layers have been identified in systematic studies (Inda et al., 2006, 2007, 2009). For the first time, we report a variety of molecular markers associated with the AAIS in the PC. Among these markers, VGAT, GAD67, and the calcium-binding protein PV (only partially) were associated with the presynaptic GAT-1⁺ varicosities, whereas gephyrin, NKCC₁, and GABA_A α 1 (but not KCC₂) were associated with the postsynaptic sites (Fig. 6). This is consistent with features of AAIS reported for neocortical regions (Lewis, 1998; DeFelipe, 1999; Howard et al., 2005). Recently, several studies have demonstrated that the AAIS were different from basket synapses in that the actions of GABA were mostly depolarizing in the AAIS synapses, largely because of a higher concentration of chloride in these terminals (Szabadics et al., 2006; Khirug et al., 2008; Woodruff et al., 2010). Our results provide anatomical evidence for this idea, in that NKCC₁, but not KCC₂, is highly expressed in presumed postsynaptic sites opposing the GAT-1⁺ terminals, which might contribute to the higher intracellular chloride in AAIS (e.g., Fig. 6H, I). However, further electrophysiological recordings are required to validate this finding in the PC.

Interneurons that provide the AAIS within PC

Together with basket cells, chandelier cells belong to fast-spiking GABAergic interneurons that express the calcium-binding protein PV. These cells are thought to regulate the timing of hyperpolarization, the firing of pyramidal cells, and the oscillations involved in various states of consciousness via AAIS (Cobb et al., 1995; Whittington and Traub, 2003; Howard et al., 2005; Inda et al., 2009). For the PC, previous physiological and anatomical studies on interneurons have not included chandelier cells (Zhang and others, 2006; Young and Sun, 2009; Stokes and Isaacson, 2010; Suzuki and Bekkers, 2010). It is also not entirely clear which exact cell types provide AAIS in the PC. In a recent study using the rapid Golgi staining method, Larriva-Sahd described, for the first time, typical “candle-like” axonal cartridges provided by chandelier cells and the morphology of these neurons in the PC (Larriva-Sahd, 2010). However, because of the limitations of the Golgi method, detailed and quantitative information regarding the density of chandelier cells and AAIS synapses in different subdivisions of PC is still lacking. Here, by using IHC staining methods, we describe the morphological differences of PV cells and argue that a very small population of PV cells could provide AAIS (Fig. 8). Furthermore, our single-cell labeling analysis confirmed IHC studies regarding the dendritic morphology of chandelier synapses in PC (Fig. 9). Together with earlier studies, morphological and molecular features of chandelier cells in the PC clearly emerged. Despite the high density of AAIS synapses in the PC, the density of chandelier cells providing AAIS was very low. Using a crude estimate based on the density of specific PV cells, the hit rate (4%) during random intracellular recordings from interneurons, and the total density of AAIS synapses in PC, we speculate that one chandelier cell may provide AAIS to 40–100 glutamatergic cells in the PC. This is probably why this cell type has been neglected in previous electrophysiological and anatomical studies. In the neocortex, although information regarding chandelier cell number/innervation ratio is not available, it was suggested that a similar situation existed based on morphology of chandelier cells (DeFelipe, 1999). Because of the very low yield of positive cells (four of 100 interneurons), future attempts to obtain meaningful data from these cells may rely on specific genetic markers for identifying chandelier cells (e.g., GAT-1-GFP, or Nkx2.1-MADM mice as described by Woodruff et al. 2010).

Implications for understanding olfactory cortical processing and oscillations

AAIS are important players in hippocampal network synchrony and oscillations, such as theta rhythm (4–8 Hz) and fast ripples (120–220 Hz) occurring during slow-wave sleep (Cobb et al., 1995; Klausberger et al., 2003). Based on the assumption that the AIS is the

initiation site of action potentials (Catterall, 1981), current dogma holds that the role of AAIS is to gate pyramidal cell output by shutting down the cell to prevent unnecessary firing. Indeed, for the somatosensory cortex, data suggest that neocortical AAIS dampen excessive excitability (Zhu et al., 2004). However, recent data suggest a different role for AAIS. The mechanism proposed is that action potentials are generated at least ~30 μm away from the soma and that AAIS prevent back-propagation of action potentials from the axon into the soma (Howard et al., 2005). This notion is supported by our anatomical evidence, which showed that the locations of GAT-1 on the AIS are some distance away from the soma (e.g., Figs. 2, 5). In addition, because of the abundance of the AAIS synapses in the PC as well as the robust inhibitory innervation per AAIS as demonstrated by this study, AAIS are likely to play a key role in regulation of olfactory-related oscillations and other important functions in the PC.

The olfactory system of mammals generates field potential oscillations in the presence of odorants (Freeman, 1978b). Both gamma (30–100 Hz) and theta (3–12 Hz) frequency oscillations occur in the olfactory bulb (Eeckman and Freeman, 1990; Kay and Freeman, 1998) as well as the PC (Illig and Haberly, 2003; Kay et al., 2009). Studies have also shown that oscillations in the bulb and cortex are correlated (Eeckman and Freeman, 1990; Kay and Freeman, 1998; Illig and Haberly, 2003). However, the precise inhibitory mechanisms have yet to be identified. Given the robust expression of AAIS in the PC shown in this study, AAIS are likely to be a main player in the inhibitory control of PC oscillations. We found, more importantly, that the dendrites of many bitufted PV cells extend through layer Ia, making these cells perfect candidates for inhibition between MOB and PC. Future *in vivo* studies similar to recent experiments by Zhan and Luo (2010) may help to elucidate the precise role of chandelier cells in olfactory cortical processing.

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Figure 1.

Western blotting of mouse cortical tissue lysate using different antibodies included in this study. **A:** The GABA_A α 1 antibody largely recognized a single expected band at about 51 kDa. **B:** The GAD67 antibody recognized a single expected band at about 67 kDa. **C:** The KCC₂ antibody recognized a single expected band at about 140 kDa, together with an unexpected (but very weak) band at about 250 kDa. **D:** The VGAT antibody detected only one expected band at about 51 kDa.

Figure 2. Confocal photomicrograph of ankyrin-G-IR (magenta, A,D,G) or GAT-1-IR (green, B,E,H), and merged photos (C,F,I) in APC (A–C), PPC (D–F), and neocortex motor area (G–I). Arrowheads mark cartridge locations where the ankyrin-G-IR is surrounded by the GAT-1⁺ boutons. Asterisk, soma of a presumed pyramidal cell. Scale bars = 10 μm.

Figure 3.

Confocal photomicrograph of a coronal section through APC (A–C) and PPC (D–F) stained with antibodies against ankyrin-G (magenta, A,D) and GAT-1 (green, B,E). G–I: Higher magnification photo through an APC area to show strong colocalization between ankyrin-G-IR with GAT-1-IR. Arrowheads mark ankyrin-G-IR or GAT-1-IR cartridges, which essentially overlapped (C,F,I). J: Percentage of cells expressing ankyrin-G cartridges in different PC and cortical areas (n = 4 mice). Scale bars = 50 μ m in C (applies to A–C); 50 μ m in F (applies to D–F); 20 μ m in I (applies to G–I).

Figure 4. Confocal photomicrographs of a coronal section through neocortex (motor area) stained with antibodies to ankyrin-G (magenta, **A**), and GAT-1 (green, **B**). Dashed lines mark boundaries of layers I–VI. Arrowheads mark ankyrin-G-IR or GAT-1-IR cartridges, which essentially overlapped (**C**). Scale bar = 50 μ m.

Figure 5.

Confocal microphotograph of ankyrin-G-IR (magenta) or GAT-1-IR (green) cartridges in two APC areas (A). The graph on the left is an orthogonal view of 3-D z-stack images showing close colocalization (arrowheads) of the GAT-1 puncta with ankyrin-G cartridge at two dimensions (x: top; y: right). B: The number of GAT-1 boutons at each ankyrin-G cartridges plotted against the length of the AIS in APC, PPC and neocortex motor area (M.Cx). Dashed lines, linear regression of the scatterplots. Histogram (C) and probability plot (D) of the same data as shown in B. Histogram (E) and probability plot (F) of the AIS lengths (i.e., ankyrin-G-IR cartridges) data in PC and neocortex motor area. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

< 0.01 , *** $P < 0.001$ using single-factor ANOVA followed by HSD analysis in this and all other graphs. Scale bars = 1 μm .

Figure 6.

A–C: Confocal microphotographs of ankyrin-G-IR (magenta, A), gephyrin-IR (green, B), and a merged image (C). **D–F:** Confocal photographs of ankyrin-G-IR (magenta, D), VGAT-IR (green, E) and a merged image (F). **G–I:** Confocal photographs of merged image: ankyrin-G-IR (magenta) with GABA_Aα1-IR (green, G), NKCC1-IR (green, H) and KCC2-IR (green, I). Scale bars = 2 μm. Arrowheads mark regions of colocalization between ankyrin-G and Gephyrin, VGAT, GABA_Aα1, NKCC1, KCC2, respectively.

Figure 7.

A–C: Confocal photomicrograph of ankyrin-G-IR (magenta, A) cartridges with FG (blue, B), and GAD67-GFP (green, C) in APC. Arrowheads mark locations where the ankyrin-G-IR cartridges were originated from FG-positive (asterisk) or -negative (+) cells. Note that ankyrin-G-IRs were associated with most of the FG-positive (marked by an asterisk) and -negative (marked by +) cells. **D:** Confocal photomicrograph of ankyrin-G-IR (red) cartridges with FG (blue) in PPC. **E:** Confocal photomicrograph of ankyrin-G-IR (red) cartridges with FG (blue) and GAD67-GFP (green) in APC. **F:** Bar graph showing percentage of cells in which ankyrin-G-IRs associated with centrifugal vs. noncentrifugal cells from slices obtained in three mice. * $P < 0.05$. Scale bars = 20 μm in A–D; 5 μm in E.

Figure 8.

A,B: Photomicrograph of PV-IR in layers I–II (A) and II–III (B) of APC. Note that the dendrites of the two bitufted PV cells (soma marked with a) in layer II extend to layer I. **C:** Merged image of PV-IR (magenta) and CamKII-GFP (green). Arrowheads in C mark abundant PV-IR near AIS regions of CamKII⁺ cells (i.e., glutamatergic cells). Two bitufted, a single-tufted, and a multipolar PV cells are marked with a, b, and d, respectively. **D,E:** Confocal images of PV-IR (green) with ankyrin-G-IR (magenta) in two APC slices. Note that in D the PV⁺ presynaptic boutons (arrowheads) surrounded the cell body region of a presumed pyramidal cell (asterisk); however, PV⁺ boutons were not found in the ankyrin-G-IR AIS region. In an adjacent section (E), the ankyrin-G-IR was partially associated with PV⁺ puncta. **F:** Diagram showing different types of PV-expressing interneurons according to their dendritic morphologies and their percentages of total PV populations of PC from four mice. Bipolar neurons (a) showed two principal dendrites emanating from two ends of the soma (usually elongated). Single-tufted cells (b) had one main dendritic tree arising from the soma and branching at close proximity of the soma. Bitufted cells (c) had two main dendritic trees arising from two opposite sides of the soma. Multipolar neurons (d) had more than three principal dendrites arising from the soma. Somata of representative cells of each type are marked accordingly in A–E. Scale bars = 50 μ m in A–C; 5 μ m in D,E.

Figure 9.

Photomicrograph of an intracellularly labeled (via patch-clamp recording and neurobiotin loading) chandelier cell in a PC slice. An intracellularly labeled PPC layer II interneuron. The dendritic and axonal morphology of this cell resembles the chandelier cells described for neocortex (A). Arrowheads in B indicate presynaptic varicosities, which may form presumed AAIS. A high-magnification photograph of presynaptic terminals that may form an AAIS (arrowheads, C). D–F: Membrane responses and action potentials induced by a series of current steps in the same cell of A. Representative traces of action potential induced by step currents are shown in D,E, respectively. The spike frequency–current plot for this cell is shown in F. The dashed line is a linear regression, where $P < 0.0001$. Scale bars = 20 μm in A; 50 μm in B; 3 μm in C.

TABLE 1

Primary Antibodies Used in This Study

Antigen	Immunogen	Species	Manufacturer and catalogue No.	Dilution used
GAT-1	C-terminus (amino acids 588–599) of GAT-1	Rabbit	Millipore; AB1570	1:500
GAD-67	Recombinant his-tag-containing human GAD67 protein in which his-tag is fused with the unique N-terminal region of GAD67 (amino acids 4–101)	Mouse	Millipore; MAB5406	1:500
PV	Parvalbumin purified from frog muscle	Mouse	Millipore; MAB1572	1:500
Gephyrin	Recombinant N-terminal domain corresponding to amino acids 1–175 of human gephyrin	Rabbit	Alexis Biochemicals; ALX 210–400	1:1,000
Calbindin	Synthetic peptide corresponding to the C-terminal region of rat calbindin-D28K (amino acids 225–244 with N-terminally added Lys-Gly)	Rabbit	Sigma; C2724	1:500
NKCC (H-110)	Amino acids 475–585 of Na-K-Cl cotransporter 2 of human origin	Rabbit	Santa Cruz Biotechnology; SC-20980	1:200
KCC ₂	KCC ₂ fusion protein (the carboxyl terminus of rat KCC ₂ protein amino acids 932–1043)	Rabbit	Millipore; 07–432	1:100
GABA _{Aα1}	Synthetic peptide (QPSQDELKDN TTV FT-C) corresponding to amino acids 1–15 of the mature rat GABA _{Aα1}	Rabbit	Upstate; 06–868	1:1,000
VGAT	Peptide sequence 17 amino acids near the carboxy terminal region of rat VGAT (VHSLEGLIEA YRTNAED)	Rabbit	Millipore; AB5062P	1:100
Ankyrin-G	Synthetic peptide corresponding to the spectrin-binding domain (amino acids 893–1474) of human ankyrin-G	Mouse	Santa Cruz Biotechnology; SC-12719	1:200