Anterior Cingulate Synapses in Prefrontal Areas 10 and 46 Suggest Differential Influence in Cognitive Control

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Dorsolateral prefrontal areas 46 and 10 are involved in distinct aspects of cognition. Area 46 has a key role in working memory tasks, and frontopolar area 10 is recruited in complex multitask operations. Both areas are innervated by the anterior cingulate cortex (ACC), a region associated with emotions and memory but is also important for attentional control through unknown synaptic mechanisms. Here, we found that in rhesus monkeys (*Macaca mulatta*) most axon terminals labeled from tracers injected into ACC area 32 innervated spines of presumed excitatory neurons, but ~20–30% formed mostly large synapses with dendritic shafts of presumed inhibitory neurons in the upper layers (I–IIIa) of dorsolateral areas 10, 46, and 9. Moreover, area 32 terminals targeted preferentially calbindin and, to a lesser extent, calretinin neurons, which are thought to be inhibitory neurons that modulate the gain of task-relevant activity during working memory tasks. Area 46 was distinguished as a recipient of more (by ~40%) area 32 synapses on putative inhibitory neurons. Area 10 stood apart as recipient of significantly larger (by ~40% in volume) area 32 terminals on spines of putative excitatory neurons. These synaptic specializations suggest that area 32 has complementary roles, potentially enhancing inhibition in area 46 and strengthening excitation in area 10, which may help direct attention to new tasks while temporarily holding in memory another task.

Introduction

Our thoughts are constantly bombarded by a multitude of stimuli, yet we can keep track of sequences in tasks and juggle multiple operations, though not always successfully. These functions, which broadly engage working memory mechanisms, are mediated by distinct dorsolateral prefrontal areas. In particular, areas 46 and 9 are involved in classic working memory functions, and their damage severely impairs the ability to keep track of information for the task at hand (for review, see Levy and Goldman-Rakic, 2000; Petrides, 2005). Neurons in dorsolateral prefrontal areas have sustained activity during the delay period of delayed-response tasks, which is thought to be a physiologic correlate of working memory (Fuster, 1973; Niki and Watanabe, 1976) (for review, see Goldman-Rakic, 1995; Fuster, 2001; Constantinidis and Procyk, 2004; Funahashi, 2006). However, when one task must be temporarily suspended to attend to another task, frontopolar area 10 is also recruited (for review, see Smith et al., 2007; Burgess et al., 2007; Koechlin and Hyafil, 2007; Badre and D'Esposito, 2009). The mechanism that underlies the functional dissociation of area 10 from other dorsolateral prefrontal areas in cognitive processing is unknown.

An equally important region in cognitive control is the anterior cingulate cortex (ACC), which interacts robustly with dorsolateral prefrontal areas (Barbas et al., 1999) but is functionally distinct (for review, see Devinsky et al., 1995; Carter et al., 1999; Bush et al., 2000; Paus, 2001; Matsumoto and Tanaka, 2004; Rushworth et al., 2007). Unlike dorsolateral areas, the ACC has strong bidirectional connections with limbic structures involved in emotions and long-term memory. The ACC is engaged during demanding cognitive tasks and is thought to have a key role in cognitive–emotional interactions (for review, see Barbas, 1995; Barbas and Zikopoulos, 2007; Botvinick, 2007; Lee et al., 2007; Schall and Boucher, 2007; Pessoa, 2008). Previously, we have shown that when axons from ACC area 32 form synapses with putative inhibitory neurons in the upper layers (I–IIIa) of dorsolateral area 9 the boutons are larger than terminals from the functionally related dorsolateral area 46 (Medalla and Barbas, 2009). Large boutons have more synaptic vesicles, a feature correlated with a higher probability of neurotransmitter release with each action potential (Tong and Jahr, 1994; Murthy et al., 1997, 2001). Thus, the synaptic interaction of these pathways suggests that, compared with area 46, area 32 can potentially engage more effectively inhibition in area 9 and help suppress noise and reverse decisions during demanding cognitive tasks (Medalla and Barbas, 2009).

Here, we investigated whether ACC area 32 of rhesus monkeys has a similar or different synaptic interaction with putative excitatory and inhibitory neurons in other dorsolateral prefrontal areas. Specifically, we focused on synapses formed by area 32 with neuronal elements in the functionally distinct dorsolateral area 46 and the dorsolateral part of frontopolar area 10. We provide evidence of synaptic specificity in the pathways from area 32 to areas 46 and 10, in circuits that support their specialization in distinct aspects of working memory and cognitive control.

Received April 5, 2010; revised Sept. 17, 2010; accepted Sept. 20, 2010.

This work was supported by National Institutes of Health grants from National Institute of Neurological Disorders and Stroke and National Institute of Mental Health; and in part by the Center of Excellence for Learning in Education, Science, and Technology, a National Science Foundation (NSF) Science of Learning Center (NSF Grant SBE-0354378). We thank Marcia Feinberg and Sue Paul for technical assistance; Kiran Bhai, Brittany Bolton, and Stephanie Jackson for help with data analysis; Dr. John Fiala for help with three-dimensional reconstruction; Dr. Alan Peters, Dr. Jennifer Luebke, and Dr. Michael Hasselmo for helpful comments; and Dr. Ron Killiany for help with surgery and imaging.

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DOI:10.1523/JNEUROSCI.1773-10.2010

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Figure 1. Tracing anterior cingulate and dorsolateral prefrontal pathways. **A**, Sites of injection of distinct neural tracers in the ACC (area 32; cases AY, BGr, BI, and Ble) are shown on the medial surface of the rhesus monkey brain. **B**, Lateral surface shows the location of sites examined in areas 10, 46, and 9 (dotted-filled squares) for labeled axonal boutons from area 32 (black arrowheads). For comparison, pathways connecting the related dorsolateral areas 46 and 9 (silhouette arrowheads) were labeled by placing distinct tracer injections in dorsal area 46 (cases Blr and BH; extending into the dorsal bank of the principal sulcus demarcated by long dashes) and the dorsal part of area 9 (cases BJ and BGI; extending from the lateral surface to the dorsomedial surface in **A**). Short dashed lines mark areal boundaries. A, Arcuate sulcus; C, central sulcus; Cg, cingulate sulcus; P, principal sulcus; Ro, rostral sulcus.

Materials and Methods

Surgical procedures and injection of neural tracers

Tracer injections were made in normal rhesus monkeys (*Macaca mulatta*; 2–3 years of age; $n = 5$), using magnetic resonance imaging (MRI), surgical, and tissue-processing protocols described previously (Medalla et al., 2007; Medalla and Barbas, 2009). Animals were obtained from the New England Primate Research Center (NEPRC), and protocols were approved by the Institutional Animal Care and Use Committee at NEPRC, Harvard Medical School, and Boston University in accordance with National Institutes of Health (NIH) guidelines (Department of Health, Education, and Welfare Publication No. NIH 80–22, revised 1987, Office of Science and Health Reports, Division of Receipt and Referral/NIH, Bethesda, MD).

Figure 1 summarizes the overall experimental design and distinct injection sites in ACC area 32 and dorsolateral prefrontal areas 46 and 9.

Injection sites were based on stereotaxic coordinates calculated from MRI scans obtained before surgery, as described previously (Zikopoulos and Barbas, 2007). To calculate coordinates for the injection sites, we used as reference points for the dorsoventral dimension the pial surface, for the mediolateral dimension the midline, and for the rostrocaudal dimension the ear bar tips, which had been filled with a marker (beta-dine) that is visible in the MRI.

About a week after the MRI, surgery was performed under aseptic conditions while heart rate, muscle tone, respiration, and pupillary dilatation were closely monitored. The monkeys were anesthetized with ketamine hydrochloride (10–15 mg/kg, i.m.) followed by isoflurane anesthetic until a surgical level of anesthesia was accomplished. The monkeys were then placed in a stereotaxic apparatus and a small region of the prefrontal cortex was exposed over the injection site. Using a microsyringe (5 or 10 μ l syringes, Hamilton) mounted on a microdrive, we injected the bidirectional neural tracers biotinylated dextran amine (BDA, Invitrogen) (cases AY, BGI, BGr, BH, BI, and BJ), fluoroemerald (FE, dextran fluorescein, Invitrogen) (case Ble), or fluororuby (FR, dextran tetramethylrhodamine, Invitrogen) (case Blr). All tracers were dextran amines of 10 kDa molecular weight, optimized for anterograde labeling, as described (Veenman et al., 1992; Reiner et al., 2000) (our personal observations). These dextran amines label the entire extent of axonal fibers and boutons. They also label neurons retrogradely, but labeling is sporadic and restricted to the cell bodies and proximal dendrites of projection neurons and does not appear to enter axon collaterals (Veenman et al., 1992; Reiner et al., 2000). For each injection site, the dye was diluted to 10 mg/ml in distilled water and delivered in two to four penetrations spaced 0.5 mm apart, to inject a total volume of $\sim 5 \mu$ l of dye.

Perfusion and tissue processing

After a survival period of 18 d, the animals were given an overdose of anesthetic (sodium pentobarbital, >50 mg/kg) until a deep level of anesthesia was achieved, then transcardially perfused with freshly depolymerized 4% paraformaldehyde and 0.2% glutaraldehyde (EM grade; Ladd Research Industries) in 0.1 M phosphate buffer (PB; with 9% NaCl, pH 7.4). The fixative solution was warmed to body temperature (37°C) at the time of perfusion. The brains were then removed from the skull, cryoprotected in graded solutions of sucrose (10–30%), frozen in -70°C isopentane (Rosene et al., 1986), and cut on a freezing microtome in the coronal plane at 50 μ m to produce 10 series per hemisphere. To preserve the ultrastructure, tissue was stored until use at -20°C in antifreeze solution (30% ethylene glycol, 30% glycerol, 40% 0.05 M PB, pH 7.4, with 0.05% azide).

Immunohistochemistry for bright-field microscopy. To visualize the neural tracers BDA, FE, and FR using bright-field microscopy, we used the avidin-biotin (AB) with horseradish peroxidase (HRP) method (Vectastain PK-6100 ABC Elite kit, Vector Laboratories), as described previously (Medalla et al., 2007; Medalla and Barbas, 2009). Assays were conducted on free-floating sections (at 4°C). Tissue sections were first incubated in 0.05 M glycine and preblocked in 5% normal goat serum (NGS) and 5% bovine serum albumin (BSA) with 0.2% Triton-X. Sections with BDA label were incubated in AB-HRP solution for 1 h (1:100 in PBS with 0.1% Triton X), and then processed using the peroxidase-catalyzed polymerization of diaminobenzidine (DAB) for 2–3 min (DAB kit, Vector Laboratories or Zymed Laboratories). Sections with fluorescent tracers were incubated overnight in antibodies against FE or FR (1:800 in PBS, 1% NGS, 1% BSA, 0.1% Triton-X; rabbit polyclonal, Invitrogen), followed by incubations in secondary biotinylated goat anti-rabbit IgG (1:200, for 2 h; Vector Laboratories), then in AB-HRP and DAB, as described above. For FE and FR cases, where there was also an injection of BDA in the same animal, sections were incubated in avidin-biotin blocking reagent (AB blocking kit, Vector Laboratories) before immunobinding, to prevent cross-reaction with BDA, as described previously (Medalla et al., 2007). After processing, sections were mounted on gelatin-coated slides, dehydrated in alcohol, and coverslipped with Entellan (EMD Chemicals). To delineate areas and layers, every other section in a series was counterstained with thionin (Sigma).

Pre-embedding immunohistochemistry for electron microscopy. We used pre-embedding immunohistochemistry to view synapses of labeled pathways in the electron microscope (EM; 100CX JEOL), as described previously (Medalla et al., 2007; Medalla and Barbas, 2009). We labeled neurons for the expression of the calcium-binding proteins parvalbumin (PV), calretinin (CR), and calbindin (CB), which label distinct and non-overlapping neurochemical classes of inhibitory neurons in the cortex in primates (for review, see DeFelipe, 1997). We used triple immunohistochemical methods to label neural tracers with DAB (as described above), and PV, CR, or CB neurons either with silver-enhanced, gold-conjugated secondary antibodies or tetramethylbenzidine (TMB), as described previously (Medalla et al., 2007). The three methods show distinct labeling at the EM: DAB appears as a dark uniform precipitate, silver-enhanced gold particles as circular clumps of variable size, and TMB as rod-shaped crystals (Gonchar and Burkhalter, 2003; Pinto et al., 2003; Moore et al., 2004; Medalla et al., 2007; Zikopoulos and Barbas, 2007) (our personal observations).

After labeling fibers with DAB (as above, except for reduced amounts of Triton-X, 0.025%), sections were incubated in AB blocking reagent to prevent cross-reaction with TMB. For BDA-labeled tissue, sections were coincubated overnight in the primary antibodies for PV or CR (1:2000, rabbit polyclonal) and CB (1:2000, mouse monoclonal; Swiss Antibodies), and then in biotinylated anti-mouse IgG, followed by AB-HRP. For FE- or FR-labeled tissue, we used a combination of two mouse monoclonal primary antibodies for PV, CB, or CR, processed successively, using the Mouse-on-Mouse blocking kit (M.O.M. basic kit; Vector Laboratories) in between to prevent cross-reaction. Sections were incubated overnight in the appropriate gold-conjugated IgG (1:50, 1 nm gold particle diameter; GE Healthcare). Sections were then postfixed in 6% glutaraldehyde with 2% paraformaldehyde using a variable wattage microwave (for 3–6 min at 150 W in the Biowave; Ted Pella) until the fixative temperature reached 30°C. Gold labeling was intensified with silver (6–12 min; IntenSE M kit; GE Healthcare), which results in aggregates of gold particles of variable sizes. Sections were processed for TMB then stabilized with DAB-cobalt chloride solution, as described previously (Medalla et al., 2007). In control experiments, we omitted primary antibodies to test the specificity of secondary antibodies, and used the AB blocking kit before AB binding, and the M.O.M. kit before secondary antibody binding. In all control experiments, there was no immunohistochemical labeling.

After immunohistochemical procedures, we cut small pieces of cortex (all layers) with anterograde label in areas 10, 46, and 9. The tissue pieces were postfixed in osmium, rinsed, dehydrated in increasing concentrations of ethanol (50–100%), stained with 1% uranyl acetate (EM Science), infiltrated with propylene oxide, and flat embedded in Araldite (Fullam) or LX112 resin (Ladd Research Industries) using Aclar plastic (Ted Pella), as described previously (Medalla et al., 2007; Medalla and Barbas, 2009). Pieces of Aclar-embedded tissue were cut and re-embedded in resin blocks, and sectioned at 50 nm with a diamond knife (Diatome US) using an ultramicrotome (Ultracut; Leica), as elaborated below. Serial ultrathin sections were collected on single slot Pioloform-coated grids.

Data analysis

Mapping labeled boutons at the light microscope. We identified and mapped at the light microscope labeled axonal boutons in dorsolateral areas 46 and 9, and the dorsolateral part of area 10 in a series of coronal sections (spaced 1 in every 10, with every other section counterstained with Nissl) ipsilateral to the injection site. We placed areal and laminar boundaries from Nissl (thionin)-stained coronal sections, based on architectonic maps (Barbas and Pandya, 1989). We first viewed the slides using a bright-field microscope (BX51; Olympus America) and scanned sections (Perfection 4990 Photo Scanner; Epson America) to qualitatively map labeled boutons within areas 10, 46, and 9, and to identify sites with dense and sparse label.

Based on the qualitative analysis, we used quantitative stereologic sampling of labeled boutons from all sites with label to estimate their density in different layers using the optical fractionator method. This was accomplished with the aid of a computer-microscope interface using commercial

software (StereoInvestigator; MicroBrightField), as described previously (Medalla and Barbas, 2006; Zikopoulos and Barbas, 2006, 2007). We used three to four evenly spaced (1 in every 20) Nissl-stained coronal sections through the entire extent of the part of each area with labeled boutons, and viewed sections at 1000 $\times$ magnification under oil-immersion. We made separate counts for each layer or laminar group as follows: I, II-IIIa, IIb, IV, Va, and Vb-VI. The 50- μ m-thick sections shrank to $\sim$ 15–30 μ m after histochemical processing and mounting on gelatin-coated slides. We counted boutons using an optical disector restricted to the central fraction of the tissue thickness (11 μ m). The top and bottom of each section (minimum of 2 μ m for 15 μ m sections) thus were used as guard zones to avoid error due to uneven tissue sectioning. The actual thickness of the mounted section was measured by the program at each counting site. The counting frame/disector size (area = 40 $\times$ 40 μ m; height = 11 μ m) and grid spacing (ranging from 100 $\times$ 100 to 400 $\times$ 400 μ m) were set to employ a sampling fraction to yield a coefficient of error of $\leq$ 5% (for review, see Gundersen et al., 1988; Howard and Reed, 1998; Schmitz and Hof, 2005). The average sampling fraction for labeled boutons was $\sim$ 1/400 of the total volume of each area with label, based on pilot studies, as described previously (Zikopoulos and Barbas, 2006). Density of boutons per μ m³ in each layer was calculated by dividing the number of boutons counted using the optical fractionator by the corresponding volumes estimated with the Cavalieri method. We normalized data to account for the variability of labeling due to differences in the size of the injections. This was accomplished by expressing the density of labeled boutons in each layer or laminar group as a fraction of the total density of labeled boutons from all layers of each area with label. Overall density in a given prefrontal area (all layers) was expressed as a fraction of the total density of labeled boutons from all areas examined in each injection case.

Mapping and measurement of labeled boutons and synapses. Photomicrographs of labeled boutons in prefrontal areas were captured under the light microscope using a high-resolution CCD camera (Olympus DP70). We acquired image stacks of several focal planes every 0.5 μ m to create pictures through the entire thickness of the sections in the z-axis using ImageJ (version 1.32j for Windows, NIH), as described previously (Medalla and Barbas, 2006; Zikopoulos and Barbas, 2006). We created montages of images captured at low magnification (200 $\times$) using Adobe Photoshop (CS2) to show the distribution of label throughout the depth of the cortex (layer I to the white matter) (Fig. 2). To obtain population estimates of bouton size, which is correlated with the number of synaptic vesicles (Germuska et al., 2006; Zikopoulos and Barbas, 2006), we captured images at high magnification (1000 $\times$) of one to two random sites within a region of anterograde label in layer I and in layers II-IIIa of areas 10, 46, and 9. Within each stack of images, we manually traced the profile of each labeled bouton to measure the major diameter using ImageJ (Fig. 2Aa1, red outlines). Our sampling included measurement of a total of $\sim$ 6000 boutons per pathway ($n \sim$ 3000 from layer I and $n \sim$ 3000 from layers II-IIIa, from 2 to 3 cases).

To map synapses formed by prefrontal axons, we identified two to three coronal sections with dense anterograde label through areas 10, 46, and 9 based on bright-field maps. We processed for EM sections from matched rostrocaudal levels in adjacent series of sections, and cut blocks of tissue (through all layers) from sites with label in areas 10, 46, and 9. The tissue blocks were flat embedded in resin (as described above). For each pathway examined, we cut four to six small pieces of embedded tissue from layer I (extending to $\sim$ 200 μ m below the pial surface), and from the same blocks we cut four to six pieces of tissue from layers II-IIIa (extending $\sim$ 500 μ m below the top of layer II; total number of pieces of tissue: area 32 $\rightarrow$ 10, $n = 10$; 32 $\rightarrow$ 46, $n = 11$; 32 $\rightarrow$ 9, $n = 10$; 46 $\rightarrow$ 9, $n = 12$). Each piece of tissue ($\sim$ 200 $\times$ 400 to $\sim$ 600 $\times$ 400 μ m) was then re-embedded in resin blocks and trimmed with a diamond trim tool (Diatome) to include most of the laminar depth (average block face area of 180 $\times$ 300 μ m for layer I; and 460 $\times$ 350 μ m for layers II-IIIa) for serial sectioning, as described previously (Medalla et al., 2007).

Ultrathin sections were examined at 60 or 80 kV with a transmission EM (100CX JEOL). Boutons labeled with tracer were identified and photographed at 10,000 $\times$ or 6600 $\times$ using either a digital camera (Digital-Micrograph, Gatan) or film camera (negatives were scanned using Epson

Figure 2. Photomicrographs of labeled axonal boutons from ACC (area 32) and dorsolateral pathways. **A**, Coronal section, counterstained with Nissl (blue), through the dorsal part of area 10 shows BDA-labeled boutons (brown) from ACC (area 32) distributed throughout the depth of the cortex but are more concentrated in the upper layers (I–IIIa). Laminar labels are placed at the beginning of each cortical layer. Insets show labeled fibers and boutons at higher magnification in layer I (**a1**), layer IV (**a2**), and layers V–VI (**a3**). **a1** shows examples of large (yellow arrows) and small (blue arrows) labeled boutons. Some tracings (red outline) of labeled boutons show measurement of major diameters. **B**, **C**, Coronal sections through dorsolateral area 46 in the upper bank

Perfection 4990 Photo Scanner), from ~50 to 100 serial sections from each piece of tissue, as described previously (Medalla et al., 2007). Overall, we identified an average of >300 labeled boutons systematically sampled from layers I–IIIa in each pathway (1554 total boutons analyzed: $n = 533$ from $32 \rightarrow 10$; $n = 351$ from $32 \rightarrow 46$; $n = 345$ from $32 \rightarrow 9$; $n = 325$ from $46 \rightarrow 9$). For each pathway, we used ~200 boutons (from layers I and II–IIIa) for two-dimensional (2D) analysis of major diameter, ~80 boutons (from layers II–IIIa) for three-dimensional (3D) reconstruction and analysis of volume, and the rest to only identify postsynaptic targets.

For 2D analysis in the EM, we used series of 50–80 sections and sampled exhaustively all labeled boutons in every 10 adjacent sections at intervals of $1 \mu\text{m}$ (skipping ~20 sections) to yield a comparable number of data points from each pathway, as described previously (Germuska et al., 2006; Medalla et al., 2007). We used ImageJ to measure the major diameters of labeled boutons at the level of the synapse within the 10 section sampling interval. For 3D analysis, we used series of 100 sections and identified all labeled boutons in every section to yield a comparable sample of boutons from each pathway. We followed and photographed each bouton throughout the adjacent serial sections (30–50 sections for each synapse), and reconstructed the boutons and their postsynaptic targets in 3D using the open source program Reconstruct (www.bu.edu/neural) (Fiala, 2005). We estimated the thickness of sections using the method of cylindrical diameters (Fiala and Harris, 2001a). We traced object contours of boutons and postsynaptic elements manually section by section, and used the tracings to generate a 3D model and calculate volume and surface area. The 3D model was imported into 3D Studio Max (version 3, Autodesk) for additional rendering.

Analysis of postsynaptic targets and the surrounding neuropil. We used classic criteria to identify synapses with spines, which are more numerous on excitatory neurons, or with aspiny (smooth) or sparsely spiny dendritic shafts, which are characteristic of presumed inhibitory neurons in the cortex (for review, see Peters et al., 1991; Fiala and Harris, 1999). For a subset of postsynaptic spines, we followed each spine through the series to identify and analyze the parent dendrite. We measured the average cross-sectional major diameter of each dendrite, estimated spine density (number of spines per micrometer length) using stereologic methods (Fiala and Harris, 2001b), and measured the volume of the spine heads emanating from each dendrite. We identified putative inhibitory postsynaptic targets labeled

of the principal sulcus. Each section shows labeled boutons (brown): from area 32 (**B**); and from area 9 (**C**). Insets show labeled fibers and boutons in layer I (**b1**, **c1**) and layers V–VI (**b2**, **c2**) at higher magnification. Scale bars: **A–C**, 100 μm ; **a1–a3**, **b1**, **b2**, **c1**, **c2**, 10 μm .

with CB, CR, or PV by the presence of gold or TMB label found in about a fourth of the sections through the postsynaptic site in the series. CB and CR are expressed in a minority of pyramidal neurons (DeFelipe et al., 1989a; del Río and DeFelipe, 1997). We considered postsynaptic targets as belonging to presumed inhibitory CB, CR, or PV neurons if they were labeled, and also were aspiny or sparsely spiny. The latter were characterized by computing a density index for spines (number of spines/ μm) and synapses (number of synapses/ μm) of reconstructed dendrites (supplemental Fig. 1, available at www.jneurosci.org as supplemental material), as described previously (Fiala and Harris, 2001b; Medalla and Barbas, 2009). On average, smooth dendrites had a higher number of asymmetric synapses per micrometer (average: 1.4 ± 0.1 synapse/ μm) compared with sparsely spiny dendrites (0.9 ± 0.2 synapse/ μm). The latter overlapped with spiny dendrites in terms of spine density (sparsely spiny: range = 0.2 – 1.0 spines/ μm ; mean = 0.4 ± 0.1 spines/ μm ; spiny: range = 0.4 – 3 spines/ μm ; mean = 1.5 ± 0.2 spines/ μm). However, sparsely spiny dendrites had a significantly higher density of synapses on shafts, which were virtually absent on spiny dendrites, consistent with previous studies (Feldman and Peters, 1978; Kawaguchi et al., 2006; Medalla and Barbas, 2009) (for review, see Fiala and Harris, 1999). Measuring the synapse density, spine density, and diameter of dendrites helped characterize dendritic morphology but could not be used to determine whether a dendritic segment was distal or proximal.

We used stereologic methods to investigate whether labeled boutons from area 32 differed from unlabeled boutons that formed asymmetric synapses in the surrounding neuropil in areas 10 and 46 (White, 1989). We used systematic random sampling of the neuropil surrounding 1 in every 30 labeled boutons, each spanning 30–50 adjacent serial sections, in area 10 (average volume sampled = 1.65 mm^3 ; $n = 6$ sites sampled, from 2 cases) and area 46 (average volume = 1.68 mm^3 ; $n = 5$ sites sampled, from 2 cases). We photographed labeled boutons at the center of the frame and counted all unlabeled boutons around them ($n = 1140$ boutons in area 10; $n = 1286$ in area 46). We reconstructed in 3D a subset of unlabeled boutons that were complete in the series ($n = 275$ in area 10; $n = 218$ in area 46).

Statistical analysis. For each injection site, we obtained measurements of labeled boutons from multiple sites with tracer label in areas 10, 46, and 9, in two to three monkeys. The analysis of major diameters at the light microscope, and proportion of postsynaptic targets and 3D volumes at the EM, included measurements for the pathway from $32 \rightarrow 9$ and $46 \rightarrow 9$ reported from a previous study (Medalla and Barbas, 2009) for comparison. Statistical comparisons were made using one-way or two-way ANOVA and Bonferroni's or Tukey's *post hoc* test in Statistica (version 7 for Windows, StatSoft). We used linear regression using least-squares approximation in Sigma-Plot (version 7.0 for Windows, SPSS) to determine relationships between presynaptic (bouton volume) and postsynaptic features (postsynaptic density area, spine volume, and dendrite diameter). We used K-means cluster analysis on the major diameters and volumes of labeled boutons using Statistica, with parameters set to maximize initial between-cluster differences, as described previously (Zikopoulos and Barbas, 2006). The cluster analysis statistically generated a cutoff point based on the major diameters or volumes and separated the population of labeled boutons into size groups (large and small). We used the resulting groups generated by the analysis to determine the proportion of large and small boutons in each pathway.

Results

We investigated the terminations of labeled pathways from ACC area 32 to the functionally distinct dorsolateral prefrontal areas 10, 46, and 9 (Fig. 1, black arrowheads) after injection of anterograde tracers in area 32 ($n = 4$). For comparison, we examined pathways connecting the functionally related and neighboring dorsolateral areas 46 and 9 (Fig. 1B, silhouette arrowheads) ($n = 4$; 2 in area 46; 2 in area 9). Figure 1 shows the injection sites within the central part of area 32 (Fig. 1A, cases AY, BGr, BI, and BLe), in area 46 within the dorsal bank of the principal sulcus (Fig. 1B, cases BH and BIr), and the dorsal part of area 9 (Fig. 1A, B, cases BGI and BJ). The findings were similar across cases for each pathway examined.

Figure 3. Distribution of labeled axonal boutons from ACC (area 32) and dorsolateral pathways. **A**, **B**, Relative regional density (**A**) and relative laminar density (**B**) of labeled boutons from area 32 to dorsolateral areas 10, 46, and 9. **C**, Relative laminar density of labeled boutons in pathways connecting the functionally related dorsolateral areas 46 and 9. Error bars = SEM.

Common features of ACC (area 32) pathways

Large boutons were prevalent in area 32 pathways to distinct dorsolateral prefrontal areas

Qualitative analysis at the light microscope showed that the axons and boutons from area 32 were widely distributed in dorsolateral prefrontal areas 10, 46, and 9. Labeled axons and boutons from area 32 spanned the entire rostrocaudal extent of the dorsolateral part of frontopolar area 10, and the anterior two-thirds of areas 46 and 9, consistent with previous findings (Barbas and Mesulam, 1985; Barbas and Pandya, 1989; Barbas et al., 1999). The labeled boutons appeared as axonal swellings, which were either terminal ("terminaux") or continuous ("en passant") varicosities along the axonal length (Fig. 2). Qualitative and quantitative analysis using unbiased stereologic methods showed that labeled boutons from area 32 were more prevalent in areas 10 and 9 than in area 46 (Fig. 3A). Labeled boutons were found in all layers but

Figure 4. Axonal boutons from area 32 terminating in dorsolateral prefrontal areas are larger than in pathways connecting areas 46 and 9. **A–C**, Size of labeled boutons from area 32 ($32 \rightarrow 10$, $46 \rightarrow 9$) and dorsolateral ($9 \rightarrow 46$, $46 \rightarrow 9$) pathways measured at the light microscope: average major diameters of total, large, and small labeled boutons in layers I–IIIa (**A**); proportion of small and large labeled boutons (**B**); and normalized frequency distribution histogram of major diameters of labeled boutons (**C**). **D, E**, Size of labeled boutons measured at the EM: average major diameters of labeled boutons in layers I–IIIa in all pathways measured using 2D electron microscopy ($32 \rightarrow 10$, $n = 266$ boutons; $32 \rightarrow 46$, $n = 134$; $32 \rightarrow 9$, $n = 180$; $46 \rightarrow 9$, $n = 171$) (**D**); and average volume of labeled boutons in layers II–IIIa in all pathways from 3D serial EM analysis ($32 \rightarrow 10$, $n = 66$; $32 \rightarrow 46$, $n = 102$; $32 \rightarrow 9$, $n = 45$; $46 \rightarrow 9$, $n = 33$) (**E**). Black asterisks show significant differences between area 32 and dorsolateral pathways; gray asterisks show significant differences among the area 32 pathways to dorsolateral areas 10, 46, and 9 ($p < 0.05$). Δ Data from (Medalla and Barbas, 2009) used for comparison. Error bars = SEM.

were somewhat denser in the upper cortical layers ($\sim 60\%$ in I–IIIa) than in the middle-deep layers ($\sim 40\%$ in IIIb–VI). Within the upper layers, boutons were densest in layer I (Figs. 2A, B, 3B). Boutons from pathways connecting dorsolateral areas 46 and 9 were about evenly distributed across layers (Figs. 2C, 3C). Here, we focused analysis of synapses in layers I–IIIa, which included robust labeling in all pathways examined.

The size of boutons is positively correlated with the number of synaptic vesicles (Germuska et al., 2006; Zikopoulos and Barbas, 2007) and the probability of transmitter release with each action potential (Tong and Jahr, 1994; Murthy et al., 1997). We thus measured the size of boutons at the light microscope ($n = 31,009$ total boutons measured for major diameters) and at the ultrastructural level (2D EM major diameter, $n = 751$; 3D EM volume, $n = 246$ total boutons). Cluster analysis segregated boutons into small (Fig. 4A–C, major diameters $< 1.0 \mu\text{m}$ at the light microscope; or Figs. 4D, E, 5E–G, 2D EM diameters $< 0.94 \mu\text{m}$, and 3D EM volumes $< 2 \mu\text{m}^3$) and large (greater than the above sizes). There was a trend for a higher prevalence of large boutons that terminated in layers II–IIIa than in layer I, consistent with previous findings (Germuska et al., 2006; Medalla et al., 2007),

Figure 5. Large area 32 boutons target preferentially dendritic shafts of putative inhibitory neurons in all dorsolateral areas and spines of putative excitatory neurons in area 10. **A**, Schematic diagram shows the three main types of postsynaptic targets of labeled boutons from area 32 to dorsolateral areas. Labeled boutons form synapses with spines of presumed excitatory (exc) neurons (green filled circle), with dendritic shafts of presumed inhibitory (inh) neurons (red filled circle), or with multiple postsynaptic sites: with more than one spine (dark green triangle), or with a spine and an aspiny shaft (red-green triangle). **B**, Scatter plot shows volumes of individual labeled boutons from area 32 with distinct postsynaptic targets in layers II–IIIa of areas 10 and 46. **C**, Average volume of boutons that target spines (green bars) and dendritic shafts (red bars) in layers II–IIIa of all pathways. Black asterisks show significant differences between area 32 and dorsolateral pathways ($32 \rightarrow 10$, $46 \rightarrow 9$ versus $46 \rightarrow 9$); gray asterisks show significant differences among the area 32 pathways to distinct dorsolateral areas 10, 46, and 9 ($p < 0.05$). Δ Data from (Medalla and Barbas, 2009) used for comparison. **D**, Average major diameters of boutons with distinct postsynaptic targets in layers I–IIIa, measured in 2D EM. **E–G**, Proportions of large and small boutons with distinct postsynaptic sites based on cluster analysis of 2D (layers I–IIIa) and 3D (layers II–IIIa) EM data: **E**, boutons forming a synapse with a spine; **F**, boutons forming a synapse with a dendritic shaft; **G**, boutons with multiple postsynaptic targets. Error bars = SEM.

but the laminar differences were not statistically significant in all pathways examined so data from the upper layers (I–IIIa) were pooled.

Boutons from area 32 that terminated in areas 46, 9, and, especially area 10, were larger than in pathways connecting the related dorsolateral areas 46 and 9. The difference was due to greater overall size ($p < 0.05$, ANOVA, *post hoc* test) (Fig. 4A, D, E), as well as the proportion of large boutons ($p < 0.01$) (Fig. 4B, C). Large boutons were associated with large postsynaptic density (PSD) areas on spines ($R^2 = 0.41$) and dendritic shafts ($R^2 = 0.42$, $p < 0.01$) (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). The pathways from area

32 to distinct dorsolateral areas had in common larger boutons than in the pathways connecting the neighboring dorsolateral areas 46 and 9.

Area 32 boutons formed large synapses with putative inhibitory neurons in distinct dorsolateral prefrontal areas

Further analysis at the ultrastructural level revealed that the differences in bouton size across pathways were related to the type of postsynaptic target (Fig. 5*A,B*), confirming and extending previous findings (Medalla and Barbas, 2009). Like other prefrontal pathways, boutons from area 32 mainly innervated single spines of presumed excitatory neurons in all dorsolateral areas examined (~ 58 – 80%) (Figs. 6*A*, 7*A,B*, 8*A*; supplemental Table 1, available at www.jneurosci.org as supplemental material). A smaller but significant proportion of boutons innervated aspiny (or sparsely spiny) dendritic shafts of presumed inhibitory neurons (~ 13 – 27%) (Figs. 6*E,F*, 7*D–I*, 8*A*), or multiple postsynaptic sites of either two or more spines (~ 5 – 8%) (Figs. 6*B,C*, 7*C*, 8*A*) or a spine and a dendritic shaft (~ 3 – 8%) (Figs. 6*D*, 7*J*, 8*A*). The pathways from area 32 to dorsolateral areas 10, 46, and 9 included in common significantly larger boutons that innervated putative inhibitory neurons, compared with the pathway connecting the related dorsolateral areas $46 \rightarrow 9$ ($p < 0.05$) (Fig. 5*C*, red bars, *D*). There was a similar trend for single boutons that innervated multiple postsynaptic sites (Fig. 5*D*). About half of the dendritic shaft-targeting boutons (Fig. 5*F*) and $\sim 80\%$ of multisynaptic boutons (Fig. 5*G*) from area 32 to dorsolateral areas (10, 46, and 9) were large (2D EM major diameter $\geq 0.94 \mu\text{m}$; 3D EM volumes $\geq 2 \mu\text{m}^3$). In contrast, most dendritic shaft-targeting ($\sim 90\%$) and multisynaptic ($\sim 65\%$) boutons from dorsolateral area 46 to area 9 were small (major diameters $< 0.94 \mu\text{m}$; volumes $< 2 \mu\text{m}^3$). The high prevalence of large boutons that innervated either dendritic shafts of presumed inhibitory neurons or multiple postsynaptic sites is a feature that distinguished the ACC (area 32) pathways to dorsolateral areas 10, 46, and 9 from the pathways that connect the related dorsolateral areas 46 and 9.

Specialized synaptic features of ACC (area 32) pathways to dorsolateral areas 10 and 46

Area 32 boutons formed large synapses with spines in area 10
Beyond the above common features of ACC (area 32) pathways to distinct dorsolateral areas, the ACC pathway to area 10 ($32 \rightarrow 10$) was distinguished further by large boutons that innervated spines of presumed excitatory neurons ($p < 0.05$) (Figs. 5*C*, green bar with asterisk, 5*D,E*, 6*A,B*, 7*A*). These large area 32

Figure 6. Examples of synapses formed by ACC (area 32) in areas 10 and 46 reconstructed in 3D. *A*, A large bouton (axon terminal, At, blue) from area 32 forms a synapse (postsynaptic density, PSD, yellow) on a spine (sp) in area 10 that branches from a dendrite (den, green). *B–D*, Large multisynaptic boutons (At): *B*, bouton forms synapses (PSD1 and 2) with two spines (sp1 and 2, branching from den1 and 2) in area 10; *C*, bouton forms synapses (PSD1–3) with three spines (sp1–3) in area 46, sp1 branches from dendrite (den); *D*, bouton forms one synapse with a spine (sp) and another with an aspiny dendrite of a presumed inhibitory neuron (den, pink translucent) in area 46. *E, F*, Large boutons (At) from area 32, each forming a synapse with an aspiny dendrite of a presumed inhibitory neuron in area 46. In *D–F*, note the prevalence of unlabeled synapses (gray patches) scattered throughout the surface of each aspiny dendrite reconstructed in 3D, with some located on the foreground (appearing dark gray) and others on the back surface visible through the translucent dendrite (appearing gray-purple). Scale cube = $0.5 \mu\text{m}^3$.

boutons exceeded in size even boutons that innervated dendritic shafts of presumed inhibitory neurons in all other pathways examined ($p < 0.05$) (Fig. 5*C*, compare red and green bars). This synaptic specialization accounted for the overall larger size of boutons from area 32 to area 10 evident at the population level (Fig. 4), and provided a sharp contrast to the other pathways examined because spines were the majority of postsynaptic targets. Spines are the most prevalent targets in other corticocortical pathways as well (Anderson et al., 1998; Melchitzky et al., 1998, 2001; Anderson and Martin, 2002, 2005, 2006; Medalla et al.,

Figure 7. Electron photomicrographs of boutons from ACC (area 32) and their synapses in areas 10 and 46. **A, B**, FE-labeled boutons (axon terminal, At) from area 32, each forming a synapse (black arrowhead) with a spine (sp): large bouton in area 10 (**A**); small bouton in area 46 (**B**). **C**, A large multisynaptic bouton (At) labeled with BDA forms synapses with three spines (sp1–3) in area 46. **D**, A large FE-labeled bouton (At) forms a synapse (black arrowhead) with a CB+ dendrite (CB+ den), which has mitochondria (m) and is labeled with silver-enhanced gold (black arrows) in area 10. **E–I**, Labeled boutons (At) from area 32 that form synapses (black arrowheads) with dendritic shafts of presumed inhibitory neurons in area 46. **E**, A large BDA-labeled bouton (At) forms a

synapse with a CB+ dendrite (CB+ den) labeled with gold (black arrows). **F1, F2**, Two consecutive images of a large FE-labeled bouton (At) that forms a synapse (black arrowheads) with a CB+ dendrite labeled with TMB (black arrows), which also receives synapses from unlabeled boutons (silhouette arrowheads). In **F1**, note the nearby dendrite with gold label (silhouette arrow) in the surrounding neuropil in triple-labeled tissue. **G**, A small bouton (At) forms a synapse with a CB+ dendrite labeled with TMB (black arrow). **H**, A small bouton (At) forms a synapse with a PV+ dendrite (PV+ den) labeled with gold (black arrow). **I**, A large bouton (At) lightly labeled with FE forms a synapse with a CR+ dendrite (CR+ den) labeled with gold (black arrows). **J1, J2**, Two consecutive images show a large FE-labeled multisynaptic bouton in area 10 (At) forming one synapse with a spine (sp) and another synapse with a CR+ dendrite (CR+ den) labeled with gold (black arrows). Scale bars, 0.5 μ m.

Area 32 boutons innervated more presumed inhibitory postsynaptic sites in area 46

The ACC pathway to area 46 (32 $\rightarrow$ 46) showed a different specialization by forming significantly more synapses with presumed inhibitory neurons ($\sim 33\%$ of all synapses) than in the other pathways ($p < 0.05$; $\sim 23\%$ in 32 $\rightarrow$ 10, $\sim 22\%$ in 32 $\rightarrow$ 9). As described above, area 32 innervated a higher proportion of dendritic shafts of presumed inhibitory neurons in all pathways examined (32 $\rightarrow$ 10, 32 $\rightarrow$ 46, 32 $\rightarrow$ 9) than in the pathway connecting the related dorsolateral areas 46 $\rightarrow$ 9 ($p < 0.05$) (Fig. 8A, black asterisk), but this pattern was most pronounced for the pathway from area 32 to area 46 (32 $\rightarrow$ 46) (Figs. 7E–I, 8A, gray asterisk). The targeted dendrites of presumed inhibitory neurons in all pathways were mostly aspiny (Fig. 6E,F), but some ($\sim 24\%$) were sparsely spiny with a relatively high density of asymmetric synapses on their shafts (supplemental Fig. 1, available at www.jneurosci.org as supplemental material).

Most (~ 57 – 78%) of the innervated dendrites of presumed inhibitory neurons were also labeled for one of the calcium-binding proteins, CB, CR, or PV (Fig. 7D–J). Axonal boutons from area 32 formed synapses with a higher proportion of CB positive dendrites (~ 6 – 10% of all targets, $\sim 47\%$ of neurochemical targets) than the pathway from dorsolateral area 46 to area 9 ($p < 0.05$; $\sim 4\%$ of all targets, $\sim 26\%$ of neurochemical targets) (Figs. 7D–G, 8B, dark gray). Innervation of CR (4–9% of all

synapse with a CB+ dendrite (CB+ den) labeled with gold (black arrows). **F1, F2**, Two consecutive images of a large FE-labeled bouton (At) that forms a synapse (black arrowheads) with a CB+ dendrite labeled with TMB (black arrows), which also receives synapses from unlabeled boutons (silhouette arrowheads). In **F1**, note the nearby dendrite with gold label (silhouette arrow) in the surrounding neuropil in triple-labeled tissue. **G**, A small bouton (At) forms a synapse with a CB+ dendrite labeled with TMB (black arrow). **H**, A small bouton (At) forms a synapse with a PV+ dendrite (PV+ den) labeled with gold (black arrow). **I**, A large bouton (At) lightly labeled with FE forms a synapse with a CR+ dendrite (CR+ den) labeled with gold (black arrows). **J1, J2**, Two consecutive images show a large FE-labeled multisynaptic bouton in area 10 (At) forming one synapse with a spine (sp) and another synapse with a CR+ dendrite (CR+ den) labeled with gold (black arrows). Scale bars, 0.5 μ m.

Figure 8. Synapses with presumed inhibitory postsynaptic sites were most prevalent in the area 32 pathway to dorsolateral area 46. **A**, Proportion of labeled boutons in area 32 (32 → 10, 46, 9) and dorsolateral (46 → 9) pathways that formed synapses with specific postsynaptic elements presumed to be excitatory (spines) or inhibitory (shafts). **B**, Proportion of synapses with putative inhibitory neurons labeled with CB, CR, or PV. Black asterisks show significant differences between area 32 and dorsolateral pathways; gray asterisks (in **A**) show significant differences among the area 32 pathways to distinct dorsolateral areas 10, 46, and 9 ($p < 0.05$). ^ΔData from (Medalla and Barbas, 2009) used for comparison. Error bars = SEM.

targets) (Figs. 7*I*, 8*B*, light gray) and PV (2–6% of all targets) (Figs. 7*H*, 8*B*, crosshatch) positive dendrites was comparable in the pathways examined. Axons from area 32 formed synapses with a significant proportion of CR positive dendrites (~35% of neurochemical targets), but synapses with PV postsynaptic sites were fewer (~18% of neurochemical targets) than for CB or CR in all areas examined (10, 46, and 9). Area 32 axons innervated all three neurochemical classes of presumed inhibitory neurons more frequently in area 46 than in areas 10 and 9, consistent with the overall higher prevalence of area 32 synapses with putative inhibitory postsynaptic sites in area 46.

Pathway-specific distinction of ACC (area 32) boutons from surrounding unlabeled boutons

The neuropil in the vicinity of synapses from labeled area 32 axonal boutons in areas 10 and 46 had a comparable distribution of unlabeled synapses with spines and dendritic shafts ($p > 0.05$) (Fig. 9*A*, silhouette bars; supplemental Table 1, available at www.jneurosci.org as supplemental material). This evidence suggests that areas 46 and 10 have similar microcompartments (White, 1989). Moreover, in area 10, unlabeled synapses were similar to labeled synapses from area 32 with respect to the proportion of specific postsynaptic targets (Fig. 9*A*, compare black and silhouette bars). On

Figure 9. Comparison of synapses from area 32 with the surrounding neuropil in layers II–IIIa of areas 10 and 46. **A**, Proportion of synapses on spines and dendritic shafts. Labeled synapses formed by area 32 boutons in area 46 differed significantly from unlabeled synapses in the surrounding neuropil ($*p < 0.01$), but not in area 10. **B**, Volumes of labeled boutons from area 32 were significantly larger ($*p < 0.01$) than nearby unlabeled boutons in areas 10 and 46, especially for boutons forming synapses with spines in area 10. Error bars = SEM.

the other hand, in area 46, labeled boutons from area 32 formed synapses more often on dendritic shafts of presumed inhibitory neurons than the unlabeled synapses in the surrounding neuropil ($p < 0.01$) (Fig. 9*A*, asterisks).

In addition, labeled boutons from area 32 were significantly larger in volume than unlabeled boutons in the surrounding neuropil in both areas 46 and 10, especially boutons that targeted spines in area 10 ($p < 0.01$) (Fig. 9*B*; supplemental Table 1, available at www.jneurosci.org as supplemental material). A similar pattern was seen for individual segments of aspiny (presumed inhibitory) (Fig. 10*A*, *C*, *D*) and spiny (presumed excitatory) dendrites targeted by area 32 boutons (Fig. 10*B*, *E*–*G*). PSDs and spines that were innervated by labeled boutons from area 32 were larger than those innervated by nearby unlabeled boutons even within the same dendritic segment ($p < 0.05$) (Fig. 10*A*, *B*, asterisks). In contrast, there were no significant differences in the size of presynaptic and postsynaptic elements between labeled and unlabeled boutons in the pathway connecting the two related dorsolateral areas 46 and 9 ($p > 0.05$). Interestingly, in all pathways studied, spines that were innervated by labeled boutons were situated on parent dendrites that had overlapping spine densities and diameters (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). This evidence suggests that all pathways studied targeted morphologically similar spiny dendritic segments (Larkman, 1991) but varied in the volume of the innervated spines, which were largest in the pathway from

Figure 10. Postsynaptic elements innervated by labeled area 32 boutons were larger than unlabeled boutons targeting the same dendritic segment in dorsolateral prefrontal areas. **A**, Labeled (red bars) and unlabeled (silhouette bars) boutons and the average surface area of their associated PSDs on shafts of the same aspy/sparsely spiny dendrite of presumed inhibitory neurons. **B**, Average volumes of spines innervated by labeled (green bars) and unlabeled (silhouette bars) boutons on the same spiny dendrites of presumed excitatory neurons. PSDs and spines that were innervated by labeled boutons from ACC area 32 were significantly larger ($p < 0.05$) than those innervated by nearby unlabeled boutons. Error bars = SEM. **C, D**, 3D reconstruction (**C**) and EM photomicrograph (**D**) of an aspy dendrite (aspy den, pink) receiving a synapse (yellow in **C**; black arrows in **C** and **D**) from a labeled bouton or axon terminal from area 32 (At-32; blue in **C**). The aspy dendrite also receives synapses from nearby unlabeled boutons (gray patches and silhouette arrows in **C**; silhouette arrowheads in **D**). **E1, E2**, Photomicrographs of adjacent sections show a large labeled bouton from area 32 (At-32) forming a synapse (black arrow) with a large spine in area 10 (sp-10) that branches from a dendrite (spiny den, green). Note the smaller nearby spine (Unlab-sp) branching from the same dendrite and forming a synapse (silhouette arrowhead) with an unlabeled bouton. **F, G**, 3D reconstructions of spines branching from spiny dendrites targeted by labeled boutons in prefrontal pathways: A labeled bouton from area 32 (At-32, blue) forms a synapse with a spine in area 10 (sp-10, green) (**F**), which is larger than the nearby spines targeted by unlabeled boutons (silhouette arrows) and branching from the same spiny dendrite (spiny den, green); and a labeled bouton from dorsolateral area 46 (At-46, blue) forms a synapse with a spine in area 9 (sp-9, green) (**G**), which is comparable in size to nearby spines targeted by unlabeled boutons. Scale cube = $0.5 \mu\text{m}^3$.

area 32 to area 10 (Fig. 10B). These findings further highlight the higher prevalence of ACC (area 32) innervation of dendritic shafts of putative inhibitory neurons in area 46, and specialized large axonal boutons that formed synapses with spines of putative excitatory neurons in area 10, which stood apart from synapses in the surrounding neuropil.

Discussion

Common features of ACC synapses in several dorsolateral prefrontal areas

The pathways from ACC (area 32) to layers I–IIIa of dorsolateral areas 10, 46, and 9 had in common a higher prevalence of large boutons, compared with pathways connecting neighboring dorsolateral areas 46 and 9. The significance of this feature is based on evidence that large boutons have more vesicles (Germuska et al., 2006; Zikopoulos and Barbas, 2007), a feature correlated with a higher probability of neurotransmitter release with each action potential (Tong and Jahr, 1994; Murthy et al., 1997). Further, large boutons form synapses with large postsynaptic densities, which have more receptors (for review, see Nimchinsky et al., 2002; Bourne and Harris, 2008). This combination of presynaptic and postsynaptic features leads to increased synaptic efficacy (Murthy et al., 2001).

In particular, large boutons from area 32 preferentially innervated in common presumed inhibitory neurons in areas 46, 9 and 10, consistent with previous findings (for area 32 to 9, see Medalla and Barbas, 2009). This evidence suggests that the ACC engages efficiently inhibitory mechanisms, which are thought to help reduce noise in dorsolateral prefrontal areas (Wang et al., 2004; Medalla and Barbas, 2009), consistent with functional studies (MacDonald et al., 2000; Badre and Wagner, 2004; Johnston et al., 2007; Mitchell et al., 2007; Ng et al., 2007; Brown and Braver, 2008; Chen et al., 2008; Mulert et al., 2008). Beyond these common synaptic features, area 32 axons had specialized synapses in areas 46 and 10, as elaborated below.

The ACC targeted preferentially distinct postsynaptic elements of areas 46 and 10

Area 32 axons showed two area-specific synaptic interactions. The first was a significantly higher prevalence of synapses with presumed inhibitory neurons in area 46, compared with areas 9 or 10, or between areas 46 and 9. The second was preferential innervation of spines in area

Figure 11. Summary circuit of distinct synaptic area 32 pathways to dorsolateral prefrontal cortex (DLPFC). The schematic highlights the main findings, showing the common and unique synaptic features of ACC (area 32) pathways to distinct dorsolateral prefrontal areas 10 and 46 (light and dark green filled arrows). Line thickness represents prevalence of pathways, and size of arrowhead represents size of boutons. ACC (area 32) pathways innervated presumed inhibitory neurons (inh, red) in all dorsolateral areas with large boutons (filled arrows with dotted lines), distinguishing them from pathways within dorsolateral prefrontal cortex (area 46 → 9, light green open arrows). **A**, The ACC (area 32) pathway to area 10 was distinguished by large boutons (dark green, larger arrowhead) on spines of putative excitatory neurons (light green open circles), poised to enhance excitation. **B**, The ACC (area 32) pathway to area 46 was distinguished by more prevalent synapses (dark green, thicker dotted line) on putative inhibitory neurons, poised to enhance inhibition.

10 through large boutons, a feature not previously seen for other prefrontal pathways (Medalla and Barbas, 2009). The large boutons that innervated spines in layers I–IIIa of area 10 formed synapses that were comparable in size to “driving” cortical pathways that transmit signals efficiently (Anderson et al., 1998; Anderson and Martin, 2002, 2005). This evidence belies the widely held view that the upper cortical layers, and especially layer I, are uniformly associated with “feedback” or “modulatory” input (for review, see Felleman and Van Essen, 1991). Rather, distinct corticocortical pathways may show synaptic specificity, even when they innervate the same area and layer, which may reflect their functional specialization.

Specifically, dorsolateral areas 46 and 9 are involved when one must remember information momentarily to perform a task with sequential components (for review, see Miller and Cohen, 2001; Curtis and D’Esposito, 2004; Müller and Knight, 2006; Tanji and Hoshi, 2008), such as cooking. But suppose that while cooking, a visitor interrupts. Frontopolar area 10 is recruited when juggling multiple tasks (Koechlin et al., 1999; Burgess et al., 2000; Braver et al., 2003; Dreher et al., 2008; Tsujimoto et al., 2010), keeping on hold the main goal (cooking) to momentarily direct attention to the visitor. Sustained multitask representations are thought to be mediated by excitatory connections within dorsolateral prefrontal cortex (Wang, 1999; González-Burgos et al., 2000; Constantinidis et al., 2001), and specifically between areas 46 and 10 (Koechlin et al., 2003; De Pisapia et al., 2007), but the mechanism is unknown. The specific synapses from area 32 may be key for this process, consistent with ACC-mediated attentional and multitask functions (Gehring and Knight, 2000; Koechlin et al., 2000; Ito et al., 2003; Badre and Wagner, 2004; Emeric et al., 2008). Area 32 terminals may thus engage presumed excitatory neurons in area 10, involved in selecting and sustaining multiple tasks, and at the same time modulate activity in area 46 through CB inhibitory neurons, as summarized in Figure 11A,B (dark green arrows). CB

inhibitory neurons innervate the distal dendrites of pyramidal neurons (DeFelipe et al., 1989a; Kawaguchi and Kubota, 1997; Peters and Sethares, 1997), a pattern that is well suited to suppress noise at the fringes of active neurons during working memory tasks, based on physiologic and computational studies (Wang et al., 2004; Zaitsev et al., 2005). Preferential innervation of CB (and presumed inhibitory) neurons in area 46 by area 32 axons may help defer temporarily the main goal (e.g., cooking) in multitask operations. In contrast, engagement of the powerful perisomatic inhibition of pyramidal neurons by PV inhibitory neurons (Somogyi et al., 1983; DeFelipe et al., 1989b) could lead to catastrophic forgetting of the deferred goal (e.g., burn the food). Interestingly, area 32 innervation of PV neurons was far less frequent in the upper layers of areas 9, 46, or 10, but was the most common innervation pattern in the upper layers of auditory association cortex (Medalla et al., 2007). Pyramidal neurons with sustained activity during working memory tasks (Wang, 1999; González-Burgos et al., 2000; Constantinidis et al., 2001) and CB inhibitory neurons are most prevalent in the upper layers (Condé et al., 1994; Gabbott and Bacon, 1996; Dombrowski et al., 2001), the same layers of areas 9, 46, and 10 that receive projections from ACC (Barbas and Rempel-Clower, 1997). The upper layers of area 46 are also preferentially affected in age-related cognitive decline (for review, see Luebke et al., 2010).

Relationship of prefrontal cortices with auditory association areas

Areas 32, 10, 46, and 9 have in common robust connections with auditory association cortices (Barbas and Mesulam, 1985; Petrides and Pandya, 1988; Barbas et al., 1999; Hackett et al., 1999; Romanski et al., 1999; Medalla et al., 2007) and show significant metabolic (2-deoxyglucose) activation in response to auditory stimuli (Poremba et al., 2003). Microstimulation of ACC areas 32 and 25 evokes species-specific vocalization in monkeys and affects activity in auditory cortices (Müller-Preuss et al., 1980; Müller-Preuss and Ploog, 1981) (for review, see Vogt and Barbas, 1988). In humans, correlated gamma-band activity in ACC and auditory areas suggests functional coupling between these cortices (Mulert et al., 2007). Moreover, the relative activation of ACC and auditory cortices distinguishes actual from inner speech in humans, in functions that are disrupted in schizophrenic patients who experience auditory hallucinations (Frith et al., 1995; McGuire et al., 1995).

Among prefrontal cortices, area 10 has the strongest connections with anterior and posterior auditory association cortices (Barbas and Mesulam, 1985; Medalla et al., 2007), mapped physiologically in monkeys (Rauschecker et al., 1997; Poremba et al., 2004). These connections are comparable in strength and extent to the connections of the frontal eye fields with visual association cortices (for review, see Barbas et al., 2002; Lynch and Tian, 2006). Here, we showed that area 10 receives dense and potentially strong synaptic projections from area 32, which is also robustly connected, and functionally coupled, with auditory association cortices. These pathways may help select relevant auditory stimuli and suppress noise, allowing communication even in a noisy environment (for review, see Conway et al., 2001; Denham and Winkler, 2006; Jääskeläinen et al., 2007; Micheyl et al., 2007).

Similar mechanisms may apply for keeping track of internal thoughts during working memory and problem solving tasks (Brewin and Smart, 2005) (for review, see Wenzlaff and Wegner, 2000). This hypothesis is consistent with findings that the ACC and area 10 are activated during mental tracking of multiple tasks

(for review, see Burgess et al., 2007). Area 10 may have a role in symbolic representation of information in organized thought, aided by ACC, which also has a role in monitoring inner thoughts (McGuire et al., 1996; Wyland et al., 2003). Combined behavioral and functional studies that employ auditory-related tasks are needed to investigate the role of auditory input, and the specialized projections from area 32, to cognitive processing in area 10.

Pathology in ACC and dorsolateral prefrontal cortices is thought to disrupt the balance of excitation and inhibition in schizophrenia (for review, see Benes, 2000; Volk and Lewis, 2002; Beasley et al., 2002; Vogels and Abbott, 2007; Fornito et al., 2009; Eisenberg and Berman, 2010). For example, the ACC shows reduced activity in schizophrenia (Fletcher et al., 1999; Kerns et al., 2005; Snitz et al., 2005; Allen et al., 2007; Leicht et al., 2010). Based on the synaptic circuits here, and previous findings in non-human primates (Medalla and Barbas, 2009), we suggest that pathologic hypofunction in ACC may weaken its output to the neurochemical class of CB inhibitory neurons in areas 9, 46, and 10, which are thought to suppress noise (Wang et al., 2004). In addition, hypofunction in ACC may reduce excitation of frontopolar area 10, which is engaged when tackling multiple tasks and keeping track of internal thoughts. Weakening of ACC output from area 32 may thus account for the high distractibility as well as the disordered thought patterns described for schizophrenic patients (for review, see Cohen et al., 1996; Honey and Fletcher, 2006; Allen et al., 2008). While these relationships may not be easily extrapolated from monkeys to humans, the specific synaptic interactions found here provide the circuit basis to test these hypotheses in behavioral and functional studies in normal and schizophrenic subjects.

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