

# Three-Dimensional Synaptic Analyses of Mitral Cell and External Tufted Cell Dendrites in Rat Olfactory Bulb Glomeruli

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## ABSTRACT

Recent studies have suggested that the two excitatory cell classes of the mammalian olfactory bulb, the mitral cells (MCs) and tufted cells (TCs), differ markedly in physiological responses. For example, TCs are more sensitive and broadly tuned to odors than MCs and also are much more sensitive to stimulation of olfactory sensory neurons (OSNs) in bulb slices. To examine the morphological bases for these differences, we performed quantitative ultrastructural analyses of glomeruli in rat olfactory bulb under conditions in which specific cells were labeled with biocytin and 3,3'-diaminobenzidine. Comparisons were made between MCs and external TCs (eTCs), which are a TC subtype in the glomerular layer with large, direct OSN signals and capable of mediating feedforward excitation of MCs. Three-dimensional analysis of labeled apical dendrites

under an electron microscope revealed that MCs and eTCs in fact have similar densities of several chemical synapse types, including OSN inputs. OSN synapses also were distributed similarly, favoring a distal localization on both cells. Analysis of unlabeled putative MC dendrites further revealed gap junctions distributed uniformly along the apical dendrite and, on average, proximally with respect to OSN synapses. Our results suggest that the greater sensitivity of eTCs vs. MCs is due not to OSN synapse number or absolute location but rather to a conductance in the MC dendrite that is well positioned to attenuate excitatory signals passing to the cell soma. Functionally, such a mechanism could allow rapid and dynamic control of OSN-driven action potential firing in MCs through changes in gap junction properties. *J. Comp. Neurol.* 000:000–000, 2016.

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Within the mammalian olfactory bulb, olfactory sensory neurons (OSNs) transmit information onto output mitral cells (MCs) and various subpopulations of tufted cells (TCs) that differ in location of their cell bodies and arborization (Macrides and Schneider, 1982; Orona et al., 1984; Schoenfeld et al., 1985; Antal et al., 2006; Imai, 2014). Recent physiological studies have suggested that MCs and TCs in fact have quite distinct odor-evoked responses. Compared with TCs, MCs are less sensitive to odors, more odor selective, and display significantly delayed action potentials (spikes; Nagayama et al., 2004; Igarishi et al., 2012; Fukunaga et al., 2012; Otazu et al., 2015). Differences have also been observed in synaptic responses in brain slice experiments. For example, in response to stimulation of OSNs at a given intensity, MCs display much weaker somatic current and voltage responses with the rapid

time course expected for direct OSN-to-MC transmission (De Saint Jan et al., 2009; Najac et al., 2011; Gire et al., 2012; Burton and Urban, 2014; Vaaga and Westbrook, 2016). MCs also have much smaller optogenetically evoked currents that are insensitive to the sodium channel blocker tetrodotoxin (Gire et al., 2012), often used to assay monosynaptic excitation (Petreanu et al., 2009). Among TCs, the largest direct OSN currents appear to be in superficial TCs, a class of output cells

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with cell bodies in the outer external plexiform layer, and external TCs (eTCs), which are glutamatergic interneurons in the glomerular layer that lack lateral dendrites. The difference in synaptic responses between MCs and eTCs also extends to inhibitory synapses; eTCs have substantially larger inhibitory currents that reflect inputs from glomerular layer interneurons in direct comparisons (Gire and Schoppa, 2009; Whitesell et al., 2013; Banerjee et al., 2015).

At an ultrastructural level, very little is known about the synaptic organization of MC and TC apical dendrites that could give rise to the different physiological responses. One limitation has been that the vast majority of ultrastructural studies of OSN synapses have not differentiated whether the postsynaptic dendrites were from MCs or TCs (Pinching and Powell, 1971; White, 1972; Hinds and Hinds, 1976; Kasowski et al., 1999). Thus, simple issues such as the number of OSN synaptic contacts onto MCs (Kosaka et al., 2001; Najac et al., 2011) or TCs have never been addressed. A dendrite's complement of ion channels can also impact the size of synaptic signals at the cell soma, as reflected in both current and voltage measurements (Rall, 1967; Williams and Mitchell, 2008). In this context, it is interesting that MCs have a large ( $\sim 1$  nS) gap junctional conductance in their apical dendritic tufts (Schoppa and Westbrook, 2002; Christie et al., 2005; Pimental and Margrie, 2008; Maher et al., 2009) that is present at a much lower level in eTCs (Hayar et al., 2005; Gire et al., 2012). Furthermore, knockout (KO) of the gap junction protein connexin (Cx) 36 results in the emergence of large, direct OSN currents in MCs and rapid spiking (Gire et al., 2012). However, the distribution of gap junctions in MC apical dendrites is not known.

Here we conducted ultrastructural analyses to understand the synaptic organization of the apical dendrites of MCs and eTCs. eTCs, and not other TC subtypes, were chosen for the analysis because of their much larger monosynaptic OSN current vs. MCs in paired-cell recordings (Gire et al., 2012; Vaaga and Westbrook, 2016) and large inhibitory synaptic currents. eTCs can also mediate feedforward excitation of MCs (OSN-to-eTC-to-MC; De Saint Jan et al., 2009; Najac et al., 2011; Gire et al., 2012), so their comparative synaptic organization vs. that of MCs will likely contribute to whether MCs are excited via eTCs or directly by OSNs. We labeled MCs and eTCs in olfactory bulb slices with biocytin, which was then converted into an electron-dense substrate using an avidin-biotin complex and 3,3'-diaminobenzidine (DAB). Serial section electron microscopy was used to create three-dimensional reconstructions of DAB-labeled MC and eTC dendrites in which the density and distribution of OSN synapses

as well as other chemical synapse types could be quantified. In addition, for a population of unlabeled putative MC dendrites, we examined the distribution of gap junctions and their spatial relationship with respect to OSN synapses and presynaptic sites of neurotransmitter release.

## MATERIALS AND METHODS

### Cell labeling in olfactory bulb slices

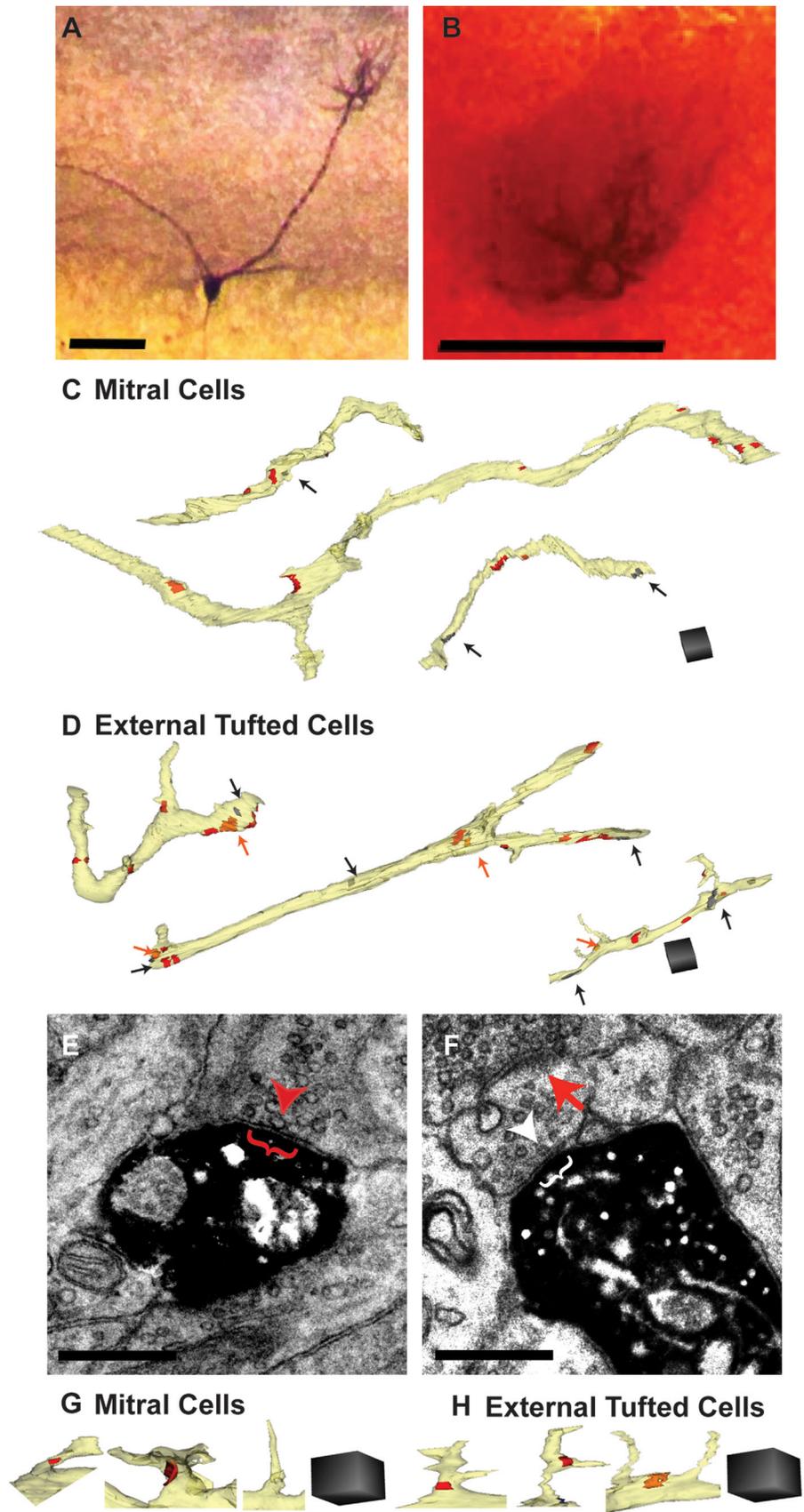
Horizontal olfactory bulb slices (330- $\mu$ m thickness) were prepared from three young Sprague Dawley rats (postnatal day [P]8–14; male and female) following general isoflurane anesthesia and decapitation. Slices were then transferred to a recording chamber with oxygenated (95% O<sub>2</sub>, 5% CO<sub>2</sub>) extracellular solution containing (in mM): 125 NaCl, 25 NaHCO<sub>3</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 25 glucose, 3 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub> (pH 7.3). Patch pipettes for whole-cell fills contained (in mM): 125 K-gluconate, 2 MgCl<sub>2</sub>, 0.025 CaCl<sub>2</sub>, 1 EGTA, 2 NaATP, 0.5 NaGTP, 10 HEPES (pH 7.3 with KOH), and biocytin (1%). Intracellular solutions were supplemented with 100  $\mu$ M Alexa 488 dye (Invitrogen, Carlsbad, CA) to establish cellular morphology during the fills.

Olfactory bulb slices were visualized on an upright fluorescence microscope (Zeiss Axioskop) equipped with DIC optics. MCs and eTCs were patched and allowed to fill for 10–15 minutes. Patch pipettes were then gently removed from the cell body, allowing the membrane to reseal. All cell fills were performed between 32 °C and 35 °C. Once cells with intact dendritic arbors had been filled in a given slice, slices were fixed overnight at 4 °C in 1% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4).

On the next day, slices were rinsed in 0.1 M phosphate buffer and incubated in a 3% H<sub>2</sub>O<sub>2</sub> solution for 30 minutes. The tissue was then cryoprotected in increasing concentrations of sucrose solution (10%/20%/30%) and freeze/thawed in liquid nitrogen five or six times. Slices were incubated in 1% ABC Solution (Vectastain Elite ABC kit; Vector, Burlingame, CA) at room temperature for 1 hour and then overnight at 4 °C. After rinsing of the tissue in 0.1 M phosphate buffer, slices were incubated in a 1% DAB solution for 30 minutes before 3% H<sub>2</sub>O<sub>2</sub> was added to catalyze the reaction. Once cells filled with biocytin had turned brown/black (30–60 seconds), the reaction was stopped by transferring the slices back to 0.1 M phosphate buffer (Fig. 1A).

### Electron microscopy

Slices were embedded in 5% agarose in 0.1 M phosphate buffer and sliced on a vibratome into 80- $\mu$ m-thick



**Figure 1.** Characterization of apical dendrites of mitral cells (MCs) and external tufted cells (eTCs). **A:** Example MC labeled with biocytin following incubation with an avidin-biotin complex and reaction with DAB. **B:** Apical dendritic tuft from same MC shown in A after treatment with osmium tetroxide. **C,D:** Example reconstructions of DAB-labeled dendrites (light yellow) of MCs (C) and eTCs (D). Olfactory sensory neuron (OSN; red) synapses are indicated, as are non-OSN putative excitatory axonal synapses (orange; orange arrows) and putative inhibitory dendrodendritic synapses (gray; black arrows). **E:** Example electron micrograph (EM) of a DAB-labeled MC dendrite receiving an axodendritic synapse from an OSN as indicated by the docked presynaptic vesicles (example at red arrowhead) and active zone (red bracket). **F:** Example EM of a DAB-labeled MC dendrite receiving a putative inhibitory dendrodendritic synapse as indicated by docked presynaptic vesicles (example at white arrowhead) and active zone (white bracket). The putative inhibitory dendrite is also receiving an asymmetric axodendritic synapse from an OSN (red arrow). **G,H:** Spine-like protrusions with putative excitatory synapses and nonsynaptic filopodia-like structures emerging from the dendritic shaft of MCs (G) and eTCs (H). Scale bars = 100  $\mu\text{m}$  in A,B; 0.5  $\mu\text{m}$  in E,F; scale cubes = 1  $\mu\text{m}^3$ .

sections. The 80- $\mu\text{m}$  vibraslices containing the DAB-labeled processes were rinsed in 100 mM cacodylate buffer and then immersed in 1% osmium and 1.5% potassium ferrocyanide for 15 minutes, rinsed five times in cacodylate buffer, immersed in 1% osmium for 1 hour (Fig. 1B), and then rinsed five times for 2 minutes each in buffer and twice briefly in water. Slices were then transferred to graded ethanols (50%, 70%, 90%, and 100%) containing 2% uranyl acetate for 15 minutes each. Finally, slices were transferred through propylene oxide at room temperature and then embedded in LX112 and cured for 48 hours at 60°C in an oven (modified from Harris et al., 2006).

Serial sections (50-nm thickness) were cut from a small trapezoid positioned over glomeruli with DAB-labeled dendrites and were picked up on Formvar-coated slot grids (EMS, Fort Washington, PA). Sections were imaged either on an FEI Tecnai G2 transmission electron microscope at 80 kV with a Gatan UltraScan 1000 digital camera at a magnification of  $\times 4,800$  or a Zeiss SUPRA 40 field-emission scanning electron microscope (FE-SEM) equipped with an integrated module called ATLAS (automated large area scanning; software version 3.5.2.385; Kuwajima et al., 2013).

### Image analysis and 3-D reconstruction

The serial section images were aligned and dendrites were traced in RECONSTRUCT software (<http://synapses.clm.utexas.edu/tools/reconstruct/reconstruct.stm>; RRID:SCR\_002716; Fiala and Harris, 2001; Fiala, 2005). Individual dendritic branches were treated as unique segments. A segment had to be at least 3  $\mu\text{m}$  in length and 0.3  $\mu\text{m}$  in diameter and have one synapse to be included in most of the analyses. Terminal ends of dendrites were defined as a segment that tapered and disappeared through serial sections. Axons were characterized by small-caliber projections that would swell to form en passant boutons that contained synaptic vesicles and formed synapses. Putative excitatory and inhibitory synapses and OSN synapses onto DAB-labeled MC and eTC dendrites were categorized based on their ultrastructural appearance as described in Results. In studies of unlabeled dendrites on putative MCs, OSN synapses were identified based on their asymmetric appearance and the dark, granular appearance of the presynaptic axon terminals (Pinching and Powell, 1971; Kasowski et al., 1999). The putative excitatory presynaptic release sites were identified based on their presence at asymmetric synapses and the appearance of clear, round vesicles. Gap junctions were identified through serial sections by the dark, dense staining plaques between adjacent dendritic membranes (Brightman and Reese, 1969; Kosaka and

Kosaka, 2004). That the unlabeled processes were dendrites (vs. axons) was determined by their greater diameter, more uniform size as a function of length, presence of fewer synaptic vesicles, and the fact that they formed both afferent and efferent synapses.

In the analysis of the gap junctionally or synaptically connected partners of unlabeled putative MC dendrites (see Fig. 6), the identity of the partner dendrites (putative excitatory vs. inhibitory) was determined by following these dendrites to where they formed an asymmetric or symmetric synapse onto another (third) dendrite. Identifying a specific dendrodendritic synapse to be asymmetric, with a clear postsynaptic density that differed in thickness from the presynaptic membrane, was more difficult than for axodendritic synapses (see red arrow in Fig. 1F) and generally required imaging across multiple sections.

Synapse size was calculated by summing the product of active zone length, as determined by a darker, slightly fuzzy thickening of the presynaptic membrane (Fig. 1E,F) and section thickness over the number of sections in which they appear. Dendrite lengths; distances of synapses, gap junctions, and release sites from distal ends of dendrites; and distances between these elements were measured across serial sections using the z-trace tool in RECONSTRUCT.

### Statistical analyses

Data were graphed and statistically analyzed in Excel. Results are presented as mean  $\pm$  SEM and include the relevant *n* values for each analysis. One-way ANOVAs were used to analyze the effect of distance from the distal end of dendritic segments on synaptic, gap junction, and release site densities, followed by post hoc two-tailed Student's *t*-tests. The two-sample Kolmogorov-Smirnov (K-S) test was used to compare cumulative distributions that reflected distances from distal end of dendrites for various elements, separation between the elements, and synapse size. Remaining analyses were performed using two-tailed Student's *t*-tests where appropriate.

## RESULTS

Individual MCs and eTCs were filled with Alexa 488 (100  $\mu\text{M}$ ) and biocytin (1%) during whole-cell patch-clamp recordings in olfactory bulb slices from juvenile rats (P8–14). MCs were identified by their characteristic row of large cell bodies, whereas eTCs were identified by their juxtglomerular position; large, spindle-shaped cell bodies; lack of secondary dendrites; and large apical dendritic tufts that occupied most of an adjacent glomerulus (Macrides and Schneider, 1982; Hayar

et al., 2004). After incubation with an avidin-biotin complex, tissue was reacted with DAB to form an electron-dense substrate (see MC example in Fig. 1A,B) and then processed for electron microscopy.

### Basic characterization of DAB-labeled dendrites and synapses

Serial sections (50–150 sections, 50-nm thickness) of glomeruli containing DAB-labeled MC or eTC dendrites were cut and imaged on a transmission electron microscope. Images of the dendrites and their synapses were then aligned, traced, and reconstructed. Dendritic segments from both MCs (Fig. 1C) and eTCs (Fig. 1D) varied in length and synaptic density. Thirteen dendritic segments from two labeled MCs ranged in length from 4.1 to 34.8  $\mu\text{m}$ , with overall synaptic densities that ranged from 0.16 to 1.38 synapses (syn)/ $\mu\text{m}$ . Ten dendrites from two labeled eTC cells ranged in length from 3.5 to 22.2  $\mu\text{m}$  and had synaptic densities varying from 0.29 to 1.12 syn/ $\mu\text{m}$ . The two example cells representing MCs or eTCs did not differ in the length of their reconstructed segments ( $P \geq 0.12$ ) nor their total synaptic density ( $P \geq 0.52$ ), so their data for each cell type were pooled. The average lengths of the dendritic segments sampled were similar for MCs vs. eTCs (MC,  $11.2 \pm 2.1 \mu\text{m}$ ; eTC,  $8.6 \pm 1.4 \mu\text{m}$ ;  $P = 0.38$ ). Dendrites from eTCs were more likely to be branched than MC dendrites (83% of eTC dendrites vs. 30% of MC dendrites).

The categorization of synapses onto the DAB-labeled dendrites was complicated by the fact that the DAB precipitate obscured possible postsynaptic densities. This made it difficult to determine whether the synapses were asymmetric or symmetric, which is one of the ultrastructural criteria typically used to distinguish putative excitatory vs. inhibitory synapses (Gray, 1959; Colonnier, 1968). Instead, we defined a synapse onto labeled dendrites to be “putatively excitatory” if it had round, clear vesicles at the presynaptic membrane (Fig. 1E). Additionally, when the processes containing these presynaptic elements were followed, they formed asymmetric synapses onto unlabeled dendrites (for examples of asymmetric synapses see Figs. 1F [red arrow], 6Aii). Similarly, “putatively inhibitory” synapses had pleomorphic, flattened vesicles (Fig. 1F), and their associated processes formed symmetric synapses onto unlabeled dendrites (for example of symmetric synapse see Fig. 6B2). These methods for categorizing synapses assumed that an individual process making synapses onto the DAB-labeled dendrite was homotypic for functional type (either excitatory or inhibitory), but this was generally reasonable given available data. Both

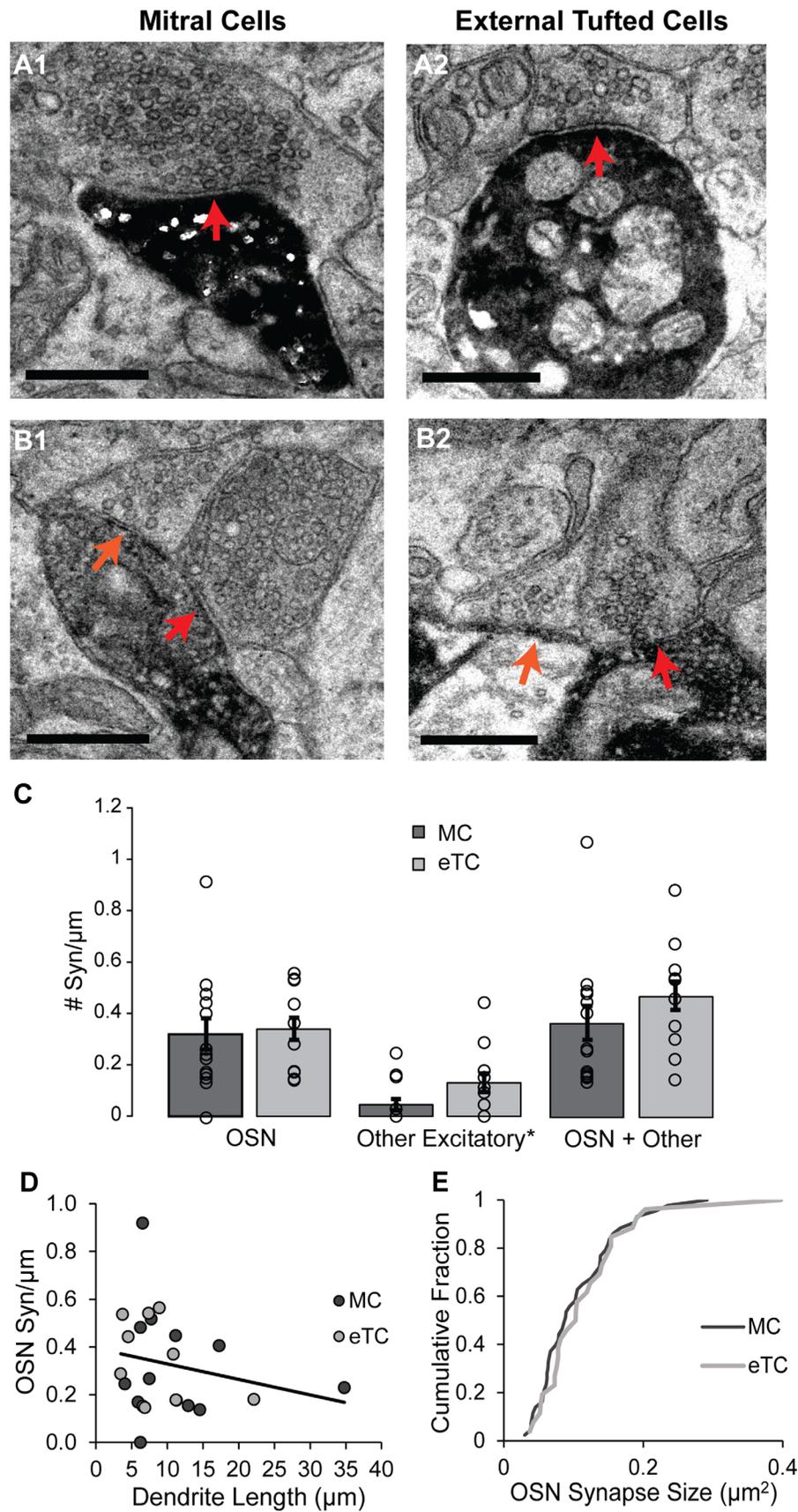
putative excitatory and inhibitory synapses were intermingled on the dendritic segments from MCs (Fig. 1C) and eTCs (Fig. 1D).

The large majority of putative excitatory synapses were formed directly onto the dendritic shafts of both cell types (90% of synapses on MCs, 92% of synapses on eTCs), although examples on spine-like protrusions were also observed (Fig. 1G,H). All of the putative excitatory synapses on the spine-like protrusions had the ultrastructural characteristics of OSN synapses (see below). Spine-like protrusions were characterized by a bulbous head, some extending small membranous projections, and a narrower neck that connected back to the dendritic shaft. Unlike typical dendritic spines in the cortex or hippocampus (Bourne and Harris, 2008), the synapses were usually not on the end of the spine-like protrusions. Putative inhibitory synapses were always on the dendritic shaft. In addition, both MC and eTC dendrites had nonsynaptic filopodia-like structures that were long ( $>0.5 \mu\text{m}$ ), thin projections arising from the dendritic shaft (Fig. 1G,H), although they were more common on MCs (46% of MC dendrites vs. 20% of eTC dendrites).

Our analysis of the apical dendrites of MCs and eTCs focused on the ultrastructure of dendrites in the glomerular tuft and did not examine properties of the apical dendritic trunk. Prior dendritic patch recordings (Urban and Sakmann, 2002) indicated that the relatively long trunk of the MC apical dendrite attenuates excitatory synaptic signals that originate in the tuft only modestly, approximately 30–40%, as they pass to the MC soma. This implies that a difference in trunk length or ultrastructure does not contribute significantly to the physiological phenomena that motivated our studies, including the much smaller glomerular synaptic signals recorded in MCs vs. eTCs.

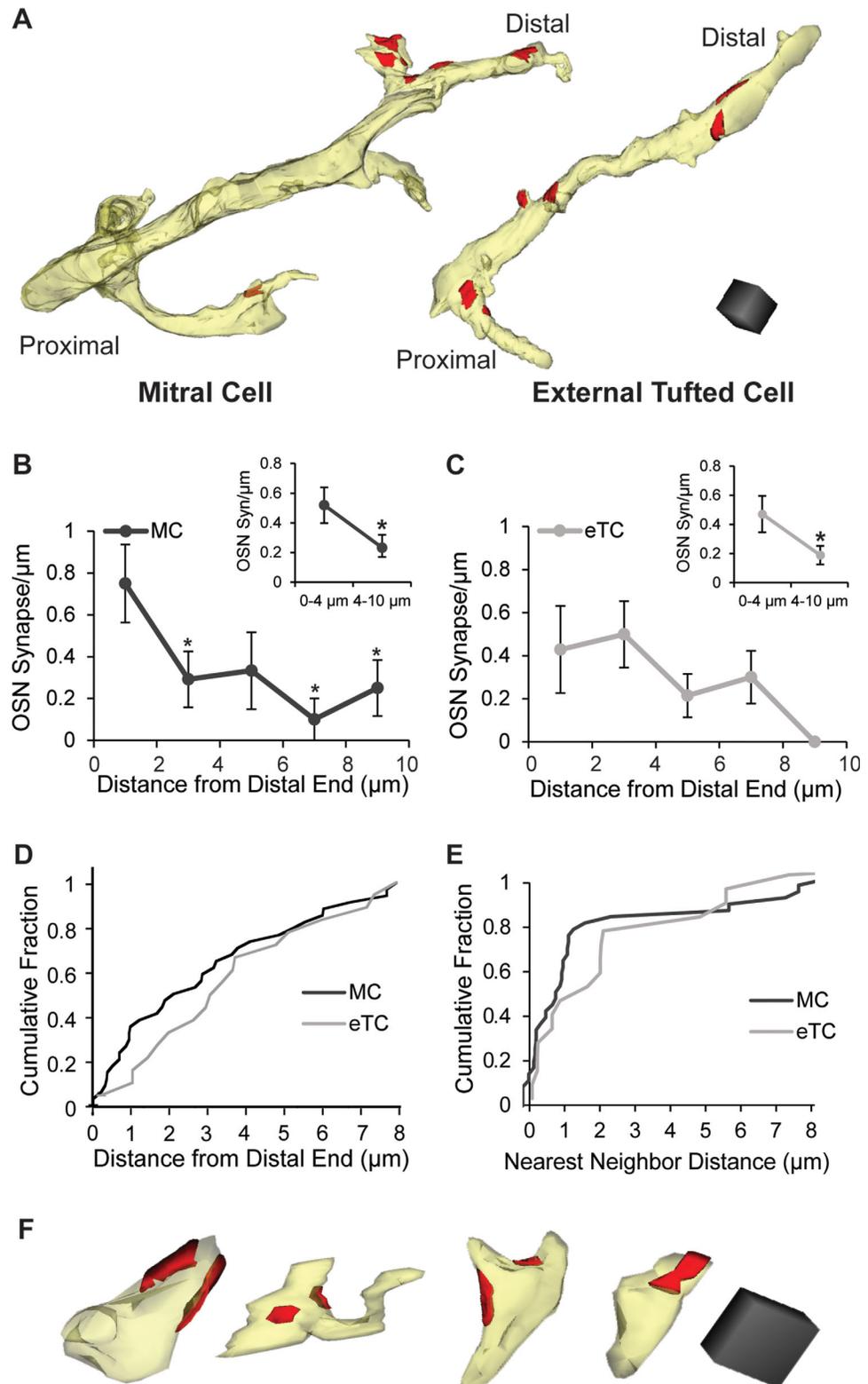
### Subtypes of putative excitatory synapses

In electron micrographs, the en passant boutons associated with presynaptic axon terminals of OSNs are characterized by a dark, granular appearance and contain a large number of round, clear vesicles (Pinching and Powell, 1971; Kasowski et al., 1999). The DAB-labeled dendrites of both MCs and eTCs displayed numerous OSN contacts by these criteria (Fig. 2A,B; MC, 43 OSN synapses across 13 dendritic segments; eTC, 26 OSN synapses across 10 dendritic segments), and the overall OSN synaptic density was similar in the two cell types (Fig. 2C; MC,  $0.32 \pm 0.06 \text{ syn}/\mu\text{m}$ ; eTC,  $0.34 \pm 0.04 \text{ syn}/\mu\text{m}$ ;  $P = 0.82$ ). The lengths of the dendritic segments that were analyzed varied considerably (see above), but we observed no correlation between segment length and the OSN synapse density estimate



**Figure 2.** MC and eTC dendrites have similar densities of OSN synapses. **A:** Examples of OSN synapses onto an MC (A1; red arrow) and eTC (A2), as characterized by docked synaptic vesicles at the presynaptic membrane and the relatively dark appearance of the axon terminal. **B:** Unidentified putative excitatory axons (orange arrows) synapse on MCs (B1) and eTCs (B2) next to OSN synapses (red arrows). For the eTC example, the DAB-labeled dendrite extends across the bottom of the image. In the imaged plane, the lighter-colored region is a mitochondrion within the dendrite. **C:** MC and eTC dendrites have similar densities of both OSN synapses (left) and unidentified, putative excitatory axonal synapses (middle). Histogram bars reflect mean values ( $\pm$ SE); density estimates from each analyzed dendritic segment are also overlaid. Asterisk on “Excitatory” emphasizes putative assignment. **D:** Estimates of OSN synapse densities were independent of the length of the dendritic segments analyzed.  $R^2 = 0.05$ . **E:** No difference was observed in the distributions of OSN synapse size between MCs and eTCs either. Scale bars =  $0.5 \mu\text{m}$ .

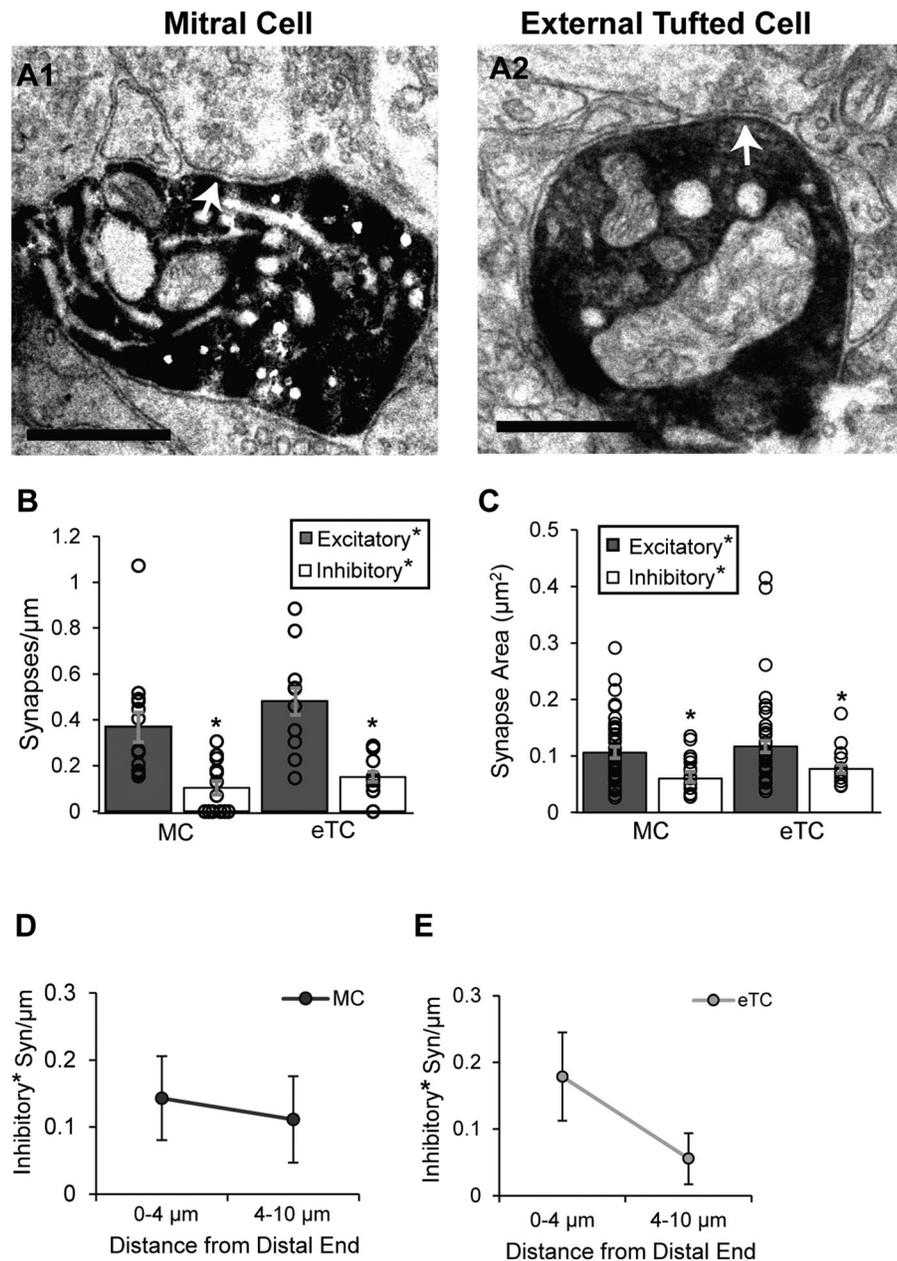
**Figure 3.** Spatial distribution of OSN synapses on MC and eTC dendrites. **A:** Reconstructions of an MC and eTC dendrite showing the distribution of OSN synapses (red). In these examples, no other types of synapses were identified. **B:** On MC dendrites, the density of OSN synapses was higher at the most distal 2  $\mu\text{m}$  of the segments vs. more proximal regions. Asterisks reflect significant differences ( $P < 0.05$ ) in OSN synapse densities in the indicated intervals vs. 0–2  $\mu\text{m}$  (in pairwise tests following an ANOVA). This pattern was also observed when the intervals were grouped together (inset;  $*P < 0.05$ ). **C:** OSN synapse density did not significantly vary along eTC dendrites when analyzed in 2- $\mu\text{m}$  intervals, but a distal preference was observed when intervals were grouped together (inset;  $*P < 0.05$ ). **D:** Cumulative distributions of OSN synapse distances from the distal ends of dendritic segments. The MC and eTC distributions were quite similar (K-S,  $P = 0.46$ ). **E:** Cumulative distributions of distances between nearest neighboring OSN synapses for MCs and eTCs. Note the large number of closely spaced OSN synapses on MCs, suggestive of clustering. **F:** Reconstructions of terminal ends of MC dendrites, all of which have OSN synapses (red) within 0.5  $\mu\text{m}$  of the tip. Scale cubes = 1  $\mu\text{m}^3$  in A; 0.125  $\mu\text{m}^3$  in F.



derived from each segment ( $R^2 = 0.05$ ; Fig. 2D). In addition, synapse size can be correlated with synaptic strength (Harris and Stevens, 1989; Schikorski and Stevens, 1997; Bartol et al., 2015), but we found no

difference in the areas of OSN synapses between MCs and eTCs (Fig. 2E; K-S test,  $P = 0.63$ ).

Both cell types also displayed presynaptic axon terminals that lacked the characteristic dark appearance of



**Figure 4.** Analysis of putative inhibitory synapses on MCs and eTCs. **A:** Electron micrographs of putative inhibitory dendrodendritic synapses onto a DAB-labeled MC (A1; white arrow) and eTC (A2). Note the vesicles that appear to be docked at the presynaptic membranes. In these examples, the presynaptic processes were followed to confirm that they formed a symmetric (inhibitory) synapse on an unlabeled dendrite (not shown). **B:** In both MCs and eTCs, putative inhibitory synapses (dendrodendritic plus axonal) were much fewer than putative excitatory synapses ( $*P < 0.05$ , all types). At the same time, the two cell types displayed similar densities of putative inhibitory synapses. Histogram bars reflect mean values ( $\pm$ SE); density estimates from each analyzed dendrite are also shown overlaid. Asterisks on “Excitatory” and “Inhibitory” emphasize putative assignments. **C:** Putative inhibitory synapses were significantly smaller than putative excitatory synapses on both cell types ( $*P < 0.05$ ). **D,E:** The distribution of putative inhibitory synapses on MCs (D) and eTCs (E) dendrites did not significantly vary with distance from the distal end. Scale bars = 0.5  $\mu\text{m}$ .

OSN axons but still had the round, clear vesicles consistent with excitatory synapses (Fig. 2B, orange arrows; see also orange synapses/arrows in Fig. 1C,D). There was a trend for the synaptic density of the unidentified axonal inputs to be higher in eTCs vs. MCs (Fig. 2C), but the difference was not significant across our samples (MC, four synapses across 13 dendritic segments, density =  $0.04 \pm 0.02$  syn/ $\mu\text{m}$ ; eTC, nine synapses across 10 dendritic segments, density =  $0.13 \pm 0.04$  syn/ $\mu\text{m}$ ;  $P = 0.12$ ). Combining all axonal inputs revealed that the overall density of these synapses was not significantly different between MCs and eTCs (Fig. 2C;  $0.36 \pm 0.07$  syn/ $\mu\text{m}$  for MCs,  $0.47 \pm 0.05$  syn/ $\mu\text{m}$  for

eTCs;  $P = 0.30$ ). Both cell types displayed a single example that appeared to be an incoming excitatory dendrodendritic synapse (not shown). These numbers for putative excitatory dendrodendritic synapses onto MC/eTC dendrites correspond to a density of only  $\sim 0.01$  syn/ $\mu\text{m}$ .

In addition to the density of synapses, the position of excitatory synapses on a cell’s dendritic arbor can impact the size of the excitatory signal at the cell body (Rall, 1967; Williams and Mitchell, 2008). The distal/proximal orientation of the dendrites was tracked on low-magnification images by their position within the glomerulus relative to the main trunk of the apical

dendrite of the labeled neuron. To analyze the distribution of OSN synapses on MCs and eTCs, dendritic segments at least 6  $\mu\text{m}$  in length were selected and broken down into 2- $\mu\text{m}$  intervals beginning at the most

distal end of the reconstructed segments (Fig. 3A). On MCs, the density of OSN synapses was highest at the most distal 2  $\mu\text{m}$  of the dendritic segments and decreased with distance from the distal end (Fig. 3B;

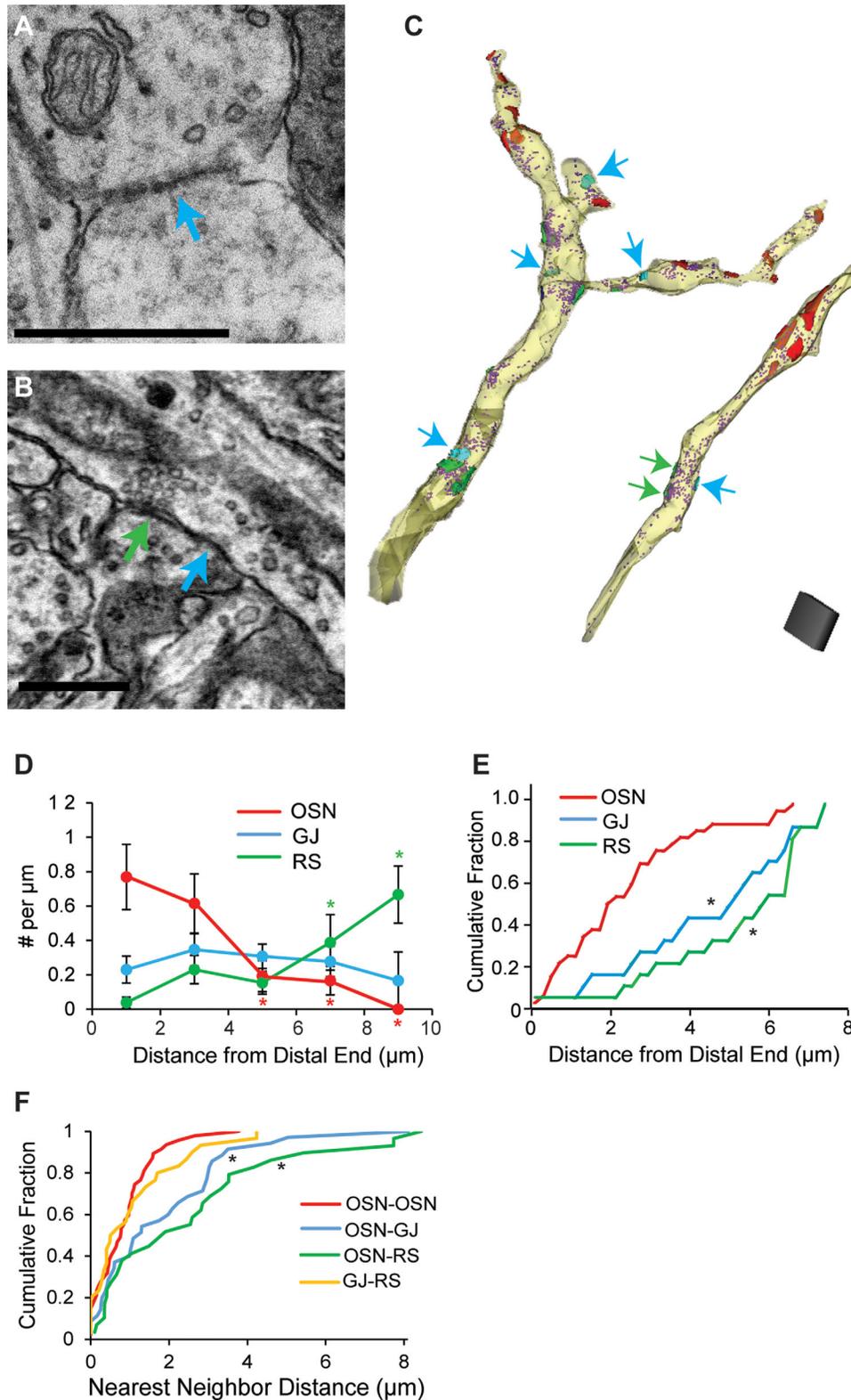


Figure 5.

$n = 12$  segments;  $F_{5,56} = 3.19$ ,  $P = 0.013$ ). For most of the MC segments analyzed, we were unable to determine definitively whether the most distal  $2\ \mu\text{m}$  reflected the terminal ends of dendritic branches. However, in four additional short MC dendritic segments that were definitively determined to be at the terminal end (see Materials and Methods), we always observed one or two OSN synapses within  $0.5\ \mu\text{m}$  of the ends (Fig. 3F). Thus, OSN synapses were preferentially located at distal ends of MC apical dendrites. On dendritic segments of eTCs, the distribution of OSN synapses did not significantly vary with distance when the dendritic segments were analyzed in  $2\text{-}\mu\text{m}$  intervals (Fig. 3C;  $n = 7$  dendrites;  $F_{5,28} = 1.87$ ,  $P = 0.13$ ). However, to account for the lower sample size for eTCs, we also analyzed the dendritic segments in longer section intervals, comparing the densities between  $0\text{--}4\ \mu\text{m}$  and  $4\text{--}10\ \mu\text{m}$ . In this analysis, eTCs as well as MCs displayed higher OSN synapse densities in the most distal  $4\ \mu\text{m}$  of the dendritic segments (Fig. 3B,C [insets]; eTCs,  $0\text{--}4\ \mu\text{m} = 0.46 \pm 0.12\ \text{syn}/\mu\text{m}$ ,  $4\text{--}10\ \mu\text{m} = 0.19 \pm 0.06\ \text{syn}/\mu\text{m}$ ,  $P = 0.046$ ; MCs,  $0\text{--}4\ \mu\text{m} = 0.52 \pm 0.12\ \text{syn}/\mu\text{m}$ ,  $4\text{--}10\ \mu\text{m} = 0.23 \pm 0.09\ \text{syn}/\mu\text{m}$ ,  $P = 0.028$ ). We also determined the distribution of distances from OSN synapses to the distal end of the dendritic segments (Fig. 3D) and found no difference between MCs and eTCs (K-S test,  $P = 0.46$ ; pooled distance measurements from MC or eTC dendrites  $\geq 8\ \mu\text{m}$ ; 34 MC synapses, 18 eTC synapses).

MCs displayed evidence for clustering of OSN synapses. This was evident in many example segments (Figs. 1C,D, 3A) and in plots of nearest-neighbor distances between synapses (Fig. 3E). For MCs, most OSN synapses ( $\sim 75\%$ ) had a nearest neighbor within  $\sim 1.2\ \mu\text{m}$ , and the distributions did not appear to follow the single exponential function expected if the synapses were distributed by a single random Poisson process. In eTCs,

some OSN synapses were within  $\sim 1\ \mu\text{m}$  of each other (Fig. 3A,D), although whether these reflected a distinct clustering process was not clear. Between MCs and eTCs, the distributions of nearest-neighbor distances for OSN synapses were not significantly different (K-S test,  $P = 0.084$ ).

### Subtypes of putative inhibitory synapses

Among putative inhibitory synapses terminating on the DAB-labeled processes, most appeared to be dendrodendritic rather than axonal in both MCs and eTCs (Fig. 4A; MCs, 11 dendrodendritic, two axonal across 13 dendritic segments; eTCs, nine dendrodendritic, one axonal across 10 dendritic segments). The density of all putative inhibitory synapses (dendrodendritic plus axonal) did not differ between MCs and eTCs (Fig. 4B;  $0.10 \pm 0.03\ \text{syn}/\mu\text{m}$  for MCs,  $0.15 \pm 0.02\ \text{syn}/\mu\text{m}$  for eTCs;  $P = 0.28$ ), although there were significantly fewer of them vs. putative excitatory synapses for both cell types ( $P \leq 0.0019$ ). We also calculated the synaptic area for the putative inhibitory synapses (all subtypes), finding that they were somewhat smaller than putative excitatory synapses for MCs (Fig. 4C; excitatory,  $0.11 \pm 0.01\ \mu\text{m}^2$ ,  $n = 48$ ; inhibitory,  $0.060 \pm 0.010\ \mu\text{m}^2$ ,  $n = 13$ ;  $P = 0.011$ ) and eTCs (excitatory,  $0.12 \pm 0.01\ \mu\text{m}^2$ ,  $n = 36$ ; inhibitory,  $0.077 \pm 0.007\ \mu\text{m}^2$ ,  $n = 10$ ;  $P = 0.020$ ). The putative inhibitory synapses were similar in size to axodendritic synapses found in regions such as area CA1 of the developing hippocampus (Watson et al., 2015) and lateral amygdala (Ostroff et al., 2010). Finally, we analyzed the distribution of putative inhibitory synapses along MC and eTC dendrites and did not observe a significant pattern in their localization along dendrites of either cell type (Fig. 4D,E; density comparisons between  $0\text{--}4\ \mu\text{m}$  and  $4\text{--}10\ \mu\text{m}$ ,  $P = 0.45$  for MC,  $P = 0.12$  for eTCs).

**Figure 5.** Distribution of OSN synapses, gap junctions, and release sites on unlabeled putative MC dendrites. **A:** Example of a gap junction (teal arrow) between two unlabeled dendrites. **B:** Putative MC dendrite forming a synapse (green arrow) and gap junction (teal arrow) with the same postsynaptic process. The presynaptic putative MC dendrite runs diagonally across the image. **C:** Reconstructions of two putative MC dendrites with OSN synapses (red), gap junctions (teal, teal arrows), presynaptic release sites (green), and vesicles (purple). Note examples of release sites that are close to (within  $\sim 1\ \mu\text{m}$  of) gap junctions. In the dendrite at right, two release sites across from a gap junction that are difficult to visualize are indicated with green arrows. **D:** Summary of spatial distributions on the distal-proximal axis. Unlike distal-favoring OSN synapses (red), gap junctions (teal) were evenly distributed, whereas release sites (green) were more highly localized on proximal portions of the dendritic segments. Colored asterisks reflect significant differences ( $P < 0.05$ ) in densities for the indicated intervals vs.  $0\text{--}2\ \mu\text{m}$  (in pairwise tests following an ANOVA). **E:** Cumulative distributions of distances from the distal ends of putative MC dendrites, plotted for OSN synapses, gap junctions, and presynaptic vesicular release sites. OSN synapses were located more distally than both gap junctions and release sites (K-S,  $*P \leq 0.005$ ), but the positions of gap junctions and release sites were not significantly different (K-S,  $P = 0.77$ ). **F:** Summary of nearest-neighbor distances. OSN synapses (red) were clustered together, as were gap junctions with release sites (yellow). OSN-to-gap junction (blue) and OSN-to-release site (green) distances were significantly longer ( $*P \leq 0.002$ ) compared with OSN synapse-to-OSN synapse distances. Scale bars =  $0.5\ \mu\text{m}$  in A,B; scale cube =  $1\ \mu\text{m}^3$ .

## Distribution of OSN synapses, gap junctions, and release sites on unlabeled dendrites

Previous studies in Cx36 KO mice suggested that direct OSN synaptic signals may be strongly attenuated by gap junctions that occur at a much higher density on the apical dendrites of MCs vs. eTCs (Gire et al., 2012). For analyzing the distribution of gap junctions on MCs, we had difficulty identifying them in our DAB-labeled dendrites, so we instead examined unlabeled dendritic segments in the same slices that appeared to be excitatory and that included gap junctions. We categorized these processes as putative MC dendrites because MCs display a 10–30-fold larger gap junctional conductance than eTCs (Hayar et al., 2005; Gire et al., 2012). A subset of the gap junction-containing dendrites could have reflected eTCs or other classes of TCs, but we considered that their classification as putative MC dendrites was reasonable given available data. The segments reflected the dendrites of MCs or TCs, and not inhibitory cells, based on the presence of round, clear vesicles and their formation of asymmetric synapses onto the dendrites of other cells. Gap junctions were identified by the appearance of densely staining plaques between two adjoining dendritic membranes (Brightman and Reese, 1969; Kosaka and Kosaka, 2004; Fig. 5A,B).

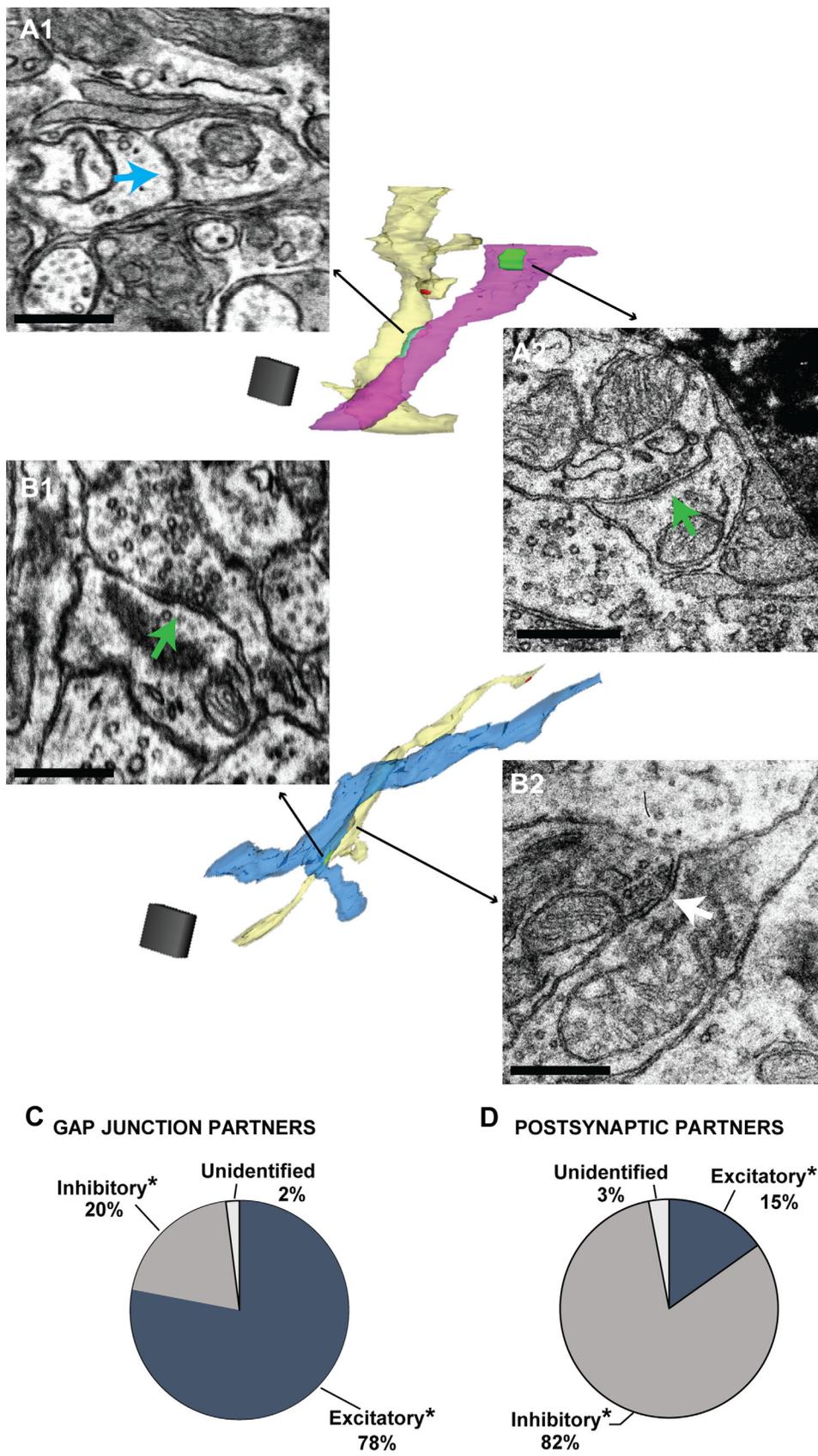
We found that, within the putative MC dendritic segments that were  $\geq 6 \mu\text{m}$  in length (examples shown in Fig. 5C), OSN synaptic density was highest on the distal-most  $2 \mu\text{m}$  of the segments and decreased with distance from the end (13 segments;  $F_{4,46} = 3.1$ ,  $P = 0.023$ ; Fig. 5D), similar to what was observed in DAB-labeled MCs (Fig. 3B). Gap junctions (overall density  $0.28 \pm 0.4/\mu\text{m}$ ), however, appeared to have a much more uniform distribution, with no significant differences in density between distal and proximal ends ( $F_{4,46} = 0.35$ ,  $P = 0.84$ ; Fig. 5D). Similarly, the position of the gap junctions on the dendritic segments was more proximal than OSN synapses, as reflected in the distributions of distances from the distal end (K-S,  $P = 0.005$ ; pooled data from all putative MC dendrites  $\geq 8 \mu\text{m}$ ; 30 OSN synapses, 18 gap junctions; Fig. 5E). We also measured the distances between OSN synapses and gap junctions (Fig. 5F). As was observed in the DAB-labeled MC dendritic segments, a large fraction of the OSN synapses in the putative MC segments was clustered together, within  $1\text{--}2 \mu\text{m}$  of each other, but OSN synapses were significantly farther from gap junctions (K-S test, OSN-OSN vs. OSN-GJ distribution,  $P = 0.002$ ). Thus gap junctions were not coclustered with OSN synapses.

We also observed, within the unlabeled dendritic segments of putative MCs, numerous presynaptic vesicular

release sites that appeared to be excitatory (overall density  $0.19 \pm 0.02$  release sites/ $\mu\text{m}$  across 13 dendritic segments). These were indicated by the presence of docked round, clear vesicles positioned across a synaptic cleft from a postsynaptic density (Fig. 5B, green arrow; Pinching and Powell, 1971). By activating other excitatory and GABAergic cells at a glomerulus, neurotransmitter released from these sites can modulate MC/TC activity downstream of OSN inputs. The density of these release sites increased from distal to more proximal regions of the dendrites (Fig. 5D;  $F_{4,46} = 3.6$ ,  $P = 0.012$ ), differing from both the distal-favoring OSN synapses and the evenly distributed gap junctions. The positioning of release sites and OSN synapses with respect to the distal end of the unlabeled dendritic segments also differed significantly (K-S,  $P = 0.0005$ ; 30 OSN synapses, 17 release sites; Fig. 5E). In terms of the distances between individual elements, presynaptic release sites were farther from OSN synapses than OSN synapses were from each other (K-S, OSN-OSN vs. OSN-RS,  $P = 0.001$ ; Fig. 5F), indicating that the release sites were not coclustered with OSNs. The release sites, however, were much closer to gap junctions (K-S, OSN-RS vs. GJ-RS,  $P = 0.006$ ). The mean separation between a release site and the nearest gap junction in fact was only  $1.1 \pm 0.2 \mu\text{m}$  ( $n = 25$ ). Thus, gap junctions and presynaptic release sites on putative MC dendrites were clustered together.

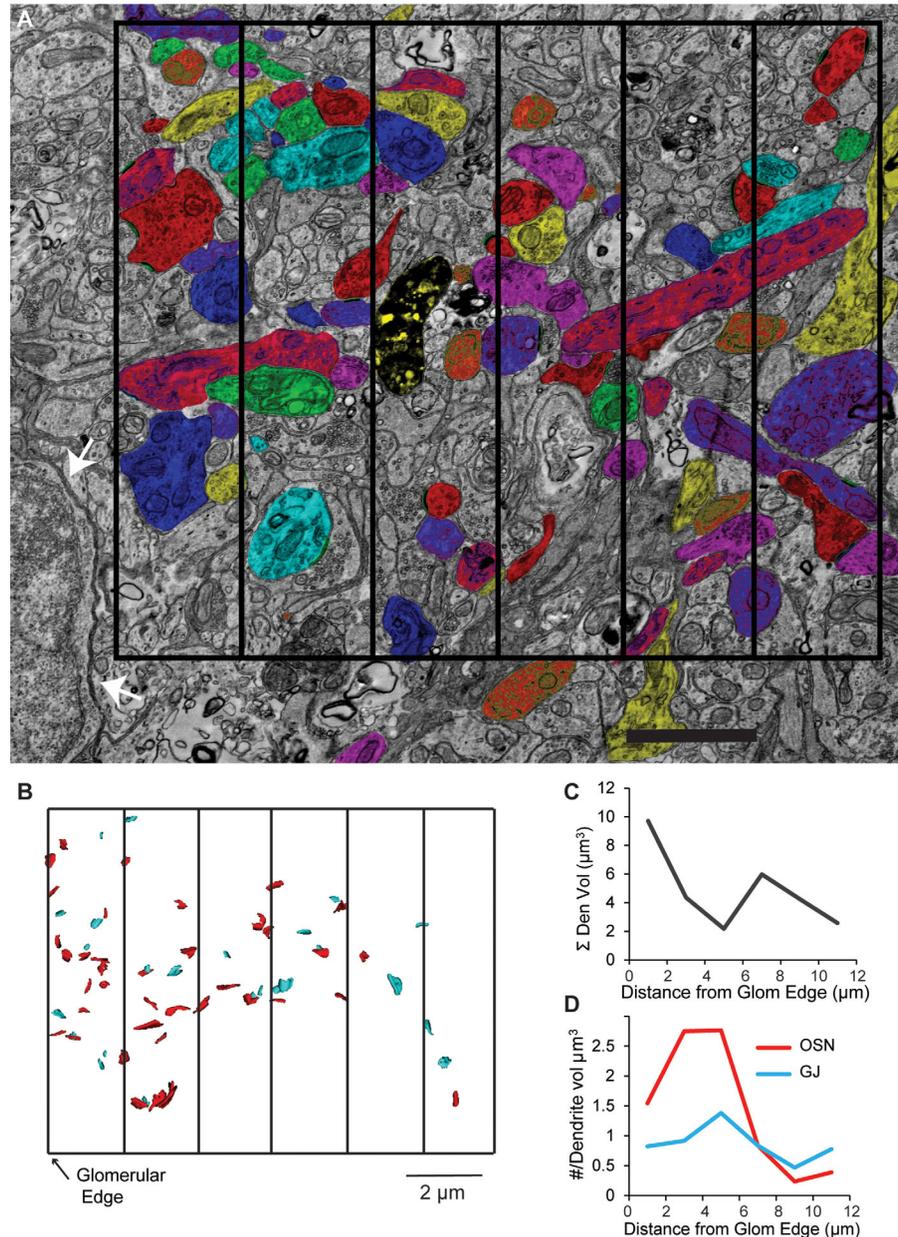
The electrically and chemically coupled partners of the gap junction-containing, putative MC dendrites were mostly identified (Fig. 6). This was done by finding dendrites that were either gap junctionally (Fig. 6A) or synaptically (Fig. 6B) connected to the putative MC dendrite and following these dendrites to where they formed either an asymmetric or a symmetric synapse onto another (third) dendrite. By these criteria, most (78%) of the dendrites that shared a gap junction with the putative MC dendrites appeared to be excitatory, although 21% were likely inhibitory (Fig. 6C). In contrast, only 15% ( $n = 4$ ) of the dendrites that received a chemical synapse from putative MC dendrites were apparently excitatory, although 82% ( $n = 21$ ) were likely inhibitory (Fig. 6D). The estimated density of synapses from putative MC dendrites onto other putative excitatory dendrites from this analysis was  $\sim 0.03$  syn/ $\mu\text{m}$ , a quite low value for dendrodendritic synapses between excitatory elements that was roughly similar to that found in the analysis of DAB-labeled dendrites described above ( $\sim 0.01$  syn/ $\mu\text{m}$ ).

There is evidence that OSN synapses occur within an “axonal compartment” located around the outer perimeter of glomeruli (Kasowski et al., 1999; but see Kosaka et al., 2001). Therefore, we wanted also to explore the



**Figure 6.** Gap junctional and chemical synaptic partners of putative MC dendrites. **A:** Putative MC dendrite (light yellow reconstructed dendrite in middle) connected through a gap junction (A1, teal arrow) to a putative excitatory dendrite (pink reconstructed dendrite), as indicated by the asymmetric dendrodendritic synapse (A2, green arrow) onto another process (not shown in image of reconstructed dendrites). **B:** Putative MC dendrite (light yellow reconstructed dendrite) that formed a putative excitatory chemical synapse (B1, green arrow) onto a putative inhibitory dendrite (blue reconstructed dendrite), as indicated by the symmetric dendrodendritic synapse (B2, white arrow) onto another process (in this case, it was the same putative MC dendrite). **C:** Putative MC dendrites formed gap junctions mainly with putative excitatory dendrites. **D:** Putative MC dendrites formed chemical synaptic contacts mainly onto putative inhibitory dendrites. Scale bars = 0.5  $\mu\text{m}$ ; scale cubes = 1  $\mu\text{m}^3$ .

**Figure 7.** OSN synapse density decreases with distance from the glomerular edge. **A:** Electron micrograph with a subset of traced dendrites in the glomerular neuropil. The cell bodies of juxtglomerular cells (example at white arrows) defined the outer edge of the glomerulus. Images were analyzed in  $2 \times 10 \times 2 \mu\text{m}$  rectangular cuboids. A two-dimensional  $12 \times 10 \mu\text{m}$  grid divided into six sections is superimposed, with the left-most vertical line reflecting the edge of the glomerulus. **B:** Reconstruction of OSN synapses (red) and gap junctions (teal) from putative MC dendrites across the six rectangular cuboids. **C:** Summed volume of analyzed dendritic segments within each rectangular cuboid plotted as a function of distance from the glomerular edge. Note that the analyzed dendritic volume was largest within the first  $2 \mu\text{m}$  from the edge. **D:** Densities of OSN synapses (red) and gap junctions (teal) normalized to the volume of dendrite analyzed plotted as a function of distance from the glomerular edge. The density of OSN synapses (red line) was highest close to the glomerular edge, whereas the density of gap junctions (teal line) remained more consistent. Scale bar =  $2 \mu\text{m}$ .



distribution of OSN synapses and gap junctions relative to position within a glomerulus. An edge of the glomerulus was overlaid with a  $12 \times 10 \times 2 \mu\text{m}$  grid that was divided into  $2 \times 10 \times 2 \mu\text{m}$  rectangular cuboids that ran parallel with respect to the glomerular edge (Fig. 7A,B; grid shown in two dimensions). We counted, within each  $2 \times 10 \times 2 \mu\text{m}$  rectangular cuboid, the number of OSN synapses and gap junctions within putative MC dendrites ( $n = 49$  OSN synapses, 26 gap junctions), and divided those values by the summed volume of analyzed dendrites in each cuboid (Fig. 7C) to obtain density estimates for dendritic volumes (Fig. 7D). We found that OSN synapse densities were highest within

$6 \mu\text{m}$  from the edge of the glomerulus, although gap junctions displayed a much more uniform distribution. These findings support the presence of an outer axonal compartment in glomeruli and also are consistent with data presented earlier indicating that OSN synapses onto MC dendrites are localized primarily to more distal portions of dendrites (Figs. 3B, 5D,E) and that gap junctions maintain a more uniform distribution (Fig. 5D,E).

## DISCUSSION

Recent studies have provided evidence that MCs and TCs have markedly different odor-evoked and synaptic

responses. To understand the anatomical bases for the physiological results, we have performed the first quantitative ultrastructural analysis of the synaptic organization within the glomerulus under conditions in which definitive information was available through biocytin and DAB labeling on whether observed dendritic elements reflected MCs vs. a class of TCs known as eTCs. We also analyzed a large sample of unlabeled putative MC dendrites to obtain information on some cellular features that could not be readily visualized in the DAB-labeled processes. Our main results were that 1) OSNs and eTCs had similar densities of OSN synapses as well as other types of chemical synapses, 2) OSN synapses were preferentially localized on distal portions of the apical dendrites on both MCs and eTCs, 3) gap junctions had a uniform spatial distribution on putative MC dendrites and on average were more proximally located than OSN synapses, and 4) gap junctions on putative MC dendrites were clustered with presynaptic vesicular release sites. We discuss our ultrastructural findings with respect to observed differences in physiological responses of MCs vs. eTCs as well as other aspects of glomerular signaling.

### What explains the difference in direct OSN signal size in MCs vs. eTCs?

In responses to both electrical and optogenetic stimulation of OSNs, eTCs display excitatory postsynaptic currents (EPSCs) that reflect direct OSN inputs ("OSN EPSCs") that are much larger than those in MCs (Gire et al., 2012; Vaaga and Westbrook, 2016). Exactly how much larger OSN EPSCs are in eTCs may depend on OSN stimulation intensity (Najac et al., 2011; Vaaga and Westbrook, 2016), with estimates ranging from a factor of 30 for weak stimuli (Gire et al., 2012) to a factor of approximately four for strong stimuli (Vaaga and Westbrook, 2016). Certainly the simplest explanation for the large differences in OSN EPSCs is that OSNs make many more synapses onto the apical dendrites of eTCs vs. MCs, but this hypothesis was not supported by our counts of OSN synapses, which showed similar densities on the two cell types ( $\sim 0.3$ – $0.4$  synapses/ $\mu\text{m}$ ). Moreover, because MCs and eTCs have similarly sized apical dendritic tufts (Macrides and Schneider, 1982), these OSN synapse density estimates imply a similar total number of OSN synapses. In our analysis, we used high electron density as a marker of OSN axonal boutons, and we did observe putative excitatory axonal boutons that were less electron dense. Thus, we might have under- or overestimated OSN synapse density depending on the criterion for electron density chosen. However, axonal synaptic markers strongly

colocalize with the OSN-specific protein olfactory marker protein (OMP; Kasowski et al., 1999; Kim and Greer, 2000), suggesting that most axonal synapses reflect OSNs, and in our experiments OSN synapses were a large fraction (84% across MCs and eTCs) of the total. In addition, MCs and eTCs displayed similar densities when we counted all putative excitatory axonal synapses. Hence, any errors in our estimates of OSN synapse density are likely to be small and cannot account for the large difference in OSN signal size between MCs and eTCs.

In many neurons, the location of excitatory synapses on the dendritic arbor can also influence the size of current and voltage signals recorded at the cell soma (Rall, 1967; Williams and Mitchell, 2008). Signals from synapses on distal dendrites are especially attenuated by the intracellular resistance and current loss through dendritic conductances. We found that synapse location is in fact likely to be important for explaining the different-sized OSN signals in MCs vs. eTCs but only as it relates to a dendritic gap junctional conductance in MCs (Schoppa and Westbrook, 2002; Christie et al., 2005; Kosaka and Kosaka, 2005; Pimental and Margrie, 2008; Maher et al., 2009) that is present at a much lower level in eTCs (Hayar et al., 2005; Gire et al., 2012). In terms of the absolute positioning of OSN synapses within the apical dendritic tuft, MCs and eTCs displayed no significant difference, but, in unlabeled putative MC dendrites, gap junctions were distributed on average between OSNs and the cell soma. Such a placement could make the gap junctional conductance well positioned to shunt OSN signals prior to their reaching the cell body of MCs. It should be noted that our results here suggesting that there are no significant differences between MCs and eTCs in the absolute positioning of OSN synapses or in OSN synapse number are in some sense not surprising. Prior studies of Cx36 KO mice showed that eliminating gap junctions causes monosynaptic OSN currents to be very similar in MCs and eTCs (Gire et al., 2012). Although KO experiments have their own caveats, those results, like our ultrastructural results here, suggested that gap junctions and not OSN synapse location or number are the major contributor to the different-sized OSN signals in the two cell types.

That a dendritic conductance intrinsic to MCs is involved in attenuating OSN signals from distal dendrites of course makes these cells like many neurons throughout the brain (Hoffman et al., 1997; Berger et al., 2001; Williams and Stuart, 2000). An attenuating effect involving gap junctions is, however, likely to be more complex than that resulting from other dendritic conductances such as potassium channels. Because at least a major component of the gap junctional

conductance is between MC dendrites, the gap junctions would allow sharing of excitatory OSN signals between MCs at the same time that the signal in any one MC is dissipated by current loss. Furthermore, the degree to which a given gap junctional conductance can shunt excitatory inputs on a particular MC will depend on the membrane potential of the dendrites of other MCs with which it is coupled (which will determine the driving force for current flow between dendrites). A modeling study of a gap junctionally connected network of interneurons in striatum (Hjorth et al., 2009) examined this latter issue, finding that the degree to which gap junctions shunt excitatory inputs decreases as the dendrites of different cells in the network become depolarized by increasingly coincident excitatory inputs.

### Other features of the synaptic organization within glomeruli

Our analysis of putative inhibitory synapses on apical dendrites showed similar densities of dendrodendritic synapses on MCs and eTCs and, on rare occasions, axonal synapses that appeared to be inhibitory. The similar density of putative inhibitory synapses was somewhat surprising given available physiological evidence. Several studies that have made side-by-side comparisons between MCs and eTCs have indicated that eTCs display much larger GABAergic electrical signals that can be attributed to neurons in the glomerular layer (periglomerular cells or short-axon cells; Gire and Schoppa, 2009; Whitesell et al., 2013; Banerjee et al., 2015). One possible explanation for a small physiological GABAergic signal at the MC soma is that those signals are attenuated by gap junctions in a manner similar to direct OSN signals. With respect to the distribution of putative inhibitory dendrodendritic synapses, we found that they were mixed with presumed excitatory axonal synapses (Kosaka et al., 2001).

Putative excitatory synapses distinct from OSNs were also found on both MCs and eTCs. These included axon terminals that lacked the electron-dense profile of OSN terminals, which most likely reflected centrifugal inputs from olfactory cortex (Pinching and Powell, 1972; Markopoulis et al., 2012; Rothermel and Wachowiak, 2014) and/or cholinergic inputs from the basal forebrain (Kasa et al., 1995; D'Souza and Vijayaraghavan, 2014). Putative excitatory dendrodendritic synapses (Kosaka and Kosaka, 2005) were also observed on both MCs and eTCs, although the estimated density of these synapses (0.01–0.03 syn/ $\mu\text{m}$ ) was markedly lower than that of other synaptic elements within glomeruli (e.g., 0.3–0.4 syn/ $\mu\text{m}$  for OSN synapses). Additionally, our

ultrastructural analysis provided evidence for mixed chemical and electrical signaling (Kosaka and Kosaka, 2005; Hamzei-Sichani et al., 2012; Vivar et al., 2012), as reflected in complexes on putative MC dendrites that contained vesicular release sites and gap junctions in close proximity ( $<1\mu\text{m}$  away). Based on the overall density of release sites ( $\sim 0.19/\mu\text{m}$ ; see above) and the nearest-neighbor distribution for gap junctions and release sites (Fig. 5F), we estimate the density of such complexes to be  $\sim 0.12/\mu\text{m}$ . These mixed complexes are provocative, especially in light of prior evidence that MCs can engage in rapid lateral excitatory interactions at their apical dendrites (Urban and Sakmann, 2002; Schoppa and Westbrook, 2002; Pimental and Margrie, 2008). These interactions, which are AMPA receptor-dependent but abolished in Cx36 KO animals (Christie et al., 2005), do not appear to involve activation of presynaptic autoreceptors on MCs (Pimental and Margrie, 2008) but could involve AMPA receptors on GABAergic interneurons that couple to MCs (see Fig. 6B; Kosaka and Kosaka, 2005) or nonsynaptic AMPA receptors on other MCs/TCs.

Our ultrastructural analyses revealed one other interesting feature of OSN synapses, perhaps important in synapse development. Although we observed that most OSN synapses occurred directly on dendritic shafts (Pinching and Powell, 1971, White, 1972, Hinds and Hinds, 1976), both MCs and eTCs displayed numerous examples of synapses positioned on spine- or filopodia-like structures. One possibility is that these synapses are involved in synaptogenesis. In the hippocampus, where most mature excitatory synapses are on dendritic spines, the progression of synaptogenesis during the first postnatal week has been proposed to involve the extension of dendritic filopodia that then form one or multiple synaptic contacts before retracting back to the dendritic shaft and finally re-emerging as a mature dendritic spine (Fiala et al., 1998; Harris, 1999; Maletic-Savatic et al., 1999). Perhaps MCs and TCs use similar mechanisms when forming excitatory synaptic contacts with OSNs, but, rather than maturing into a typical dendritic spine, the mature synapses remain on the dendritic shaft. Ongoing synaptic refinement could also explain the fact that we did not observe a strict segregation of dendrodendritic synapses from OSN axonal synapses (see above), which differed from findings of some previous studies (Kasowski et al., 1999; Kim and Greer, 2000). Our ultrastructural experiments were in rats at postnatal P8–14, when the organization of glomerular synapses might still be in flux (Hinds and Hinds, 1976).

### Broader implications

Returning to the issue of direct OSN signal size discussed above, our ultrastructural results here have

implications that go beyond just explaining why the signals are much smaller in MCs vs. eTCs in brain slice recordings. First, the fact that OSN signals at the cell body are relatively small in MCs itself has immediate functional significance. Because spikes are generally initiated at the MC soma (except when many OSNs are excited simultaneously; Chen et al., 1997), the small direct OSN signal at the soma means that direct OSN inputs will activate MCs with low efficacy. This will be true regardless of the number of OSN-to-MC synapses. Furthermore, because MCs can be activated through a multistep OSN-to-eTC-to-MC pathway (De Saint Jan et al., 2009; Najac et al., 2011; Gire et al., 2012), the small direct OSN signal in MCs compared with eTCs means that the system would generally favor multistep activation of MCs. Multistep activation of MCs would be favored all the more because TCs have greater intrinsic excitability (Liu and Shipley, 2008; Burton and Urban, ). In the natural situation, a low efficacy for direct OSN signals in MCs and a preference for multistep activation could underlie the relatively low sensitivity of MCs to odor as well as their delayed spike responses (Nagayama et al., 2004; Igarishi et al., 2012; Fukunaga et al., 2012; Otazu et al., 2015).

Our results also raise the obvious question of why MCs have a significant complement of OSN synapses if their associated signals at the soma are greatly attenuated by dendritic gap junctions. Among the possible explanations is that direct OSN synapses in fact drive only local depolarizations that are restricted to the apical dendrites of MCs. These could control functions such as synaptic plasticity. Also, it is possible that the shunting effect of the gap junctions is dynamically modulated such that direct OSN signals emerge in MCs in certain situations. As discussed above, the shunting capacity of gap junctions may decrease as excitatory inputs are increasingly coincident (Hjorth et al., 2009), as might occur when OSNs at a glomerulus are highly active or if the gap junctions themselves are downregulated (Bloomfield and Volgyi, 2009). These mechanisms could operate without any change in the number of expressed OSN synapses or gap junctions, allowing rapid changes in the efficacy of direct OSN signals in driving MC spiking.

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## CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest in the studies described in this article.

## ROLE OF AUTHORS

JNB and NES had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: JNB, NES. Acquisition of data: JNB. Analysis and interpretation of data: JNB, NES. Drafting of the manuscript: JNB, NES. Critical revision of the manuscript for important intellectual content: JNB, NES. Statistical analysis: JNB, NES. Obtained funding: NES.

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