Chapter 2

3D axon morphologies of individual Layer 5 neurons indicate cell type-specific intracortical pathways for whisker motion and touch

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Published in: Proceedings of the National Academy of Sciences, 2011, 108 (10), 4188-4193
3D reconstructions of L5 axon morphology
Abstract

The cortical output layer 5 contains two excitatory cell types, slender- and thick-tufted neurons. In rat vibrissal cortex, slender-tufted neurons carry motion and phase information during active whisking, but remain inactive after passive whisker touch. In contrast, thick-tufted neurons reliably increase spiking preferably after passive touch. Reconstructing the 3D patterns of intracortical axon projections from individual slender- and thick-tufted neurons, filled *in vivo* with biocytin, we were able to identify cell type-specific intracortical circuits that may encode whisker motion and touch. Individual slender-tufted neurons showed elaborate and dense innervation of supragranular layers of large portions of the vibrissal area (total length 86.8 ± 5.5 mm). During active whisking, these long range projections may modulate and phase-lock the membrane potential of dendrites in layer 2 and 3 to the whisking cycle. Thick-tufted neurons with soma locations intermingling with those of slender-tufted ones display less dense intracortical axon projections (total length 31.6 ± 14.3 mm) that are primarily confined to infragranular layers. Based on anatomical reconstructions and previous measurements of spiking we put forward the hypothesis that thick-tufted neurons in rat vibrissal cortex receive input of whisker motion from slender-tufted neurons onto their apical tuft dendrites and input of whisker touch from thalamic neurons onto their basal dendrites. During tactile-driven behavior, such as object location, near coincident input from these two pathways may result in increased spiking activity of thick-tufted neurons and thus enhanced signaling to their subcortical targets.
3D reconstructions of L5 axon morphology
Introduction

Based on classification of dendrite morphology, cortical layer 5 (L5) contains two primary excitatory cell types: slender- and thick-tufted neurons (Hallman et al., 1988; Larkman and Mason, 1990). These two types are considered the main output neurons of a cortical column (Jones, 1984; Meyer et al., 2010a). Slender- (or thin-) tufted pyramidal neurons project to the striatum and are commonly referred to as corticostriatal neurons. Thick- (or tall-) tufted pyramidal neurons project to the posterior nucleus of the thalamus, brain stem, superior colliculus and pons (Alloway, 2008; Groh et al., 2008; Aronoff et al., 2010). The two neuron types have been characterized across cortical areas, including somatosensory, visual, auditory, motor and prefrontal cortices and therefore represent canonical elements of the cortical micro-circuitry (Hubener et al., 1990; Ojima et al., 1992; Binzegger et al., 2004; Brecht et al., 2004; Gao and Zheng, 2004; Martinez et al., 2005; Morishima and Kawaguchi, 2006; Larsen et al., 2007; Schubert et al., 2007; Brown and Hestrin, 2009; Sakata and Harris, 2009).

We recently showed that slender- and thick-tufted neurons in L5 of vibrissal cortex differentially increase spiking activity depending on the behavioral state (de Kock et al., 2007b; de Kock and Sakmann, 2009a). The majority of slender-tufted neurons carry phase information upon free-whisking (self-motion of whiskers). Their modulation depth is highest among all excitatory cell types in vibrissal cortex. During quiet (non-whisking) periods or passive whisker deflection (touch), however, slender-tufted neurons remain relatively inactive. In contrast, thick-tufted neurons are reliably activated upon passive whisker touch, but show almost no increased spiking during free-whisking periods.

The pathways that underlie these differences in context-dependent spiking remain controversial (Ahissar et al., 2008; Masri et al., 2008), but could at least in part be the result of cell type-specific thalamocortical excitation (Bureau et al., 2006a; Yu et al., 2006; Urbain and Deschenes, 2007; Petreanu et al., 2009b; Meyer et al., 2010b). Thick-tufted neurons receive input onto their basal dendrites primarily from excitatory neurons in the ventral posterior medial nucleus of the thalamus (VPM), while basal dendrites of slender-tufted neurons are primarily innervated by neurons in the medial division of the posterior thalamic nucleus (POm) (Meyer et al., 2010b). Using artificial whisking in anaesthetized rats, it was suggested that the POm (paralemniscal) pathway may carry information on whisker motion, while the VPM (lemniscal) pathway may encode whisker touch (in addition to whisker motion). Thus, cell type- and state-specific spiking in L5 during
active somatosensation (Knutsen et al., 2006; Mehta et al., 2007) could be mediated by these two functionally and anatomically segregated thalamocortical pathways (Yu et al., 2006).

Finally, functional and morphological studies of slender- and thick-tufted neurons indicate that these output neurons also contribute to intracortical circuits (Schubert et al., 2001; Shepherd et al., 2005; Shepherd and Svoboda, 2005; Schubert et al., 2006a; Larsen et al., 2007). Here, we reconstructed the entire 3D axon projections in the vibrissal area of individual, in vivo filled slender- and thick-tufted neurons in rat vibrissal cortex, using a semi-automated tracing technique (Oberlaender et al., 2007). We quantified the cell type-specific 3D innervation patterns with respect to the laminar and columnar architecture of the vibrissal cortex and found highly significant, cell type-specific differences in total axon length and intracortical projection patterns.

Considering that slender- and thick-tufted neurons are primarily involved in processing of signals related to the phase of whisker motion and whisker touch, respectively, our anatomical quantification of their intracortical targets leads to a hypothesis on cell type-specific microcircuits that may encode active whisker motion, passive whisker touch and object location.

![Figure 1: 3D reconstructions of in vivo filled slender-tufted pyramidal neuron in L(ayer) 5 of rat vibrissal cortex. A) View on cortical surface, approximately tangential to the cortical surface above the vibrissal area (dorsal axis points out of the paper plane). Neuronal processes (axon = blue, basal dendrites = red, apical dendrites = orange) are shown with reference to the barrel field in L4 (letters A-E refer to whisker rows, numbers to whiskers](image-url)
within the same row). Note the wide lateral spread and dense innervation of slender-tufted axon into multiple barrel columns surrounding the principal column, which contains the cell’s soma. Further, this neuron displays long range projections outside the vibrissal area into surrounding higher order dysgranular cortices. B) Semi-coronal view along the D-whisker row in A). Note that the wide ranging lateral projections are primarily confined to supragranular layers. Projections in dysgranular cortex originate from a single branch and end in clusters of columnar dimensions.

Results

3D intracortical projections of L5 slender-tufted pyramidal neurons

Figure 1 shows one example of five reconstructed slender-tufted neurons in rat vibrissal cortex (Fig. S1). The 3D neuron morphologies (dendrites and axon) were obtained from individual in vivo preparations and are shown in tangential (Fig. 1A) and semi-coronal view (Fig. 1B), respectively. Originally classified by common dendrite morphology (Hallman et al., 1988), slender-tufted neurons also share cell type-specific and characteristic intracortical axon projection patterns. First, slender-tufted neurons displayed elaborate and dense lateral projections, innervating large portions of the vibrissal area (Fig. 1A). Second, these lateral projections were mostly confined to supragranular layers, while granular and infragranular projections remained primarily within the principal column or septa in its immediate vicinity (Fig. 1B). Third, two out of five neurons displayed significant innervation of higher order dysgranular cortices adjacent to the vibrissal area (Fig. S1). In the dysgranular zones, projections ended in supragranular depths in clusters of columnar dimensions, originating from a single projecting axon branch, indicating highly specific outgrowth and branching.
Semi-automated axon reconstructions

*Figure 2 (previous page): Quantification of 3D intracortical axon innervation of slender-tufted neurons (I).* A) Semi-coronal view of 3D axon density of the five neurons shown in Supplementary Figure 1. The dashed box renders the approximate dimensions of the respective principal column of each neuron tracing. Note that innervation is not restricted to the principal column, particularly in supragranular layers. B) The total axon length of individual neuron tracings exceeds previous reports by one order of magnitude. This is due to the fact that most axon is found outside the principal column, or even extends into dysgranular cortices outside the vibrissal area, which cannot be recovered in in vitro preparations. C) Axon length profiles along the vertical column axis: within the principal column, L5 slender-tufted neurons display two innervation zones: one in supragranular L2/3 and one in infragranular L5; outside the principal column innervation is largely restricted to supragranular layers, indicating that L5 slender-tufted – L5 projections remain locally (intracolumnar), whereas L5 slender-tufted – L2/3 projections comprise primarily long range projections (i.e. leaving the principal column).

To discriminate quantitatively between the short (columnar) and long range (vibrissal area) projections, we measured the total axon length within the principal column, as well as within and outside the vibrissal area (Fig. 2A, B). Total axon length of cortical projections from slender-tufted neurons ranged from 79.5 mm to 94.8 mm (Fig. 2B; 86.8 ± 5.5 mm) of which 16.4 ± 6.7 mm (19%) was confined to the principal column. The majority of axon (59.9 ± 9.7 mm; 69%) was found within the vibrissal area, but outside the principal column, with similar amounts in septa (28.0 ± 7.5 mm) and columns (31.9 ± 7.6 mm). A significant amount of axon (10.6 ± 9.9 mm; 12%) was located in dysgranular zones.

Furthermore, the vertical innervation profiles of axon projections within and outside the principal column displayed significant differences (Fig. 2C, 3A). Within the principal column, innervation peaks similarly in supragranular layers (6.2 ± 3.6 mm) and infragranular L5 (8.1 ± 2.7 mm). Outside the principal column, innervation is primarily restricted to supragranular depths (Fig. 2C; 43.4 ± 9.7 mm), exceeding granular (7.1 ± 3.6 mm) and infragranular projections (9.4 ± 3.8 mm) outside the principal column by a factor of three (Fig. 3B).
Figure 3: Quantification of 3D intracortical axon innervation of slender-tufted neurons (II).
A) Tangential view of 3D axon density of the five neurons shown in Supplementary Figure 1 in supragranular, granular and infragranular layers (from left to right). As indicated by the semi-coronal view in Figure 2A, projections from L5 slender-tufted neurons remain largely confined to the principal column in granular and infragranular layers, but innervate almost the entire vibrissal area in supragranular layers. B) Quantification of axon length within and outside the principal column reveals that most axon is found in supragranular layers outside the principal column.

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<th>Thick-tufted</th>
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<td>Outside vibrisal cortex</td>
<td>10.6 ± 9.9*</td>
<td>2.9 ± 1.8</td>
<td>&lt;0.001</td>
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*Dysgranular zones.
Semi-automated axon reconstructions

**Figure 4: 3D reconstructions of in vivo filled thick-tufted pyramidal neuron in L5 of rat vibrissal cortex.** A) Tangential view and notation as in Figure 1A. Note that the intracortical axon innervation by thick-tufted cell is far less elaborate when compared to slender-tufted neurons. Further, innervation remains largely confined to the principal column and a limited number of surrounding columns in its immediate vicinity. B) Semi-coronal view as in Figure 1A. Please note that the horizontal projections to surrounding columns remain primarily confined to infragranular layers, whereas projections to granular and supragranular layers remain within the lateral boundaries of the principal column.

**3D intracortical projections of L5 thick-tufted pyramidal neurons**

To compare the cell type specificity of intracortical axon projection patterns of layer 5 output neurons (Table 1), we also reconstructed five axons of individual thick-tufted pyramidal neurons (Fig. S2). Figure 4A and 4B show one example neuron in tangential and semi-coronal view, respectively. Somata of slender- and thick-tufted neurons were found at overlapping cortical depths, 1036 ± 46 μm and 1090 ± 97 μm, respectively. Thus, classification based solely on cortical depth or layer, i.e. into L5A and L5B neurons was not predictive of morphology. We found that axon branches of thick-tufted neurons were shorter and less complex than those of slender-tufted neurons, with total length ranging from 11.4 mm to 46.5 mm (Fig. 5B; 31.6 ± 14.3 mm, p < 0.0001 t-test) of which 10.1 ± 6.4 mm (32%) was confined to the principal column. Long range projections of thick-tufted neurons within the vibrissal area, but outside the principal column, were less elaborate and less dense when compared to slender-tufted neurons (18.6 ± 7.5 mm, 59%, p < 0.0001 t-test), and present in equal amounts in septa (9.3
± 3.9 mm) and columns (9.3 ± 3.8 mm). Furthermore, thick-tufted neurons did not innervate dysgranular zones. Projections to areas outside the vibrissal cortex were restricted to its immediate vicinity and significantly less than slender-tufted ones (2.9 ± 1.8 mm, 9%, p < 0.001 t-test).

Figure 5: Quantification of 3D intracortical axon innervation of thick-tufted neurons (I). A) Semi-coronal view of 3D axon density of the five neurons shown in Supplementary Figure 2. Please note that innervation in granular and supragranular layers respects the lateral borders of the principal column, whereas innervation in infragranular layers extends to surrounding columns. B) Thick-tufted neurons display much less axon when compared to slender-tufted neurons (see Figure 2B). Whereas the amount of axon is similar for slender-tufted and thick-tufted neurons within the principal column (16.4 ± 6.7 vs. 10.1 ± 6.4; p = 0.17), long range projections are significantly less elaborate for thick-tufted neurons (59.6 ± 9.7 vs. 18.6 ± 7.5; p < 0.0001). C) The axon length profile of thick-tufted neurons within the principal column reveals that intracolumnar innervation is almost homogeneous throughout all layers, reaching a peak in infragranular L5 around 1250 µm below the pia surface. The profile outside the principal column shows that long range projections are confined to infragranular layers.
(previous page) Figure 6: Quantification of 3D intracortical axon innervation of thick-tufted neurons (II). A) Tangential view of 3D axon density of the five neurons shown in Supplementary Figure 2 in supragranular, granular and infragranular layers (from left to right). As shown in Figure 5, projections of thick-tufted neurons remain almost completely confined to the lateral column boundaries in granular and supragranular layers, but spread to adjacent columns in infragranular layers. B) Quantification of axon length within and outside the principal column reveals that most axon is found in infragranular layers outside the principal column.

With respect to layer-specific innervation profiles, thick-tufted neurons were also significantly different from slender-tufted neurons (Fig. 5C). Within the principal column, innervation was rather homogeneous throughout all layers and reached a maximum in infragranular layers (6.6 ± 4.1 mm). Outside the principal column most axon was located in deep infragranular layers, where axon branches innervated multiple surrounding columns (Fig. 6B; 12.2 ± 4.4 mm). Almost no axon was present in supragranular layers (3.0 ± 1.9 mm), marking the most conspicuous difference with respect to slender-tufted neurons (p < 0.0001).

Bouton distributions of L5 slender- and thick-tufted pyramidal neurons

To relate the obtained 3D axon projection patterns to innervation densities that may allow speculating on synaptic connectivity, we determined the distance between boutons (n=956) along axons in dys-, supra-, infra- and granular regions for slender- and thick-tufted neurons (n=4), respectively. We found swellings that are likely to correspond to en passant and in some cases terminaux boutons (De Paola et al., 2006) along all axon branches and in all regions (Fig. S3). The ratio between the two bouton classes was layer- and cell type-specific, which may help to distinguish between these axon types during functional imaging studies (De Paola et al., 2006). However, the small total number of terminaux boutons and the limited resolution of brightfield microscopy prevent us from making absolute statements on bouton statistics.
More importantly, we found that the inter-bouton distance was similar for slender- and thick-tufted neurons (2.84 ± 1.36 vs. 2.90 ± 1.19 µm) and independent of axon location (Fig. S3).

**Figure 7: Cell type-specific microcircuits in L5 are involved during different behavioral states.** A) During active whisking (self motion of whiskers), slender-tufted neurons display spiking activity that carries phase information (p). Due to their intracortical axon projection pattern, slender-tufted neurons from a single column convey this information i) via local circuits to L5 neurons within the principal column (not shown), yielding whisker-specific output of slender-tufted neurons to the striatum and ii) via long range circuits to supragranular layers of the entire vibrissal area and adjacent dysgranular zones, diminishing whisker-specificity in supragranular layers. B) During passive whisker touch, thick-tufted neurons display the highest increase in spiking activity in vibrissal cortex, primarily caused by thalamocortical input from relay neurons in VPM (t). Due to their 3D intracortical axon pattern, thick-tufted neurons from a single cortical column convey information of whisker touch i) via local circuits to all layers within the principal column (not shown), ii) via long range circuits to L5 of surrounding columns and (iii) to subcortical targets (e.g. POm and pons). C) Object location during active whisker motion may be encode by simultaneous input from slender-tufted neurons to apical tufts and from VPM to basal dendrites of L5 thick-tufted neurons, causing increased spiking activity (bold arrow) and enhanced output to subcortical targets (e.g. corticothalamic feedback to POm).

**Discussion**

The results indicate that slender- and thick-tufted pyramids in layer 5 have cell type-specific intracortical axon distributions, which differ in (i) total axon length, (ii) local and long-range projection patterns within different layers of the vibrissal area, and (iii) in their cortical targets outside the vibrissal area. The axonal
length measurements can be directly converted into 3D bouton distributions (see results above). In the neocortex, synapses of excitatory neurons are associated either with en passant or terminaux boutons (De Paola et al., 2006). Thus, for the present study, axonal length measurements are regarded predictive of intracortical connectivity (Peters, 1979). Since the two neuron types in L5 are differentially involved in whisker motion or passive whisker touch (Fig. S4), the data suggest that cell type-specific intracortical circuits are activated by slender- and thick-tufted neurons depending on the behavioral state, as schematically shown in Figure 7.

**Contribution of slender-tufted neurons to cortical signaling during active whisking**

Spiking in a subset of L5 slender-tufted neurons reliably carries information on position and, more importantly, phase upon active whisker motion (Fig. S4) (Curtis and Kleinfeld, 2009; de Kock and Sakmann, 2009a). This information is conveyed subcortically to striatum (Groh et al., 2010a), which is thought to participate in sensory-motor integration to fine-tune whisker movements (Alloway, 2008). In addition, our data suggests that information on active whisker motion is relayed via three distinct intracortical pathways (Fig. 7A).

The first pathway may target dendrites of L5 neurons within the same principal whisker column, explaining in part the narrow receptive field observed for slender-tufted neurons (Manns et al., 2004; de Kock et al., 2007b). This column-restricted spread of excitation mediated by slender-tufted neurons within the infragranular layers suggests a column- and therefore whisker-specific corticostriatal output during whisker motion.

The second pathway targets dendrites located within supragranular layers of a large portion of the vibrissal area, which primarily contain basal and apical dendrites of L2 and L3 pyramidal neurons, apical tufts of L5 thick-tufted and slender-tufted neurons (Meyer et al., 2010b), as well as dendrites from L2 and L3 inhibitory interneurons (Helmstaedter et al., 2008). Axon projections of L5 slender-tufted neurons have been shown to establish functional connections with L2/3 pyramidal neurons (Shepherd et al., 2005; Shepherd and Svoboda, 2005). However, as spiking in supragranular layers does not increase significantly upon active whisking (Crochet and Petersen, 2006b; de Kock and Sakmann, 2009a), the sensory information carried by these intracortical long range projections is likely within fine-scale synaptic activity (Alenda et al., 2010). During active whisking, the membrane potential of L2 and L3 pyramidal neurons may therefore be locked to
the phase of individual slender-tufted neurons (Crochet and Petersen, 2006b). However, more importantly, slender-tufted projections to supragranular layers may also target the apical tuft dendrites of L5 thick-tufted neurons, present at high density in supragranular layers (Meyer et al., 2010b). These long range L5 slender-tufted projections may then serve to depolarize and phase-lock the membrane potential of apical tuft dendrites of L5 thick-tufted neurons to whisker motion.

The third target population of L5 slender-tufted neurons comprises a previously unknown pathway to supragranular depths of higher order dysgranular zones adjacent to the vibrissal area. We hypothesize that this direct cortical pathway from vibrissal to dysgranular cortex underlies the activity observed previously in dysgranular zones after stimulation of the whiskers or inter-vibrissal skin and fur (Nussbaumer and Van der Loos, 1985; Brett-Green et al., 2001; Takashima et al., 2005). Dysgranular zones are thought to participate in multisensory integration of auditory, visual and somatosensory stimuli (Brett-Green et al., 2001), rendering L5 slender-tufted neurons as one potential cell type that signals whisker motion and position to higher order sensory areas, in a phase-sensitive way.

**Contribution of thick-tufted neurons to cortical signaling after passive whisker touch**

L5 thick-tufted neurons reliably increase spiking after passive whisker touch (de Kock et al., 2007b). This increase may be primarily caused by thalamocortical input to basal dendrites from VPM neurons via the lemniscal pathway (Manns et al., 2004; Bureau et al., 2006a; Yu et al., 2006; Petreanu et al., 2009b; Meyer et al., 2010b; Wimmer et al., 2010).

Our data suggest that thick-tufted neurons may contribute significantly less to signaling via intracortical pathways (Fig. 7B) than slender-tufted neurons. Axon projections into granular and supragranular layers are sparser and mostly confined to the principal whisker column, suggesting an almost column-restricted spread of intracortical excitation. However, thick-tufted neurons project more densely to infragranular layers of the principal and surrounding columns, explaining in part the broad sub- and supra-threshold receptive fields of thick-tufted neurons (Ito, 1992; Manns et al., 2004; de Kock et al., 2007b) and suggesting that output to subcortical targets from thick-tufted neurons may be less column- and therefore whisker-specific than that of slender-tufted neurons.
**Thick-tufted neurons may function as coincidence detectors during object location**

*In vitro*, L5 thick-tufted neurons have been shown to act as coincidence detectors when basal and apical trees are simultaneously depolarized within a critical time window (Larkum et al., 1999a; Schaefer et al., 2003; Spruston, 2008). Input to the basal dendrites evokes action potentials back propagating into the apical dendrite, which, when coinciding with depolarization from the apical tuft, results in an increased number of spikes and evokes bursts of somatic and axonal action potentials (Larkum et al., 1999a, 2001).

We suggest that *in vivo*, L5 thick-tufted neurons of vibrissal cortex are activated under two different conditions. First, excitatory input from VPM to basal dendrites drives L5 thick-tufted neurons to spiking threshold after passive whisker touch and results in single or short trains of spikes (de Kock and Sakmann, 2008). Second, near simultaneous activation of L5 slender-tufted and VPM pathways during sensory-motor behavioral paradigms, such as object location during active whisking, may coincidentally depolarize basal and apical dendrites of L5 thick-tufted neurons and cause increased (burst) activity (Fig. 7C) (Curtis and Kleinfeld, 2009; O’Connor et al., 2010a), which is then conveyed to intra- and subcortical targets.

We put forward the hypothesis that individual L5 slender-tufted neurons serve primarily as intracortical hubs of cell-specific information on whisker motion and phase. They potentially modulate the membrane potential of all dendrites in supragranular layers (including those of inhibitory neurons) within large portions of the vibrissal area according to their phase and lock them to specific parts of the whisking cycle. This cell-specific phase-locking may facilitate encoding of object location, which comprises simultaneous, unspecific input on whisker touch from VPM neurons to the basal dendrites and specific input on whisker phase from L5 slender-tufted neurons to the apical tufts of L5 thick-tufted neurons (Fig.7C).

**Additional pathways for integration of whisker motion and active touch**

Other intra- and thalamocortical mechanisms for integration of whisker motion and touch may exist in parallel to the pathways described here. L5 slender-tufted neurons may also function as coincidence detectors when lemniscal input from L4 (Feldmeyer et al., 2005; Schubert et al., 2006a) and paralemniscal input occur simultaneously (Schubert et al., 2007). However, somatic action potentials only back propagate into the apical dendrite for short distances during spiking frequencies that are observed *in vivo* (Grewe et al., 2010b). It seems therefore...
more likely that L5 slender-tufted neurons integrate input on whisker motion and touch at the soma (Curtis and Kleinfeld, 2009; O’Connor et al., 2010b), but coincidence detection via near simultaneous input to basal and apical tuft dendrites is less likely to occur.

Furthermore, direct innervation from POM onto apical dendrites of L5 thick-tufted neurons could serve in a similar way as the input from slender-tufted neurons suggested here (Zhu and Zhu, 2004; Meyer et al., 2010b). Another possibility for simultaneous input on whisker motion and touch could involve direct innervation from the primary motor cortex (Petreanu et al., 2009b; Aronoff et al., 2010) onto apical dendrites of L5 thick-tufted neurons.

Conclusion
Considering that slender-tufted neurons predominantly carry phase information during active whisker motion and that thick-tufted neurons are preferentially activated upon passive whisker touch, we argue that the pathways we described here are realistic candidates for encoding object location. The emergence of cell type-specific genetic labels for slender- and thick-tufted neurons (Groh et al., 2010a) sets the stage to specifically manipulate the activity of slender- and/or thick-tufted neurons, which may ultimately prove or disprove the functional contributions of the pathways described here during whisker-mediated behavior.

Methods
Juxtasomal labeling with biocytin
Recordings from urethane (1.6-1.7 g.kg⁻¹) anaesthetized Wistar rats (postnatal day 28 ± 1, bodyweight 78 ± 10 gr, ♂ / ♀) were made as described previously (de Kock et al., 2007b). Cytochrome C oxidase staining was used to label barrel contours in L(ayer 4) (Horikawa and Armstrong, 1988). The recorded neurons were filled with biocytin (Molekula, Munchen, Germany) and labeled with the chromogen 3,3’-diaminobenzidine tetrahydrochloride (DAB) (Wong-Riley, 1979) using the avidin-biotin-peroxidase method. Selection criteria for reconstructions included adequate labeling, sufficient Cytochrome C signal to reconstruct the barrel/septum pattern, and reliable serial reconstruction of long-range axon branches (Kaspirzhny et al., 2002).
Semi-automated axon reconstructions

3D semi-automated reconstruction of neuron morphology

Neuron tracings were performed on 100 µm thick vibratome sections, cut approximately tangential to the D2 barrel column. Ranging from the pia surface to the white matter, 20 sections were reconstructed per neuron. DAB-stained neuronal branches were detected and traced using a previously described automated pipeline (Oberlaender et al., 2007). For each section a tissue volume of 1.5 mm x 1.5 mm x 0.1 mm was imaged using optimized mosaic/optical-sectioning microscopy (Oberlaender et al., 2009) and an oil immersion objective (Olympus 100x UPLAN S APO, NA 1.4), yielding a voxel size of 0.184 µm x 0.184 µm x 0.5 µm. In cases where neuronal branches reached the borders of the imaged volume, additional image stacks were taken at adjacent areas. Manual post-processing of individual sections (Dercksen et al., 2010), as well as automated alignment of reconstructed branches across sections (Dercksen et al., 2009), were performed using a custom designed 3D editing environment based on Amira visualization software (Stalling et al., 2005). Pia and barrel outlines were manually drawn on low resolution images (Olympus 4x UPLAN S APO, NA 0.16) and added to the tracings in Neurolucida software (MicroBrightfield, Williston, VT, USA).

Data analysis

Before analysis, all neurons were transformed into a standardized coordinate system, having its origin at the center of the L4 barrel containing the neuron’s apical dendrite (“principal barrel”). The z-axis was chosen to point dorsally, parallel to the vertical axis of the principal barrel, the x-axis laterally towards the center of the first neighboring L4 barrel within the same whisker row. Analysis of these standardized neurons was performed in Amira software, using custom written tools. All resultant total length measurements were double checked with analysis tools in NeuroExplorer software (MicroBrightfield, Williston, VT, USA).

Axon length per individual column was determined by extrapolating the respective L4 barrel contours along the vertical axis towards the pia and white matter. No standardized barrel or column dimensions were used for analysis. Axon structures that are not contained within any column were counted as septal projections. Supragranular, granular and infragranular projections were measured for each column individually, since barrel size or thickness of the cortex may vary between columns.
Average axon density distributions were determined by aligning all axons to the D2 column, but preserving vertical and lateral position of the soma as well as orientation with respect to the barrel center. The maximal 3D bounding box surrounding all axons was subdivided into 100 µm voxels. Density is presented as axon length per such voxel.

Average inter-bouton distances were obtained manually by using a transmitted light brightfield microscope equipped with a 100x oil immersion objective (N.A. 1.4) and Neurolucida Software (MicroBrightfield, Williston, VT, USA). Preferentially, horizontally projecting axons were chosen for analysis. Inter-bouton distances were determined by using the ‘Quick Measure’ tool in Neurolucida. Focusing on one bouton, the distance to the next neighboring bouton along the axon trajectory was measured for samples from two slender- and thick-tufted neurons, respectively.

Data is presented as mean ± stdev and Graphpad Instat 3 (San Diego, CA, USA) was used for statistical analysis. Significance level was set at \( p < 0.05 \).

Author Contributions
M.O., B.S. and C.P.J.d.K. conceived and designed the project. Z.S.R.M.B. and C.P.J.d.K. carried out the juxtasomal labeling experiments. M.O. and T.K. performed the 3D reconstructions. M.O. developed the data analysis routine. All authors were involved in data analysis and writing the manuscript.

Acknowledgments
This work was supported by the Max-Planck Society, CNCR and a VENI grant (Netherlands Organization for Scientific Research - NWO) to C.P.J.d.K. We thank Andrea Weber and Sebastiano Bellanca for Neurolucida reconstructions during initial stages of the project, Vincent J. Dercksen for implementing analysis tools in Amira and Marlies Kaiser, Ellen Stier, and Brendan Lodder for excellent technical support, Robert Egger for his help on the bouton counts and Hanno-Sebastian Meyer and Jason Christie for fruitful discussions.
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Supplementary figures

Figure S1. Gallery of five reconstructed 3D axon and dendrite morphologies of slender-tufted pyramidal neurons in L5 of rat vibrissal cortex. (A) Tangential view. (B) Semicoronal view.

Figure S2. Gallery of five reconstructed 3D axon and dendrite morphologies of thick-tufted pyramidal neurons in L5 of rat vibrissal cortex. (A) Tangential view. (B) Semicoronal view.
Figure S3. Measurements of interbouton distances in dysgranular, supragranular, infragranular, and granular layers of axons from slender- and thick-tufted pyramidal neurons in L5 of rat vibrissal cortex. (A) Minimum z-projections of image stacks of axon branches from slender-tufted neurons obtained by high-resolution mosaic/optical sectioning microscopy (sampling, 0.092 × 0.092 × 0.25 μm). Blue and red arrows indicate examples of en passant and terminaux boutons, respectively. (B) Minimum z-projections of image stacks of axon branches from thick-tufted neurons. (C) The interbouton distance was independent of neuron type or branch location. The ratio of terminaux versus en passant boutons was layer- and cell type-specific.
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Figure S4. In vivo spiking activity of slender- and thick-tufted neurons (1, 2). (A) Spiking activity of reconstructed slender- and thick-tufted neurons within 50 ms after passive whisker touch. Thick-tufted neurons are reliably activated by passive touch, whereas slender-tufted neurons remain primarily inactive. (B) On the population level, spiking activity during active whisker motion does not display any significant differences between slender- and thick-tufted neurons and is similar to spontaneous spiking during anesthesia or nonwhisking periods (note that significant differences between nonwhisking and whisking periods can be observed for individual recordings, preferentially in L5A). Neuron identities were determined by reconstruction or cell type-specific parameters (i.e., recording depth and spontaneous spiking frequency) obtained from the dataset presented in A). (C) Phase modulation during active whisker motion is present in slender- and thick-tufted neurons. However, the size of the subset of slender-tufted neurons that carry phase information, as well as their modulation depths, exceed that of thick-tufted neurons.
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