

Extensive Connections of the Canine Olfactory Pathway Revealed by Tractography and Dissection

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The olfactory sense of the domestic dog is widely recognized as being highly sensitive with a diverse function; however, little is known about the structure of its olfactory system. This study examined a cohort of mixed-sex mesaticephalic canines and used diffusion tensor imaging (DTI), an MRI technique, to map connections from the olfactory bulb to other cortical regions of the brain. The results were validated using the Klingler dissection method. An extensive pathway composed of five white matter tracts connecting to the occipital lobe, cortical spinal tract, limbic system, piriform lobe, and entorhinal pathway was identified. This is the first documentation of a direct connection between the olfactory bulb and occipital lobe in any species and is a step toward further understanding how the dog integrates olfactory stimuli into their cognitive function.

Key words: canine; DTI; MRI; occipital; olfactory; white matter

Significance Statement

The highly sensitive olfactory system of the domestic dog is largely unexplored. We applied diffusion tractography and dissection techniques to evaluate the white matter connections associated with the olfactory system in a large cohort of dogs. We discovered an extensive white matter network extending from the olfactory bulb to form novel connections directly to other cortices of the brain. This is the first documentation of these novel olfactory connections and provides new insight into how dogs integrate olfactory stimuli in their cognitive functioning.

Introduction

The impressive sense of smell of the domestic dog (*Canis familiaris*) is well known and has been deployed in a variety of settings, from identifying individuals with disease, including COVID-19 and cancerous tumors (Gordon et al., 2008; Angeletti et al., 2021; Sakr et al., 2022), to drug (Hayes et al., 2018) and explosive detection. (Gazit and Terkel, 2003; Fischer-Tenhagen et al., 2017) This has led to dedicating much research to understanding their scent ability through anatomic and behavioral studies. The canine sense of smell is optimized by having specialized primary and secondary olfactory systems and by employing specific sniffing patterns to maximize airflow and stimuli detection (Barth et al., 2003; Craven et al., 2007). Many scent breeds, such as the bloodhound, have been selectively bred for olfactory tasks such as tracking, an attribute reflected in their ear and head shape, which optimizes odor collection into the nostrils. Similarly,

much attention has been paid to the importance of scent behavior in dogs. There is increasing evidence for the olfactory system playing a dominant role in dog cognition rather than a more complementary role as is often described in human functioning. Research has shown that canines use olfactory markers to communicate using behavior such as marking and sniffing as well as to perceive pheromones for mating (Pageat and Gaultier, 2003). The olfactory system is consistently used in tracking, interacting with other dogs and animals, and responding to social stimuli with humans (Wells and Hepper, 2003; Hepper and Wells, 2005; Gadbois and Reeve, 2014; Horowitz, 2020). Studies have also found that trained dogs primarily use olfaction in detection settings, regardless of any available visual stimuli, demonstrating the dominance of the olfactory system on dog cognition (Gazit and Terkel, 2003). Despite this evidence that olfaction plays a key role in canine cognition and behavior, the structure of the dog olfactory system within the brain remains a mystery.

In humans, the cerebral representation of the olfactory system is small when compared with other sensory systems. In common with other mammals, the olfactory system is composed of sensory neurons that transmit information from olfactory epithelium to the olfactory bulb, from where signals are then relayed to the olfactory cortex of the brain (Shepherd, 2006). In terms of neural connectivity, it is widely accepted that olfaction influences

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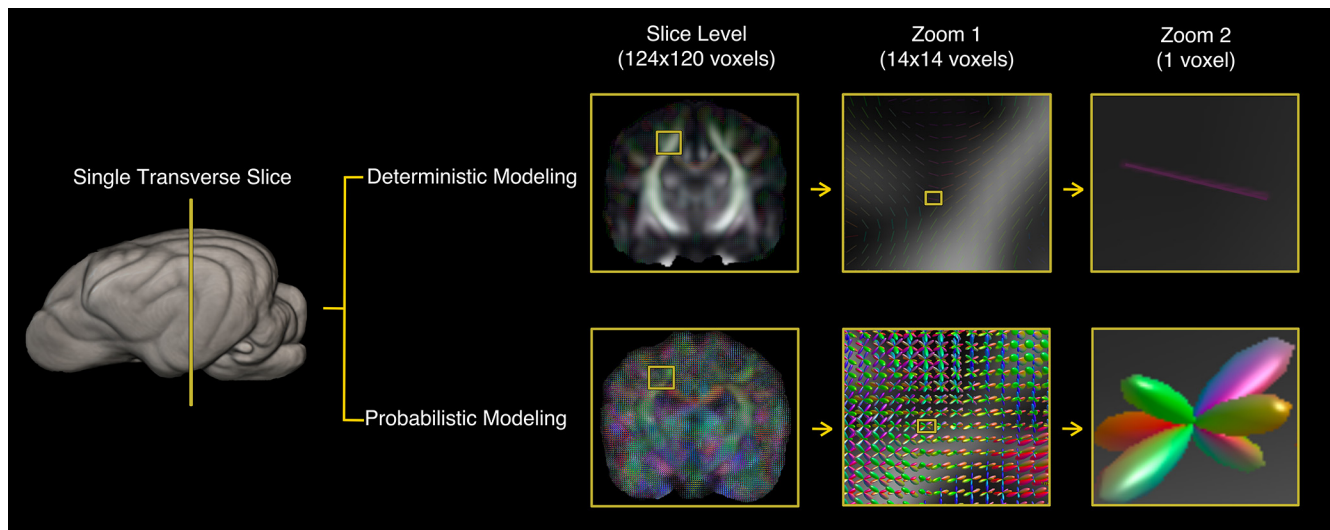


Figure 1. Deterministic and probabilistic modeling of diffusion data beginning at a single-slice level and zooming in to a section and voxel level to show directionally modeling at each level.

cognitive function in humans, with the olfactory tract being integrated with many regions of the brain associated with emotional and memory processing such as the limbic system and entorhinal cortex (Biella and De Curtis, 2000; Burmeister et al., 2012). We know that the canine olfactory system, when compared with humans, shows dramatic differences both anatomically, functionally, and behaviorally. Anatomically the olfactory bulb is ~30 times larger, and the olfactory epithelium contains between 200 million and 1 billion olfactory receptors (depending on the breed), considerably more than the 5 million receptors typically found in humans (Moulton, 1976). Behavior studies have shown that unlike humans, dogs use olfaction for many more functions including spatial awareness demonstrated in tracking studies (Wells and Hepper, 2003; Hepper and Wells, 2005) and social behavior by means of recognition by scent (Horowitz, 2017, 2020).

Despite the consensus that canine olfaction is significant for their cognitive processing and integral to successful detection of stimuli, there has been little research investigating the role of olfaction in canine brain function and the location of olfaction networks within the canine brain. In addition, reviews have found that most canine cognition studies did not account for olfactory stimuli and used human-centric visual stimuli to test their cognitive abilities (Pongrácz et al., 2017; Horowitz, 2020). Previous research explored the cellular structure of the olfactory bulb and the role of the piriform lobe in cognition in rats, but similar neurophysiological studies are not found in the canine literature (Xu et al., 2003; Sheperd, 2006). Although research into the primary olfactory structure of the dog, from the nostrils to the epithelium then onto the olfactory bulb (Barth et al., 2003), has been well documented, there is a distinct lack of knowledge of the connections from the olfactory bulb to the cerebrum in the canine brain. Although axonal tracing studies have been performed in dogs (Stanton et al., 1986; Han et al., 2012), there is limited work on the olfactory pathways. A functional MRI (fMRI) study mapping the olfactory system in both awake and anesthetized dogs found activation in the piriform and olfactory bulb in anesthetized dogs and activation in the frontal cortex in awake dogs (Jia et al., 2014, 2016). However, this study was reliant on the self-control of awake highly trained subjects, which could activate inhibition and top-down processing regions rather

than the olfactory network. Although the number of fMRI studies is increasing in the canine brain literature, limited information is available on the structure of the dog brain, with few studies focusing on creating structural brain atlases and characterizing white matter tracts (Datta et al., 2012; Nitzsche et al., 2019; Jacqmot et al., 2020; Johnson et al., 2020).

Diffusion tensor imaging (DTI) is an advanced MRI technique that uses the movement of water to model white matter tracts within the brain (Alexander et al., 2007). To our knowledge, DTI has never been used to investigate the olfactory system in dogs. These white matter tracts are large bundles of fibers or groups of neuronal axons, similar to very small wires, that connect different parts of the brain (Fig. 1). There are different methods of modeling white matter fibers (known as tractography), which generally fall under two categories, deterministic, where the direction of water is modeled on an ellipsoid, and probabilistic, where the movement of water is modeled on a spherical surface (Fig. 1; Smith et al., 2004; Behrens et al., 2007; Jbabdi et al., 2010). In humans, much research has been done to identify white matter connections responsible for exchanging information between brain regions via large tracts like the corpus callosum, corticospinal tract, and uncinate fasciculus (Basser and Jones, 2002).

Tractography techniques have been used in human studies to better understand the olfactory system within the brain. Studies have shown that the relatively small olfactory tract in humans is integrated with regions of the brain associated with emotion and memory as well as activation of the spatial what/where network featuring the occipital, piriform, and temporal lobes (Porter et al., 2005). These studies provide evidence that DTI can successfully be applied to model even relatively small olfactory tracts. To complement these virtual techniques, studies have suggested using the Klingler method or cortex-sparing fiber dissection to remove the outer cortex from an *ex vivo* sample to reveal the underlying white matter tracts in an anatomic dissection (Klingler, 1935; Martino et al., 2011; Pascalau et al., 2016). This method provides an anatomic validation for white matter tracts found via DTI tractography. Because olfaction is known to be an important source of information for canines, and the structural connections from the olfactory bulb are unknown, DTI with gross Klingler dissection validation would be a useful method for

Table 1. Overview of canine participants detailing demographic data, husbandry, and experimental information

Variable type	Variable	Group 1	Group 2	Group 3	Group 4
Demographic data	1. Species, common name	Dog	Dog	Dog	Dog
	1. Species, Latin name	<i>Canis familiaris</i>	<i>Canis familiaris</i>	<i>Canis familiaris</i>	<i>Canis familiaris</i>
	1. Sample size	4	6	10	3
	1. Sex of individuals	Male ($n = 4$)	Female ($n = 6$)	Male ($n = 4$), female ($n = 6$)	Female ($n = 5$)
	1. Age (mean $\pm$ SD)	4.5 $\pm$ 0.5	5.7 $\pm$ 0.47	10.4 $\pm$ 0.49	2 ($\pm$ 0)
	1. Names	Jack, Rocky, Buzz, Axel	Ginny, Mocha, Caramel, Butterscotch, Molly, Dottie	Siris, Wanda, Nomad, Cashew, Quick, Streak, Jane, Zest, Lucy, Maggie	Faraday, Flair, Tilly
	1. Breed	Medium to large mixed breed	Beagle crossbreed	Alaskan sled dogs	Beagles
	1. Inclusion criteria	Mesaticephalic skull conformation, neurologically and clinically healthy			
	1. Source of animals	Bowman Lab	Boesch Lab	Vaika Foundation	Clinical Sciences Teaching Animals
	1. Housing of animals	Cornell Center for Animal Resources and Education		Cornell Aging Canine Facility	CARE
Husbandry	1. Enrichment provided	Kenneling with outdoor or indoor runs allowing for supervised play in compatible groups			
	1. End point for animals	Returned to original lab protocol after MRI procedure. Groups 1, 2, and 4 have been subsequently adopted out to families for a home life. Group 3 is being monitored to a natural end of life.			
	1. Experimental outcome measures	Diffusion tensor imaging and T1-weighted 3D MRI sequences			
	1. Procedure/experiment type	MRI of the brain			
Experimental details	1. Justification of protocol	3T GE Discovery MRI unit			
	1. Termination criteria	Limited research has gone into understanding the structure and function of the normal canine brain			
	1. Criteria for assent of animal to participate	If any animal showed signs of pain or distress during the procedure while awake or demonstrated signs of clinical deterioration during anesthesia, they were immediately terminated from the study.			
	1. Ethics approval reference	Any animal that demonstrates obvious distress because of any experimental procedure will be removed from the study			
		IACUC Protocol No. 2015-0115			

CARE, Cornell Center for Animals Resources and Education.

investigating the structural connection between the olfactory bulb and the rest of the brain.

In this study we aimed to delineate the olfactory white matter system in canines via DTI tractography in a cohort of dogs. We hypothesized that in line with the previous literature, white matter tracts from the olfactory bulb would be identified extending to regions of the cortex traditionally associated with olfactory function including the piriform lobe, entorhinal cortex, frontal lobe, and limbic system. In addition, in light of the enhanced use of the olfactory system observed consistently in dog behavior studies, we expected to identify novel pathways to additional cortical regions. We used both deterministic and probabilistic DTI tracking to compare consistency of results over two different tracking techniques and provided a diffusion metric, fractional anisotropy (FA), for each tract found with consistency across subjects. To validate these tracking techniques, a Klingler dissection was performed in two *ex vivo* samples to confirm whether these tracts exist anatomically as well as virtually in alignment with previous validation studies (Pascalau et al., 2016). We hypothesized that all consistent white matter tracts found virtually using DTI tractography methods would be replicated in the Klingler dissection validation.

Materials and Methods

Subject recruitment

Twenty-three canine subjects were recruited from the research populations at Cornell University. To be included in the study, all subjects were required to be clinically and neurologically healthy after physical and neurologic evaluations to exclude any possible confounding conditions and were required to have a neurocephalic index that conformed to a dolichocephalic or mesaticephalic skull conformation (Carreira and Ferreira, 2015; Hecht et al., 2019). The resulting cohort included mixed breeds ($n = 20$, weighing 10–31 kg) and beagles ($n = 3$, weighing 7–9 kg), 16 females (six neutered) and 7 males (two neutered), with an age ranging from 2 to 11 years old (median age of 6 years). The protocol for this study was approved by Cornell University Institutional Animal Care and

Use Committee (IACUC; Protocol No. 2015–0115). A full description of the canine participants detailing demographic data, husbandry, and experimental information is provided in Table 1.

Scanning protocol

Anesthesia

All subjects underwent general anesthesia administered by a board-certified anesthesiologist while undergoing MRI. Subjects were premedicated with dexmedetomidine (3 mcg/kg; Dexdomitor 0.5 mg/ml). General anesthesia was induced with propofol (3.2–5.4 mg/kg), and subjects were then intubated. Anesthesia was maintained with inhalant isoflurane and oxygen with a dexmedetomidine continuous rate infusion (1 mcg/kg/h; Dexdomitor 0.5 mg/ml). While the animals were under anesthesia, the following parameters were monitored: pulse rate, respiratory or ventilation rate, systolic and diastolic blood pressure, oxygen saturation, end tidal carbon dioxide level, and electrocardiogram.

MRI Acquisition

MRI was performed in a 3.0T General Electric Discovery MR750 (GE Healthcare) whole-body scanner (60 cm bore diameter), operating at 50 mT/m amplitude and 200 T/m/s slew rate. Subjects were placed in dorsal recumbency with their head centered in a 16-channel medium flex radio-frequency coil (NeoCoil). A high-resolution T1-weighted 3D inversion recovery fast spoiled gradient echo sequence (Bravo program) was performed in each subject with the following parameters: isotropic voxels 0.5 mm³, TE = 3.6 ms, TR = 8.4 ms, TI = 450 ms, NEX = 3, 12° flip angle, and acquisition matrix size = 256 × 256. Diffusion tensor images were acquired in the transverse plane (TR = 7000 ms, TE = 89.6 ms, flip angle = 90°, isometric voxel size of 1.5 × 1.5 × 1.5 mm, in-plane field of view = 135 × 35 mm, matrix size 90 × 90 with 60 gradient directions, b = 800 s/mm² and a single unweighted, b = 0, diffusion image, total acquisition time 9 m 42 s, bipolar diffusion encoding scheme).

Data processing

DTI MRI preprocessing. DWI data were corrected for noise (Veraart et al., 2016), phase distortion (Andersson et al., 2003; Smith et al., 2004), eddy current distortion, and motion correction (Andersson and Sotiropoulos, 2016) using the Functional MRI of

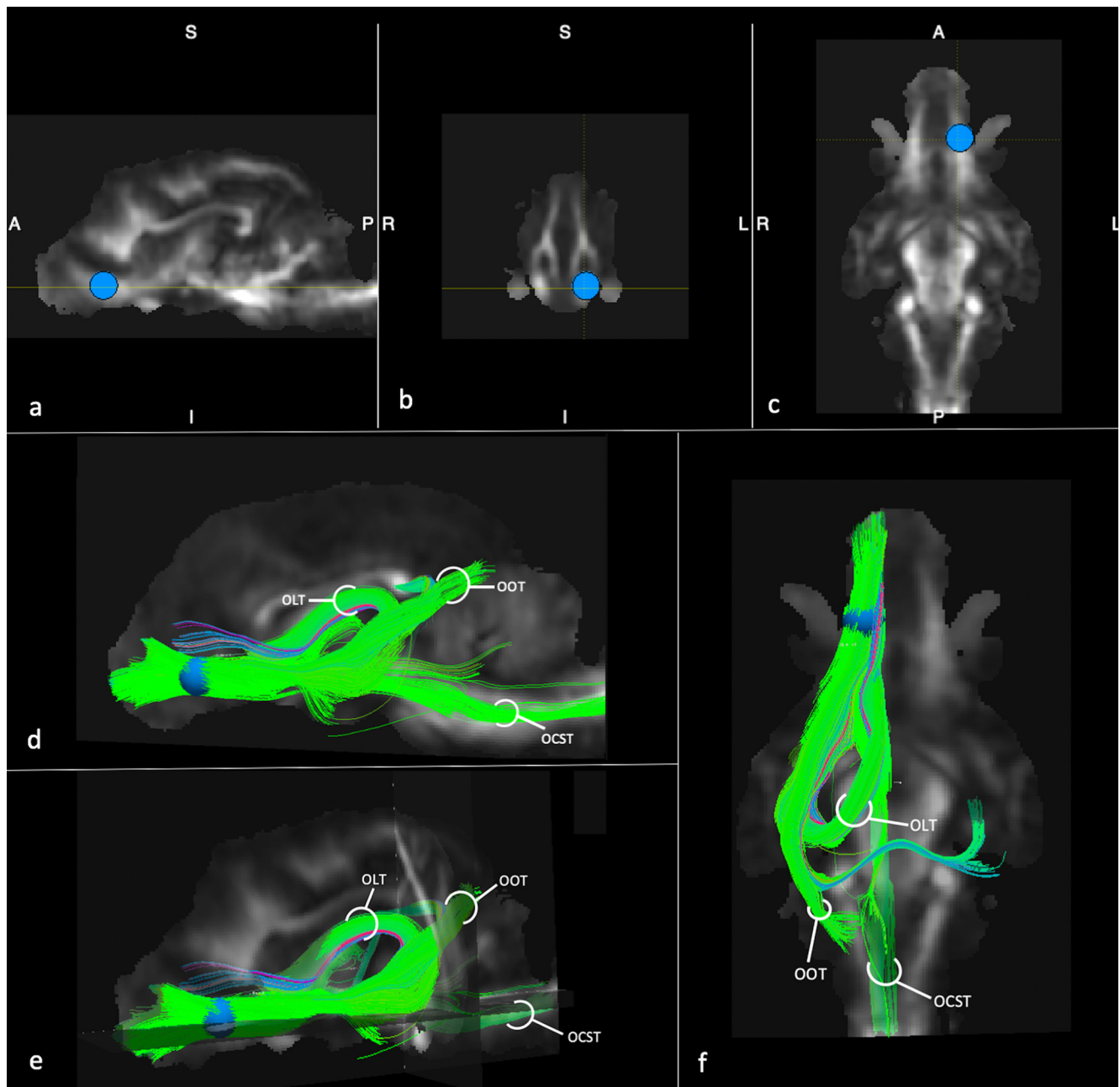


Figure 2. The exploratory dissection technique. A single spherical region of interest was placed over the rostral olfactory peduncle in the 23 dogs evaluated. This primary seed region was toggled with the whole brain tractogram and the resulting connections evaluated. Three long-range connections were consistently identified in subjects, the OLT, the OOT, and the OCST. This figure documents the placement of the seed spherical region on the left peduncle in parasagittal (a), transverse (b), and dorsal (c) planes and the resulting white matter tracts in three-dimensional reconstructions in sagittal (d), oblique (e), and dorsal (f) planes. A, anterior/rostral; P, posterior/caudal; I, inferior/ventral; S, superior/dorsal; R, right; L, left.

the Brain Software Library (FSL; <https://fsl.fmrib.ox.ac.uk/>) and MRtrix (<https://www.mrtrix.org>) software packages. A brain mask for each subject was created using the MRtrix dwi2mask command (Tournier et al., 2019) to exclude all nonbrain tissue from analysis. Brain masks for each subject were examined and manually adjusted, when necessary, to ensure masking accuracy. Diffusion tensors were then modeled using the FSL dtifit function from the FSL diffusion toolbox (Behrens et al., 2003, 2007). Each tensor was defined by three principal eigenvalues (i.e., λ_1 , λ_2 , λ_3). Tensor maps were then calculated for FA as follows:

$$\sqrt{\frac{(\lambda_1 - \lambda_2)^2 + (\lambda_2 - \lambda_3)^2 + (\lambda_1 - \lambda_3)^2}{2(\lambda_1^2 + \lambda_2^2 + \lambda_3^2)}} \quad (\text{Basser and Jones, 2002; Beaulieu, 2002}).$$

Diffusion tensor maps for FA were generated for each subject and visually inspected to ensure the quality of the

preprocessing, volume registration, orientation, and preprocessing (Fig. 1).

DTI tractography

Deterministic tractography. A whole-brain tractogram was created for each subject using Diffusion Toolkit and TrackVis (Wang and Benner, 2007). Tensor modeling using Fiber Assignment by Continuous Tracking algorithm (Mori et al., 1999; Mori and Van Zijl, 2002) with a 45° threshold angle, binary brain mask, and spline filtering. Lower cutoff for the whole-brain tractogram was set at 10%. For manual dissection, a spherical seed region of interest (ROI) was placed on the rostral olfactory peduncle, and its size was tailored to match the margins of this region in each animal (radius 5–7.5 mm). For each tract, a seed (olfactory ROI) and target approach was used for dissection. The olfactory-occipital tract (OOT) was isolated by using an inclusion mask placed over the occipital

Table 2. The classic Klingler dissection protocol

Step	Classic dissection procedure	Exposed tracts
1	The brain was split in two hemispheres by a midsagittal cut	none
2	Removal of the cortex on the medial surface	Short association fibers Dorsal cingulum
3	Removal of the cortex of the parahippocampal gyrus	Ventral cingulum Olfactory-parahippocampal tract
4	Removal of cingulum and short fibers on the medial surface	Corpus callosum Commissural olfactory fibers joining forceps minor
5	Removal of olfactory bulb cortex	Stem of olfactory tract Olfactory-basal forebrain connections
6	Removal of ependymal layer of the third ventricle and septum pellucidum	Body and pillars of fornix Mammillothalamic tract
7	Dissection of ventral striatum area	Rostral commissure
8	Removal of anterior perforate substance	Medial olfactory tract Lateral olfactory tract
9	Removal of corpus callosum and opening of the temporal horn of the lateral ventricle	Column of fornix
10	Removal of fornix and caudate nucleus	Olfactory-stria terminalis tract Olfactory-spinal tract
11	Removal of the cortex on the lateral surface	Short association fibers Lateral olfactory frontal connections
12	Removal of the short association fibers of the lateral surface	Superior longitudinal fasciculus Uncinate fasciculus
13	Removal of the superior longitudinal fasciculus, the uncinate fasciculus, and the lenticular nucleus	Fronto-occipital fasciculus Olfactory-occipital tract

lobe. The olfactory-piriform tract (OPT) was isolated by using exclusion masks to remove any tracts that extended past the piriform cortex. The olfactory-limbic tract (OLT) was isolated by using an inclusion mask placed over the dorsal surface of the thalamus. The olfactory-cortical spinal tract (OCST) was isolated by using an inclusion mask over the caudal brainstem. The olfactory-entorhinal tract (OET) was isolated by using an inclusion mask to include tracts entering the medial temporal lobe and exclusion masks to remove tracts with alternate pathways. For all tracts an exclusion mask was placed on the midline of the brain to ensure tracking was confined to a single hemisphere. Resultant tract masks were binarized and used to measure intersubject variation in FA and volume. A further description of the identification of long-range tracts is provided in Figure 2.

Probabilistic tractography. MRI resolution is limited, and it is estimated that many voxels contain fibers that cross or kiss, which can make modeling those underlying tracts difficult (Behrens et al., 2007; Tournier et al., 2012). Therefore, in addition to the deterministic tractography of each subject, which was used for virtual dissection, a probabilistic tractogram was also generated. This method of tracking uses a sophisticated method called BEDPOSTX (Bayesian Estimation of Diffusion Parameters Obtained using Sampling Techniques) to model crossing fibers within a voxel (Behrens et al., 2007; Jbabdi et al., 2010). In contrast to deterministic tracking, probabilistic tracking accounts for many possible directions the fibers could track and the probability of each tracking outcome. The following settings were used for BEDPOSTX: number of fibers per voxel = 3, automatic relevance detection weight = 1, burn-in period = 1000, number of jumps = 1250, sampled every 25, with sticks with a range of diffusivities model. The FSL probtrackx2 was used to model probabilistic tracking from an ROI seed mask containing the entire olfactory bulb of one hemisphere. The following parameters were used for probtrackx2 modeling: single hemisphere olfactory bulb mask was used as a seed, a loop check was performed on paths to allow a lower curvature threshold, the one-way condition was set to exclude self-looping tracts, curvature threshold = 0.2, step length = 0.5, angle threshold 50°, number of streamlines = 5000, and tracking was constrained to within the binary brain mask of the subject. To limit manipulation of the final tractogram only a single seed mask was used for each olfactory bulb. The resulting tractogram, therefore, included all pathways extending from this region. These whole olfactory probabilistic tractograms were compared with the deterministic dissections of each tract to see whether these two techniques corroborate. The outcome of the probabilistic tracking of all subjects was consistent with the deterministic

dissections in that the connections from the olfactory bulb tracked to the same regions in both methods.

Population template creation

The preprocessed diffusion data and brain masks of all subjects were used to create fiber orientation distributions (FODs) using MRtrix tools (Tournier et al., 2012, 2019). The MRtrix population_template was then used to create an unbiased average FOD dataset for this sample of canine subjects (Tournier et al., 2019). The average FOD was then converted back into diffusion data using the MRtrix fod2dwi function (Tournier et al., 2019) to create an average diffusion dataset. Average population data were then deterministically modeled using Diffusion Toolkit (Wang and Benner, 2007). Virtual dissection of the template diffusion tractogram was then performed in TrackVis (Wang and Benner, 2007). This process was used to create a population average diffusion dataset representative of our canine sample, which could be used for figure creation purposes.

Statistics

The volume and FA of each tract were plotted for each hemisphere and statistically analyzed using Tukey's procedure to assess for statistically significant hemispheric differences while correcting for familywise error rate. Given that FA can be affected by healthy aging (Barry et al., 2021; Radhakrishnan et al., 2021) and that our cohort included subjects older than 8 years of age, we statistically evaluated whether the mean FA of the whole olfactory system was significantly different between young (<8 years, $n = 13$) and old (≥ 8 years, $n = 10$) groups using a Student's t test.

Klingler dissection

Preparation

Two young adult mesocephalic canine brains sourced from the Cornell University College of Veterinary Medicine Anatomy Group were used for tridimensional white matter fiber dissections based on the Klingler (1935) method. The brains were extracted together with the meninges and surrounding skull base structures and preserved for several months in 4% formaldehyde solution. They were dissected out from the dura mater, and the arachnoid membrane and blood vessels were removed and immersed in 10% formaldehyde in water solution and kept for 2 weeks at -20°C . They were then allowed to thaw at room temperature for 24 h.

Tridimensional fiber dissections

Dissections were performed with blunt instruments, in a tridimensional fashion, following the trajectories of the fibers plane by plane, which was

Table 3. The modified Klinger dissection protocol

Step	Modified dissection procedure	Exposed tracts
1	Removal of the olfactory bulb cortex	Stem of olfactory tract
2	Removal of cortex on rostral part of the temporal and parahippocampal gyri	Olfactory-basal forebrain connections
3	Removal of cortex on the frontal lobe	Olfactory-parahippocampal tract
4	Removal of short association fibers of temporal and occipital lobes	Lateral olfactory frontal connections
5	Removal of the superior longitudinal fasciculus, the uncinate fasciculus, and the lenticular nucleus	Olfactory-parahippocampal tract
6	Removal of olfactory-occipital tract and fronto-occipital fasciculus and opening of the temporal horn of the lateral ventricle	Olfactory-occipital tract
7	Removal of fornix, olfactory-parahippocampal tract, and olfactory-basal forebrain connections	Fronto-occipital fasciculus
8	On the other hemisphere, removal of basal surface olfactory bulb cortex	Ventral cingulum
9	Removal of parahippocampal gyrus cortex	Body and column of fornix
10	Removal of anterior perforated substance	Rostral commissure
12	Removal of rostral commissure, accumbens nucleus and thalamus	Olfactory-spinal tract
13	Opening the rostral horn and body of the lateral ventricles on both hemispheres	Olfactory-basal forebrain connections
14	Removal of fornix and caudate nucleus	Olfactory-parahippocampal tract
		Medial and lateral olfactory tracts
		Rostral commissure
		Olfactory-spinal tract
		Fornix
		Olfactory-stria terminalis tract

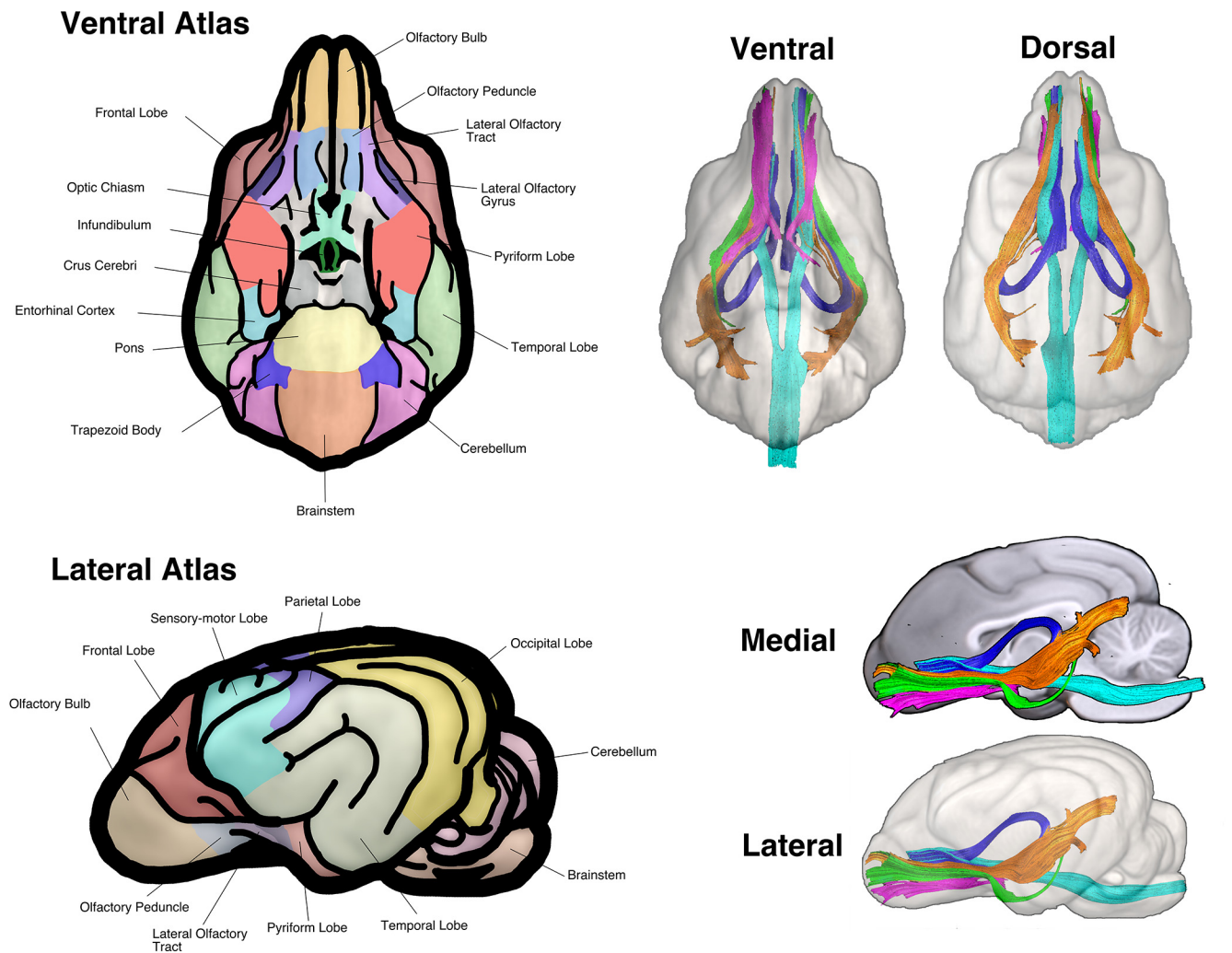


Figure 3. Left, Ventral and lateral atlas of the canine brain. Right, Anatomic placement of the five olfactory tracts including the OOT in orange, the OSCT in turquoise, the OPT in green, the OLT in blue, and the OET in pink from the ventral, dorsal, medial, and lateral views.

made possible by the freezing step in the preparation. Two different approaches were used. One brain was dissected with a classical Klinger technique (a full description is provided in Table 2), starting by splitting the two hemispheres and performing stepwise

lateromedial and mediolateral dissections, as described in previous works (Pascalau et al., 2016; Pascalau and Szabo, 2017). The fiber tracts were exposed in a superficial to deep order, starting with the complete removal of the cortex and continuing by peeling

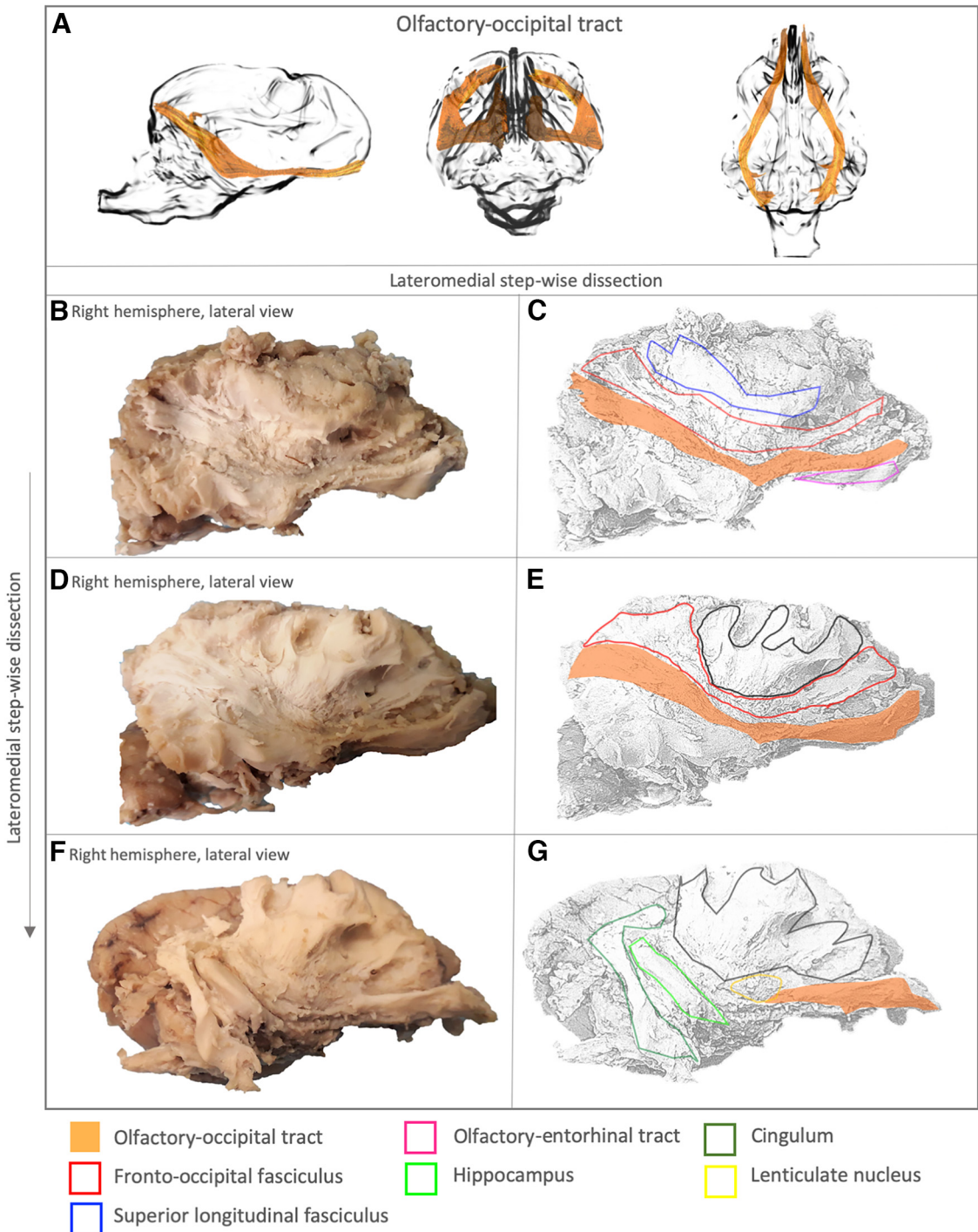


Figure 4. Documents the sequential dissection of the OOT. **A**, Demonstrates the three-dimensional image of the tract dissected using deterministic tractography and overlain on a glass brain in lateral, rostral, and dorsal views (left to right). **B–G**, Documents the photographs and associated annotated schematic diagrams of the progressive lateral to medial anatomic Klingler dissection. After removing the short arcuate and inferior longitudinal tract fibers from the temporo-occipital area, some fibers that belonged to the fronto-occipital fasciculus were followed in a rostrocaudal direction and were found to leave the fronto-occipital fasciculus and enter the olfactory bulb. They were identified as the superficial portion of the OOT. The dissection continued to expose the whole tract by extracting some of the fronto-occipital fasciculus and the OET. To better understand the anatomic relations of the OOT with the lateral ventricle and the limbic structures, its occipital part was removed along with the white matter of the parietal and occipital lobes, and the temporal horn of the lateral ventricle was opened. Comparing with the previous images, we can say that the OOT is located lateral to the hippocampus (separated by the lateral ventricle) and dorsolateral to the temporal segment of the cingulum.

away the successive layers of fibers, following their trajectories. The other brain was dissected without splitting the hemispheres. For this brain we followed a modified Klingler dissection technique known as cortex-sparing fiber dissection (Martino et al., 2011), which means keeping the cortex on the top or the gyri for orientation and focusing the dissection on areas of interest instead of removing all the tracts that form a particular layer in the same

dissection stage. The dissection steps were also adapted to the tracts of interest (A full description is provided in Table 3.). The dissection proceeded in a laterodorsal to ventromedial direction in the right hemisphere and in the opposite order in the left hemisphere. After the dissections were completed, or during pauses in dissection, the specimens were stored in 4% paraformaldehyde in PBS at 4°C.

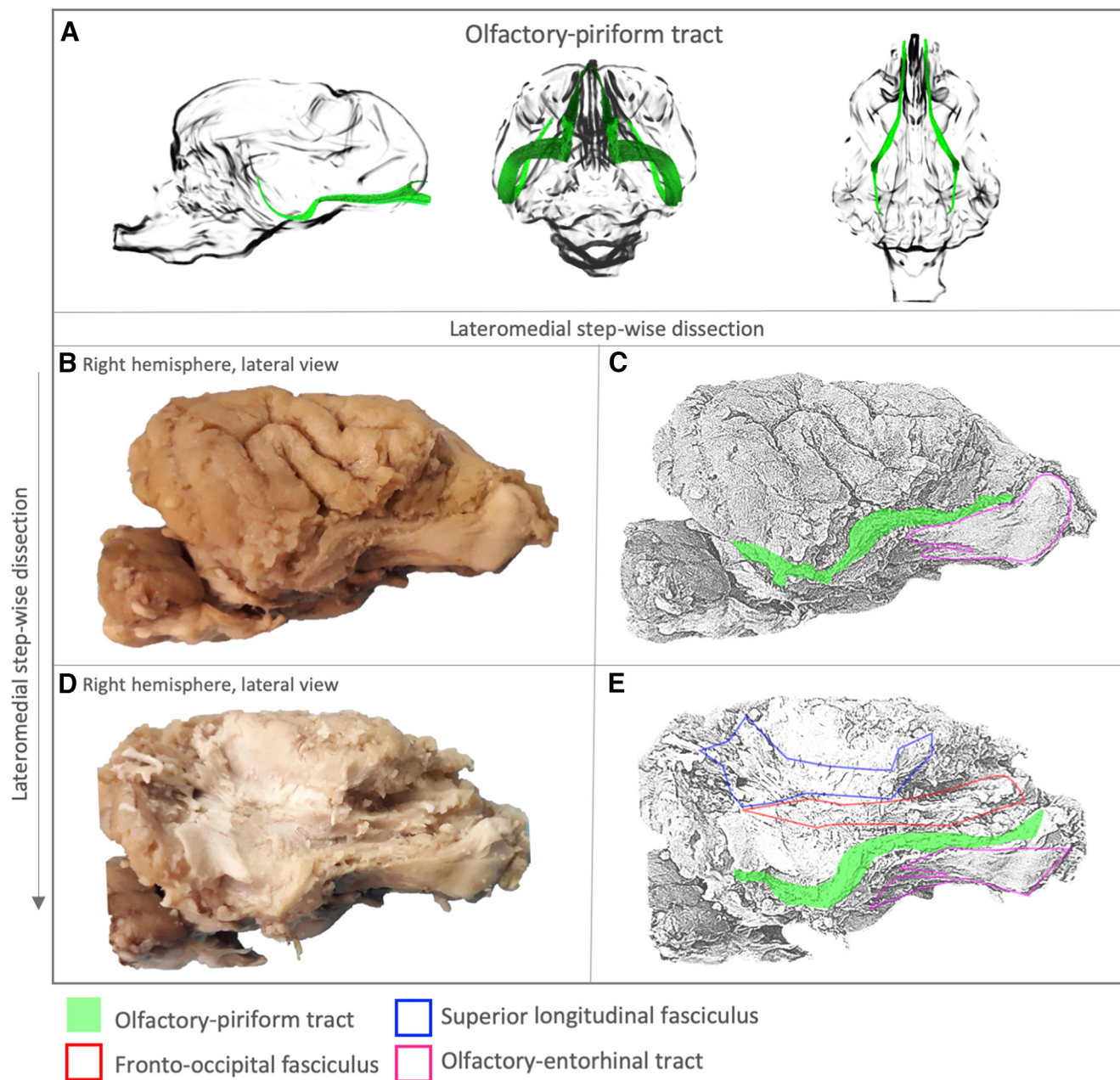


Figure 5. Documents the sequential dissection of the OPT. **A**, Demonstrates the three-dimensional image of the tract dissected using deterministic tractography and overlain on a glass brain in lateral, rostral, and dorsal views (left to right). **B–E**, Documents the photographs and associated annotated schematic diagrams of the progressive lateral to medial anatomic Klingler dissection. The OPT is a superficial tract, lying underneath the cortex and short fibers of the piriform gyrus. It follows the longitudinal direction of the piriform gyrus and enters the dorsal aspect of the olfactory bulb. It was dissected from the dorsolateral aspect of the olfactory bulb, and its deeper fibers were exposed by removing some of the OET.

Data availability

All data used in this project are available for collaborations subject to a materials transfer agreement.

Results

Five olfactory-cortical tracts found consistently using two tracking methods

Consistent tracking patterns from the olfactory bulb to the cortex of the brain were found using deterministic and probabilistic tractography. In addition, Klingler dissection found all five tracts in two dissected brain samples (Figs. 3, 4, 5, 6, 7, 8, 9, 10). These tracts included the following: OOT, OCST, OLT, OPT, and OET.

OOT

The OOT is a large, novel tract that extends between the olfactory bulb and the occipital cortex. To our knowledge, this direct tract linking the olfactory and visual processing hubs has not been demonstrated reliably in other species, including human, primate, or mouse models. This tracking pattern was consistently identified in both hemispheres across subjects and was confirmed with Klingler dissection. It was found to begin in the dorsal and rostral portion of the olfactory bulb and to run through the dorsal portion of the olfactory peduncle. The OOT then runs through the medial portion of the piriform white matter, continues caudal through the posterior internal capsule, then turns dorsally through the retrolenticular internal capsule, ending in the

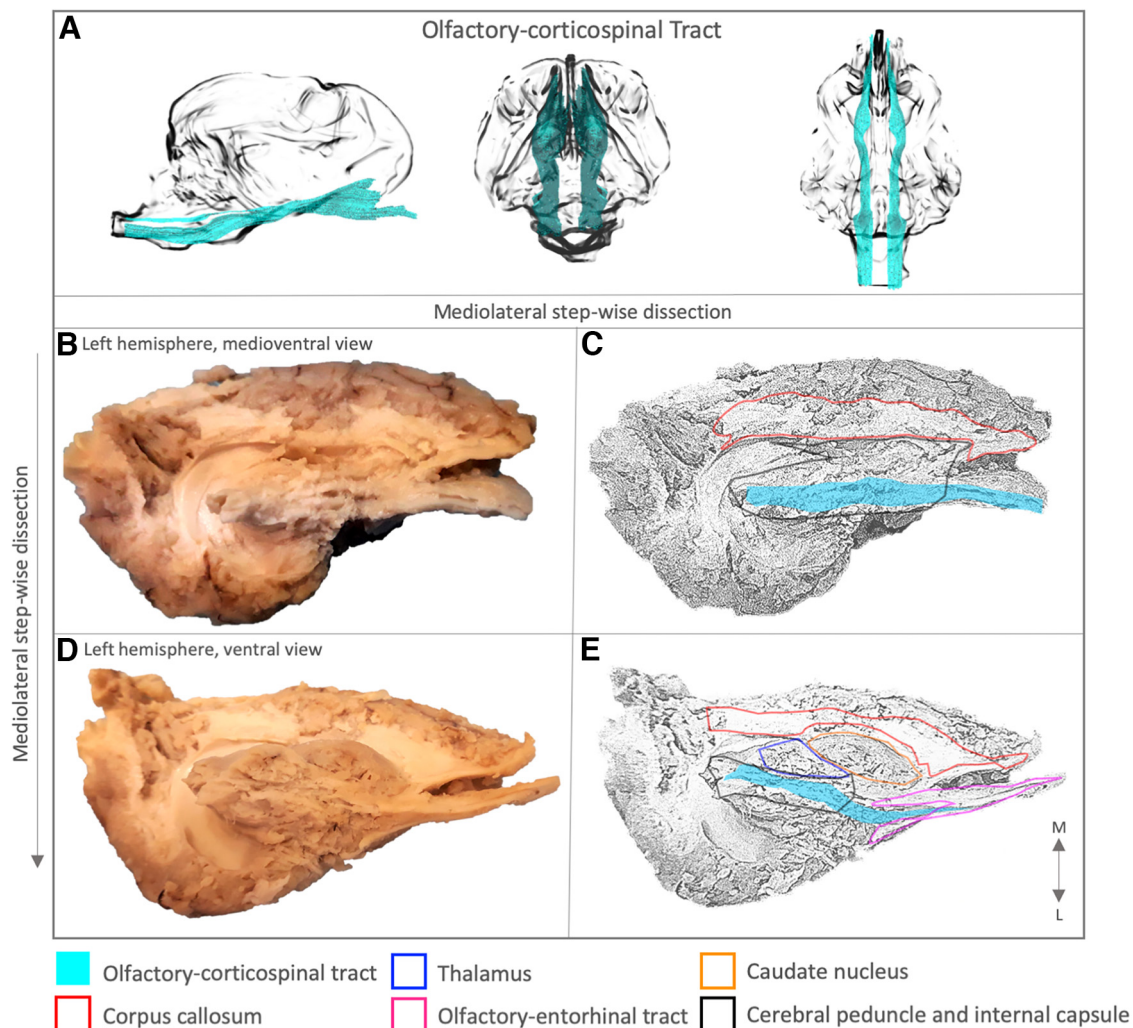


Figure 7. Documents the sequential dissection of the OCT. **A**, Demonstrates the three-dimensional image of the tract dissected using deterministic tractography and overlain on a glass brain in lateral, rostral, and dorsal views (left to right). **B–E**, Documents the photographs and associated annotated schematic diagrams of the progressive medial to lateral anatomic Klinger dissection. The OCT is a tract that originates in the ventral portion of the olfactory peduncle, in the opening of the OET bifurcation, and joins the corticospinal tract fibers as they leave the internal capsule and the thalamus to form the cerebral peduncle at the level of the midbrain. The tract becomes visible after some superficial structures are removed, such as the pillars of fornix, the optic tract, and the gray substance of the ventral striatum area. The fibers can be followed from the olfactory bulb down to the brainstem. They have a ventral position within the cerebral peduncle. L, Lateral; M, medial.

this is the first evidence of a specific white matter tract connecting these parts of the brain.

OPT

The OPT runs from the olfactory bulb laterally into the piriform lobe and terminates around the lateral ventricle. More specifically, this tract begins in the dorsal olfactory bulb, runs through the olfactory peduncle, and continues into the anterior internal capsule. The OPT then branches laterally into the white matter of the piriform lobe, turning to a more ventral path shortly after entering and running below the amygdala and the hippocampus. The OPT then turns and runs dorsally posterior along the posterior wall of the lateral ventricle (Figs. 3, 5, 9). There was some variation across subjects, with some OPTs of subjects truncating in line with the dorsal wall of the lateral ventricle and OPTs of other subjects continuing medially toward the splenium of the corpus callosum. This tract was successfully virtually dissected in TrackVis in 100% of subjects in this right hemisphere and in 96% of subjects in the left hemisphere. Probabilistic modeling identified this tract in 100% of subjects, and the OPT was

dissected in both *ex vivo* samples (Fig. 10). The mean FA values in the OPT ranged from 0.28 in the right hemisphere to 0.26 in the left hemisphere with no significant difference in mean FA because of laterality. The OPT has an average volume of 463 mm³ (minimum = 105 mm³, maximum = 1355 mm³, SD = 285).

OLT

The OLT is a large tract that begins in the olfactory bulb and forms a loop around the limbic system to terminate in the frontal lobe. This tract begins in the medial olfactory bulb, runs through the olfactory peduncle, and continues ventrally, passing through the anterior internal capsule and amygdala before turning dorsally surrounding the dorsal surface of the thalamus. It then loops medially around the interthalamic adhesion following the small stria terminalis white matter tracts where it terminated on Klinger dissection (Fig. 7). On tractography dissection, the OLT then dips ventrally and continues rostrally underneath the caudate nucleus and terminates in the pregenual gyrus of the frontal lobe (Figs. 3, 6, 9). This tract was successfully virtually dissected in TrackVis in 87% of subjects in this right hemisphere and in

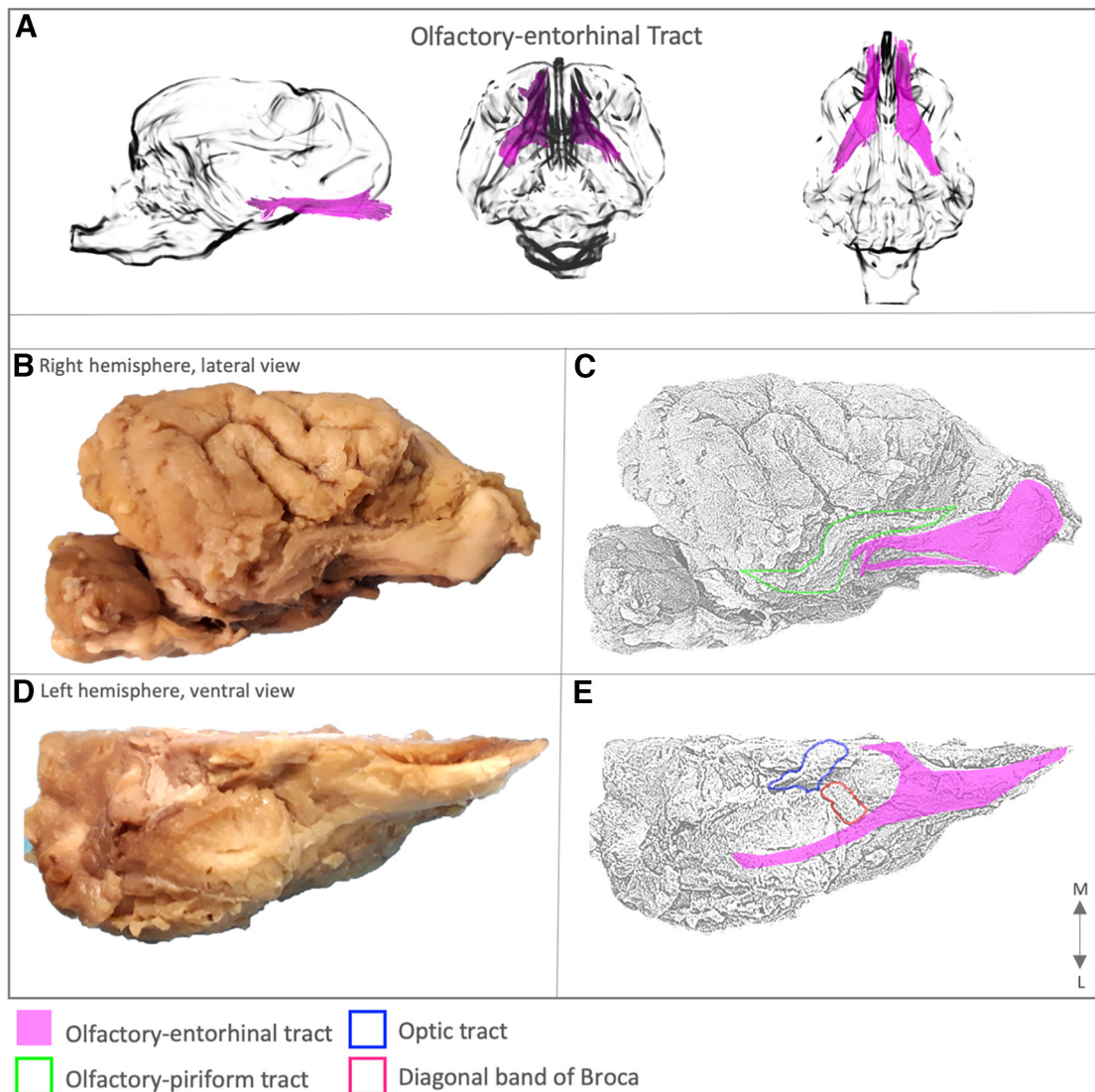


Figure 8. Documents the sequential dissection of the OET. **A**, Demonstrates the three-dimensional image of the tract dissected using deterministic tractography and overlain on a glass brain in lateral, rostral, and dorsal views (left to right). **B–E**, Documents the photographs and associated annotated schematic diagrams of the tract after anatomic Klinger dissection in lateral and ventral projections. The OET is the most superficial tract, visible before any dissection on the ventral and lateral aspects of the olfactory bulb and peduncle. Its projections were followed as they bifurcated on the ventral aspect of the hemisphere. The lateral projection ended in the entorhinal cortex, and the medial one entered the ventral subthalamic region. L, Lateral; M, medial.

92% of subjects in the left hemisphere. Probabilistic modeling identified this tract in 100% of subjects, and the OLT was dissected in both *ex vivo* samples (Fig. 10). A significant difference was found ($t_{(37)} = -2.78$, $p < 0.01$) in mean FA values in the OLT between the left (mean = 0.25, SD = 0.02) and right (mean = 0.28, SD = 0.03). The OLT has an average volume of 1137 mm³ (minimum = 114 mm³, maximum = 2797 mm³, SD = 227).

OCST

The OCST is a ventral tract that runs from the olfactory bulb along the bottom of the brain into the brainstem. This tract begins in the central olfactory bulb and runs through the center of the olfactory peduncle. The OCST then runs briefly through the dorsal corona radiata before continuing into the anterior internal capsule. The tract then continues caudally, running through the posterior internal capsule, the ventral section of thalamus, the ventral section of the mesencephalon, and medulla oblongata (Figs. 3, 7, 9). This tract was successfully virtually dissected in TrackVis in 73% of subjects in this right hemisphere

and in 70% of subjects in the left hemisphere but failed to track in 27% and 30% of subjects, respectively. Probabilistic modeling identified this tract in 100% of subjects, and the OCST was dissected in both *ex vivo* samples (Fig. 10). The mean FA values in the OCST was 0.34 in both hemispheres with no significant difference in mean FA because of laterality. The OCST has an average volume of 577 mm³ (minimum = 174 mm³, maximum = 1282 mm³, SD = 340).

OET

The OET is small ventral tract. It begins in the ventral olfactory bulb, runs through the middle of the olfactory peduncle, and terminates in the entorhinal cortex. This tract was successfully virtually dissected in TrackVis in 57% of subjects in this right hemisphere and in 43% of subjects in the left hemisphere (Figs. 3, 8, 9). Probabilistic modeling identified this tract in most subjects, and the OET was dissected in both *ex vivo* samples (Fig. 10). The mean FA values in the OET was 0.25 in both hemispheres with no significant difference in mean FA because of

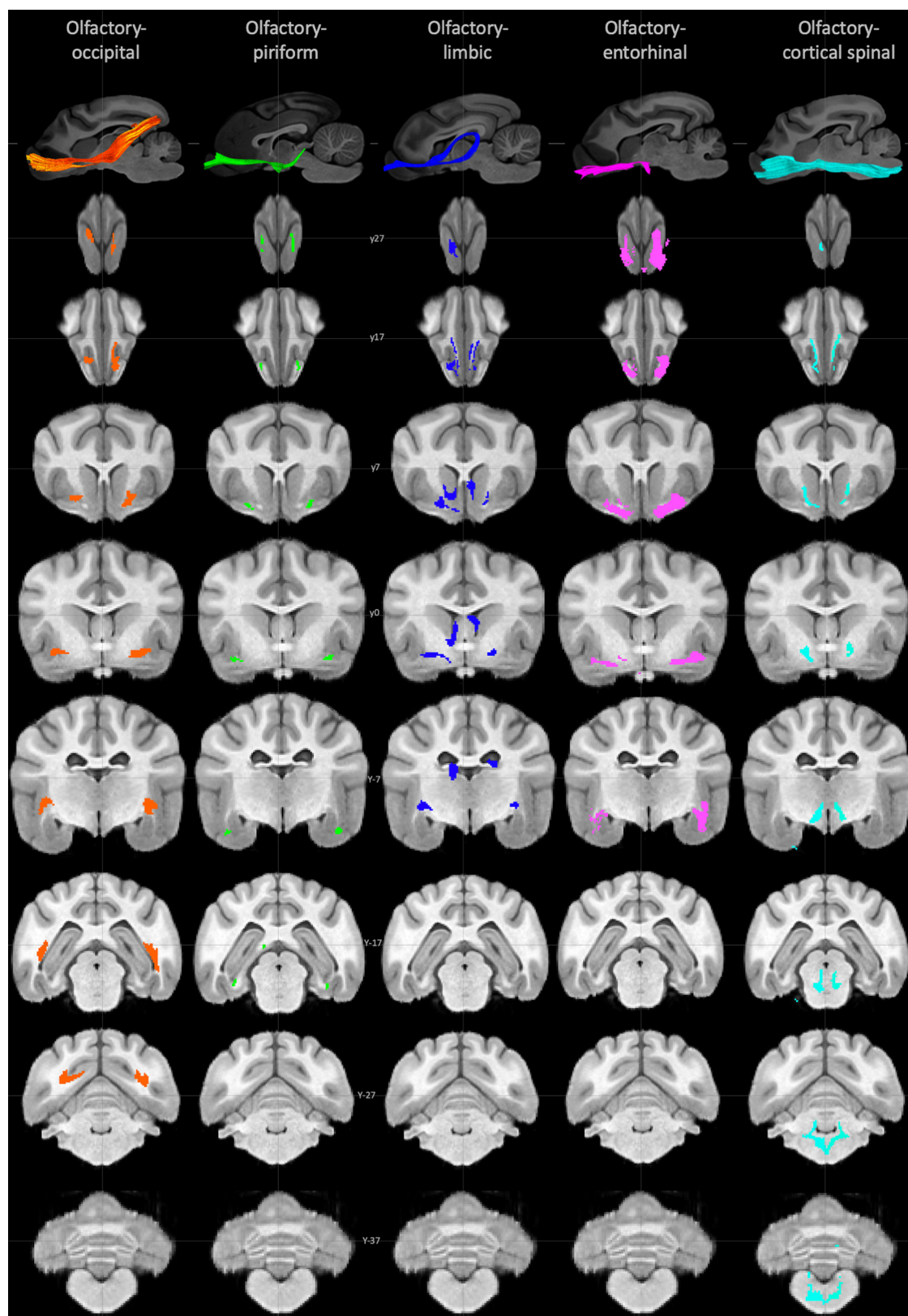


Figure 9. Five major white matter pathways were identified extending from the olfactory bulb. This figure provides the three-dimensional reconstruction of the tract, dissected from the population average tractogram, overlaid on a parasagittal T1-weighted template image (top line). The location of each tract is demonstrated on sequential transverse images of the T1-weighted template. *y*: *y*-axis orientation.

laterality. Of all the tracts, the OET had the most variation in size across subjects (minimum = 72 mm³, maximum = 930 mm³) with an average volume of 227 mm³, although the tracking was consistent across hemispheres.

Variation in olfactory tracts across subjects

Variation in volume and mean FA values across hemispheres is demonstrated via box plots in Figure 11. No statistically significant differences were identified in volume or FA between

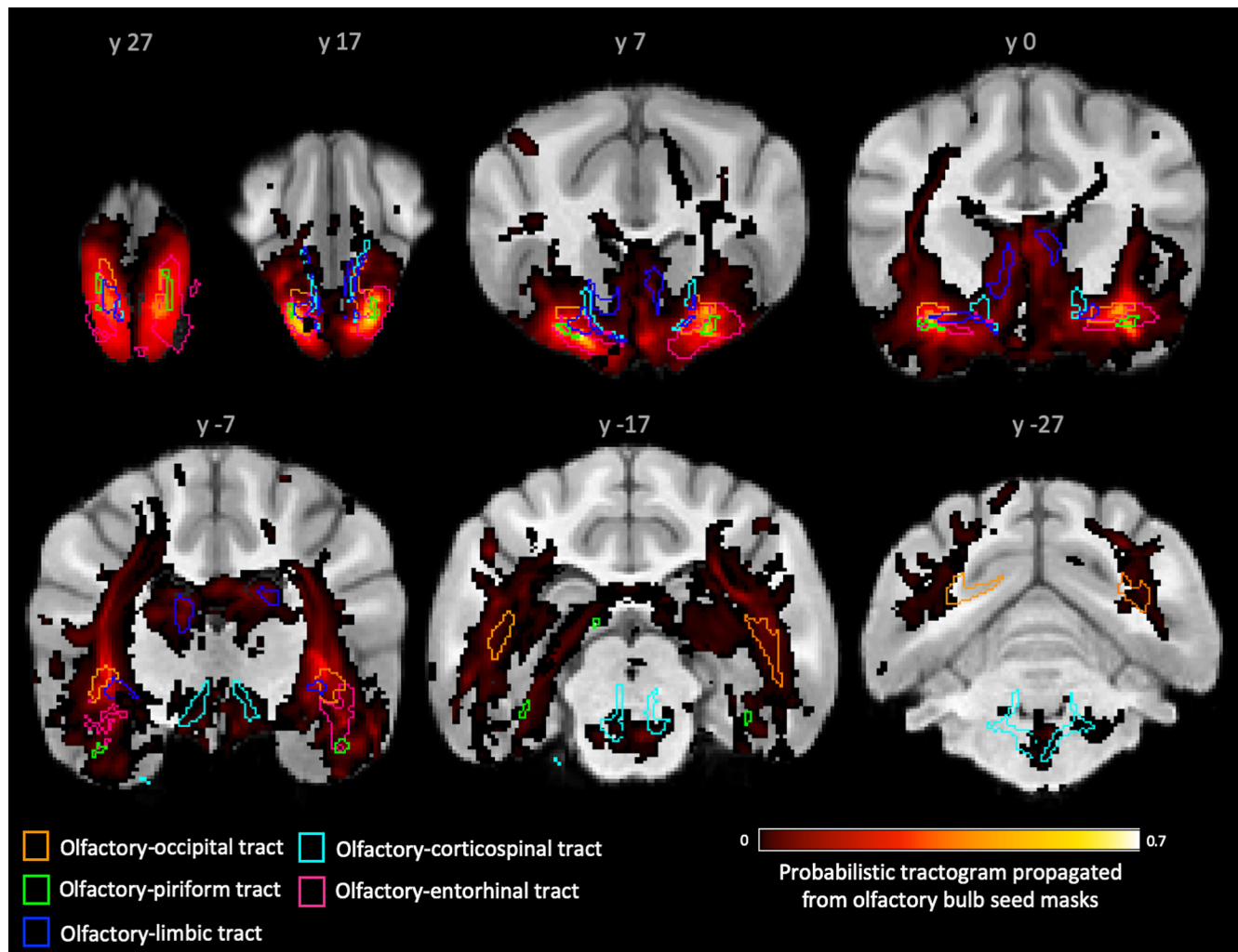


Figure 10. Population average probabilistic tractograms generated from single seed regions placed within the right and left olfactory bulbs. Registered to and overlaid on a population average T1-weighted brain template. The outline of each olfactory tract, dissected using deterministic tractography, is depicted on the tractogram to show how the deterministic dissections corroborate to the probabilistic technique. The location these tracts are demonstrated on sequential transverse images of the T1-weighted template. y: y-axis orientation.

hemispheres for any of the tracts. In addition, statistically significant differences in FA were not identified between aged and young groups. For demonstration purposes, the population average tractogram was virtually dissected to document the OOT, OCST, OLT, OPT, and the OET (Figs. 3, 9). In addition, these tracts were overlain on the population average probabilistic tractogram demonstrating the whole olfactory pathway (Fig. 10).

Discussion

In this study, we found extensive white matter connectivity from the olfactory bulb to the cerebral cortex of the canine brain using DTI MRI and Klingler dissection. Five white matter tracts were found across subjects—the OOT, OPT, OLT, OET, and OCST. Although an integration of smell and vision in dog cognition has been previously theorized (Gazit and Terkel, 2003; Byosiére et al., 2018), the olfactory–occipital connection provides a novel structural finding to support these assumptions. Even though the OPT, OLT, OET, and OCST were more expected results, the scale of these olfactory connections within the brain shows how truly integrated olfaction is in canine cognitive processes.

We used probabilistic tractography and the *ex vivo* anatomic Klingler dissection method to further validate our deterministic

tractography findings. All tracts were found to be present via probabilistic tractography methods and in two *ex vivo* brain samples. This level of validation provides strong evidence that the tracts found virtually through DTI dissection are anatomically valid as found in the *ex vivo* Klingler dissections. Although there was a trend variation in track volume across participants, there was no significant difference between the left and right hemispheric tracts. Similarly, FA values were consistent across hemispheres in all tracts bar the OLT, which had significantly higher FA values in the right hemisphere. To our knowledge, this study is the first to use DTI to investigate the structural connections of the olfactory bulb in the canine brain. Although there were some inconsistencies in the smaller tracts, the use of two tracking methods and anatomic dissection validation provides evidence that these white matter tracts are present and integral to the olfactory system.

Virtual dissection with deterministic DTI found extensive white matter tracts in the dog brain from the olfactory bulb. The percentage of tracts found across subjects varied, with the most consistent tracts, the OOT and OPT, being the largest tracts, and the least consistent tract, the OET, being the smallest. These findings are expected as smaller white matter tracts like the OET may be more difficult to dissect at the current MRI resolution. In

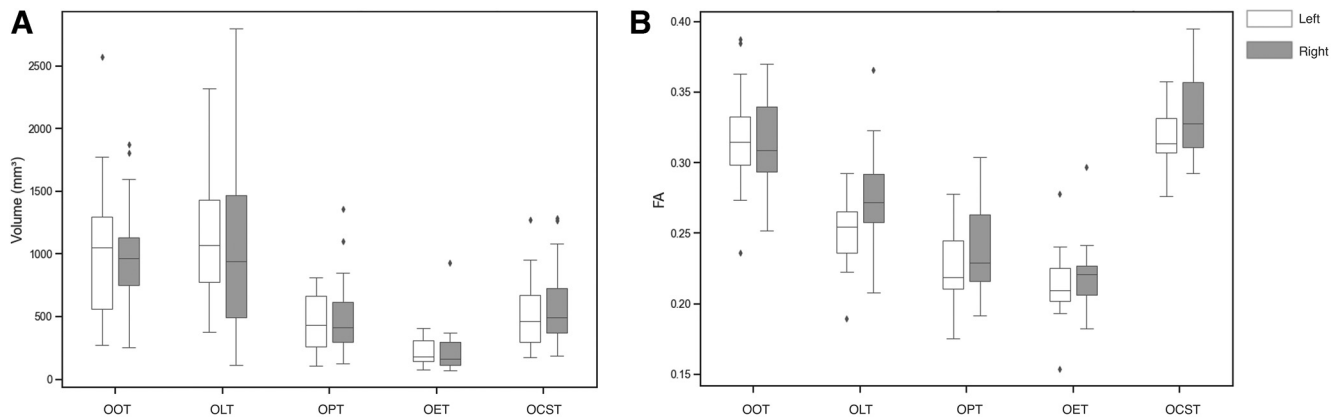


Figure 11. *A, B*, The variation in volume (*A*) and mean FA (*B*) values for each tract per hemisphere via box plots in 23 subjects. No statistically significant differences were detected between hemispheres for FA or volume for any tract.

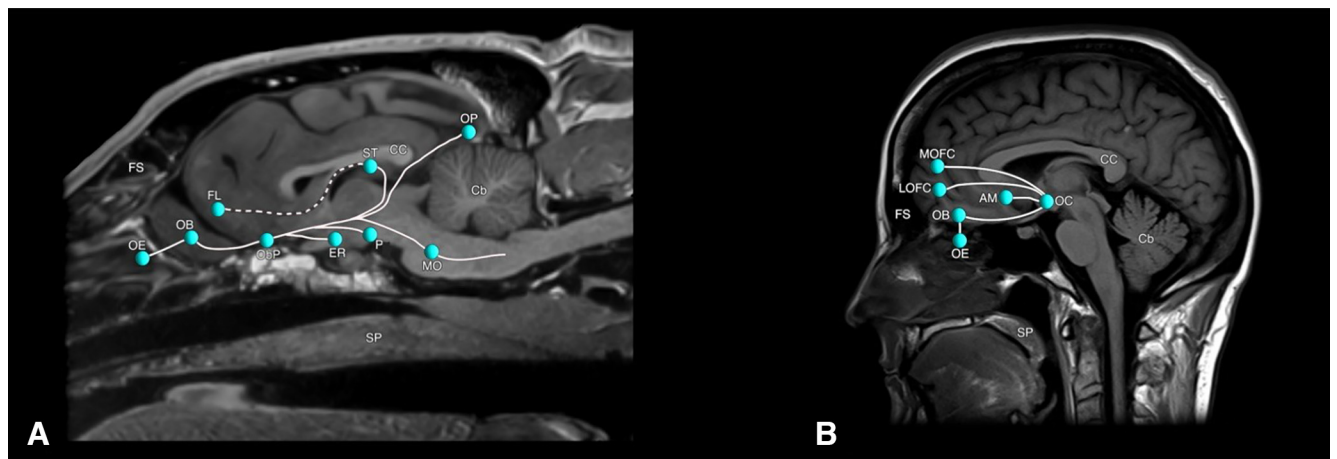


Figure 12. Comparative olfactory systems. The canine (*A*) and human (*B*; Sheperd, 2006) brain olfactory system from a sagittal MRI midline slice. Solid line depicts connections identified on Klingler and tractography dissections. Dashed line shows connections identified only on tractography dissections. A.M., Amygdala; Cb, cerebellum; CC, corpus callosum; FL, frontal lobe; FS, frontal sinus; ER, entorhinal cortex; LOFC, lateral orbitofrontal cortex; MO, medulla oblongata; MOFC, medial orbitofrontal cortex; OB, olfactory bulb; ObP, olfactory bulb peduncle; OC, olfactory cortex; OE, olfactory epithelium; OP, occipital pole; P, piriform lobe; SP, soft palate; ST, stria terminalis.

addition, the OET is the most ventrally positioned tract, which could make it more susceptible to artifacts and warping in MRI acquisition because of its position on the edge of the brain. In contrast, the OOT, OPT, and OLT tracked consistently across subjects, potentially because these were the largest connections. The Klingler dissection anatomic validation of DTI tractography provides further confidence in our olfactory tract findings. By using probabilistic tractography, not only are the tracts found with another method of modeling white matter fibers but it provides a probabilistic heatmap showing the areas where the white matter tracking is most likely. Our probabilistic tracking with the FSL toolbox aligned with the deterministic virtual dissections. Tractography has been a topic of much debate in the neuroimaging literature. Limited resolution, modeling techniques, and acquisition artifacts can all affect the outcome of DTI tractography. Therefore, we used two *ex vivo* samples to anatomically dissect the canine brain using the Klingler method. The finding that these tracts are consistent in size, shape, and orientation across subjects is vital to establishing these tracts as anatomically sound. Klingler dissection involves carefully peeling back the gray matter tissue of a postmortem brain to reveal underlying white matter tracts beneath. By using this method, the tracts found through virtual dissection have been validated in an anatomic sample.

Although the discovery of the structure of the dog olfactory system in the brain and novel white matter tracts is exciting and suggests a novel functional connection between the olfactory bulb and the cortex, there are limitations to the functional inferences that can be made from these findings. Even though progress has been made with MRI and other canine brain atlases (Johnson et al., 2020), it is unclear whether these regions function similarly to those in the human literature or are related by anatomic location and name only. Electrophysiological research on brain function in dogs is limited, and fMRI studies in canines mainly rely on awake unrestrained subjects, which could lead to biases in recruitment and confounding factors like attention and inhibition in activation patterns. However, what is clear from these results is that the olfactory system in the dog is widespread and has pronounced connections to many important regions of the brain.

The olfactory white matter tracts found with DTI MRI and validated with Klingler dissection are particularly intriguing. They are consistent with findings from canine cognition studies and previous assumptions made about the canine olfactory system. Although the OPT and OLT findings are in line with the previous literature in humans that showed the olfactory system being incorporated into the processing

of memory and emotion (Biella and De Curtis, 2000; Burmeister et al., 2012), the size and consistency of the OOT was less expected (Fig. 12). This OOT finding is particularly significant as cognition studies in dogs have proposed an integration of the visual and olfactory system (Pongrácz et al., 2017), but there has been little structural evidence to support this assumption (Jia et al., 2014). These findings propose an information expressway between the olfactory and visual system that may process information independently of higher order cognitive regions such as the frontal lobe. This integration of olfaction and vision is in line with the previous behavioral canine literature that has shown not only the combined use of these senses but also the dominance of the olfactory system over visual stimuli in some cases (Gazit and Terkel, 2003).

The other white matter connections are also informative. The OCST shows a direct connection between the olfactory bulb and the brainstem, potentially allowing for integration of information with the cranial nerves, permitting more instinctual behaviors to occur. Regarding the OPT, there has been much research into the role of the piriform lobe in olfactory perception. Studies suggest the piriform lobe plays a key role in processing and integrating olfactory stimuli with the rest of the brain to create a perception of scent (Courtillot and Wilson, 2017), which aligns with the white matter structural findings of this study. We found that the OLT had a consistent structural connection among the olfactory bulb, limbic system, and frontal cortex with both tractography techniques; however, this connection was not definitively identified on Klinger dissection where the tract terminated at the striatum terminalis. This disparity is likely because of the Klinger dissection technique, which requires removal of the medial frontal lobe to expose the OLT. This would have removed fibers extending into the frontal lobe, artifactually terminating the tract at the striatum terminalis level. Therefore, connection of the OLT with the frontal lobe is likely to be present and is in line with canine behavioral studies where the integration of olfactory information processing with frontal executive functions has been demonstrated through canine cognition tasks (Gadbois and Reeve, 2014; Horowitz, 2017; Horowitz and Franks, 2020). The OET shows a direct connection between the olfactory bulb and entorhinal cortex, a brain region associated with olfactory and memory processing (Biella and De Curtis, 2000; Dahmani et al., 2018). These findings suggest the olfactory system plays a prominent if not dominant role in canine cognition and has connections in most of the major processing pathways, making it a vital network to consider when studying canine cognition.

As part of our project we provided mean volume and FA parameters for each olfactory pathway. FA is a diffusivity parameter that acts as a general measure of axonal coherence and microstructural integrity (Basser and Jones, 2002; Niogi et al., 2007). As the brain ages, white matter degeneration and demyelination causes reductions in FA (Barry et al., 2021; Radhakrishnan et al., 2021). As our cohort included dogs up to 11 years of age, and although when tested we found no significant difference between aged and young dogs, it is important to take into consideration that the metrics provided may have been affected by age-related changes.

Conclusion

This study identified intrabrain connections composing the extensive canine olfactory system with novel cortical connections

that expands our understanding of the dominant role olfaction has in canine cognition. The olfactory system consisted of five white matter tracts connecting the olfactory bulb to the OOT, OPT, OLT, OCST, and OET. Tracts were found to be consistent across tractography methods and were successfully dissected in two *ex vivo* samples. These findings show the olfactory system of the dog is integrated with many different parts of the brain. The OOT connection is of particular interest because of its size and relevance to understanding canine cognition. The presence of an olfactory–occipital information highway provides structural evidence for the theorized olfactory–vision integration proposed in canine cognitive research. Although more research is needed to explore the function of these cortical regions and to access the potential brain networks involved in olfactory-related behaviors, this research provides the first exploration of the dog olfactory network in the brain.

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