Building a connectome of the insect brain's navigational center

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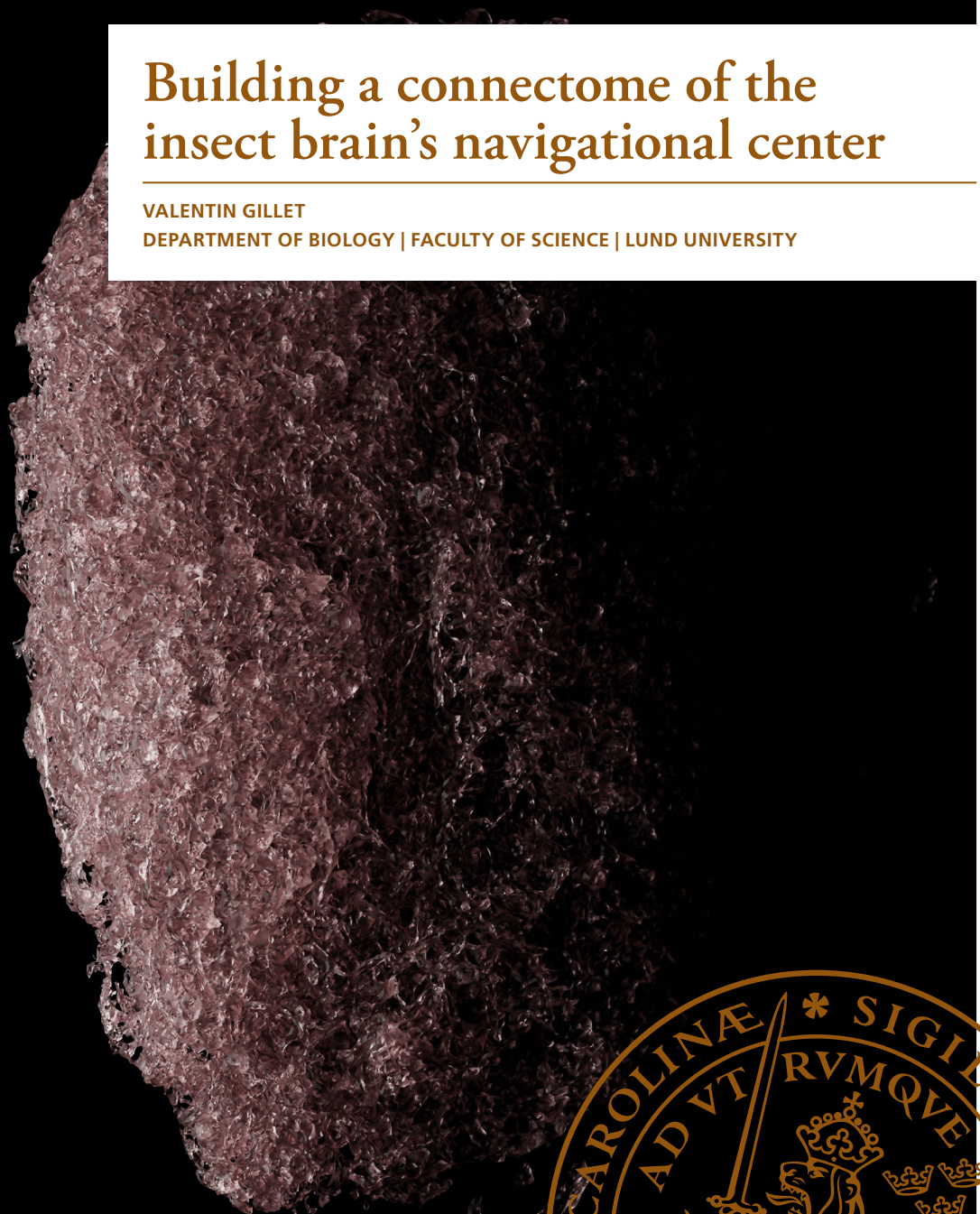

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by Valentin Gillet

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Abstract
The central complex is a conserved brain region at the core of insect navigation, integrating sensory information to guide behavior across species spanning over 400 million years of evolution. While connectomes of the fruit fly have provided unprecedented detail about its circuitry, understanding how this neural architecture varies across insects with diverse navigational capabilities requires comparative approaches. This thesis presents a multiresolution imaging, processing, and analysis pipeline designed to make comparative connectomics accessible at a reduced cost. By leveraging the predictable neuroarchitecture of the central complex, we are able to produce a global projectome with embedded local connectomes describing entire computational units.

Applying this pipeline, we reconstructed head direction circuits across multiple hymenopteran species, revealing remarkable conservation in cell types and projection patterns despite over 300 million years of divergence from the fly. Circuit-level analysis, however, uncovered fundamental differences in the feedback loops underlying the ring attractor, demonstrating functionally convergent solutions to the same computational problem. Extending this analysis to downstream traveling direction neurons in the sweat bee, we identified both ancestral pathways homologous to the fly and novel circuits specific to bees, including a new cell type that may contribute to enhanced vector navigation capabilities. Together, these findings illuminate how evolution shapes neural circuits to produce behavioral diversity while maintaining core computational functions.

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central complex, connectomics, volume electron microscopy, segmentation, sweat bee

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Cover illustration front: Neurons in the nodulus of *Megalopta genalis*

Cover illustration back: Nearest neighbor affinity prediction highlighting membranes of neurons of the central complex of *Megalopta genalis*

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Contents

List of papers

I **Gillet V***, **Kluge J***, & Patel RN (2025). **A historical perspective on the insect central complex: Anatomy, development, and function**. *Molecular Psychology: Brain, Behavior, and Society*, https://doi.org/10.12688/molpsychol.17564.3

II **Gillet V**, Sayre ME, Badalamente GS, Schieber N, Tedore K, Funke J, & Heinze S. **A multiresolution imaging and analysis pipeline for comparative circuit reconstruction in insects**. (*Submitted to eLife*)

III Dorkenwald S[1], Schneider-Mizell CM[1], Brittain D, Halageri A, Jordan C, Kemnitz N, Castro MA, Silversmith W, Maitin-Shephard J, Troidl J, Pfister H, **Gillet V**, Xenes D, Bae JA, Bodor AL, Buchanan J, Bumbarger DJ, Elabbady L, Jia Z, Kapner D, Kinn S, Lee K, Li K, Lu R, Macrina T, Mahalingam G, Mitchell E, Mondal SS, Mu S, Nehoran B, Popovych S, Takeno M, Torres R, Turner NL, Wong W, Wu J, Yin W, Yu Sc, Reid RC, da Costa NM, Seung HS, & Collman F. **CAVE: Connectome Annotation Versioning Engine**. *Nature Methods*, https://doi.org/10.1038/s41592-024-02426-z

IV Sayre ME, **Gillet V**, Pinzon-Rodriguez A, Badalamente GS, Ceberg N, Griggs N, Serratosa Capdevila L, Roberts R, Gunnarsson ES, Ceberg N, Szadaj F, Ellendula S, Honkanen A, Narendra A, & Heinze S. **Functional convergence of distinct head direction circuits in bees, ants and flies**. (*Manuscript*)

V **Gillet V**, Sayre ME, Badalamente GS, Pinzon-Rodriguez A, Zadel A, Griggs N, Monteleone A, Langreiter M, Nerme V, & Heinze S. **A novel navigation circuit in the bee brain**. (*Manuscript*)

Author contributions

Paper 1 **Valentin Gillet***: Conceptualization, Visualization, Writing - Original Draft Preparation, Writing - Review Editing; **Janka Kluge***: Conceptualization, Visualization, Writing - Original Draft Preparation, Writing - Review Editing; **Rickesh N. Patel**: Conceptualization, Writing - Review Editing

Paper 2 **Valentin Gillet**: Conceptualization, Methodology, Software, Investigation, Data curation, Writing—original draft, Writing—review and editing, Visualization; **Marcel E. Sayre**: Conceptualization, Methodology, Investigation, Data curation, Writing—review and editing; **Griffin S. Badalamente**: Methodology, Software, Investiga-

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tion; **Nicole Schieber**: Methodology; **Kevin Tedore**: Software; **Jan Funke**: Supervision;**Stanley Heinze**: Conceptualization, Methodology, Resources, Data curation, Validation, Writing—review and editing, Supervision, Project administration, Funding acquisition

Paper 3 **S.D.**, **F.C.** and **C.M.S.-M.** designed CAVE's core functionalities, service interactions and layout. **S.D.** and **A.H.** implemented the ChunkedGraph and the L2-Cache. **M.A.C.**, **S.D.**, **W.S.** and **A.H.** implemented the ChunkedGraph meshing logic. **C.M.S.-M.** implemented the improved splitting logic. **C.J.**, **N.K.**, **J.M.-S.** and **D.X.** extended neuroglancer for proofreading. **J.M.-S.**, **V.G.**, **J.T.** and **H.P.** implemented adapters for and tested CAVE with supervoxel graphs produced by other segmentation pipelines. **C.J.**, **S.D.** and **F.C.** implemented the authentication system. **F.C.** and **D.B.** implemented the annotation service and the annotation schema system. **D.B.**, **F.C.** and **S.D.** implemented the Annotation database and the materialization service. **S.D.** and **F.C.** implemented the neuroglancer state server. **F.C.**, **C.M.S.-M.**, **S.D.** and **D.B.** implemented the CAVEclient. **F.C.**, **C.M.S.-M.** and **S.D.** implemented MeshParty. **C.M.S.-M.** and **F.C.** implemented Neuroglancer-AnnotationUI. **A.H.** and **S.D.** implemented datastoreflex. **C.M.S.-M.** and **F.C.** implemented PCGskel and skeletonization processing. **C.M.S.-M.** and **F.C.** implemented the dash apps. **W.S.** provided support and tools for fast cloud storage access. **F.C.**, **S.D.**, **C.M.S.-M.**, **D.B.**, **C.J.** and **A.H.** maintained the kubernetes deployments. **A.H.**, **A.L.B.**, **B.N.**, **C.J.**, **C.M.S.-M.**, **D.B.**, **D.J.B.**, **D.K.**, **E.M.**, **F.C.**, **G.M.**, **H.S.S.**, **J.A.B.**, **J.B.**, **J.W.**, **K. Lee**, **K. Li**, **L.E.**, **M.A.C.**, **M.T.**, **N.K.**, **N.L.T.**, **N.M.d.C.**, **R.C.R.**, **R.L.**, **R.T.**, **S.-c.Y.**, **S.D.**, **S.K.**, **S.M.**, **S.P.**, **S.S.M.**, **T.M.**, **W.S.**, **W.W.**, **W.Y.** and **Z.J.** created the structural MICrONS65 dataset. **S.D.**, **F.C.** and **C.M.S.-M.** wrote the paper with contributions from all authors.

Paper 4 **Marcel E. Sayre**: Conceptualization, Methodology, Software, Formal Analysis, Investigation, Visualization, Data Curation, Writing - Original Draft, Writing - Review and Editing; **Valentin Gillet**: Investigation, Software, Writing - Review and Editing; **Atticus Pinzon-Rodriguez**: Investigation; **Griffin Badalamente**: Software, Investigation; **Nils Ceberg**: Methodology, Formal Analysis; **Nina Griggs**: Investigation; **Laia Serratosa Capdevila**: Investigation; **Ruairí Roberts**: Investigation; **Ebba S. Gunnarsson**: Investigation; **Felicia Szadaj**: Investigation; **Saroja Ellendula**: Investigation; **Anna Honkanen**: Resources; **Ajay Narendra**: Resources, Writing - Review and Editing, Supervision; **Stanley Heinze**: Conceptualization, Methodology, Investigation, Resources, Data Curation, Validation, Writing – Review and Editing, Supervision, Project administration, Funding acquisition.

Paper 5 **Valentin Gillet**: Conceptualization, Methodology, Software, Formal analysis, Investigation, Data curation, Writing—original draft, Writing—review and editing, Visualization; **Marcel E. Sayre**: Conceptualization, Methodology, Investigation,

Data curation, Writing—original draft, Writing—review and editing; **Griffin S. Badalamente**: Software, Investigation; **Atticus Pinzon Rodriguez**: Investigation; **Ana Zadel**: Investigation; **Nina Griggs**: Investigation; **Arianna Monteleone**: Investigation; **Matilda Langreiter**: Investigation; **Vytautas Nerme**: Investigation; **Stanley Heinze**: Conceptualization, Methodology, Investigation, Resources, Data curation, Validation, Writing—review and editing, Supervision, Project administration, Funding acquisition.

Popular summary

When we think of insects, we probably often picture tiny flying pests that land on our food, tickle our necks, or bump into our windows. To be fair to them, insects have evolved without windows to bump into for the major part of their reign over the world, which started some 500 million years ago. In that span of time, they have colonized almost every ecological niche on Earth, something that we have only started to match recently, in comparison. Insects are, among other things, great navigators capable of traversing half a continent or orient themselves with an amount of information that would leave us stranded in the middle of the desert. Like for most animals, their great capabilities mostly stem from their brain, which in this case is the size of a mere bread crumb. Contrary to popular belief that windows are smarter, insect capabilities are, indeed, great.

The brain center for insect navigation is called the central complex (CX). It is a beautiful structure situated right at the center of the brain that is orders of magnitude older than modern humans and has been studied for more than a century (as we discuss in Paper 1). More importantly, we know through studies of many species that the CX is largely conserved across insects, and that it guides navigation using sensory information coming from all over the brain. The CX is an integrative center of the brain that represents a probe into brain evolution across hundreds of millions of years, which can probably help us elucidate some fascinating questions. How can the CX allow the great navigational feats that insects exhibit when we do not put windows in their way? And more importantly, how can evolution shape tiny brains to produce the great diversity that insects are capable of?

In Paper 2, we provide tools to start elucidating some of these questions about the CX (notably using the software described in Paper 3). We use a combination of electron microscopy, machine learning, and elbow grease to reconstruct entire neuronal circuits of the CX of several insect species. We started with neurons involved in a network that we call the head direction circuit, because it acts like a literal compass tracking where the animal is looking at all times, a function that is at the core of the CX role. In Paper 4, we demonstrate that this circuit is largely conserved across bees and ants, with some variations at the level of fine connectivity. It indicated that some circuits of the CX are likely common to all insects. We then looked closer into other neurons responsible for encoding the direction in which the animal travels. In Paper 5, we show that these neurons compose several parallel pathways of information: some that likely fulfill the essential function of computing traveling direction, and some that may have specialized across insects to contribute to their diversity.

There are still more unanswered questions about the CX and the brain overall. Enough, surely, to feed our curiosity for another century at least.

Populärvetenskaplig sammanfattning på svenska

När vi tänker på insekter föreställer vi oss förmodligen oftast små flygande plågor som landar på vår mat, kittlar oss i nacken, eller flyger in i våra fönster. Å deras försvar har insekter utvecklats utan fönster att flyga in i under större delen av sin tid på jorden, vilken började för cirka 500 miljoner år sedan. Under den tidsperioden har de koloniserat nästan varje ekologisk nisch, något som vi i jämförelse bara nyligen har börjat matcha. Insekter är bland annat skickliga navigatörer som kan korsa en halv kontinent eller orientera sig med information så begränsad att vi skulle hamna strandsatta mitt i öknen. Liksom för de flesta djur härstammar deras förmågor mestadels från deras hjärna, som i detta fall är stor som en ynka brödsmula. I motsats till den allmänna uppfattningen att fönster är smartare, är insekters förmågor faktiskt storartade.

Hjärncentret för insekters navigation kallas "the central complex" (CX). Det är en vacker struktur som är belägen precis i hjärnans mitt, är storleksordningar äldre än moderna människor och som har studerats i mer än ett sekel (som vi diskuterar i Paper 1). Vad som är ännu viktigare är att studier av många arter visar att CX är högst konserverat bland insekter och att det styr navigering med hjälp av sensorisk information från hela hjärnan. CX är ett integrativt centrum i hjärnan som erbjuder en inblick i hjärnans evolution över hundratals miljoner år vilket förmodligen kan hjälpa oss belysa några fascinerande frågor. Hur kan CX möjliggöra de storartade navigationsbedrifter som insekter uppvisar när vi inte sätter fönster i deras väg? Och kanske ännu viktigare: hur kan evolutionen forma små hjärnor för att producera den stora mångfald som insekter är kapabla att uppvisa?

I Paper 2 tillhandahåller vi verktyg för att börja belysa några av dessa frågor om CX (framför allt med hjälp av mjukvaran beskriven i Paper 3). Vi använder en kombination av elektronmikroskopi, maskininlärning och hårt arbete för att rekonstruera hela neuronala kretsar i CX hos flera insektsarter. Vi började med neuroner involverade i ett nätverk som vi kallar "the head direction network", eftersom det fungerar som en bokstavlig kompass som kontinuerligt spårar vart djuret tittar, en funktion som är central för CX:s roll. I Paper 4 visar vi att denna krets till stor del är konserverad bland bin och myror med vissa variationer på nivån av fin konnektivitet. Det tyder på att vissa kretsar i CX sannolikt är gemensamma för alla insekter. Vi tittade sedan närmare på andra neuroner som ansvarar för att koda djurets färdriktning. I Paper 5 visar vi att dessa neuroner utgör flera parallella informationsvägar: några som sannolikt uppfyller den väsentliga funktionen att beräkna färdriktning och några som kan ha specialiserats bland insekter och därmed bidragit till deras mångfald.

Det finns fortfarande fler obesvarade frågor om CX, och hjärnan överlag. Tillräckligt många för att mätta vår nyfikenhet i åtminstone ytterligare ett sekel.

Résumé populaire

Quand nous pensons aux insectes, nous imaginons probablement des petits nuisibles volants qui se posent sur notre nourriture, nous chatouillent la nuque, ou se cognent contre nos fenêtres. Il faut dire que les insectes ont évolué sans fenêtres contre lesquelles se cogner pendant la majeure partie de leur règne sur le monde, qui a commencé il y a environ 500 millions d'années. Durant cette période, ils ont colonisé presque toutes les niches écologiques sur Terre, quelque chose que nous n'avons commencé à faire que récemment. Les insectes sont, entre autres, d'excellents navigateurs capables de traverser la moitié d'un continent ou de s'orienter avec une quantité minime d'informations qui nous laisserait perdus au milieu du désert. Comme pour la plupart des animaux, leurs capacités impressionantes proviennent principalement de leur cerveau, qui, dans ce cas, est de la taille d'une tête d'aiguille. Contrairement à la croyance populaire les capacités des insectes sont en effet remarquables.

Le centre de la navigation dans le cerveau de l'insecte s'appelle le complexe central (CX). C'est une structure intriguante située en plein centre du cerveau qui est bien plus ancienne que l'humanité elle-même, et qui a été étudiée depuis plus d'un siècle (comme nous le discutons dans Paper 1). Plus important encore, nous savons grâce aux études de nombreuses espèces que le CX est largement conservé chez les insectes, et qu'il guide la navigation en utilisant des informations sensorielles issues de multiple régions du cerveau. Le CX est un centre intégratif qui offre une perspective privilégiée pour comprendre l'évolution du cerveau à travers des centaines de millions d'années. Cela peut notamment nous aider à élucider certaines questions fascinantes. Comment le CX peut-il permettre les grands exploits de navigation dont les insectes font preuve quand nous ne mettons pas de fenêtres sur leur chemin ? Et plus important encore, comment l'évolution peut-elle façonner une telle diversité chez les insects à partir d'aussi miniscules cerveaux ?

Dans Paper 2, nous proposons des outils pour commencer à élucider certaines de ces questions sur le CX (notamment en utilisant le logiciel décrit dans Paper 3). Nous utilisons une combinaison de microscopie électronique, de machine learning, et d'huile de coude pour décrire des circuits neuronaux entiers présent dans le CX de plusieurs espèces d'insectes. Nous avons commencé par les neurones impliqués dans un réseau que nous appelons le circuit de direction de la tête (ce qui sonne bien mieux en anglais), parce qu'il agit comme une véritable boussole qui indique où l'animal regarde à tout moment, une fonction qui est au cœur du rôle du CX. Dans Paper 4, nous démontrons que ce circuit est largement conservé chez les abeilles et les fourmis, avec quelques variations au niveau de la connectivité fine. Cela indique que certains circuits du CX sont probablement communs à tous les insectes. Nous avons ensuite examiné de plus près d'autres neurones responsables de l'encodage de la direction dans laquelle l'animal se déplace. Dans Paper 5, nous montrons que ces neurones composent plusieurs voies parallèles d'informations : certaines remplissent probablement

la fonction essentielle de calculer la direction de déplacement, et d'autres se sont peut-être spécialisées pour contribuer à la diversité des insectes.

Il reste encore de nombreuses questions sans réponse sur le CX et le cerveau en général. Suffisamment, certainement, pour nourrir la curiosité des scientifiques pendant au moins un siècle encore.

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"Word of advice, try to accumulate a few quotes in the next couple of years."

- Marcel Sayre, four years ago, as he finished writing his thesis in the middle of the night, and did not find quotes

"We do these things not because they are easy, but because we thought they were going to be easy."

- Somebody on the internet, probably not a doctoral student

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Preamble: Cool brains in a small package

Probably like most reading this thesis, I have always been full of questions about the world, ever since I was a kid. As I grew up and started learning about biology, I realized that what truly excited me was simple things. I find it fascinating that nature, through the process of evolution, can come up with such elegant solutions that they just become self-evident when we start understanding them deeply enough. I like to believe that all things, at their core, are just a bunch of simple bits put together that, by chance, became something more. I also like to believe that the brain is not as fancy as we tend to think, that it just does a bunch of simple things, that together, become something more.

One of the main functions of the brain is to extract information from its sensory environment and integrate it with the organism's internal state. From peripheral sensors to the decision-making centers, processing layers treat increasingly high-level information as they filter sensory data to highlight relevant features for decision-making. Through the process of learning, the right behavior is selected when the right ramification of filters is activated. In some way, neurons are these filters, and they can transform data between layers. They perform simple computations, creating complexity when assembled into circuits. The more neural substrate, the more complex the circuit can become, and thus the tree of possibilities from which flexible behavior can emerge. Perhaps that is how we, with our big human brain and its innumerable number of neurons [1], gained the ability to spend most of our time writing overly complicated descriptions of the world for each other.

Even tiny brains with approachable numbers of neurons can produce great complexity. On the other side of the spectrum, the estimated 5.5 million insect species existing on Earth (Stork, 2018) have colonized most of its ecological niches, from lush forests to Antarctica and dry deserts. Their behavioral repertoire is as diverse as insects are widespread, despite having a brain the size of a bread crumb (or a pinhead, or a grain of rice).

Dragonflies are some of the most successful predators in the animal kingdom, capable of

[1] 86 billion neurons and as many non-neuron cells according to Herculano-Houzel (2012)

predicting the trajectory of their prey while flying during high speed chases (Mischiati et al., 2015). Bogong moths (Warrant et al., 2016) and monarch butterflies (Reppert et al., 2016) are both long distance migrators, accurately traveling thousands of kilometers every year by using celestial cues and the Earth's magnetic field for guidance. Eusocial insects, such as termites, bees, and ants, have colonized wildly different environments by distributing tasks and communicating among conspecifics. Cockroaches alone can be found in almost all ecologies, including in aquatic and arctic habitats.

These feats are impressive, and even more so when factoring in the size of the insect brain. It contains roughly between about 7 400 neurons in a microscopic parasitic wasp (*Megaphragma mymaripenne*; Makarova et al., 2022) and 1 million neurons in the honeybee (Menzel and Giurfa, 2001). This limited amount of neural substrate necessarily restricts the potential of the brain, yet it must be able to exploit all the sensory worlds that insects evolve in and produce their wide array of behavioral repertoires.

Processing sensory signals is one of the missions of the central complex, the very topic of this thesis. This brain region only represents about one percent of the insect brain volume (calculated using volumes from `https://insectbraindb.org/`, and Adden et al., 2020), and is nonetheless essential for most of the behaviors that amaze us, such as courtship, sleep, and most importantly for this work, spatial navigation. Throughout more than a hundred years of research on the central complex, it became clear that it integrates visual, olfactory, and mechanosensory information with the animal's internal state to produce motor commands.

Perhaps the central complex, and the insect brain as a whole, should be highly evolvable and vary greatly between species to create such behavioral diversity. Paradoxically, the central complex appears to be highly conserved across all insect species and across arthropods. The question is then, how can flexibility emerge from this stereotypical brain structure? What circuits are ancestral, and how much of them must be specific to the lifestyle of the species?

Chapter 1

A Complex and Central Brain Region

The central complex is a sensorimotor transformation center in the insect brain. It is generally accepted that it exists in some shape in the brain of nearly all arthropods (Homberg, 2008, Strausfeld, 2012). This notably includes malacostracan crustaceans such as the crayfish (Utting et al., 2000) and stomatopods (Chou et al., 2022, Thoen et al., 2017), and chelicerates where it is more often called the arcuate body (Loesel et al., 2011, Strausfeld et al., 1993). Although its homology is not yet confirmed across all these taxa, its presence in all of them suggests the central complex originated over 500 million years ago (Strausfeld et al., 2016, Thoen et al., 2017).

A word about nomenclature

Throughout years of study, neuroanatomists have come up with two parallel nomenclatures for cell types and neuropils of the insect brain, depending on their model of predilection. Historically, these were the fruit fly (Ito et al., 2014, Wolff et al., 2015) or other insects (Althaus et al., 2022, Heinze et al., 2013, Hensgen et al., 2021a, Homberg, 2008, el Jundi et al., 2018, Stone et al., 2017). In this thesis work, I will use the fruit fly names, which generally allow an easier comparison with the multiple published *Drosophila* connectomes (Berg et al., 2025, Dorkenwald et al., 2024, Hulse et al., 2021), by far the most complete accounts of the central complex anatomy. This nomenclature intuitively assigns neuron names based on their projection patterns. For example: PFN cells, which are central to this thesis, get their name from their innervation of three neuropils of the central complex called Protocerebral bridge, Fan-shaped body, and Nodulus (hence P-F-N). When possible, I provide equivalent names for other species in Table 1.

Table 1: Table of equivalent names between the fruit fly nomenclature (Wolff et al., 2015) and other insects (Heinze and Homberg, 2008).

Drosophila melanogaster	Other insects
Columnar cells	
EPG/PEG	CL1a-d
PEN	CL2
PFL1	CPU1-type1
PFL2	CPU2
PFL3	CPU1-type2
PFN	CPU4/5
PFRa/b	*Unknown**
PFG	*Unknown**
Fx	CU
*Unknown**	CP1/2
Tangential cells	
Δ7	TB1
IbSpsP	*Unknown**
SpsP	*Unknown**
*Unknown**	TB2-8
FBx	TU
ER	TL
LNO	TN
Interneurons	
hΔ	Pontines
vΔ	PoUv
Neuropils	
PB	PB
FB	CBU
EB	CBL
NO	NO

*Homologs have not been found at the date of publication

Where sensing and acting converge

The central complex has been studied since the end of the 19th century (Dietl, 1876, Flögel, 1878, Viallanes, 1887), and for good reason (also reviewed in paper 1). It represents a collection of the few neuropils that do not have one copy per hemisphere of the insect brain, as they are situated at its very center across the midline. More importantly, the central complex sits at the crossroads between sensory and pre-motor pathways, in a privileged position

for integrating sensory signals and directing behavior.

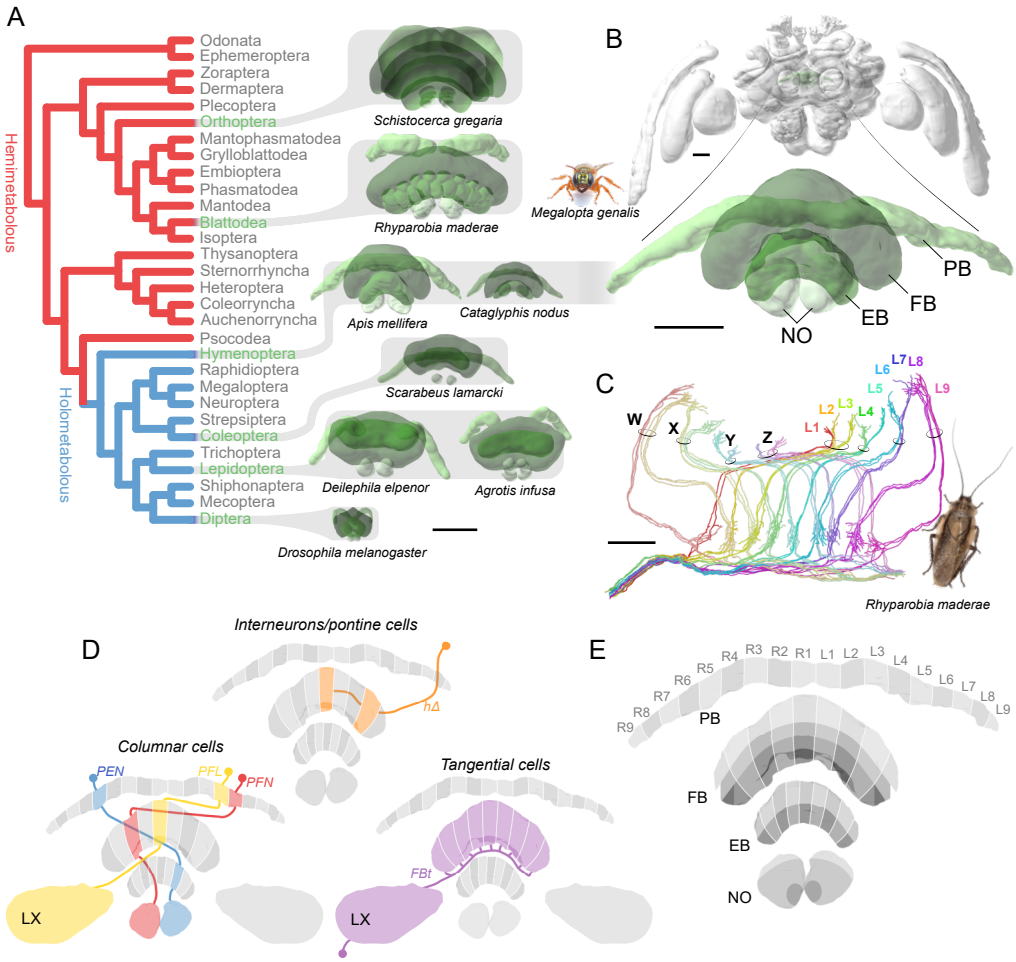


Figure 1: (**A**) Phylogenetic tree illustrating the conserved organization of the central complex across insect species. Modified from Paper 1 (Gillet et al., 2025). (**B**) Volume showing the brain of the sweat bee *Megalopta genalis* (extracted from the insectbrain database). The central complex can be found on the midline of the brain in the protocerebrum. It is composed of the protocerebral bridge (PB), fan-shaped body (FB), ellipsoid body (EB), and the noduli (NO). (**C**) Reconstruction of the head direction cells in the central complex of the Madeira cockroach. EPG colored by the column of the PB they innervate illustrate the columnar arrangement of cells of the central complex. Columnar neurons moreover project in four developmental bundles called W, X, Y, and Z which are visible in the adult and conserved across insect species. This data is also shown in Paper 2. (**D**) Neurons innervating the central complex can be divided into 3 classes: columnar, tangential, and interneurons/pontine neurons. (**E**) With the exception of the NO, neuropils of the central complex are divided into vertical units called columns. In the PB, they are labeled by the hemisphere side, R (right) or L (left), and their position from medial-most to lateral-most (from 1 to 9 in the sweat bee). A dorsal view of columnar neurons manually traced in the central complex of the bee shows the four developmental bundles termed WXYZ. Note that the W bundle is missing due to a cropping of the image data making it impossible to trace neuron projections.
Scale bars: 100 µm

Across insect species, it is composed of five neuropils (Heinze, 2024, Honkanen et al., 2019): the fan-shaped body (FB), the ellipsoid body (EB), the protocerebral bridge (PB), and the paired noduli (NO). The PB is an elongated neuropil shaped like a handlebar,

sometimes split into two parts connected by nerve fibers across the midline (like in the monarch butterfly). In an anterior-ventral position to the PB lies the FB, the largest and most salient neuropil of the central complex, itself enveloping the smaller EB. Finally, the NO are two spherical structures directly ventral to the EB. In the fruit fly, the central complex also comprises the asymmetrical bodies (Wolff et al., 2015, Wolff and Rubin, 2018), a pair of small neuropils situated between the FB and EB, with the right one consistently four times larger than the left one. They have so far not been found in any other insect species, and are still poorly understood except for the fact that they have been associated with long-term memory abilities (Lapraz et al., 2023, Pascual et al., 2004).

The central complex is generally associated with other neuropils of the central brain which provide it direct input. Most of the sensory input to the central complex comes from the visual system, originating from the optic lobes in each hemisphere of the brain (Honkanen et al., 2019, Hulse et al., 2021). Light captured by photoreceptors in the retina is processed and transmitted through the anterior visual pathway until it reaches the bulbs and the lateral accessory lobes (Homberg et al., 2003). In all insects studied to date, neurons of the bulbs project from segregated visual pathways to the EB, carrying information related to visual features, polarized light e-vectors, or circadian rhythm (Hulse et al., 2021).

The bulbs belong to the lateral complex, a group of neuropils also including the lateral accessory lobes (LAL) and the gall. Although the function of the gall is unclear, the LAL is known to provide multisensory information related to self-motion to both the EB and the NO, such as signals encoding the speed of optic flow (Hulse et al., 2023, Lu et al., 2022a, Lyu et al., 2022, May et al., 2025, Stone et al., 2017), wind flow (Currier et al., 2020, May et al., 2025, Okubo et al., 2020), and self-motion information through mechanosensation (Hulse et al., 2023). The LAL is most importantly a pre-motor region innervated by descending neurons, involved in producing motor commands to guide steering (Rayshubskiy et al., 2025, Steinbeck et al., 2020). As such, it is the main output target of the central complex, via neurons projecting from the PB and FB (Dan et al., 2024, Hulse et al., 2021, Mussells Pires et al., 2024, Namiki and Kanzaki, 2016, Westeinde et al., 2024). Moreover, the LAL provides the central complex with proprioceptive and efferent information in return (Hulse et al., 2021).

The FB of the central complex also integrates information originating in the mushroom bodies, directly or via neurons projecting mainly from the protocerebrum and crepine (Hulse et al., 2021, Kandimalla et al., 2023). The mushroom bodies are the associative memory centers of the insect brain (Buehlmann et al., 2020, Heisenberg, 2003, Kamhi et al., 2020). They likely provide the central complex with information related to context and valence, notably essential for goal-directed navigation. Their inputs to the central complex are likely neuromodulatory and could influence the activity of circuits based on prior experience and context. However, these pathways are not well understood and are mainly characterized in the fruit fly with connectivity data (Hulse et al., 2021).

This list only comprises some of the most important and most studied upstream partners of the central complex. Other sources of input were identified in the fruit fly thanks to the exhaustive hemibrain connectome (Hulse et al., 2021). Some notable mentions are the rubus, the round body, and the ovoid body, where central complex neurons make recurrent connections with each other. However, the rubus and round body were so far only described in the fruit fly (Hulse et al., 2021, Wolff and Rubin, 2018) and the cockroach (Althaus et al., 2022), while the ovoid body is a locust-specific neuropil (Hensgen et al., 2021b).

The neuroarchitecture of the central complex

What makes the central complex so remarkable is its highly defined neuroarchitecture, made of an intricate array of vertical columns and horizontal layers, often strikingly clear even at low resolution (see Strausfeld, 2012). As it turns out, this architecture is a great example of relationship between structure and function in the brain.

Columnar neurons make up the first of three major central complex neuron classes (Figure 1D). As their name would suggest, they innervate specific columns of the central complex across neuropils, thus giving it its characteristic look. Most subtypes project from a single column of the PB towards a column of the EB or FB, clustering into four bundles per hemisphere called W, X, Y, and Z in most insects (from lateral-most to medial-most, Figure 1C, Boyan and Williams, 1997; DPMm1/DM1, DPMpm1/DM2, DPMpm2/DM3, and CM4/DM4 in the fruit fly, Ito et al., 2013). These bundles are the adult remnants of highly conserved developmental processes that shape the central complex in a similar way across all insect species by guiding the projection patterns of columnar neurons (reviewed in paper 1). The major subtypes of columnar neurons additionally innervate a third neuropil: the NO, LAL, gall, crepine, or rubus (Hulse et al., 2021). One of the main output channels of the central complex is notably a FB columnar neuron projecting to the LAL called PFL, which has been shown in flies to control steering, and which I touch upon in Chapter 2 (Mussells Pires et al., 2024, Westeinde et al., 2024). Finally, specific types of columnar neurons termed Fx ignore the PB and only connect individual columns of the FB to various neuropils outside of the central complex (Hulse et al., 2021). Depending on the species, projection fields of columnar neurons in the PB divide it into 16 to 18 glomeruli distributed equally between the two hemispheres (Heinze and Homberg, 2007, Honkanen et al., 2019, Wolff et al., 2015). These glomeruli are commonly called by their hemisphere and position relative to the midline: R or L (for right and left), and numbered from the medial to lateral-most. As columnar neurons from both hemisphere of the PB converge on the FB and EB, they generally split these two neuropils into 8 or 9 columns (Hulse et al., 2021, Figure 29,32). Only in *Drosophila melanogaster*, the EB is shaped like a doughnut and divided into 8 wedges of equal size (Figure 1A). The open EB of most insects has 9 wedges (Hensgen et al., 2021a, el Jundi et al., 2018, Sayre et al., 2021), including two half-sized segments at

the extremities, which indicate that they could be equivalent to the fly's EB segments (see paper 4)

A second neuron class represents tangential neurons, cells projecting from other brain regions to neuropils of the central complex where they generally have wide-field projections across multiple columns (Figure 1D, von Hadeln et al., 2020, Honkanen et al., 2019, Hulse et al., 2021, Jahn et al., 2024, Kandimalla et al., 2023). With the exception of a few cell types projecting to both the FB and NO (von Hadeln et al., 2020, Hulse et al., 2021, Jahn et al., 2024, Kandimalla et al., 2023), tangential neurons generally innervate a single neuropil of the CX. They project from some of the more peripheral neuropils we mentioned previously, injecting the central complex with sensory information relative to vision, olfaction, mechanosensation, or ascending pathways from pre-motor regions. They may also carry information relative to internal state or long-term memory, via direct and indirect connections to the mushroom bodies. Some types of tangential neurons have been shown to influence computations of the central complex via neuromodulation, although this aspect of its dynamics is still somewhat understudied (Fisher et al., 2022). In the FB and EB, projections of tangential neurons delimit specific layers which appear to vary greatly across species (Heinze, 2024). Layers of the FB and EB are notably orthogonal to the central complex columns, thus making it a two dimensional array of compartments.

Finally, the third class of central complex neurons encompasses interneurons which project exclusively within the FB (Figure 1D, Homberg, 1985, Hulse et al., 2021). They follow the same four bundles per hemisphere as columnar neurons, as they arise from the same neuroblasts. In fact, interneurons of the FB are the first central complex cells to emerge during development. They lay the scaffold for subsequent cells to project to the right region, which makes them the architects of the central complex columns and its most ancestral neuron types. hΔ cells connect two columns of the FB generally separated by about half of its width. Their projection fields split the FB into 6 to 12 columns depending on subtype (Hulse et al., 2021, Figure 31), adding a degree of complexity to the columnar neuron's projection patterns. Arbors in the column closest to their soma are generally mostly dendritic, while arbors furthest away are generally axonal, thus defining information flow as going away from the soma. vΔ cells are other types of interneurons of the FB, this time connecting two horizontal layers. They have only been described in the fruit fly (Hulse et al., 2021) and the cockroach (Jahn et al., 2023) at the time of writing.

Summary

The neuroarchitecture of the central complex is a beautiful example of how design can serve function. Multimodal sensory inputs reach specific neuropils and layers of the central complex via tangential neurons that form separate streams of data. Columnar neurons connect

neuropils of the central complex via parallel corridors, thus mixing and integrating sources of information from all over the brain. Interneurons of the FB finally connect columns and layers of this neuronal matrix to perform operations at the heart of the functions of the central complex.

We will see in the next section that this remarkable organization is in fact naturally suited to compute navigational decisions. The two arms of the PB and NO together discriminate left from right, while the columnar organization of the central complex forms a topological map of angular space across the PB, FB, and EB.

Chapter 2

Layers of Computations in the Central Complex

The central complex integrates sensory inputs from all over the brain and is upstream of the LAL, a pre-motor center. Today, it is well established that it extracts directional cues from sensory input and uses them to plan directed navigation. The processing layers in between are more and more understood thanks to functional and connectomics data. The overwhelming majority of information comes from the fruit fly, *Drosophila melanogaster*, notably with the publication of a comprehensive connectome of the hemibrain comprising the entire central complex (Hulse et al., 2021).

In this section, I will describe some of the major computational layers of the central complex. I will mainly use data obtained from the fruit fly, and will draw comparison to other insects where possible and relevant. Note that the fly's EB is shaped like a doughnut (Hulse et al., 2021, Strausfeld, 2012), whereas all other species studied to date have an open EB similar to the FB (Honkanen et al., 2019). This particularity is an essential feature of the head direction circuit of the fly.

The head direction circuit

At its core, the central complex hosts a neuronal circuit capable of tracking the organism's gaze relative to the outside world. The head direction circuit, spanning across the EB and PB, relies on multimodal cues to produce a dynamic representation of heading, similar to a compass (reviewed in Fisher, 2022, Hulse and Jayaraman, 2020). Rather than pointing to a unique stable feature of the world, the insect head direction circuit is a flexible representation of angular space that may change in different contexts. It is therefore more accurate

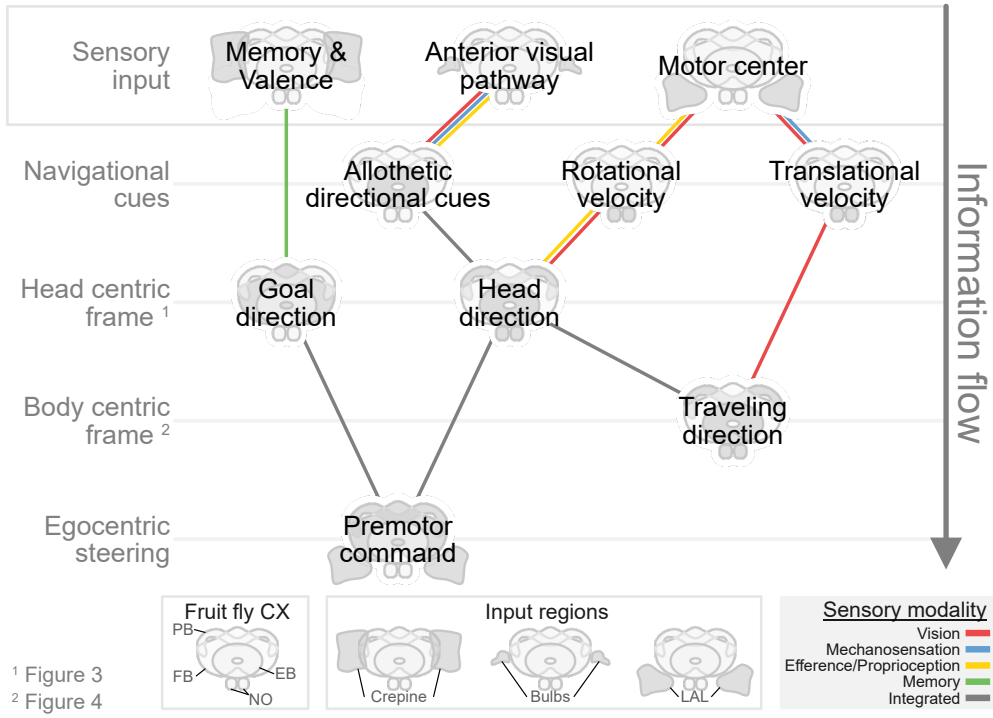


Figure 2: Schematic summary of information flow in the central complex. Multimodal sensory input reaches the central complex via connections to more peripheral brain regions. Neurons of the central complex filter these signals to extract relevant navigational information representing directional cues and self-motion velocity. They are integrated to produce an internal representation of orientation in both head-centric (head direction circuit) and body-centric frames (allocentric traveling velocity), allowing for the computation of egocentric steering commands to follow an internally represented goal direction.

to think of it as a system tracking angular rotation.

Its existence was first suggested by electrophysiological studies in the locust which found specific sets of cells that responded to polarized light e-vectors in a topological manner (Heinze and Homberg, 2007). These patterns of light are invisible to us but can be used by many animals to inform their sense of orientation. Since the locust studies, the head direction circuit was characterized in the fruit fly with calcium imaging (Kim et al., 2017, Seelig and Jayaraman, 2013, 2015), which showed that a population of neurons of the EB could track allocentric direction in their collective activity pattern. Functional studies in the monarch butterfly (Beetz and el Jundi, 2023, Beetz et al., 2022a) and anatomical evidence in other insects (El Jundi et al., 2018, Heinze et al., 2009, Hensgen et al., 2021a, Pisokas et al., 2020, Sayre et al., 2021) suggest that it is a core and conserved circuit of the insect's central complex.

Allothetic directional cues at the input

Input to the head direction circuit comes from a type of inhibitory tangential neurons called ring neurons (ER), projecting from the bulbs and the lateral accessory lobe in each hemispheres (Hulse et al., 2021). Most subtypes are sensitive to visual information via their inputs from the anterior visual input pathways terminating in the bulbs (Pegel et al., 2018,?, Seelig and Jayaraman, 2013, Sun et al., 2017, Vitzthum et al., 2002), but some respond to mechanosensation (Okubo et al., 2020) or are even involved in sleep regulation (Donlea et al., 2018, Raccuglia et al., 2025). Each ER neuron covers the entirety of the EB in parallel layers where they provide input to columnar neurons of the central complex (Figure 3A, Hulse et al., 2021). The subtypes involved in navigation encode allothetic cues providing a sense of direction, such as the orientation of polarized light e-vectors (Hardcastle et al., 2021, Pegel et al., 2018, Sun et al., 2017, Vitzthum et al., 2002), the position of salient features in the visual field (Seelig and Jayaraman, 2013), or the direction of wind flow (Okubo et al., 2020). Each neuron is sensitive to sensory cues within a receptive field that gives it spatial specificity. Together, ER neurons can thus encode the relative position of directional cues, forming a coarse spatial representation of the outside sensory world at the population level (Seelig and Jayaraman, 2013). Within the EB, they form a network with all-to-all connectivity, a mechanism of global inhibition thought to increase the signal of salient features in the environment while limiting noise levels.

In the EB, ER neurons provide input to columnar cells called EPG, which innervate the PB, EB, and contralateral gall (Figure 3A, Hulse et al., 2021). EPG projecting from a single column of the PB specifically innervate a sector of the EB that corresponds to about $1/16^{th}$ of its width (or circumference in the fly). Together, EPG from neighboring columns tile the entire EB by alternating between projections from the right and left PB hemisphere. ER neurons coming from both sides of the brain then provide every EPG with multimodal information encoding the position of allothetic navigational cues relative to the animal's field of view. As a result of this input, EPG are maximally active when the animal faces their preferred firing direction, matching the configuration of cues encoded by ER neurons (Figure 3B, Seelig and Jayaraman, 2015). These preferences emerge from plastic synaptic weights between ER and EPG neurons, which allow their activity to be flexibly tethered to directional cues in different sceneries (Fisher et al., 2019, Kim et al., 2019, Seelig and Jayaraman, 2015). Importantly, plasticity is induced by dopaminergic neurons which are active only when the animal rotates (Fisher et al., 2022), presumably allowing the circuit to only track cues sufficiently informative about angular rotations.

EPGs within a column respond to the same receptive field, mirroring the EB sector they innervate, a slice of angular space which directly neighbors that of neighboring populations. They consequently create a topographical map distributed across columns of the central complex, representing all possible azimuths around the animal with a resolution of about 22.5 degrees. Activity of EPG populations manifests as a bump of activity, centered on a

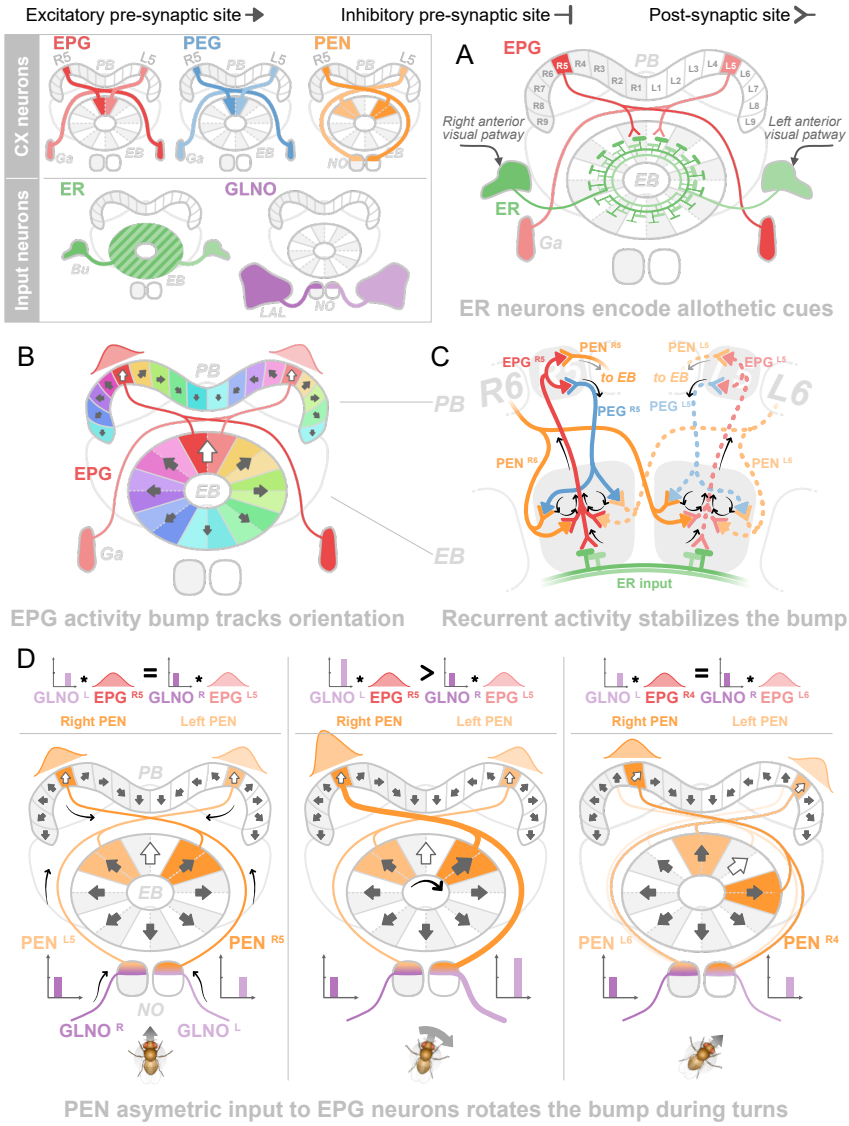


Figure 3: Step by step representation of the head direction circuit in the fruit fly central complex. (**A**) Ring neurons (ER) receive input from the anterior visual pathways in both hemispheres of the brain. As a population, they represent various directional cues extracted from multiple sensory modalities. They inhibit their downstream partners EPG neurons. (**B**) EPG neurons encode a bump of activity in the EB via the input from ER neurons. This activity pattern is duplicated in both hemispheres of the PB. EPGs in each columns of the central complex respond preferentially when the animal faces specific azimuths, thus representing a map of all angles around it with a 45 degrees resolution. (**C**) EPG, PEG, and PEN neurons form a recurrent circuit between the PB and EB, which likely stabilizes the head direction bump. (**D**) GLNO neurons provide input relative to angular velocity to PEN neurons. PEN project from the PB to the EB with a shift of one wedge. When the animal rotates, asymmetrical input from GLNO onto PEN therefore produces an imbalance in activity between the EPG populations directly neighboring the bump. This pushes the bump of activity to the next EB wedge, allowing it to dynamically track the animal's rotation.

wedge of the EB (Seelig and Jayaraman, 2015), and replicated in each hemisphere of the PB via their projection patterns. This bump dynamically encodes the animal's current head

direction, and is the basis for the compass representation that tracks orientation and angular rotation.

Producing a compass representation

The bump of activity carried by EPG neurons is formed by the collective action of four neuron types, together constituting a ring attractor: a network that can adopt multiple stable states and freely transition from one to the next (Turner-Evans et al., 2020; attractors also reviewed in Khona and Fiete, 2022). In the head direction circuit, the ring attractor is characterized by local excitation loops that produce and reinforce a persistent bump of activity tracking the animal's gaze direction, and long-range inhibition that suppresses activity in columns representing opposing directions.

EPG, PEG, and PEN neurons together form local excitatory networks within each column of the central complex. They all have projections to the PB and EB, thus forming a loop of recurrent connectivity between the two neuropils which is responsible for the formation of the bump. PEN neurons differ from EPG and PEG in two key aspects. First, they send neurites to a wedge of the EB shifted contralaterally compared to EPG and PEG from the same column of the PB, thus bridging neighboring columns. Second, they also project to the contralateral NO where they receive input relative to rotational self-motion, carried by inhibitory neurons called GLNO (also called LNOs in Paper 5 and Paper 4). These tangential neurons encode the animal's rotational velocity using both visual and motor signals (Hulse et al., 2023). That is, the activity of GLNO correlates with optic flow and motor information (likely efferent or proprioceptive signals) congruent with their preferred rotation direction, either clockwise or counter-clockwise. PEN cells from the right hemisphere consequently decrease in activity when the animal turns clockwise, and vice versa for PEN in the left hemisphere (Seelig and Jayaraman, 2015). As PEN neurons in each column contact the neighboring EB wedge on the contralateral side, their asymmetric activity during a turn causes an imbalance, thus shifting the bump to the corresponding column (Green et al., 2017, Seelig and Jayaraman, 2015, Turner-Evans et al., 2017). Proprioceptive input via GLNO allows PEN neurons to keep tracking angular rotations even in darkness (Green et al., 2017, Hulse et al., 2023, Turner-Evans et al., 2017), although the head direction bump, only guided by error-prone idiothetic cues, tends to drift in the absence of visual input to tether it to external cues.

The long-range inhibition component of the ring attractor is implemented by inhibitory neurons of the PB called $\Delta 7$. These neurons arborize across both hemispheres of the PB, with processes in all glomeruli with either input or output sites (Hulse et al., 2021). They were notably the very first neurons observed to exhibit a compass representation of polarized e-vectors orientation in the locust (Heinze and Homberg, 2007). We know today that this signal is inherited from EPG neurons in the PB, which provide input to $\Delta 7$ in all glomeruli

of the PB except at two output sites (or three for the lateral-most Δ7). In the fly, these two output sites are systematically separated by 7 glomeruli, thus innervating the columns corresponding to the same angular position in each hemisphere of the PB. Importantly, connections from EPG neurons vary in synaptic weights sinusoidally across the PB, such that they are strongest in columns opposite to Δ7 output sites and gradually decreasing in strength with proximity. These connectivity patterns cause the population activity of Δ7 to be shifted by 180 degree compared to EPG neurons. By inhibiting neurons more strongly with distance to the bump, they suppress activity in columns representing opposite directions. As Δ7 are pre-synaptic to each other and to most columnar neurons in the PB, they ensure the presence of a single head direction bump across the central complex. This sinusoidal distribution of synaptic weights between EPG and Δ7 creates a similarly shaped bump of activity at Δ7 output sites. This shape is likely essential to all downstream computations, as it can represent a vector whose direction is the phase of the sinusoid and its length the amplitude (Hulse et al., 2021).

In a striking example of convergent evolution, similar head direction dynamics were described in rats (Taube, 1995), bats (Finkelstein et al., 2015), and zebrafish (Petrucco et al., 2023), suggesting that the ring attractor is a common biological solution to tracking an organism's orientation. Our mechanistic understanding of the head direction circuit is however still limited to the fruit fly. We contribute to filling this knowledge gap by describing the head direction circuit of hymenopteran insects in Paper 4.

Closing the loop in other insects

In the fruit fly, the EB hosting some of the core connectivity of the head direction circuit is a toroid that loops onto itself, thus emulating a literal compass capable of representing an activity bump that can shift infinitely between wedges (Hulse et al., 2021, Seelig and Jayaraman, 2015, Turner-Evans et al., 2020). Despite their open EB, evidence suggests that this circuit is anatomically conserved across insect species (Hensgen et al., 2021a, el Jundi et al., 2018, Pisokas et al., 2020, Sayre et al., 2021, Stone et al., 2017), in addition to functional studies in the monarch butterfly indeed showing the presence of neurons tracking orientation (Beetz and el Jundi, 2023, Beetz et al., 2022a,b). For the head direction circuit to be functional, non fruit fly central complexes must therefore have implemented circuit solutions to close the loop between the two ends of the EB, using cell types that appear to be conserved.

In both the locust (Pisokas et al., 2020) and the bumblebee (Sayre et al., 2021), components of the head direction circuit show equivalent projection patterns, with the exception of some differences in EPG cells tiling the two midline glomeruli of the PB (called CL1a in non fruit fly insects). In the locust, EPG innervating the two lateral-most sectors of the open EB each innervate both midline columns of the PB: R1 and L1. In the bumblebee,

EPGs from these two PB glomeruli instead swap their projection fields in the EB, thus tiling the ipsilateral sector at the end of the EB, instead of the contralateral one. R1 and L1 notably correspond to the same angle of space but at the opposite extremes of the loop: -180 and +180 degrees. These anatomical variations could provide a channel between the two PB hemispheres, allowing the head direction activity bump to travel across the midline and thus jump from one end of the EB to the other.

Vector computations in the central complex

The head direction representation encoded by EPG and $\Delta 7$ is broadcasted to the rest of the central complex via the PB. Columnar neurons that project from the PB to other regions inherit a preferred orientation from EPG and $\Delta 7$ neurons that correspond to the glomerulus they innervate (Hulse et al., 2021). All columnar cells from one PB glomerulus therefore share the same preference, and at the population level, encode an activity bump reflecting that of EPG neurons or its sinusoidal equivalent, the head direction signal.

Most subtypes of columnar neurons also project to the FB (Hulse et al., 2021, Sayre et al., 2021). Most notably, PFG and PFR neurons projecting from equivalent glomerulus in each hemisphere of the PB meet in the same column of the FB (Figure 4B). We say that they project to the FB with a 0 degree phase shift, or with no phase shift. That is, the bump conserves its topology and reflects the same angular space when transported to the FB.

On the contrary, other columnar neurons project with an anatomical phase shift from the PB, relative to a projection pattern where equivalent columns meet in the same region of the FB (Hulse et al., 2021, Sayre et al., 2021). For example, PFN neurons project with a contralateral shift of one column from the PB to the FB. PFN neurons meeting in the same column of the FB therefore have angular tuning preferences separated by about 90 degrees, obtained by adding up angular shifts of -45 and +45 degree. Various columnar types come with various phase shifts, all of which are likely essential to downstream computations because they allow the comparison of signals coming from different directions. In addition, hΔ cells are FB interneurons that project between two of its columns and can add a layer of computations downstream of columnar neurons by shifting signals across half of the FB corresponding to a 180 degree angular shift.

As a reminder, the sinusoidal signal shape of head direction activity can also be a mathematical representation of a vector (Figure 4). As we will see in the next section, the phase of the sinusoid can encode a direction, while its amplitude can represent velocity or distance. Via morphology, anatomical phase shifts, excitation and inhibition, neurons of the central complex can work together to perform complex vector computations essential for insect navigation (also see Webb, 2025)

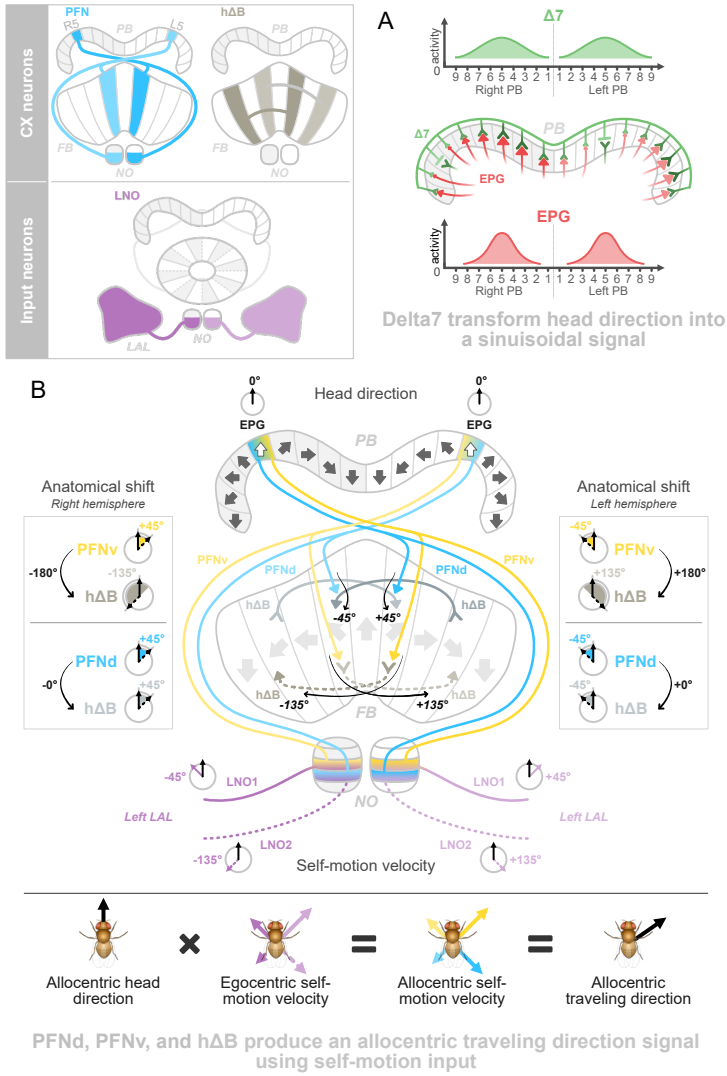


Figure 4: Vector computations within circuits of the central complex. **(A)** $\Delta 7$ neurons project to all glomeruli of the central complex where they inhibit all other $\Delta 7$ and receive input from EPG neurons. Their two output sites are separated by 7 glomeruli in the fruit fly, and they receive input more strongly away from their output sites. Via their all-to-all global inhibition, they transform the EPG activity bumps into a sinuisoidal signal suited for vector computations. **(B)** PFNd and PFNv neurons receive the head direction signal from EPG neurons in the PB, and translational optic flow velocity information from LNO cells in the NO. They project to the FB with a contralateral shift of one column, corresponding to a 45 degree shift in angular space. In the FB, they synapse onto hΔB neurons, interneurons carrying information from one column of the FB to another one shifted by 180 degrees. PFNd synapse close to the local output sites of ΔB thus shift the head direction bump by +- 45 degrees. PFNv synapse close to the input sites of hΔB, thus transmitting the bump to columns shifted by +- 135 degrees from the original PB glomerulus. This modulates the head direction bump depending on translational velocity, thus creating an allocentric representation of traveling direction.

From head direction to traveling velocity

The head direction circuit computes a compass representation mainly using directional cues acquired by the eyes or the antennae, making it a head-centric system. However, insects can

fly or jump in any direction regardless of where they are facing, or can simply be displaced by external forces such as the wind. Such motion is called holonomic, when the direction of movement does not match the direction faced by the animal. This mismatch between head direction and traveling direction is resolved by integrating self-motion velocity (Lu et al., 2022a, Lyu et al., 2022).

Self-motion input

On the ventral side of the central complex, the NO are the only paired neuropils of the central complex. They act as a hub receiving information related to self-motion velocity, or how fast the animal is moving through space (Currier et al., 2020, Hulse et al., 2021, Lu et al., 2022a, Lyu et al., 2022, Stone et al., 2017). This input comes through multiple types of LNO cells, inhibitory tangential neurons projecting from the LAL.

We already mentioned GLNO neurons in the context of the head direction circuit. These neurons encode rotational velocity, as they are tuned to optic flow and mechanosensory signals congruent with rotations. They innervate a lateral region of the LAL and the most dorsal compartment of the NO in the fly (Hulse et al., 2021, Kandimalla et al., 2023), where they provide input to PEN neurons that is key to their function rotating the head direction bump (Hulse et al., 2023, Seelig and Jayaraman, 2015, Turner-Evans et al., 2017, 2020).

Other LNO cells also project from the LAL to diverse compartments of the NO that are not innervated by GLNO. They encode translational velocity via different sensory modalities, and provide input to PFN neurons. LNO1 and LNO2 respectively respond to front-to-back and back-to-front optic flow (Lu et al., 2022a, Lyu et al., 2022, Stone et al., 2017), congruent with forward and backward motion through the environment. The remaining types encode mechanosensory information. LNOa encodes the direction of wind flow but not velocity (Currier et al., 2020, May et al., 2025). LCNOp and LCNOpm encode wind flow direction and speed (May et al., 2025). Finally, LNO3 is a peculiar type that innervates both NO with most of its neurites in the contralateral NO (Hulse et al., 2021). Its function is unclear but it may encode proprioceptive information via efferent copies. Curiously, it was only found in the hemibrain connectome (Hulse et al., 2021), but not the full brain connectomes of the female (Dorkenwald et al., 2024) or male (Berg et al., 2025) flies.

An essential feature of the visual LNO neurons is that they respond to stimuli with a preferred tuning offset by about 45 degrees from either side of the animal's body axis (Currier et al., 2020, Ishida et al., 2025, Lu et al., 2022a, Lyu et al., 2022, May et al., 2025, Stone et al., 2017). Considering the front of the animal facing 0 degree, LNO2 neurons thus respond maximally to optic flow expanding from a +-135 degree angle, and LNO1 respond to points of expansion offset by +-45 degrees. These properties mean that visual LNO together encode four orthogonal vectors together capable of encoding all possible directions of translational motion. This was in fact first described functionally in the sweat bee, where

LNO1 and LNO2 are respectively (and confusingly so) called TN2 and TN1 (Stone et al., 2017). A representation of direction made with four orthogonal vectors notably disambiguates the two hemispheres, thus allowing to faithfully track motion even during holonomic movements when the animal does not face its traveling direction.

Computing allocentric traveling direction

All non-GLNO LNO cells target PFN neurons in the NO (Currier et al., 2020, Hulse et al., 2021, Lu et al., 2022a, Lyu et al., 2022). These columnar neurons innervate the PB and FB, and project to the contralateral NO, avoiding the most dorsal compartment occupied by GLNO and PEN neurons. Multiple subtypes exist which are distinguished by their projections to distinct compartment of the NO, where specific LNO neurons provide them with sensory input related to self-motion. In the PB, PFN neurons inherit the head direction signal from EPG and/or $\Delta 7$ neurons (Lu et al., 2022a, Lyu et al., 2022). Different subtypes of PFN neurons are therefore perfectly suited to integrate multimodal translational velocity and head direction signals in parallel information channels. They inherit directional tuning from sources of input, which makes their activity maximal when the animal experiences translational motion along their preferred direction in allocentric head direction space.

Two of these subtypes were in fact shown to contribute to encoding an allocentric traveling velocity in the FB, by encoding a vector in their combined population activity pointing in the direction of movement regardless of head direction (Lu et al., 2022a, Lyu et al., 2022). These subtypes, called PFNd and PFNv, respectively encode forward and backward motion with a +- 45/135 degree offset from their preferred head direction (Lu et al., 2022a, Lyu et al., 2022), a tuning that is anti-correlated to that of their inhibitory upstream partners: LNO2 and LNO1. As we mentioned in Chapter 2, they project to the FB with a columnar phase shift of 45 degree towards the contralateral hemisphere. In the FB, they contact interneurons called hΔB, which themselves project in columns separated by half the width of the neuropil, thus performing an anatomical phase shift of 180 degree. Crucially, PFNd and PFNv synapse onto hΔB neurons at different locations along their arbors. PFNv contact them in the opposite hemisphere, on arbors closest to their soma where neurites are predominantly dendritic and thus accepting input. In principle, information within hΔB therefore flows to the other side of the FB. The 45 degree anatomical shift performed by PFNv combines with the -180 degrees shift by hΔB, resulting in a transformation of -135 degree or +135 degree. In contrast, PFNd neurons contact hΔB away from their soma, on axonal neurites providing output to other cells. PFNd thus promote activity in the same column where they provide input to hΔB, resulting in a transformation of 45 degrees corresponding to their own angular phase shift alone.

Given the tuning preferences of their LNO input partners in the NO, corresponding to

four orthogonal directions of translational motion, PFNd and PFNv thus project egocentric translational velocity vectors into the allocentric head direction reference frame. At their output sites, hΔB produce a sinusoidal population activity encoding a bump across columns of the FB. This bump encodes the allocentric traveling velocity of the animal, regardless of head direction, and proportionally to speed. Further studies determined that PFNd neurons also respond to wind flow direction (May et al., 2025), suggesting that multiple sources of information are integrated to produce a more accurate estimate of traveling direction.

PFN circuit have to this date only been characterized in the fruit fly, which has the great advantage of coming with multiple full connectomes and genetic tools enabling functional imaging. We however describe homologous circuits in the sweat bee for the first time, in Paper 5.

Expanding the central complex toolkit with mechanosensation

LNO1 and LNO2 only represent two of the six types of LNO innervating the central complex, and their downstream partners, PFNd and PFNv, are only two of the ten subtypes of PFN neurons. As we mentioned previously, the remaining types of LNO (excluding the cryptic LNO3) all appear to encode properties of wind flow. LNOa are maximally excited by wind flow arriving at a 90 degree angle relative to the animal's midline, encoding the direction of wind but not its speed (Currier et al., 2020). LCNOp and LCNOpm are instead transiently inhibited by airflow, suggesting they encode wind direction and acceleration (May et al., 2025). These observations notably indicate that the downstream populations of PFN neurons, comprising most PFN neurons split among ten subtypes, likely also respond to wind flow.

PFNa were in fact shown to encode wind flow direction coming from directions offset by 90 degrees from the front of the animal, reflecting the directional tuning of their upstream partners LNOa (Currier et al., 2020, Ishida et al., 2025, May et al., 2025). Remarkably, Ishida et al. (2025) showed that, unlike the PFNd-PFNv network which encodes optic flow direction using four orthogonal vectors, PFNa could encode wind direction with only two invertible vectors. When receiving weak input from LNOa, PFNa depolarize in phase with EPG input in the PB, thus producing conventional sodium spikes. When instead inhibited by strong LNOa activity signalling wind from the opposite direction, oscillatory hyperpolarization of PFNa elicits calcium spikes that are anti-phase with EPG signal, effectively performing a vector inversion. This provides an elegant solution for encoding directions with orthogonal vectors, using two populations of neurons instead of four. Coincidentally, Currier et al. (2020) reported oscillatory fluctuations in membrane potential in multiple PFN and PEN neurons, including PFNa. Although vector inversion was not considered at the time, the authors mention that cells exhibiting oscillatory frequencies also presented

larger membrane potential distributions. This on its own is inconclusive, but it suggests that vector inversion could be used by other cell types, thus expanding the toolkit of the central complex to perform vector operations.

More potential functions for PFN neurons

Further work examining the encoding of wind flow among PFN neurons demonstrated that PFNa, PFNd, and PFNp_c could together compute the direction of wind flow, regardless of that elicited by self-motion, thus representing an external variable (May et al., 2025). This may in fact be another mechanism by which the central complex and PFN neurons could contribute to spatial navigation. Although this study, as many functional studies, was only performed in the fly, one could imagine the ability for the central complex to encode the movement of external objects to be extremely useful for many insect species. This could for example enable the amazing ability that dragonflies have to track and predict the movements of preys (Mischiati et al., 2015), or the high speed chases performed by courting house flies (Land and Collett, 1974).

On a similar matter, Hadjitofi and Webb (2024) proposes that the central complex could be suited to decode the waggle dance. This incredible behavior unique to honeybees allows an individual to signal the direction and distance of a location of interest to their nestmates, by encoding a vector in the movement of their abdomen. Bees watching the dance are able to track it with their antennae, which were shown to be enough to decode it. As LNO neurons that encode wind flow likely do so by inheriting a signal that encode passive antennae displacement via the LAL (Currier et al., 2020, Suver et al., 2023), Hadjitofi and Webb (2024) propose that they could enable the central complex to decode the waggle dance. This would notably imply that specialized PFN neurons in the honeybee could encode the waggle dance vector in their population activity.

Other models of the central complex have in fact directly proposed novel functions for PFN neurons. Stone et al. (2017) suggested that the central complex could host the neural substrate of path integration. This strategy, often employed by central-place foragers such as bees and ants, consists in integrating distance and direction traveled to compute a home vector, the straightest path back to a starting point. The model proposed that PFN neurons could integrate activity proportional to self-motion velocity inherited from LNO neurons, thus computing an estimate of distance traveled over time, within each column of the central complex. They would therefore encode in their population activity a food vector, the inverse of a home vector which could itself be retrieved by rotating it by 180 degrees with hΔ neurons. This model was later complemented by Moël et al. (2019) who proposed that the working memory of the home vector could be immortalized in the synaptic weights of a putative FB neuron once a food location was reached. Although the neural substrate for path integration has not yet been confirmed to exist in the central complex, what we

currently know of its circuitry makes it a compelling candidate.

Steering towards a goal

Head direction and traveling direction both reflect the current orientation of the animal with respect to the world. This is however not sufficient for navigation alone, as an animal must compare its current heading to a desired heading towards a goal. In case of a mismatch between these two vectors, the animal must perform a corrective maneuver to align its current heading with the goal direction. This goal direction can be, and is in fact represented within the central complex. As a population, the goal neurons encode the direction of a goal relative to the current compass state, irrespective of changes in head or traveling direction, as shown in functional studies in the Monarch butterfly (Beetz et al., 2022b) and the fruit fly (Mussells Pires et al., 2024). In the fruit fly, the neural substrate for goal direction was identified as columnar neurons called FC2 (Mussells Pires et al., 2024). These neurons may not be the only ones doing so, as multiple goals may likely co-exist in the central complex to potentially allow for comparison between routes or based on different sensory modalities. Another study looking at odor-gated directed navigation notably suggests that hΔC neurons could fulfill this function (Matheson et al., 2022), in a circuit notably involving PFNa neurons for orientation up an odor plume.

Both of these neuron types converge onto PFL3 neurons in the FB of the fly (Hulse et al., 2021). PFL are columnar neuron types that innervate the PB and FB, and are generally assumed to promote steering via projections to the LAL (Dan et al., 2024, Mussells Pires et al., 2024, Westeinde et al., 2024). As other columnar neurons innervating the PB, PFL neurons inherit angular tuning from the head direction circuit that corresponds to the column they innervate. PFL3 project to the FB with a columnar offset of two columns compared to the PB, resulting in a phase shift of 90 degrees. PFL2 project to the FB with a columnar offset of four columns corresponding to a phase shift of 180 degrees. These neurons also differ in that PFL3 innervates the contralateral LAL, while PFL2 have bilateral projections to both LAL. According to functional recordings (Mussells Pires et al., 2024, Westeinde et al., 2024), and an anatomically constrained model (Dan et al., 2024), this phase shift likely allows PFL3 neurons to promote steering towards the goal when the animal faces away from it. In each hemisphere, their 90 degree columnar phase shift allows them to compare the mismatch between a goal direction encoded in the FB and two anti-correlated heading directions separated by a total of 180 degrees. When the animal directly faces the goal, the symmetric activity of PFL3 neurons from each hemisphere would keep the animal going forward. On the other hand, if goal and heading were to drift away from each other, the activity of PFL3 neurons tuned to head direction would sum with a misaligned goal, resulting in a disrepancy between the left and right population. As the resulting bias would travel to LAL, it would cause more activity in one hemisphere thus causing a corrective

turn aligning heading and goal direction.

PFL2 fulfill a complementary role to PFL3, likely by influencing the probability of turning in ambiguous situations (Dan et al., 2024, Westeinde et al., 2024). For example, an animal flying perfectly away from its goal would see the activity of PFL3 neurons perfectly equal, reflecting a situation where the goal and heading are aligned. With their 180 degree phase shift, PFL2 activity added to the goal signal would reach its highest activity when it is misaligned with the head direction bump, while being minimally active when they match. PFL2 activity therefore appears to correlate with the probability of turning, which would bring the insect back into a position where PFL3 can guide corrective turns.

PFL neurons in other insect species are yet to be characterized. However, pioneering studies in the cockroach showed that neurons of the FB could predict steering decisions with their activity or even cause a turn when they were artificially activated by injecting current intracellularly (Bender et al., 2010, Guo and Ritzmann, 2013, Martin et al., 2015). Although these neurons were not identified, they could indeed be PFL neurons, and at least suggest that their role is likely conserved across species.

Summary

Today, most of what is known about the intricacies of the central complex circuitry comes from functional and connectomics data extracted from the fruit fly, *Drosophila melanogaster*. It is also largely dominated by studies on visually-driven behavior, although mechanosensation is making its way more and more in central complex literature, for the better. Many insects are in fact not visually-driven, either because they are near-blind like the army ant, or are active at different times of day like the crepuscular sweat bee *Megalopta genalis*. Despite the diversity of insects, anatomical and functional data across species have already shown that neuron types (von Hadeln et al., 2020, Hensgen et al., 2021a, Jahn et al., 2023, el Jundi et al., 2018, Pisokas et al., 2020, Sayre et al., 2021) and functions (Beetz and el Jundi, 2023, Beetz et al., 2023, Heinze and Homberg, 2007, Stone et al., 2017) of the central complex are likely conserved, but what of the underlying circuitry? How much can it vary yet deliver similar functions? On the contrary, how much must it vary to produce the large range of behaviors that insects exhibit?

We attempt to answer these questions with comparative connectomics, by describing the wiring of the central complex across multiple insect species. In Paper 4, we compare hymenopteran insects to show that largely conserved head direction cells may vary in connectivity, in order to produce an accurate compass representation despite an open ellipsoid body. In Paper 5, we dive deep into the central complex of *Megalopta genalis* to instead demonstrate how flexible development may shape new pathways, and add new functionalities to the toolkit of the central complex.

Chapter 3

From Image Acquisition to Automatic Neuron Segmentation

Apart from its obvious complexity, our understanding of the central complex is highly constrained by the practical challenges that come with trying to study it. Different approaches have prevailed over the years (as reviewed in Paper 1), from anatomy to lesion study, or from intracellular recordings to genetic manipulation, all with their own advantages and limitations. The methods of predilection for this thesis work are projectomics and connectomics. The first consists in mapping the projections of neurons across brain regions to assign cell types, inventorize them, and describe information flow by building a projectome (Kasthuri and Lichtman, 2007). The goal of the second is a connectome, whereby we painstakingly reconstruct entire circuits, cell by cell and synapse by synapse. Although the cost is great, the reward is greater, because it gives access to the wiring diagram of the brain.

Volume electron microscopy

Studying individual neurons and synapses necessitates that we reach the nanometer scale, which is physically impossible using light microscopy. That is, with the exception of expansion microscopy which, rather than shrinking pixel size, stretches a sample to make it observable under visible light (Chen et al., 2015).

The current method of choice in the field of connectomics is volume electron microscopy (vEM, Peddie et al., 2022). Electron beams are generally used in biology to observe objects down to a size of 4 nm which is perfect to view neurites sometimes thinner than 100 nm of diameter. vEM allows to do just this, in three dimensions, either by cutting slices of tissue that will then be imaged or by iteratively imaging a block of tissue that is gradually shaven to

oblivion to reveal its insides. The first method consists of cutting a sample into thousands of thin slices using an ultramicrotome. With automated tape-collecting ultramicrotome (ATUM, Baena et al., 2019) the sample is then imaged with a scanning electron microscope, while the grid-tape method instead uses a transmission electron microscope (Yin et al., 2020). The state-of-the-art uses a magnetic field to passively collect slices from an ultramicrotome onto a wafer, before imaging them using 91 simultaneous electron beams (Fulton et al., 2024).

The other, more dramatic way of imaging a sample is embodied by scanning block-face electron microscopy (SBEM, Denk and Horstmann, 2004) and focused ion beam electron microscopy (FIB-SEM, Knott et al., 2008, Xu et al., 2017). With these methods, the surface (or block-face) of a sample is scanned with an electron beam in a raster pattern, before a thin slice is automatically cut from the top thus reaching a new depth that can be scanned in turn. The process is repeated hundreds or thousands of times, until the whole sample is acquired, destroying it in the process. FIB-SEM uses an ion beam to disintegrate the top of the block between scanning bouts, which allows the acquisition of images separated by less than 10 nm of empty space (Xu et al., 2017). Apart from the fact that finer resolution is better, this has the advantage of producing datasets with close to isotropic voxel size, where resolution is the same along all dimensions.

To produce the data for this thesis, we used an SBEM. This incredible microscope was born from an idea by Winfried Denk who simply decided to put an ultramicrotome inside the vacuum chamber of a scanning electron microscope (Denk and Horstmann, 2004). The result is a microscope equipped with a diamond knife that automatically cut slices off the top of a block of tissue between scanning bouts, to acquire images every 50 nm. Although the resolution for SBEM is much coarser than that of FIB-SEM, it is often more than enough to reconstruct the brains of the large insects that we are interested in.

To image them, our samples underwent a long and tedious process to be stained, embedded in resin, and shaped into rectangular blocks before they could be imaged for weeks at a time using SBEM. Obtaining the data necessary to map the circuitry of the central complex is therefore not trivial, and image acquisition, processing, and data extraction are costly and time-consuming tasks. Throughout this chapter, I describe some of the principles that make everything possible from histology to image acquisition and from images to circuitry. Our method itself is described at length in Paper 2.

Sample preparation

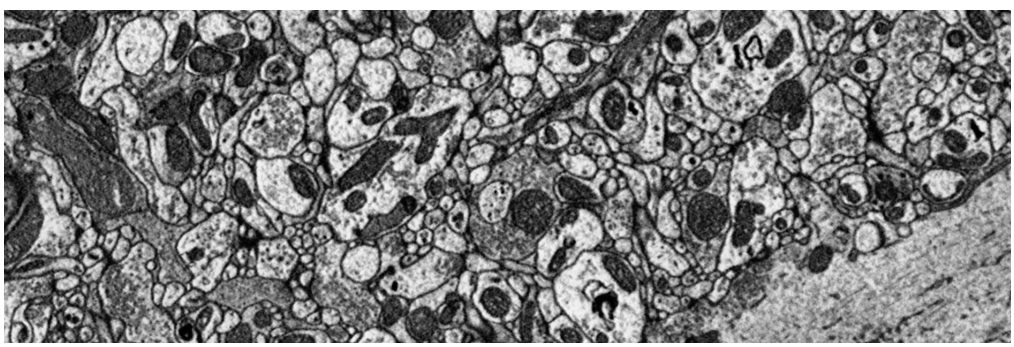
The quality of the image data, on which all downstream steps rely, is the result of two major steps going well: histology and image acquisition itself. The aim of the histological protocol for vEM is to preserve the ultrastructure of the brain and stain it so that neuron membranes and synapses are visible with electron microscopy (Hua et al., 2015). The quality of fixation

and staining define how much contrast is contained in the images and how well the sample sustains the energy of the electron beam or the action of the knife.

Our protocol for preparing insect brains is largely based on Hua et al. (2015), and follows classic steps detailed in Paper 2 (also see Lu et al., 2023). The preparation relies mainly on four substances staining the tissues: osmium tetroxide, potassium ferrocyanide, sodium thiocarbohydrazide, and uranyl acetate (Hua et al., 2015). Osmium tetroxide is the main staining agent of this cocktail. It binds to lipids, therefore labeling membrane lipid bilayers and providing contrast for electron microscopy. This reaction is complemented by potassium ferrocyanide which enhances contrast by reducing cytoplasmic staining, and sodium thiocarbohydrazide which promotes the binding of osmium to membranes. Finally, uranyl acetate is a general contrasting agent that overall contributes to tissue contrast. The full staining protocol carefully alternates between the above-mentioned compounds to balance diffusion into the block of tissue and reactivity with the targeted features. In its final steps, the tissue is dehydrated with ethanol baths before being embedded in resin. Dehydration is essential for the penetration of the hydrophobic resin into the sample, which solidifies it and ensures smooth slicing and scanning during image acquisition.

Once the staining protocol is completed, the sample is a pitch black, stained central brain embedded in a block of plastic that needs to be trimmed into a roughly trapezoidal shape, small enough to fit in a typical SBEM (a roughly 1 x 1 x 1 mm cube). To guide trimming - and later, scanning - we use micro-computed tomography (microCT).

MicroCT scanners image samples by subjecting them to X-rays. As they pass through matter, X-rays are absorbed by dense objects before they reach a detector on the other side. The denser the material is, the more attenuated the X-rays will become. The resulting image generally show a shadow of the sample, with regions of high density (that absorbed X-rays) showing up darker. By repeating this process while rotating the sample, microCT produces thousands of so-called "projections" that are then assembled to computationally reconstruct a 3-dimensional volume. As the neuropils of the prepared brain are densely packed with small neural processes, they tend to stand out in images produced via microCT. Fortunately, this is especially the case for the mushroom bodies which are remarkable landmarks, and



the central complex which is what interests us. MicroCT is now essential to our protocol as it lets us check samples for defect before they go in the SBEM, and helps us orient them to ensure smooth scanning of the central complex.

Image acquisition using block-face electron microscopy

Principles of electron microscopy

Similarly to how photography or light microscopy rely on light photons to acquire images, an electron microscope uses electrons as its energy source. While using light relies on the absorption of photons by the sample to produce an image, electrons do so by being scattered by atoms based on their atomic number. The higher its atomic number, the more an atom scatters electrons and produces contrast in the resulting image. The advantage of electrons is that they can achieve much shorter wavelengths than visible light, which allows considerably higher resolution.

An electron microscope first produces a concentrated beam of electrons focused on the surface of a sample (Suga and Hirabayashi, 2025). As the incident electrons penetrate the sample, they interact with its atoms and release various signals that can be imaged with the right detector. To scan a full image, the beam slides across the sample in a raster fashion, stopping mere microseconds per pixel, thus gradually building an image. The sample is placed in vacuum to allow electrons to travel uninterrupted by gaseous particles in the imaging chamber's atmosphere, which could also produce noise in the resultant images.

For SBEM (Denk and Horstmann, 2004), we detect high energy electrons whose trajectories are altered by atomic nuclei within the sample causing them to slingshot back towards the surface of the sample, the aptly named "back-scattered" electrons (Suga and Hirabayashi, 2025). Heavier elements divert electrons more strongly and ensure that they travel back towards the surface of the sample rather than scattering deeper, and thus create better contrast. This is the main reason why biological samples are stained with heavy metals.

Detecting back-scattered electrons produces greyscale images where high brightness values correspond to a strong signal coming from the sample. As the lipids contained in the cell membranes, synaptic vesicles, or mitochondria are bound to heavy elements, they appear bright while the cytoplasm appears dark. Images produced by scanning EM are however inverted in order to appear more similar to transmission EM results, which historically came first and where scattered electrons produce a shadow detected on the other side of thin samples. This means that neuron membranes and synapses conventionally appear dark in vEM data, while cytoplasm and empty space is light.

Apart from sample preparation, vacuum state, and working distance, image quality is

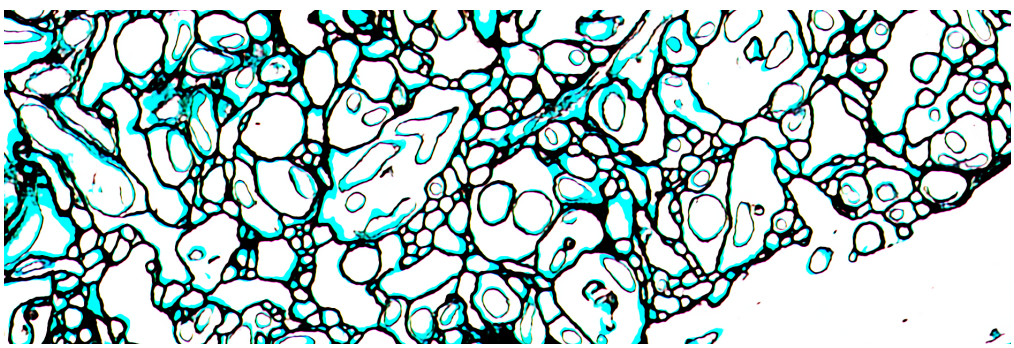
mainly determined by 3 parameters during the scan:

- **Voltage** (in kilovolt, kV) is the energy carried by the electrons. Among other things, the voltage level determines the penetration depth of electrons into the sample. Higher voltage electrons dive deeper into the tissue, while low voltage electrons scatter closer to the surface. Slice thickness must be taken into account when choosing voltage values.
- **Beam current** (in nanoAmpere, nA) represents the density of electrons per unit time. A higher beam current typically produces a higher signal-to-noise ratio.
- **Dwell time** (in microseconds, µs) is the duration that the beam stays on a pixel to produce a signal value. A longer dwell time also produces a higher signal-to-noise ratio, as signal is summed over time for each pixel.

Theoretically, increasing any of these parameters will produce better contrast in the image, as signal-to-noise ratio correlates positively with their values. In practice however, the conductivity of a sample dictates how much electron dose it can sustain. Because the electron dose is a measure of density of electrons per unit area, it increases with resolution, but also with beam current and dwell time. Highly conductive samples, or regions of a sample, can more easily dissipate electrons, while insulated regions tend to accumulate them. We say of a region that accumulates electron that it is charging.

Biological samples are typically poorly conductive until they are stained with heavy metals. They are prone to charging when conductive stained regions become over-saturated with electrons. The accumulation of electron dose causes images to be darker and oftentimes produces aberrations that make cellular structures impossible to resolve. High electron doses can also heat the sample, occasionally causing the resin to shrink or expand, resulting in loss of focus and inconsistent slicing.

Different factors can help mitigate charging and may allow for more aggressive imaging parameters to be used to increase contrast and resolution. We typically image samples under low vacuum conditions (10 Pa) by introducing water vapor inside the imaging chamber,



which helps dissipating charge from the sample. The same can be done by increasing the surface of contact between the tissue and its aluminum holder, or the use of conductive silver resin, both of which provide a path of least resistance for electrons to exit the sample. FIB-SEM inherently helps neutralizing surface charges thanks to its positively charged gallium ion beam used for milling (Suga and Hirabayashi, 2025). The focal charge compensator produces similar results by blowing nitrogen gas on the surface of the sample (Deerinck et al., 2018, Suga and Hirabayashi, 2025), and can be added to an SBEM.

In summary, the main challenge of optimizing image quality is to balance the trade-off between different combinations of scanning parameters, to optimize signal-to-noise ratio and contrast while minimizing imaging time (Lu et al., 2022b). A fast scan with short dwell time requires high energy to obtain good contrast, but risks charging. The alternative is a longer dwell time with lower energy, increasing the time needed to image the same region of interest.

Imaging strategy and considerations

For all samples acquired for this thesis work, we used a VolumeScope microscope (ThermoFisher Scientific) hosted at the Centre of Microscope and Microanalysis of the University of Queensland (Brisbane, Australia), and used the ThermoFisher MAPs software to guide image acquisition.

As the field of view of the microscope is limited, imaging large regions of interest requires multiple images, which is where the MAPs software comes in. It allows a user to place tilesets, collections of overlapping images organized on a grid, on specific regions of the sample. Multiple tilesets are organized in a job queue that the microscope cycles through after every cut, each potentially with their own size and imaging parameters such as resolution. We typically use the same voltage (2 kV) for all sample, and adapt beam current (0.1-0.2 nA) and dwell time (1-3 us) based on the charging rate of each sample.

For medium to large insects such as the ones we imaged, it would take many months to image the entire central complex at the synaptic resolution (8-12 nm pixel scale) required to extract a connectome. To reduce imaging time, we therefore use a multi-resolution approach whereby we image the entire central complex at cellular resolution while simultaneously capturing specific regions at synaptic resolution. Synaptic resolution tiles are placed such that they cover one hemisphere of the protocerebral bridge, and the contralateral hemisphere of the fan-shaped body and ellipsoid body, and the contralateral nodulus. This strategy ensures that we capture entire bundles of columnar neurons at high resolution, enabling us to build local connectome that map a few of the repeating computational units of the central complex. In particular, we attempt to capture key regions where we expect inter-species differences to occur, such as the middle columns of the protocerebral bridge or one of the medial-most columns of the central complex neuropils.

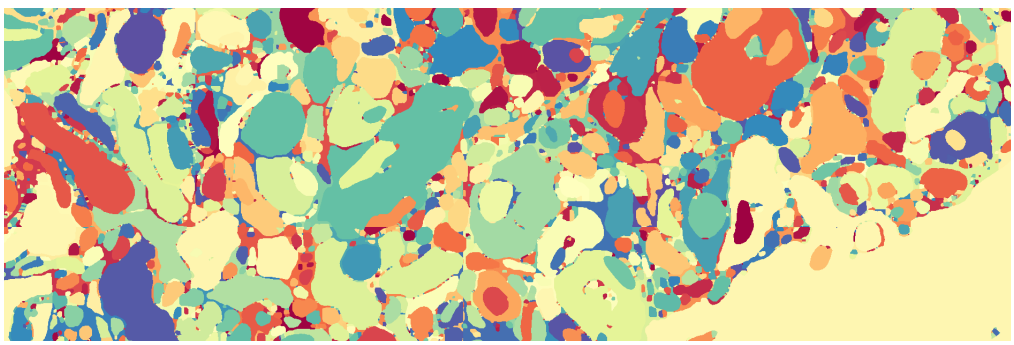
Resolution varies from sample to sample to accommodate for different animal sizes. We typically set cellular resolution between 40 and 50 nm pixel size, while synaptic resolution data is between 8 and 12 nm. Resolution along the Z axis corresponds to slice thickness and is therefore always 50 nm.

We detail this imaging strategy and all subsequent image processing steps in Paper 2. In this paper, we also estimated that it was on average 4.5 times faster to use multiple resolutions than if we had acquired the entire central complex at synaptic resolution. The intuition behind this approach is that we can exploit the repeating columnar units of the central complex to extrapolate their connectivity from only a few compartments reconstructed at connectomics resolution. We demonstrate the value of this strategy in Paper 4 and Paper 5.

Post-processing

SBEM produces tens of thousands of image tiles and multiple terabytes of data per dataset. Straight out of a scan, images are split into small tiles that overlap with each other and together map the region of interest. These tiles must be matched with each other, aligning them in regions of overlap at the interface with their neighbors. Tiles are then stitched together to form one coherent image per slice, before slices are aligned to each other to form one coherent three dimensional image stack. This is first done for the cellular resolution stack, which constitutes the common 3D coordinate space to which synaptic resolution images and neuron reconstruction will be aligned. The synaptic resolution image tiles are subsequently stitched with each other before being aligned to the cellular resolution stack. This process is not trivial, as it can be slow, computationally expensive, and is subject to image quality which varies throughout a dataset. Moreover, it is key to both manual and automatic neuron reconstruction.

To address this challenge, I wrote a custom Python pipeline adapted to our particular needs stemming from the multi-resolution imaging approach described in the previous section. It relies primarily on two libraries: OpenCV and SOFIMA. OpenCV is a powerful and well-



established computer vision library (Bradski, 2000). It is used to compute coarse alignment via affine transformations, whereby lines and parallelism are preserved while allowing rotation, translation, and scaling. These transformations correct misalignments detected by SIFT (Scale Invariant Feature Transform; Lowe, 2004), an algorithm that identifies and matches keypoints between images. SOFIMA, developed by Google Research (https://github.com/google-research/sofima), is the core of the pipeline. Rather than applying a single global transformation, it uses optical flow to estimate local displacements between images, producing vector fields that capture local fine-scale deformations. These displacement fields are then optimized over an elastic mesh, which ensure smooth and coherent deformation across the resulting image. This pipeline is described in Paper 2, and otherwise accessible at https://github.com/ValGillet/EMalign/tree/main.

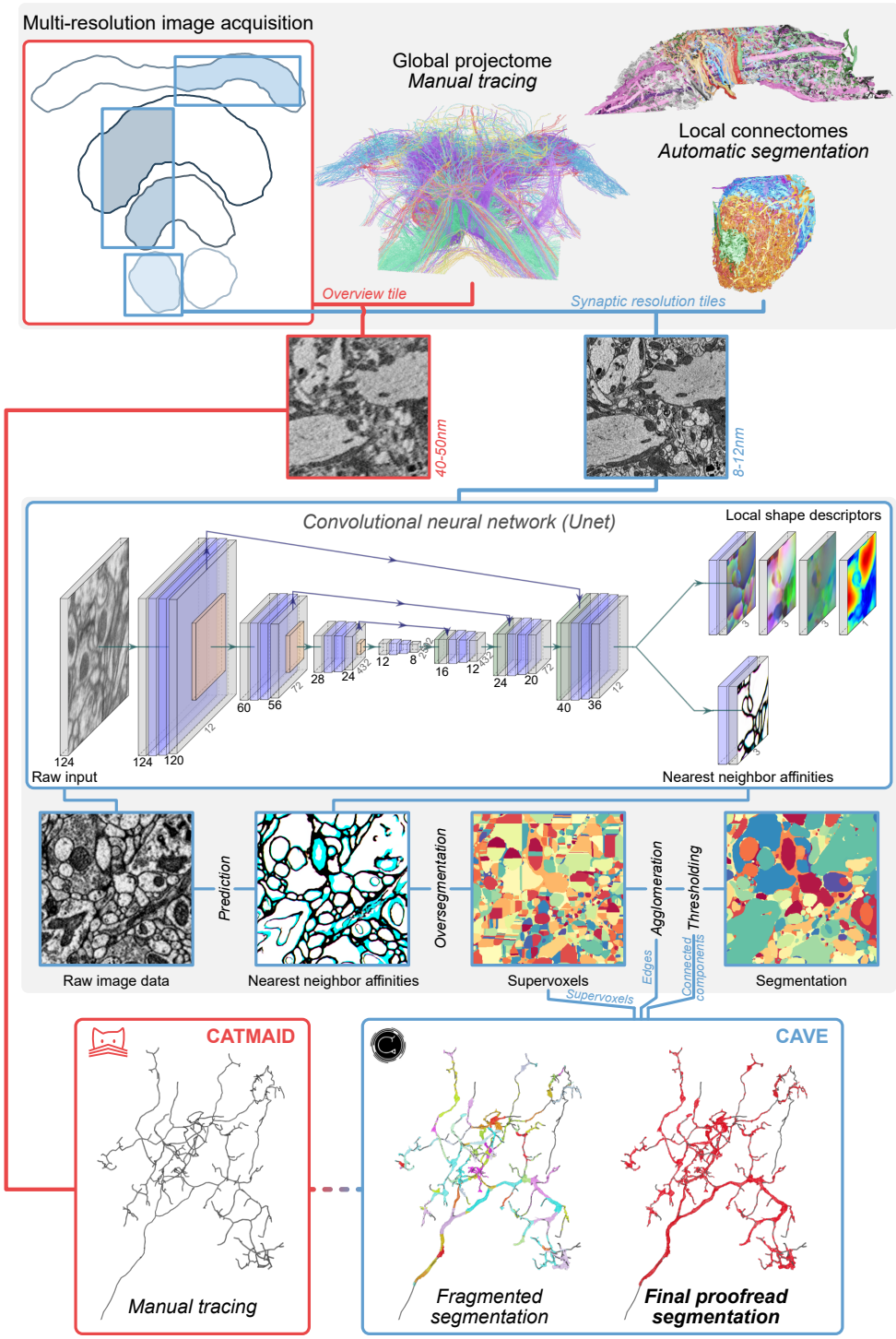

Multi-resolution image acquisition
Global projectome
Manual tracing
Local connectomes
Automatic segmentation
Overview tile
Synaptic resolution tiles
40-50nm
8-12nm
Convolutional neural network (Unet)
Local shape descriptors
Raw input
Nearest neighbor affinities
Raw image data
Prediction
Nearest neighbor affinities
Oversegmentation
Supervoxels
Agglomeration
Edges
Thresholding
Connected components
Segmentation
Supervoxels
CATMAID
Manual tracing
CAVE
Fragmented segmentation
Final proofread segmentation

Figure 5: Workflow of the image processing pipeline, from image acquisition to segmentation and proofreading. A sample is acquired at two resolutions, respectively allowing the extraction of a global projectome of the central complex, and local connectomes of selected overlapping regions. The overview image stack is used to inventory neurons of the central complex. Neuron profiles are manually traced into the overview stacks using CATMAID to obtain skeletons mapping their backbones. Image stacks at high resolutions are segmented automatically by using a convolutional neural network trained to produce nearest neighbor (NN) affinities and local shape descriptors. NN affinities are used to compute the oversegmentation and agglomeration graph of a dataset. Both are ingested into our CAVE instance where an artificially fragmented segmentation can be viewed by proofreaders. By overlapping skeletons traced in CATMAID with the fragmented segmentation in CAVE, proofreaders can efficiently merge fragments to produce the final state of a neuron.

Data extraction for connectomics

Once image data is acquired and aligned, we can finally start exploiting it to extract neuron morphology, projections, and entire circuits. Reconstruction is by far the most tedious and time-consuming part of this suite of methods. It often requires years to produce meaningful data and many people to collaborate to make it possible.

Manual tracing

Manual tracing is done using CATMAID (Saalfeld et al., 2009). This software allows multiple users to concurrently view and annotate 3D image stacks from their web browser. Annotating, or tracing a neuron, consists in following their profile across image stacks, placing nodes at regular interval that delineate their branching path. The resulting data representation is a skeleton, a graph of nodes associated with 3D coordinates that are connected by edges drawing the neuron. It minimally describes the neuron's geometry, that is, the length and orientation of every branch but not their volume. CATMAID also allows users to annotate synapses and could therefore be sufficient to build entire connectomes. We however prefer to resort to other methods for synapse annotation.

We use CATMAID to manually trace the backbone of neurons in cellular resolution image data. These images, due to their coarse resolution, do not allow the reconstruction of fine neural processes. However, an expert annotator can easily follow the path of the largest neurons, reconstructing dozens of them in a few days. We typically trace the backbone of neurons, stopping roughly when branches are too difficult to resolve in the cellular resolution stacks. This produces enough data to reconstruct a projectome and inventorize cell types, while linking synaptic resolution image stacks.

Automating data extraction

As producing massive amounts of data becomes more and more accessible, the need for automatic processing tools becomes more and more essential. In order to keep up with

our image data production, and efficiently reconstruct neurons, we turned to automatic methods that leverage machine learning with artificial neural networks.

An artificial neural network, or model, is a mathematical construct capable of learning a set of weights and extracting features from complex data by reducing its dimensionality. Similarly to a circuit of biological neurons, the network is composed of interconnected nodes organized in layers such that each layer receives input from the one upstream to it. Each node is a weighted function that filters the data and modifies it as it is relayed through the network. One simplistic way to think about it, is that an artificial neural network gradually modifies and filters the input data until it becomes the feature of interest. This is similar to how a moving object can elicit a signal in the retina, which travels to the central brain where it can become a signal encoding optic flow velocity in the central complex.

What is a convolutional neural network?

The feature to extract for our purpose is the neuronal membranes across the synaptic resolution image data, with the ultimate goal to segment neuron processes. One of the established go-tos for this sort of image processing is the convolutional neural network (CNN) which was initially inspired by biological visual systems. The CNN is typically made of alternating layers performing different operations on the input image that contribute towards extracting features of interest. At its heart is the convolution, whereby a small filter (typically 3 x 3 or 5 x 5 pixels) is slid across the image, stopping at every position to compute a dot product with the overlapping region. The resulting values form a feature map highlighting regions where the filter matched its feature of interest. This is often followed by "max pooling," which downsamples the feature map by keeping only the maximum value within small local regions, reducing dimensionality while preserving the strongest activations. In shallow layers of the CNN, the filter represents a simple geometrical shape such as a white vertical line. Similarly to a neuron, it responds more strongly when presented with its favorite stimulus in the image. In deeper layers of the neural network, filters combine to detect more and more complex features[1].

After each convolution, a nonlinear activation function (typically ReLU, which zeroes out negative values) is applied, enabling the network to learn complex, non-linear relationships between features. What is learned by the CNN is the filters weights, which allows it to learn to identify arbitrary features, as long as they can be described by a filter. At first, the filters are initiated with random values which are gradually modified to adapt to the training regime. During training, the CNN predicts an output from a small sample of raw data many times. The prediction is evaluated against a ground-truth image by a loss function, which evaluates the discrepancy between predicted and desired outcome. The loss

[1]for a great in-depth explanation, see 3Blue1Brown's video on youtube: "But what is a convolution?" (https://www.youtube.com/watch?v=KuXjwB4LzSA&t=643s)

value is then used to update the weights through the process of backpropagation, which is arguably one of the most important algorithms in modern society, as it is at the core of artificial intelligence. This is generally done via stochastic gradient descent, an optimization method used to push the network to produce a lower loss value at each iteration.

Training a model requires a large amount of ground-truth data, which for our segmentation purposes is a manually produced, accurate segmentation of neurons and mitochondria. The quality of a model depends on the amount, variety, and quality of the ground-truth, but also on parameters related to training, also called hyperparameters. Training can in fact be optimized by finding the combination of hyperparameters best adapted for a task.

The type of network that we use to segment neurons from image data is called a 3D U-net (Çiçek et al., 2016), a type of CNN whose architecture can be represented by a U-shape (see figure 5). In particular, we use an architecture and method published by Sheridan et al. (2023), specifically tailored to segment neurons in image data produced by vEM.

Local shape descriptors

The task of our model is to predict values of nearest neighbor affinity and local shape descriptors for each pixel of an image[2]. The first is a value that represents the similarity of a pixel to its neighbor. When computed over an image, it essentially works as a boundary detector, computing a highly contrasted map of neuron membranes and mitochondria. Local shape descriptors (LSD) are statistics computed for each pixel relative to its local surroundings. They represent information including size, offset to center of mass, and directionality. The model is trained to produce both affinities and LSDs for each input image. The latter is however an auxiliary task, used to enhance the primary task which in this case is the computation of affinities. The authors of the method (Sheridan et al., 2023) suggest that both metrics become correlated during training, resulting in predictions that use more local spatial information when also computing LSDs.

Once the model has produced its predictions, standard segmentation steps are performed on the resulting affinity map. Firstly, the oversegmentation consists in pooling pixels that represent similar values together under the same label. This is done using the geology-inspired watersheding algorithm, whereby the affinity map is treated like a topographical map where dark values represent high elevation. Watershedding randomly places a seed which fills up its own basin with a label that stops when it meets another label. The result is a mosaic of "supervoxels" that resemble a Voronoi diagram. Secondly, supervoxels are assembled into a graph by the process of agglomeration. Each supervoxel represents a node, which is connected to each of its direct neighbors via an edge. Each edge is assigned a score that reflects how likely pairs of supervoxels are to belong to the same object, based on the

[2]for more detail, see the blog version of the paper at: https://localshapedescriptors.github.io/

affinity values. Finally, we can obtain a final, or agglomerated, segmentation by applying a threshold to the segmentation graph, for example to prune away edges with affinity scores below a given score. The resulting connected components are islands of interconnected supervoxels that can then be assigned the same label, signifying that they belong to the same object. These labels are typically viewed thanks to a color map, which is generally more appreciable by humans than random numbers disseminated across an image.

Models are typically not infallible and can thus produce errors, which are also often caused by artifacts in the input image data. False splits occur when supervoxels that should be combined are separated, whereas false merges combine supervoxels that should belong to different connected components. Reconstructing neurons using automatic segmentation therefore still requires manual labor for proofreading its result.

This is made possible by the CAVE infrastructure, which is described in Paper 3. Similarly to CATMAID, this platform enables multiple users to simultaneously proofread neuron segmentation from their browser. It was most notably used to produce the FlyWire connectome of the entire fly brain (Dorkenwald et al., 2024, 2022). Besides reconstructing our own data, our segmentation pipeline has allowed me to process data for some of our collaborators, which we then host within our deployment of CAVE, thus mutualizing these incredible tools and enabling connectomics to be more accessible.

From neurons to connectomes

Although incredibly rich, morphological reconstruction alone is not enough to build a connectome. We therefore use a similar methods to that used for segmentation, to detect synapse locations using a CNN (Buhmann et al., 2021). This time, the model is trained to identify pre- and post-synaptic sites as coordinates corresponding to points in the image data. These coordinates can be matched with the neuron segmentation to assign synapse to neurons with known identity, thus forming a connectivity graph. As we reconstruct neurons, we gradually expand the connectivity graph and thus our abilities to study the connectome.

Chapter 4

Project Summaries

Paper 1 - A historical perspective on the insect central complex: Anatomy, development, and function

Research on the central complex is celebrating its 150th birthday this year, as one of the first mentions of the central complex in scientific literature was the 1876 paper by Dietl, to the best of my knowledge. Methods have since evolved at an increasingly rapid rate and led to the publication of multiple connectomes of the fruit fly which now give the most exhaustive account of the central complex circuitry ever achieved (Berg et al., 2025, Dorkenwald et al., 2024, Hulse et al., 2021). In this review paper, we attempt to give credit to the researchers that came before us by recounting the evolution of the field of neuroethology and some of the major findings that led the way. We describe how our understanding of the central complex became what it is today, while summarizing current knowledge on its anatomy, development, and functions across insect species.

Paper 2 - A multiresolution imaging and analysis pipeline for comparative circuit reconstruction in insects

The overwhelming majority of evidence describing the central complex comes from the fruit fly *Drosophila melanogaster*, owing to the incredible genetic tools that come with it and the colossal amount of work from large consortia that gave us multiple exhaustive connectomes (Berg et al., 2025, Dorkenwald et al., 2024, Hulse et al., 2021). This work notably provides a great point of reference for researchers working towards expanding our understanding towards more insect species. This is especially important as it is increasingly

evident that the central complex is at the core of important and fascinating behaviors that may not best be representing by the fruit fly. However, generating connectomes remains prohibitively expensive for most research groups, both in imaging time and data storage, limiting the possibility for comparative studies across species.

In this paper, we present a multi-resolution imaging and analysis pipeline designed to address this bottleneck. The core insight is that, by relying on the predictable neuroarchitecture of the central complex made of repeating computational units, we can answer mechanistic questions about its circuitry without exhaustively reconstructing it. We propose a multi-resolution approach whereby the central complex is imaged at cellular resolution with embedded compartments at synaptic resolution. This data captures global projection patterns across the whole region with local connectomes that serve to describe the detailed workings of some of its computational units. This approach reduced imaging time by a 4.5-fold on average compared to full synaptic resolution acquisition. This also came with welcome reductions in data storage, processing, and analysis burden.

We integrated this imaging approach into a complete protocol and data processing pipeline: µ CT-guided sample trimming, custom python-based alignment for multi-resolution data relying on SOFIMA, automatic segmentation and synapse detection via 3D U-nets, and collaborative neuron reconstruction through CATMAID and CAVE. We were notably able to use a single segmentation model across multiple datasets describing different insect species with variable image quality. Although this model did not perform as well as the state-of-the-art used for the fruit fly connectomes, it greatly improved our ability to rapidly reconstruct entire circuits across multiple insect species. The pipeline, code, and model are publicly available, aiming to democratize comparative connectomics and make it accessible to smaller research groups.

As proof of concept, we reconstructed head direction neurons (EPG/PEG cells) across six species spanning over 400 million years of evolution: African praying mantis, Madeira cockroach, desert locust, army ant, and sweat bee. While we only reconstructed the largest branches in all of these dataset, this data alone demonstrated deep conversation in morphology, cell numbers, and projection patterns, likely placing the emergence of the head direction cells 400 millions year ago. We furthermore reconstructed EPG and PEG neurons at synaptic resolution in the sweat bee. This revealed a conserved functional connectivity achieved differently than in the fly. EPG and PEG neurons appeared to form recurrent loops within columns as in the fly, but connected directly rather than via PEN neurons. This proof of concept demonstrates the power of our methods, which is further supported by the more in-depth analyses that resulted from it in Paper 4 and Paper 5.

Paper 3 - CAVE: Connectome Annotation Versioning Engine

This paper describes CAVE (Connectome Annotation Versioning Engine), the now standard infrastructure for neuron segmentation proofreading in connectomics. CAVE enables researcher communities to simultaneously proofread and annotate neuron reconstructions, providing a browser-based platform for efficiently splitting and merging segmentations, which has notably enabled worldwide collaborative citizen science. CAVE has supported several large-scale projects including FlyWire (the first complete adult fly brain connectome, Dorkenwald et al., 2024), FANC (fruit fly nerve chord, Azevedo et al., 2024), the H01 dataset (cubic millimeter of human cerebral cortex, Shapson-Coe et al., 2024), and the MICrONS dataset (mouse visual cortex, Bae et al., 2025).

My contribution addressed an integration challenge: bridging CAVE with the local shape descriptors (LSD) segmentation method (Sheridan et al., 2023) used to automatically reconstruct neurons in our datasets. Because CAVE and LSD rely on different data conventions, I developed Python code to efficiently translate segmentation graphs from the LSD pipeline into CAVE-compatible formats. This code is notably part of the data processing pipeline presented in Paper 2. We deployed our own CAVE instance with continuous support from the CAVE team, and I since processed multiple datasets some of which are now presented in Paper 4 and Paper 5. Additionally, we extended this deployment to collaborators, for whom I processed and ingested multiple datasets into CAVE using the pipeline presented in Paper 2.

Paper 4 - Functional convergence of distinct head direction circuits in bees, ants and flies

This paper builds directly on the pipeline presented in Paper 2 to reconstruct the head direction circuit of four hymenopteran species with diverse navigational capabilities: a tropical sweat bee, honeybee, army ant, and jumper ant. We aimed to find out whether the central complex head direction circuit described in mechanistic details in the fruit fly (Turner-Evans et al., 2020) represented a general blueprint for other insect species. At the level of cell types and projection patterns, we found remarkable conservation within our species and with the fly despite over 300 million years of divergence. This suggested that the core architecture of this brain circuit emerged early in insect evolution and has remained under strong selective constraint. Circuit-level reconstruction however reveal fundamental differences in the feedback loops that make up the ring attractor, which notably enable the open EB of most insects to emulate a toroidal structure like in the fly. Despite divergence in fine circuitry, computational models based on the anatomy of the sweat bee demonstrated that it indeed functions as a ring attractor. These results reveal functionally convergent solutions

to solve the same computational problem through flexible circuit implementation.

My contributions to this work spanned both technical development and data production. I produced the automatic segmentation for the sweat bee and army ant datasets, which formed the basis for circuit-level analysis of the hymenopteran head direction circuit. I also wrote code for efficiently assigning neuronal identity to automatically detected synapses, enabling the extraction of connectivity data. Additionally, I contributed to neuron reconstructions in the honeybee and sweat bee.

Paper 5 - A novel navigation circuit in the bee brain

This paper extends the connectomic analysis of the central complex of the sweat bee started by Paper 4, by instead looking at neurons downstream to the head direction circuit called PFN. This class of columnar cells integrates self-motion and direction signals in the fly to compute a representation of traveling direction (Lu et al., 2022a, Lyu et al., 2022). While they are increasingly characterized in the fly (Currier et al., 2020, Ishida et al., 2025, Lu et al., 2022a, Lyu et al., 2022, May et al., 2025), their circuitry remains unknown in other insect species.

Using the processing pipeline described in Paper 2, we exhaustively reconstructed all PFN neurons from one of the columnar computational units of the central complex, and their main input partners in the nodulus (self-motion) and the protocerebral bridge (head direction). We revealed a set of ancestral pathways that appeared homologous to the fruit fly's traveling direction circuits, and parallel novel pathways that are specific to the bee. Our projectome and connectome-level reconstruction highlights potential developmental mechanisms through which new sets of PFN neurons called PFNc emerged in the bee. This novel type fundamentally differed from conserved PFN by lacking input from self-motion neurons, instead forming recurrent circuits with the bee-specific FBtNOc neurons previously only described in the bumblebee (Sayre et al., 2021). The novel PFNc showed diverging projection patterns which we propose may serve enhanced vector navigation capabilities in bees. Moreover, we shed new light on data previously recorded in neurons of the sweat bee (Stone et al., 2017) which constitute the self-motion input pathways to the central complex, by highlighting circuits that may explain their response profiles and functionalities.

In future work, we will complement the current reconstruction with partners of PFN neurons within the fan-shaped body, where their circuits in the fly highlight parallel functional pathways. We will use this information to reveal conserved and divergent PFN pathways compared to the fly that may be the neural substrate of path integration in bees. We will propose functional hypothesis for how these circuits contribute to vector navigation by building computational models constrained to the anatomy of the central complex.

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Papers

Paper I: A historical perspective on the insect central complex: Anatomy, development, and function

Paper II: A multiresolution imaging and analysis pipeline for comparative circuit reconstruction in insects

Paper III: CAVE: Connectome Annotation Versioning Engine

Paper IV: Functional convergence of distinct head direction circuits in bees, ants and flies

Paper V: A novel navigation circuit in the bee brain

List of papers

I. Gillet V*, Kluge J*, & Patel RN (2025). A historical perspective on the insect central complex: Anatomy, development, and function. *Molecular Psychology: Brain, Behavior, and Society,* https://doi.org/10.12688/molpsychol.17564.3

II. Gillet V, Sayre ME, Badalamente GS, Schieber N, Tedore K, Funke J, & Heinze S. A multiresolution imaging and analysis pipeline for comparative circuit reconstruction in insects. (*Submitted to eLife*)

III. Dorkenwald S[1], Schneider-Mizell CM[1], Brittain D, Halageri A, Jordan C, Kemnitz N, Castro MA, Silversmith W, Maitin-Shephard J, Troidl J, Pfister H, Gillet V, Xenes D, Bae JA, Bodor AL, Buchanan J, Bumbarger DJ, Elabbady L, Jia Z, Kapner D, Kinn S, Lee K, Li K, Lu R, Macrina T, Mahalingam G, Mitchell E, Mondal SS, Mu S, Nehoran B, Popovych S, Takeno M, Torres R, Turner NL, Wong W, Wu J, Yin W, Yu Sc, Reid RC, da Costa NM, Seung HS, & Collman F. CAVE: Connectome Annotation Versioning Engine. *Nature Methods*, https://doi.org/10.1038/s41592-024-02426-z

IV. Sayre ME, Gillet V, Pinzon-Rodriguez A, Badalamente GS, Ceberg N, Griggs N, Serratosa Capdevila L, Roberts R, Gunnarsson ES, Ceberg N, Szadaj F, Ellendula S, Honkanen A, Narendra A, & Heinze S. Functional convergence of distinct head direction circuits in bees, ants and flies. (*Manuscript*)

V. Gillet V, Sayre ME, Badalamente GS, Pinzon-Rodriguez A, Zadel A, Griggs N, Monteleone A, Langreiter M, Nerme V, & Heinze S. A novel navigation circuit in the bee brain. (*Manuscript*)

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