The complex synaptic pathways onto a looming-detector neuron revealed using serial block-face scanning electron microscopy

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Abstract
The ability of locusts to detect looming stimuli and avoid collisions or predators depends on a neuronal circuit in the locust’s optic lobe. Although comprehensively studied for over three decades, there are still major questions about the computational steps of this circuit. We used fourth instar larvae of Locusta migratoria to describe the connection between the lobula giant movement detector 1 (LGMD1) neuron in the lobula complex and the upstream neuropil, the medulla. Serial block-face scanning electron microscopy (SBEM) was used to characterize the morphology of the connecting neurons termed trans-medullary afferent (TmA) neurons and their synaptic connectivity. This enabled us to trace neurons over several hundred micrometers between the medulla and the lobula complex while identifying their synapses. We traced two different TmA neurons, each from a different individual, from their synapses with the LGMD in the lobula complex up into the medulla and describe their synaptic relationships. There is not a simple downstream transmission of the signal from a lamina neuron onto these TmA neurons; there is also a feedback loop in place with TmA neurons making outputs as well as receiving inputs. More than one type of neuron shapes the signal of the TmA neurons in the medulla. We found both columnar and trans-columnar neurons connected with the traced TmA neurons in the medulla. These findings indicate that there are computational steps in the medulla that have not been included in models of the neuronal pathway for looming detection.

KEYWORDS
collision detection, connectivity, LGMD, Locust, looming, medulla, SBEM, TmA neuron

Abbreviations: DM, Digital Micrograph™; IP, input; LGMD, lobula giant movement detector; LOX, lobula complex; M, medulla interneuron; ML, medulla layer 1–10; OCH2, second optic chiasm; OLO, outer region of the lobula complex; OP, output; RS, reciprocal synapses; SBEM, serial block-face scanning electron microscopy; TmA, trans-medullary afferent; TmAL, trans-medullary afferent-like.
INTRODUCTION

The timely detection of an approaching predator can be critical for the survival of an animal. Throughout the animal kingdom, species have evolved different detection systems matching the constraints and needs of their respective environments (de Vries & Clandinin, 2012; Dunn et al., 2016; Klapoetke et al., 2017; Peek & Card, 2016; Rind & Simmons, 1992; Wang & Frost, 1992).

Studies on the neuronal substrate of such detection systems identified several different types of looming-sensitive neurons: the giant fiber neurons in Drosophila (Peek & Card, 2016), which have counterparts in several other species, such as crabs, (monostratified lobula giant neurons of type 1,MLG1; Oliva & Tomsic, 2014), praying mantis (tangential projecting neurons in the lobula complex; Yamawaki, 2019), or in zebra fish (periventricular neurons of the optic tectum; Dunn et al., 2016). Physiological, structural, and ultrastructural investigations of these neurons and their circuits are necessary to highlight their similarities and differences. Considering the differences in behavior and distances in the animal tree, comparative studies of the neuronal circuits and their computational solutions will improve our understanding of the different ways evolution solved the task of predator/looming detection.

For locusts, visual cues allow the animal to discriminate between dangerously approaching objects and receding or translating ones (Gabbiani et al., 2002; Rind & Simmons, 1992; Schlotzer, 1977). The underlying neuronal circuit in the locusts' visual system has been studied over several decades (Dewell & Gabbiani, 2018; Gabbiani et al., 2004; Olson et al., 2021; Rind, 1987, 1996; Sztarker & Rind, 2014). The physiology of the key neurons, the descending contralateral movement detector (DCMD) and the lobula giant movement detector (LGMD) is well documented and the whole system is used as the template for artificial collision detection models (Fu et al., 2019; Rind & Bramwell, 1996; Yue et al., 2006). There are two giant movement-detecting neurons located in the locust's lobula complex (LOX); the LGMD1, and LGMD2. For both neurons, it is characteristic that conspicuous dendritic arborizations are found in the LOX. In the case of LGMD1, this arborization can be divided into three distinct subfields, A, B, and C (O’Shea & Williams, 1974). The inputs to subfield A are of excitatory nature and involve acetylcholine release (Peron et al., 2009; Rind & Leitinger, 2000). Subfield A receives synaptic input from afferent neurons covering almost the visual field of the locust's compound eye (Krapp & Gabbiani, 2005). The inputs to the LGMD1’s subfields B and C are both inhibitory and phasic in nature mediated by GABA_A release (Gabbiani et al., 2004). The LGMD2 only has one dendritic field where it receives both excitatory and inhibitory input (Rind, 1987; Simmons & Rind, 1997).

The input neurons of the LGMD1 subfield A originate from the second neuropil, the medulla (therefore, named trans-medullary afferent [TmA] cells) and connect the medulla with the outer region of the LOX (OLO, Rosner et al., 2017). Unfortunately, knowledge about these cells is sparse. In 1981, Strausfeld and Nässel showed using cobalt staining that the cells connecting the medulla with this subfield of the LGMD have two different types of branching patterns, indicating that there may be at least two different cell populations of TmA cells (Strausfeld & Nässel, 1981). Their connection with the LGMD was shown, in 2018, light microscopically using en masse staining (Wang et al., 2018). The ultrastructural features of LGMD1 input are well documented: the chemical synapses between TmA neurons and LGMD1 and 2 are always arranged in such a way that neighboring TmA neurons have output synapses both onto the LGMD and onto their neighbors (Rind & Simmons, 1998; Sztarker & Rind, 2014). Each output synapse thus has two postsynaptic partners, the LGMD1 and the neighboring afferent neuron. Afferents make back-to-back synapses with one another and the LGMD1. The importance of this synaptic setup for the detection of looming stimuli is still debated with both lateral inhibitory and excitatory effects of one TmA on its neighbor proposed. Physiological experiments and modeling showed lateral inhibition is present and improves collision tuning (O’Shea & Williams, 1974; Pinter, 1977, 1979, 1983; Rind et al., 2016; Rind & Bramwell, 1996). However, this role for lateral inhibition, mediated by muscarinic receptors (Rind et al., 2016; Rind & Leitinger, 2000; Rind & Simmons, 1997, 1998) has been questioned as blocking muscarinic receptors near the LGMD1 with receptor antagonist decreases excitation while potentiating their action leads to increased excitation not inhibition (Zhu et al., 2018). While the TmA neurons’ terminal barbaryizations in the LOX were previously well described, it was not known in which layer in the medulla they receive synapses from upstream neurons, nor whether they have dendritic branches in the medulla, which is commonly found in medulla interneurons, or receive input synapses along only one dendrite, which so-far was described only for Tm2 neurons in layer M10 of the Drosophila medulla (Shinomiya, Huang, et al., 2019). For a better and complete understanding of the whole computation process within the looming sensing pathway, a detailed description of the synapses made by the medullary cells’ contributing to the circuit is needed. On electron micrographs of the LOX, the LGMD1 and 2 can be easily identified by their characteristic arrangement of the dendritic cross-sections within the neuropil with the LGMD2 directly posterior to the LGMD1 (Rind & Simmons, 1998; Simmons et al., 2013). This fact allows the use of the LGMD1 as a starting point of tracing the TmA neurons back to the medulla. The TmA neurons are themselves unambiguously identifiable due to their reciprocal synaptic arrangement. However, the tracing of a neuron is a challenging task. Over the last decades, the method of choice was to collect ultra-thin sections on grids for transmission electron microscopy investigations but this technique is very time consuming and prone to errors and therefore rarely used by researchers. Serial block-face scanning electron microscopy (SBEM) was (re-)introduced in 2004 (Denk & Horstmann, 2004) and became a vital tool investigating large sample volumes in life and materials science (Zankel et al., 2009), but especially in neuroscience (Helmstaedter et al., 2013; Holcomb et al., 2013; Mukherjee et al., 2016; Scheffer et al., 2020). With SBEM, an ultra-microtome is installed in the specimen chamber of a scanning electron microscope and the block-face of the specimen is imaged after each cut with the diamond knife of the microtome. This technique makes it possible to identify TmA neurons at the LGMD by their synapses and to trace the neurons over a distance of over 300 μm to the medulla.
Here, we present the first morphological description of two TmA neurons of fourth instar locusts, reconstructed along their whole length from serial electron micrographs obtained using SBEM. We also present the number and direction of their synapses and reconstructions of key parts of their pre- and postsynaptic neurons.

2 | MATERIAL AND METHODS

2.1 | Animals and sample preparation

The locusts were obtained from a gregarious culture with day/night cycle of 12/12 h at a mean temperature of 30°C at the Biosciences Institute (Biosciences Institute, Newcastle University, UK). Locusts are big insects so to reduce the distances to be reconstructed smaller fourth instar locusts were used (Figure 1a). At this stage, the LGMD1 and 2 have their mature form except at their branch extremities (Sztarker & Rind, 2014). Reconstructions were begun at dendritic subfield A in the mid region of a large branch where synapses should be mature. We scanned a data set that covered the main branches of one entire TmA in one sample, and another entire TmA in a second sample.

The animals were chilled on ice and brains were dissected and cut in half. During dissection, the brain was kept moistened with insect saline. The samples were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde (solved in 0.1 M sodium cacodylate buffer, pH 7.4) for 3 h at room temperature. To create the heavy metal contrast needed for imaging in the SBEM the samples were
prepared using the SBEM protocol by Deerinck et al. (2010) with some slight changes (e.g., usage of another embedding medium, see below). In short, after fixation the brain tissue was treated with reduced 1% osmium tetroxide (reduced with potassium hexacyanoferrate (II) mixed with sodium cacodylate buffer) for 30 min in dark. Thio carbocarbohydrate (1%) was used for contrast enhancement for 1 h at room temperature. After rinsing, the tissue was incubated in 1% osmium tetroxide for 30 min at room temperature in dark. The rinsed samples were put into 1% uranyl acetate solution for 2 h at 60°C. As a final step, the Walon’s lead aspar tic staining (Walton, 1979: 0.066 g lead nitrate [Sigma-Aldrich, USA] in 10 ml 0.03 M aspar tic acid [SERVA Electrophoresis GmbH, Germany] and pH adjusted to 5.5 with 1 N KOH) was applied over night at room temperature. Samples were dehydrated in a graded ethanol series and transferred, using propylene oxide as intermedium, into the resin (embedding resin, TAAB Laboratories Equipment Ltd., UK) and polymerized at 60°C over 3 days.

The resin blocks were mounted on an ultra-microtome Leica UC7 (Leica, Vienna, Austria) and sectioned in 500 nm steps from the optic stalk to the LOX (Figure 1b). Every 20 μm, semi-thin sections were stained with toluidine blue (Sigma-Aldrich, USA) and checked with a light microscope. The region of interest (ROI) was identified by the clear visible crescents of the LGMD1 and LGMD2 on the semi-thin sections (Figure 1c) and the resin blocks with the identified ROI were further trimmed so that their edges were smaller than 600 μm (Figure 1c) and transferred onto a metal rivet. An in-situ ultra-microtome 3View (Gatan, Inc., Pleasanton, CA, USA), mounted in the chamber of an environmental scanning electron microscope ESEM Quanta 600 FEG (FEI, Eindhoven, Netherlands) at the Graz University of Technology was used for image acquisition. The microscope settings for the recording of the electron micrographs were: 3 kV acceleration voltages, vapor as imaging gas with a pressure of 20 Pa, and a spot size of 3.2 (determines the beam current). The electron micrographs were recorded with a dwell time of 12.5 μs using a backscattered electron detector (BSED; Gatan, Inc.). Digital Micrograph™ (DM, Version 2.01.697.0. Gatan Inc.) was used as imaging software and the images were inverted to create contrast characteristics similar to those TEM bright field micrographs. The electron micrographs were recorded with a pixel size of 10 nm, giving a resolution of 20 nm which is sufficient for localizing chemical synapses because they contain a presynaptic bar and synaptic vesicles (Wernitznig et al., 2015). In the first sample throughout the whole series, an image size of 25 × 25 μm was chosen. This was less than the approximate dimensions of a single column within the medulla, so the pre- and postsynaptic neurons could not be reconstructed in entirety. Therefore, a second sample was scanned for which the image size was adapted to the region and widened when the medulla was reached. Image size was 30 × 30 μm in the LOX, 20 × 40 μm close to the second optic chiasm (OCH2), 25 × 25 μm within the OCH2 and in the medulla; it was either 30 × 30 or 35 × 35 μm to cover the approximate width of a column within the medulla. This second data set allowed reconstructions of much larger parts of pre- and postsynaptic neurons than the first data set, but the center of the field of view was continuously manually adjusted to follow the TmA’s main process, so pre- and postsynaptic neurons were not covered entirely. The recording time for a 25 × 25 μm electron micrograph took approximately 1.3 min. The section thickness of 40 nm was initially chosen for an accurate tracing and reliable identification of synapses. In the case of inconsistent cutting, the thickness was increased to 50 nm. Side branches are absent within the region of the OCH2, so for most of the OCH2 of the first specimen 60 nm section thickness was chosen due to time considerations. Section thickness between 50 and 60 nm is suitable to identify and quantify synapses in the insect nervous system (Meinertzhagen, 1996). The total number of sections was 8400 for Sample 1 and 7500 for Sample 2. The region of the OCH2 was identified by the presence of tightly packed neurons together with glial cells and cell bodies.

The protocol was suitable for the SBEM imaging, though we experienced some problems with accumulations of a contrast chemical (presumably uranyl acetate; Figure 1c). Fortunately, neither the identification of the LGMDs in the LOX (Figure 1c,d), nor the tracing of TmA cells were compromised by this artifact.

### 2.2 Segmentation of data

Reconstructions were done using the Amira® 3D Software (Version 5.6.0 FEI™). The micrographs were imported as TIFF-files after converting them from the original GATAN file type dm3 using DM. The segmentation was done on a desktop machine with an Intel®Xeon®E3-1200 (4 cores, 3.30 GHz), 16 GB RAM and NVIDIA GeForce GTX TITAN X with 12 GB of GPU RAM.

Segmentation of the LGMD, TmA neurons and input neurons to the TmA neuron in the medulla was done manually in Amira®. Presynaptic densities were used to identify synaptic connections from TmA neurons onto the LGMD dendrites (Figure 2) and between TmA neurons and other neurons in the LOX, chiasm, and in the medulla. Presynaptic densities are characterized by the presence of synaptic structures, such as a synaptic bar, synaptic vesicles, and additional structural proteins which become electron dense after contrast treatment (Leitinger et al., 2012; Leitinger & Simmons, 2002; Watson & Schürmann, 2002). For the described neurons, the number of input and output synapses was recorded. The neuron of interest is always the reference for the definition of input and output. An input (IP) synapse for a neuron means that it receives synaptic input from another neuron; hence, the neuron is a postsynaptic partner. Conversely, if the neuron has an output (OP) synapse it is the presynaptic partner. Usually insect synapses are dyadic, with processes of two neurons connected to each output synapse (Watson & Schürmann, 2002). In the LGMDs, two dyadic synapses occur back-to-back with both TmA neurons making contact with each other and with the LGMD. Both TmA neurons share the same synaptic cleft. Each TmA makes one dyadic synapse targeting its neighboring TmA and the LGMD, plus it receives an input from its neighbor TmA (Figure 2a–f). Synapses were only counted if identified by at least two independent investigators. Therefore, in contrast to other recent
connectomics studies (e.g. Scheffer et al., 2020), connections between neurons were taken in consideration even if they shared only one synapse and regardless of their numeric strength. The borders between the neuropil of the medulla and LOX, and the OCH2 that connects them was characterized by axon bundles and glial sheaths close to the TmA neuron.

The figures were created by using Adobe Photoshop® (CS4, Adobe Systems) to crop and place scale bars from Amira-Screenshots. Tables were generated in Excel (Version 2009, Microsoft, USA). Movies were generated using Amira®.

2.3  |  Skeleton reconstructions

Additional neurons around TmA1 and 2 were traced in each animal using the filament editor module in Amira®. Instead of displaying the neurons as a mesh, only the skeleton of the neurons is reconstructed. In total, 9 neurons were traced in Animal 1 and 23 neurons in Animal 2. The neurons were chosen close to the traced TmA1 and 2 neurons, which appeared to be a bundle of neurons enclosed by a glia cell. In Animal 2, neurons from two other bundles which looked similar to the TmA bundle were also traced. As initial starting point for tracing, neuronal cross-sections were chosen in the area of the transition from OCH2 to medulla.

2.4  |  Public repository

The raw data were converted into .tiffs and placed into a public repository, datadryad. They are available under https://datadryad.org/stash/share/EftiSOVz7EuZi011rB8pJXwDKDjttZ6GEb_KvTYY, doi. 10.5061/dryad.3j9kd51hc (Leitinger et al., 2021).
3 | RESULTS

A general description of the LOX, and medulla is given in (Rosner et al., 2017; Strausfeld, 1976; Strausfeld & Nässel, 1981), and a description of the LGMD1 and 2’s ultrastructure is available in (Rind et al., 2016; Rind & Simmons, 1998). The anatomy of the LGMD1 and 2’s reconstructed from silver-stained sections in a fourth instar locust is shown in Figure 1d. In this study, we used SBEM to trace two neurons, which synapsed with the LGMD1. Each TmA was from a different animal. Identification of the LGMDs was possible because the LGMD1 subfield A forms a dendritic arbor that can be clearly recognized in sections of the optic lobe where it appears as a crescent of lightly stained profiles (Figure 1c). Profiles of TmA neurons involved in looming detection are identified by their output synapses onto the LGMD1 (Figure 2a–d,f).

![Figure 3](image.png)

**Figure 3** Distribution of input and output synapses of the TmA neurons. (a) Reconstruction of TmA1 neuron (yellow), part of the LGMD1 (blue), white dotted lines separate LOX, OCH2, and medulla. Gray dotted lines mark the layers in the medulla. The thickness of each layer was calculated based on the relative thickness of the medulla layers in adult animals. All the indicated areas correspond with the same areas in (b) and (c), anatomical directions are indicated by the arrows at the bottom. ML1-3 are termed distal as they are furthest from the mid-line of the animal, ML4-5 are termed serpentine because of the many tangential fibers running parallel to the plane of section, and ML6-10 are termed proximal. (b) Bar charts of the number of TmA1 and TmA2 synapses (input [IP] and output [OP] synapses), summed over 5 μm. Location of synapses in the bar chart correspond to the TmA reconstructions shown in (a) and (c). To allow easier comparison, the shared y axis has its zero point at the start of the medulla. The x axis shows the number of synapses, IP (green) and OP (red). (c) Reconstruction of TmA2 neuron (yellow), part of the LGMD1 (blue) scale as in (a). Scale bars (a) and (c) 100 μm. LOX, lobula complex; LGMD1, lobula giant movement detector 1; OCH3, second optic chiasm; TmA, trans-medullary afferent [Color figure can be viewed at wileyonlinelibrary.com]

| TABLE 1 | Synaptic connections of TmA1 and TmA2 neuron in the LOX. Connections were counted and categorized into: input synapses (IP), or output synapses (OP). The number of synapses that had a reciprocal back-to-back partner in a neighboring neuron is also stated. Synaptic partners were either the LGMD1, the LGMD1 but not the reconstructed branch or unclassified |
|---------|------------------|-----------------|-----------------|-----------------|
| **Region** | **Total synapses** | **Input (IP) synapses** | **Output (OP) synapses** | **Reciprocal pairs** |
| TmA1 | LGMD1 | 16 | 4 | 12 | 2 |
| | LGMD (not reconstructed) | 6 | 3 | 3 | 0 |
| | Unclassified neurons | 245 | 115 | 130 | 7 |
| TmA2 | LGMD1 | 13 | 4 | 9 | 1 |
| | LGMD (not reconstructed) | 60 | 23 | 37 | 9 |
| | Unclassified neurons | 251 | 116 | 135 | 2 |

Abbreviations: LGMD, lobula giant movement detector; TmA, trans-medullary afferent.
3.1 Anatomy and synaptic arrangements of input neurons to the LGMD1: the TmAs

Two neurons, each in a different animal were traced from their synapses with the LGMD1 in the outer lobula (OLO) layer of the lobula complex (LOX; Rosner et al., 2017; Strausfeld, 1976), through the OCH2 to the medulla (Figure 3a–d). We were not able to locate the cell bodies of either TmA in the medulla probably because the neurite connecting to their cell bodies were very thin and moved out of the block-face. Because both TmA neurons were identified in the LOX and reconstructed from there toward the compound eye, the detailed synaptic examination will start in the...
TABLE 2 Synapse distribution for TmA1 and TmA2 in the two neuropils, the medulla and lobula, and in the connecting OCH2 between them; input synapses (IP), output synapses (OP)

| Region | Reconstructed depth (μm) | Total synapses | IP synapses | OP synapses | Ratio IP:OP |
|--------|--------------------------|----------------|-------------|-------------|-------------|
| TmA1   | Total                    | 405            | 490         | 271         | 219         | 1.2:1       |
|        | Medulla                  | 184            | 195         | 124         | 71          | 1.7:1       |
|        | ML6-10                   | 110            | 95          | 54          | 41          | 1.3:1       |
|        | ML1-3                    | 74             | 100         | 70          | 30          | 2.3:1       |
|        | OCH2                     | 184            | 28          | 25          | 3           | 8.3:1       |
|        | LOX                      | 37             | 267         | 122         | 145         | 1:1.2       |
| TmA2   | Total                    | 393            | 493         | 285         | 208         | 1.37:1      |
|        | ML6-10                   | 117            | 135         | 108         | 27          | 4:1         |
|        | OCH2                     | 220            | 34          | 34          | 0           | n.a.        |
|        | LOX                      | 56             | 324         | 143         | 181         | 1:1.26      |

Abbreviations: LOX, lobula complex; OCH2, second optic chiasm; TmA, trans-medullary afferent; TMAL, trans-medullary afferent-like.

FIGURE 5 TmA1 neuron and connecting neurons in the medulla. (a) TmA1 neuron (yellow) with connecting neurons TmAL1, M1, and M2 in the medulla. (b) TmAL1 alone in greater detail (violet). (c) TmA1-M1 (green), (d) TmA1-M2 (light-blue), asterisk shows where TmA1 neuron was lost entering the second optic chiasm (OCH2). TmA, trans-medullary afferent; TmAL, trans-medullary afferent like [Color figure can be viewed at wileyonlinelibrary.com]
LOX. A part of the main dendritic arbor of the LGMD1 was identified (Figure 1c) and reconstructed in both animals (Figure 3a,d). In the LOX, both TmA1 and 2 neurons ramified extensively into smaller neurites (<1 μm in diameter) that exhibited multiple synapses with the LGMD1 (Table 1). The overall number of synapses made by TmA1 or 2 onto the reconstructed LGMD1 branch, was lower than those made onto unreconstructed LGMD1 processes (Table 1). For TmA2 particularly, there were more synapses at identified LGMD1 branches that were not reconstructed. The highest number of synapses was found with additional cells which we could not classify. Despite the high number of synaptic connections, there were only a small proportion of reciprocal synapses (RS) between TmA1 and 2 neurons and other, unidentified cells in the LOX (Table 1).

In both TmA1 and 2, a single neurite enters the OCH2 and projects in it to the medulla (Figure 3a,c; TmA1 yellow profile in 4a; TmA2 yellow profile in 4d). In the OCH2, the axon of TmA1 zig-zags somewhat while the axon of TmA2 forms a straight line. In the OCH2, both TmA1 and 2 receive input synapses (Figure 3b). Major differences in morphology between them in the medulla are (i) TmA1 traverses ML6–10 and ML3–5 arborizing extensively in ML1–3 while (ii) TmA2 forms multiple, pearl-string-like varicosities but does not branch. Several little protrusions can be found throughout the medulla (Figure 4i–p) and it ends in the ML6–10 capped by another cell.

**FIGURE 6** TmA1 synaptic distribution in the medulla. (a) Reconstruction of TmA1 (yellow) and TmA1 (violet). (b) Bar chart showing distribution of input (green) and output (red) synapses of the TmA1 in the medulla. TmA, trans-medullary afferent; ImAL, trans-medullary afferent like [Color figure can be viewed at wileyonlinelibrary.com]
FIGURE 7  TmA2 neuron and connecting neurons in the medulla. (a) TmA2 (yellow) and connecting neurons in the medulla. (b) TmAL2 (blue), neuron does not terminate (distal—asterisk, proximal—arrow), (c) TmAL3 (violet), neuron does not terminate (distal—asterisk, proximal—arrow), (d) TmAL4 (pink), (e) M1 (brown), (f) M2 (light brown), (g) M3 (dark brown), (h) M4 (dark green), (i) M5 (light green), (j) M6 (green), (k) M7 (gray). TmA, trans-medullary afferent; ImAL, trans-medullary afferent like [Color figure can be viewed at wileyonlinelibrary.com]

without entering ML4,5 (TmA1: Figures 3a and 4b,c; TmA2: Figures 3c and 4e–h).

Previously the medulla had been regarded as an input area for the TmAs only (Jones & Gabbiani, 2010; Rind & Bramwell, 1996) and it was assumed that they would receive input from LMC neurons, which terminate in ML1-3 (Elphick et al., 1996). When we mapped the number and distribution of input and output synapses along the entire TmA reconstructions, we found predominantly input synapses in the medulla but both input and output synapses were identified along the entire reconstruction of the TmA neurons, indicating a much more complex synaptic arrangement than previously assumed.

A total of 490 synapses were found in the TmA1 neuron, and 493 in TmA2, including the synapses counted in the LOX, the OCH2, and the medulla (Figure 3b, Table 2). A back-to-back reciprocal synapse involving two TmAs and the LGMD counts as two synapses, even though there are two output targets and one input connection for each TmA. Each TmA synapse is characterized by a presynaptic density and two target neuron profiles (Figure 2e,f). The overall ratio of input synapses to output synapses was 1.2:1 for TmA1 and 1.4:1 for TmA2. For the LOX, the number and direction of synapses of both neurons were similar to each other and the number of IP almost equals the number of OP synapses with a ratio of 1:1.2 for TmA1 and 1:1.3 for TmA2 (IP:OP; Figure 3b, Table 2). In the medulla, many more input
FIGURE 8  Synaptic connections of TmA2 neuron with TmAL2 and TmAL3. Reconstruction from the OCH2 to medulla. (a) All three neurons are shown together. (b) Single reconstruction of TmAL2 in the medulla and chiasm. (c) Synapse distribution of TmAL2 with TmA2 corresponding to (b). (d) Single reconstruction of TmAL3 in the medulla and chiasm. (e) Synapse distribution of TmAL3 with TmA2 corresponding to (d). OCH2, second optic chiasm; TmA, trans-medullary afferent; TmA, trans-medullary afferent like [Color figure can be viewed at wileyonlinelibrary.com]

synapses than output synapses, with a ratio 1.7:1, were detected for TmA1 and 4:1 for TmA2. In layers 6–10 of the proximal medulla, there are more synapses found for the TmA2 neuron in total, with more input synapses but fewer output synapses compared to the TmA1. The arbor of TmA1 in the distal medulla exhibited additional synapses with a ratio of 2.3:1 (IP:OP) (Table 2). A small number of synapses were found in the
### Table 3: Number of synapses in the OCH2 and medulla for TmAL1, TmAL2, TmAL3, and TmAL4

| Neuron | Synapse location | Synapses with TmA1 or 2 | Input (IP) synapses onto TmA1 or 2 | Output (OP) synapses from TmA1 or 2 | All IPs onto TmA | All OPs from TmA | All IP:OP Ratios of TmA | Reciprocal pairs associated with TmA |
|--------|------------------|-------------------------|------------------------------------|-------------------------------------|-----------------|-----------------|---------------------|----------------------------------|
| TmAL1  | OCH2             | n.a.                    | n.a.                               | n.a.                                | n.a.            | n.a.            | n.a.                | n.a.                             |
|        | Medulla          | 5                       | 5                                  | 0                                   | 39              | 27              | 1:4:1               | 8                                |
| TmAL2  | OCH2             | 7                       | 3                                  | 4                                   | 7               | 5               | 1:4:1               | 3                                |
|        | Medulla          | 0                       | 0                                  | 0                                   | 103             | 27              | 3.8:1               | 5                                |
| TmAL3  | OCH2             | 15                      | 4                                  | 11                                  | 1               | 18              | 1:3:6               | 4                                |
|        | Medulla          | 21                      | 15                                 | 6                                   | 189             | 52              | 3.6:1               | 13                               |
| TmAL4  | OCH2             | n.a.                    | n.a.                               | n.a.                                | n.a.            | n.a.            | n.a.                | n.a.                             |
|        | Medulla          | 1                       | 0                                  | 1                                   | 101             | 59              | 1:7:1               | 1                                |

Note: Input (IP) and output (OP) synapse numbers for TmAL1 with TmA1 and for TmAL2–4 with TmA2 are listed followed by all identified synapses (IPs and OPs) of TmAL1–4. Lastly, the number of back-to-back reciprocal synapses (RS) associated with each TmAL is listed. Abbreviations: OCH2, second optic chiasm; TmA, trans-medullary afferent; TmAL, trans-medullary afferent-like.

### Table 4: Number of synapses reconstructed from the small cells (M1–7) in the medulla which have at least one synaptic connection with the trans-medullary afferent 2 (TmA2) neuron

| Neuron | Synapses IP OP With TmA2 total | Input (IP) | Output (OP) | Reciprocal pairs |
|--------|-------------------------------|------------|-------------|------------------|
| M1     | 7 12 5                         | 2 3        | 1           |
| M2     | 0 3 1                          | 0 1        | 0           |
| M3     | 7 4 1                          | 0 1        | 0           |
| M4     | 8 12 4                         | 0 4        | 1           |
| M5     | 8 4 2                          | 0 2        | 1           |
| M6     | 4 7 2                          | 1 1        | 0           |
| M7     | 12 8 6                         | 2 4        | 2           |

OCH2 (28 and 34, respectively; Figure 3b, Table 2), which are almost entirely input synapses, except in three cases for TmAL1.

### 3.2 TmA1 and 2 synaptic partner neurons in the medulla and OCH2 revealed

The description of the medulla interneurons focuses mainly on those associated with TmA2 as the image quality of this data set was better and allowed a more reliable tracing of finer neurites. The profiles that were found in synaptic contact with TmA2 varied in electron density and in the number of mitochondria and synaptic vesicles, indicating that several different neuron types may be involved (Figure 4e–p). They included profiles that resembled photoreceptor terminals occurring in the lamina, with dark cytoplasm and a high number of mitochondria (data not shown), however they could not be photoreceptors as the only long photoreceptor in the locust, R7, does not extend beyond ML3 (Schmeling et al., 2015).

Previously, there were only speculations about the wiring of the TmA neurons in the medulla. We traced a total of 13 synaptic partner neurons: 3 neurons connected to TmA1 (Figures 5 and 6) and ten to TmA2 (Figures 7 and 8). For TmAL1 and TmA2, we can distinguish two groups of synaptic partner neurons based on their morphology. The first group accompanies the TmA neurons over their entire length, or at least over most of the proximal medulla. They are termed “TmA-Like” (TmAL) neurons, as it is likely that all go from the medulla through the OCH2 to the LOX. In our study, four neurons in this group were identified: TmAL1–4. By comparing the morphology of those four cells, three different types (i–iii) were categorized because they exhibited differences in branching pattern:

#### 3.2.1 Group 1 (TmAL 1–4)

i. Two very similar neurons, TmAL1 and TmAL2 were both characterized by a small arborization at the OCH2/medulla border and additional branches in ML6–10 (TmAL1 in Figures 5 and 6; TmAL2 in Figure 8b,c). TmAL1 was found in Animal 1, and TmAL2 was found in Animal 2. TmAL1 was traced all the way to ML1–3 like the TmA1 neuron, and, like TmA1, exhibited another arborization there. In contrast, TmAL2 was not traced into ML1–
3 as Animal 2 does not include data for the distal medulla. However, TmAL2 was traceable through the OCH2 to ML6–10 (Figures 7 and 8a,b) and the synapse distribution there mapped (Figure 8c). In morphology and types and location of synapses, TmAL1 and TmAL2 closely resembled TmA1 and TmA2 respectively, although the number of synapses found along the branches of TmAL1 is much lower than the number found at the other TmAL2–4 neurons (Table 3). A small proportion of both input- and output synapses had a back-to-back reciprocal partner synapse in a neighboring profile.

ii. TmAL3, (Figures 7c and 8d,e) was identified once in Animal 2 and has one area of extensive branching in the ML6–10 and the synapse distribution there was mapped (Figure 8e). The main neurite left the distal block-face towards the ML1–3, and in the other direction the neurite was traced through the OCH2 to the LOX where it was lost.

iii. TmAL4 (Figures 7d and 8f) differs from the previously mentioned TmALs in its arborization pattern in the medulla. Its arborizations in the ML6–10 are not as distinct as in the other two types, but several branches along the neurite are found. Distally the neurite terminates within ML6–10 and was traced to the border of the OCH2 before it left the data set, so it is likely that its main neurite reached the OLO.

3.2.2 | Group 2 (M neurons 1–7)

The second group of synaptic partner neurons for TmA1 and for TmA2, are all bushy and run mainly perpendicular to the TmA neurons and therefore are likely to connect different columns in the medulla and are referred to as M neurons (morphology; Figures 5a,c,d and 7a–k; and synaptic connectivity with TmA2: Table 4). Because our data set was limited to approximately one column, these reconstructions are within a 15–20 μm radius around each TmA neuron so the M neurons were not fully reconstructed. The wiring diagram in Figure 9 gives an overview of the numerical strength of all the connections with TmA2 and between the neurons that connect with TmA2. The connections reveal a complex neuronal circuit around the TmA2: (i) TmAL3 and TmAL4 neurons are synapticaly connected with TmA2; (ii) TmA2 and the TmAL neurons all have many more input synapses than output synapses, revealing they mainly receive signals in the medulla; (iii) Four out of seven medulla interneurons (M2-5) are connected with input synapses to TmA2, while the other three (M1, 6, 7) are connected with both IP and OP synapses. Then there are feedback circuits involving two neurons and the TmA2, for example, neuron M7 and TmAL3 share six synapses together: three OP synapses, giving input to TmA3 and M7 respectively, then both connect with and receive inputs from TmA2. TmA4 and M1 neurons are connected by one synapse, an output synapse from M1 onto TmA4 (Tables 3 and 4.
and Figure 9). A back-to-back RS made by TmA neurons with an M neuron were only found once (shared with M7 and TmA3).

### 3.3 Neurons neighboring TmA1 and 2 in the medulla

In both animals, we traced neurons located close to each TmA neuron at the proximal border of the medulla (ML10). In Animal 1, we followed nine neurons within the same bundle as TmA1, in the OCH2. (Figure 10a). Three diverged from the TmA1 neuron and were lost shortly after entering the medulla. The other six neurons stayed close to the TmA1 neuron for a longer distance, one resembling the TmA1 neuron's morphology (Figure 10c, neuron 10). Only one of those traced neurons displayed regions with a high number of branches (Figure 10c, neuron 3). All the other neurons had no or only a few branches (Figure 10c, neurons 4, 5, 6, 9, and 10). These points are illustrated in 3D for neurons 2-10, surrounding TmA1 in Movie S1.

FIGURE 10  Skeletonized cells in the medulla. (a) Electron micrograph in the second optic chiasm, approximately 10 μm before entering the medulla in Animal 1. The over-laid colors mark all the cells that were traced using the filament editor module in Amira. (b) Reconstruction of the trans-medullary afferent 1 (TmA1) neuron (reconstruction start in the medulla, TmA1 is neuron 1) and the skeletonized cells (starting in chiasm). (c) Visualization of all the skeletonized neurons (2-10). Main branches that are leaving the field of view are marked with an “x” (color code matches the color overlay in [a]). See also Movie S1 with TmA1 and near-by neurons 2-2-10 as in (b). (d) Electron micrograph at the transition from the second optic chiasm (OCH2) to the medulla in Animal 2. (e) 3D visualization of the skeletons together with the reconstruction of the TmA2. TmA2 neuron is designated neuron 1. (f) Visualization of all the skeletonized neurons (2-24). Main branches that are leaving the field of view are marked with an “x” and main branches terminating are marked with “T.” (g) Silver stain (Blest, 1961) of a horizontal section through the medulla in Animal 2. Three diverged from the TmA1 neuron and were lost shortly after entering the medulla. The other six neurons stayed close to the TmA1 neuron for a longer distance, one resembling the TmA1 neuron’s morphology (Figure 10c, neuron 10). Only one of those traced neurons displayed regions with a high number of branches (Figure 9c, neuron 3). All the other neurons had no or only a few branches (Figure 9c, neurons 4, 5, 6, 9, and 10). These points are illustrated in 3D for neurons 2-10, surrounding TmA1 in Movie S1. [Color figure can be viewed at wileyonlinelibrary.com]
Animal 2 seven neurons within the bundle containing the TmA2 neuron and 16 more within a radius of 15 µm around this bundle were followed. Three neurons (Figure 10f, neurons 3, 4, and 22) resembled TmA2 in morphology, two of those (Figure 10f, neurons 4 and 22) terminated in the proximal medulla (ML6–10) without branching, and another one displayed two small side branches before terminating (Figure 10f, neuron 3). One more neuron without branches was traced over a longer distance but left the block-face before terminating (Figure 10f, neuron 5). In the bundle containing TmA2, most of the traced neurons did not show any or only sparse branching. This stands in contrast to those traced neurons which were not part of a clear bundle (Figure 10d, blue-labeled neurons) or were from another bundle 5–10 µm away from TmA2 (Figure 10d red-labeled neurons). Almost all of those blue- and red-labeled neurons show extensive branching (Figure 9f, neurons 12, 16–24). In Figure 10f, red neuron 21, and Figure 10c, magenta neuron 3, both indited by a red asterisk in the figure, show similar branching patterns and could be from the same neuron class. Unfortunately, the neurons from a third bundle in the same animal (Figure 10f, green-labeled cells) left the block-face after a short distance. Only one of those neurons (Figure 10f, neuron 10) was traced over a longer distance and showed some branching (Figure 10d). The relationships of the TmA1 or 2 to their neighbors are shown in Figure 10b,e respectively, and in relation to the overall neural composition of the medulla in the silver stained section (Blest, 1961) in Figure 10g. This can also be seen in 3D for cells surrounding TmA2 in Movie S2.

4 | DISCUSSION

To our knowledge, this is the first description of the anatomy and connectivity of columnar TmAs synaptically connected with the LGMD1, a well-known looming detector in the locust (Gabbiani et al., 2002; Rind & Simmons, 1992; Schlotterer, 1977). We traced two different TmA neurons (TmA1 and 2) in excess of 300 µm from their synapses with the LGMD1 back along their length within a block-face, centered on a single medulla column (Figure 1). Previously, only very few publications showed the neuroanatomy of TmA cells (Rind et al., 2016; Strausfeld & Nässel, 1981; Wang et al., 2018). Moreover, the work we present here is the first description of those cells that are presynaptic to the LGMD1 from their origin in the medulla to their terminals in the lobula complex. TmA1 and 2 have a similar overall shape to the two classes of neurons filled by trans-synaptic migration of cobalt stain from the main sub-field A of the LGMD1 (Strausfeld & Nässel, 1981). Like TmA1, one class had dendrites in ML 1–3 and the other class, had branches in ML 6–10 only (T cells in figure 67, Strausfeld & Nässel, 1981). Recently, Wang et al. (2018) also showed fluorescence images of en mass medullary interneurons in close association with the LGMD1 but individual afferents were not followed to their origin in the medulla. However, light activation of the afferents using optogenetic stimulation led to depolarization of the LGMD1 indicating a likely connection between them. Wang et al. (2018) showed that the side branches leading to the TmA cell bodies have lengths of up to approximately 100 µm, which is well beyond the width of our data set and may explain why we were not able to reconstruct the TmA’s cell bodies.

The synapses of afferents onto the LGMD1 showed some differences with previous studies. A characteristic of the TmA neurons is that they exhibit output synapses both with the LGMD1 and with their neighbors in a reciprocal arrangement, with pairs of presynaptic terminals back-to-back with one another sharing a synaptic cleft (Rind & Simmons, 1998). Our study using SBEM showed that most TmA output synapses onto the LGMD1 were not reciprocal; at least we found no presynaptic densities back-to-back in many cases. This could be due to the immaturity of the locust. However, this is not a good explanation for two reasons; previous work has shown that there is already a functional, LGMD1 driven collision avoidance behavior in L4 and RS are in evidence even in L1 (Simmons et al., 2013; Sztarker & Rind, 2014). Plus, we chose a part of the LGMD1 dendritic tree that was not adding new synapses, we began our reconstruction at medium sized branches of the LGMD1 as it is known that newly formed facets of the eye are integrated into the anterior eye margin with every instar (Anderson, 1978). The anterior eye margin is connected with the finest branches of the LGMD1 (Peron et al., 2009), and it is these finer branches which are formed during postembryonic larval development (Sztarker & Rind, 2014). In this way, we aimed to avoid newly connecting synapses that are still in development. A different explanation could lie in our very conservative approach: we only included synapses if at least two out of three independent researchers agreed that they were clearly visible. Moreover, the enhanced osmium staining protocol (Deerinck et al., 2010) applied here has been shown to stain certain proteinaceous structures like the mammal postsynaptic density less intensely than previous protocols (Capetian et al., 2020), so some presynaptic bars may have been excluded because of insufficient staining. This is a possibility, as when TEM methods with standard contrasting were used in L1 and L4 RS were abundant in the LGMDs (figure 9 in Simmons et al., 2013). This exclusion of presynaptic densities may lead to an underestimate of synaptic connectivity but will not lead to false synapse identification.

We also found chemical synapses between the TmA1 and 2 neurons and neighboring neurons in the OCH2. In the OCH2 the TmA neurons mostly received synaptic input and it would be of great interest to know what cell type is involved here. In Drosophila, synapses occur between neurons in the OCH2 but they are rare and involve amacrine cells (ME-OCH2 cell; see figure 5A,B in Shinomiya, Huang, et al., 2019). These observations, may suggest that the chiasm is not merely a bundle of axons going elsewhere, but is also functional neuropil modulating visual information dynamically.

The TmA’s synaptology in the medulla gives new insight into the processing stages of the collision detection pathway. The presence of predominantly input synapses in the medulla fits well with assumptions made for early-simplified computational models of the LGMD’s circuit, where it was assumed that the TmA neurons receive input from LMC neurons in the medulla (Rind & Bramwell, 1996; Yue et al., 2006). However, the LMC neurons terminate in the distal
medulla in ML1–3 (Elphick et al., 1996) and only TmA1 has input and output synapses there in ML1–3. TmA2 at least must interact synaptically with neurons other than the LMCs. An option that cannot be excluded is that TmA1 neurons receive direct synaptic input from the only long photoreceptor cell R7, which terminates in the distal medulla, in layer 3 (Nowel & Shelton, 1981; Schmeling et al., 2015). Particularly, as some of the processes in contact with the TmA neurons exhibited similarities with photoreceptor terminals, such as dark cytoplasm and numerous mitochondria (data not shown). But, the afferents synapsing with the LGMD are excited by both luminance increments (ON channel) and decrements (OFF channel: O’Shea & Rowell, 1976) and while LMCs can be excited by both luminance increments and decrements, photoreceptors including R7 are depolarized by luminance increments only (Stuart, 1999). Off excitation must be delivered from neurons other than R7. TmA2 has many synapses in the medulla (ML6–10) both inputs and outputs. Curiously, apart from varicosities and a few protrusions that did not exceed 3 μm, we did not find side branches from the main neurite, a situation which was shown for the Tm2 neurons in layer M10 of the Drosophila medulla (Shinomiya, Horne, et al., 2019), but is not common among the trans-medullary neurons involved in the directionally selective EMD in insects (Nowel & Shelton, 1981; Fischbach & Dittrich, 1989; Takemura et al., 2017; Shinomiya, Huang, et al., 2019; bee Ribi & Scheel, 1981; summarized in Borst et al., 2020). Looking at the synaptic arrangement in 3D, we found a small proportion of back-to-back R5 in the medulla. To our knowledge, such an arrangement has not previously been described for the medulla and its functional significance would need further studies.

In Drosophila, all optic flow-based behaviors including landing, freezing, and escape depend on the same local direction-selective motion detectors. Detection of the patterns of motion indicating looming involve precisely oriented local T4 and T5 motion-direction-selective cells in the lobula which provide specific inputs to lobula plate/lobula columnar, type 2 (LPLC2) that are then selectively excited by local outward motion and inhibited by inward. LPLC2 cells indicate the size of a looming object while lobula columnar, type 4 (LC4) visual projection neurons provide an estimate of the object’s velocity and both output onto the giant fiber driven escape pathway that determines the speed of escape or landing depending on its time course of activation (Ache et al., 2019; Klapoetke et al., 2017; von Reyn et al., 2017). This reliance on specific direction-selective inputs for looming detection is notably different from the LGMD pathway where motion in any direction is excitatory with maximum excitation if motion is spatially continuous and rapidly increases in angular velocity (Jones & Gabbiani, 2010; Peron et al., 2009; Peron & Gabbiani, 2009; Simmons & Rind, 1992; Zhu & Gabbiani, 2016).

Although we are confident in finding small side branches even in neurons that we only skeletonized, there is a possibility certain side branches were missed. Nevertheless, the fact that we found both input and output synapses concentrated on these naked neurites (Figure 3) led us to assume that the LGMD pathway is quick which may lead to simplified anatomy in its input neurons. The neurons, such as TmA2, did have regular knobby varicosities throughout the medulla that often contained mitochondria. TmA1 resembles Tm9 that provides excitation to T5, the OFF channel of the EMD for direction selective motion detection in Drosophila (Shinomiya et al., 2014; Shinomiya, Huang, et al., 2019; Takemura et al., 2017). The LGMD1 reacts most strongly to luminance decreases (Simmons & Rind, 1992).

Two classes of synaptic partners of the TmA1 and 2 neurons were traced and reconstructed in the OCH2 and medulla: first, TmA-Like (TmAL) neurons that accompany the TmAs crossing towards the LOX in the OCH2 (on two occasions, TmA2, TmA3, Figures 8 and 9) or at least enter the OCH2 (on two occasions, TmA1, TmA4, Figures 5 and 7, respectively). Based on their branching pattern in the medulla different types of these TmA neurons where distinguished, creating the possibility of additional targets downstream in the LOX, including the LGMD2 (Simmons & Rind, 1997). Second, were the intra-medulla interneurons (M neurons in the Figures 5 and 7), these were oriented in a tangential direction, perpendicularly to the axons of TmA 1 and 2 in the medulla, so only a short part of these neurons were traced. Hence, we can only speculate as to whether these inter-neurons connect different columns in the medulla and/or if the reconstructed parts are branches of neurons which connect upstream to the lamina or connect to the LOX. The M neurons of TmA1 were located in ML1-3 while those synapsing with TmA2 were located in ML6-7. The only bushy neuron with multiple tangential dendrites reconstructed from the vicinity of TmA2 arborized in ML6-10 and was restricted to one column.

We further reconstructed the outlines of 32 neurons with axons within 20 μm of TmA1 or 2 at the border between OCH2 and the medulla. Of these, three looked like either TMA1 or 2, one similar to TmA1 in Animal 1, and two similar to TmA2 in Animal 2. The latter terminated in the proximal medulla without displaying any branches. It is possible that certain branches are missed during tracing, especially if they are parallel to the section and thinner than the section thickness of 50 μm. But finding three neurons entering the medulla and terminating there without branching makes it more unlikely that branches were missed during the tracing process plus as mentioned previously synapses occurred on the neurite itself (Figure 3).

Early investigations suggested that the TmA1 or 2 would be excited by luminance changes in both directions: darkening or lightening although they would have a preference for darkening (O’Shea & Rowell, 1976; Simmons & Rind, 1992). Candidate TmA responses were described by Osorio (1987) who recorded and stained neurons in the medulla most sensitive to movement which, because of their brief responses and preference for motion he termed “transient and non-linear.” Osorio stained three examples that projected from the medulla towards the LOX. Two had Arborizations in the ML1-3 that resemble TmA1 and TmA1 in that they have a plume of dendrites, but also, like TmA1-3 but not TmA1, they have two small fields of arborization within a single column in ML6-10. Their responses were very similar to the LGMD1: they gave phasic responses, typically a single spike, to any supra-threshold luminance change and a burst of spikes to bar motion; the timing of their responses was precise, typically the standard deviation of the spike latency was only 2 ms; their spiking responses adapted so that they did not give a maintained response to stimulus frequencies above 10 Hz.
They responded equally to light increments and decrements over the range tested. They had small receptive fields compared with the LGMD1 their sensitivity to a 2° spot dropped by 50% 2°–3° from the receptive field center, and by 90% 5° from the center suggesting a receptive field of <20°.

In summary, we were able to 3D-reconstruct two TmA neurons, each in a different fourth instar locust. In the medulla, they interact with similar-shaped neurons to themselves, neurons that are classified as TmA-like, as well as with more tangentially oriented neurites. There are significant differences in branching patterns between them in both the medulla and lobula, indicating that there may be several classes of TmA neurons. Their anatomy is consistent with them being the TmA neurons termed transient and nonlinear by Osorio (1987) because they responded briefly to local luminance changes and to motion in any direction. Further studies will be necessary to confirm this. The LGMD pathway is an example of an escape/hiding behavior that is not mediated by neurons dedicated to optic flow analysis but to object detection and motion analysis, and this contrasts to the giant fiber mediated escape responses to looming optic flow in Drosophila (Ache et al., 2019; Klapoetke et al., 2017; von Reyn et al., 2017).

ACKNOWLEDGMENTS

We are very thankful for the technical support by Claudia Mayrhofer and Daniel Kummer. This work was financially supported by the Austrian Science Fund FWF-projects P 32058 and P 32376, EU-2020 RISE, STEP2DYNA (691154), and the Styrian Government—Grant HTI:SMApp program 2012. As a result of this project, a new project evolved, which enabled the build-up of a new SEM Zeiss Sigma 500 VP and an automated tape-collecting ultra-microtome (ATUMtome): “HRSM-Projekt ELMInet Graz - Korrelative Elektronenmikroskopie in den Biowissenschaften” (i.e., a cooperation within BioTechMed-Graz), which was financed by the Austrian Federal Ministry of Education, Science and Research (BMBWF).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

All authors had full access to all the data in the study and take responsibility for its integrity and the accuracy of the data analysis. Study concept: Gerd Leitinger. Experimental design: Stefan Wernitznig, Gerd Leitinger, Claire Rind, Armin Zankel, Peter Pölt. Microscopy: Stefan Wernitznig, Armin Zankel, Peter Pölt. Sample preparation, Segmenting, Visualizing: Stefan Wernitznig, Elisabeth Bock, Daniel Gütl, Ulrich Hobusch, Manuela Nikolik, Lukas Pargger, Elisabeth Pritz, Snježana Radulović, Mariella Sele, Susanne Summerauer. Analyzing the data: Stefan Wernitznig. Data interpretation, writing of the manuscript: Stefan Wernitznig, Claire Rind, Gerd Leitinger. All authors proofread and edited the manuscript.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/cne.25227.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in datadryad at https://doi.org/10.5061/dryad.3j9kd51hc.

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