Orcokinin in the central complex of the locust *Schistocerca gregaria*: Identification of immunostained neurons and colocalization with other neuroactive substances

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Abstract
The central complex is a group of highly interconnected neuropils in the insect brain. It is involved in the control of spatial orientation, based on external compass cues and various internal needs. The functional and neurochemical organization of the central complex has been studied in detail in the desert locust *Schistocerca gregaria*. In addition to classical neurotransmitters, immunocytochemistry has provided evidence for a major contribution of neuropeptides to neural signaling within the central complex. To complement these data, we have identified all orcokinin-immunoreactive neurons in the locust central complex and associated brain areas. About 50 bilateral pairs of neurons innervating all substructures of the central complex exhibit orcokinin immunoreactivity. Among these were about 20 columnar neurons, 33 bilateral pairs of tangential neurons of the central body, and seven pairs of tangential neurons of the protocerebral bridge. In silico transcript analysis suggests the presence of eight different orcokinin-A type peptides in the desert locust. Double label experiments showed that all orcokinin-immunostained tangential neurons of the lateral accessory lobe cluster were also immunoreactive for GABA and the GABA-synthesizing enzyme glutamic acid decarboxylase. Two types of tangential neurons of the upper division of the central body were, furthermore, also labeled with an antiserum against Dipallatostatin I. No colocalization was found with serotonin immunostaining. The data provide additional insights into the neurochemical organization of the locust central complex and suggest that orcokinin-peptides of the orcokinin-A gene act as neuroactive substances at all stages of signal processing in this brain area.

KEYWORDS
allatostatin, glutamic acid decarboxylase, immunocytochemistry, insect brain, RRID: AB_2307385, RRID: AB_2307443, RRID: AB_2313971, RRID: AB_2314455, RRID: AB_2314497, RRID: AB_2315017, RRID: AB_2315056, RRID: AB_2337258, RRID: AB_2337423, RRID: AB_2337925, RRID: AB_2340411, RRID: AB_2340607, RRID:
1 | INTRODUCTION

Arthropod orcokinins and molluscan pedal peptides are members of a large and diverse family of neuropeptides with occurrence in proto- and deuterostomian phyla (Jękely, 2013; Jiang, Kim, & Park, 2015; Lin, Egertová, Zampronio, Jones, & Elphick, 2018; Tanaka, 2016). In insects, alternative splicing of the orcokinin gene leads to the expression of two precursors containing orcokinin isoforms with distinctly different amino acid sequences, now commonly termed orcokinin-A type peptides, corresponding to the originally identified orcokinins from Crustacea (Bungart, Hilbich, Dircksen, & Keller, 1995; Dircksen, Burdzik, Sauter, & Keller, 2000; Dircksen et al., 2011; Yasuda-Kamatan i & Yasuda, 2000), and orcokinin B (Sterkel et al., 2012) which closely resemble orcomyotropin-like peptides but not the true orcokinins (Dircksen et al., 2000; Dircksen et al., 2011). While in Dro sophila melanogaster the prepropeptides of both isoforms contain only a single biologically active orcokinin A and orcokinin B peptide (Chen et al., 2015), eight different orcokinin A and six different orcokinin B peptides have been predicted in the locust Locusta migratoria (Hou, Jiang, Yang, Wang, & Kang, 2015). Immunostaining and in situ hybridization studies suggest that orcokinins are widely distributed in interneurons and neurosecretory cells of the insect brain and ventral nerve cord, as well as in neuroendocrine cells of the midgut (Chen et al., 2015; Hofer, Dircksen, Tollböck, & Homberg, 2005; Hofer & Homberg, 2006; Jiang et al., 2015; Wulff et al., 2017). The physiological roles of orcokinins are still poorly understood, but appear to be pleiotropic and species-specific. At the neuroendocrine level, orcokinin has prothoracicotropic activity in the moth Bombyx mori (Yamanaka et al., 2011), a role in oogenesis in the cockroach Blattella germanica (Ons, Belles, & Maestro, 2015), and is involved in signaling pathways regulating ecdysis in the kissing bug Rhodnius prolixus (Wulff et al., 2017; Wulff, Capriotti, & Ons, 2018). RNAi knockdown of orcokinins (A and B) in larval and adult beetles (Tribolium castaneum) promoted death feigning responses, suggesting that orcokinins have “awakening” effects (Jiang et al., 2015). In the cockroach Rhyparobia maderae, injections of orcokinin near the accessory medulla, the internal circadian clock, led to phase-dependent shifts in circadian locomotor activity, indicating an involvement in the circadian system of the animals (Hofer & Homberg, 2006).

Besides the circadian system, the central complex is a focus of neuroendocrine-containing interneurons in the insect brain (Nässel & Homberg, 2006; Pfeiffer & Homberg, 2014). The central complex plays a key role in the control of spatial orientation and navigation. The central complex spans the midline of the insect brain and is composed of four substructures, the protocerebral bridge, the upper division of the central body (CBU), also termed fan-shaped body (FB), the lower division of the central body (CBL), also termed ellipsoid body (EB), and a pair of globular shaped noduli (Pfeiffer & Homberg, 2014). Based on data from several insect species the central complex is a brain region controlling spatial orientation during flight and walking based on sky compass signals, landmark signals, proprioceptive input, as well as spatial memory (Green & Maimon, 2018; Honkanen, Aden, da Silva Freitas, & Heinze, 2019; Pfeiffer & Homberg, 2014; Varga, Kathman, Martin, Guo, & Ritzmann, 2017). As shown in D. melanogaster and the desert locust Schistocerca gregaria, the central complex has an internal compass-like organization with topographic 360° representations of space (Green et al., 2017; Heinze & Homberg, 2007; Pegel, Pfeiffer, Scholtyssek, & Homberg, 2019; Zittrell, Pfeiffer, & Homberg, 2020; Seelig & Jayaraman, 2015). Its anatomical and neurochemical organization has been studied in great detail in the desert locust (Homberg, 2002; Pfeiffer & Homberg, 2014). Immunostaining using antisera against neuroactive substances provided evidence for the involvement of γ-aminobutyric acid (GABA), serotonin, dopamine, nitric oxide, and neuropeptides related to tachykinins, allatostatins, and allatotropins in central-complex circuits involved in sky compass coding (Wendt & Homberg, 1992; Vitzthum, Agricola, & Homberg, 1996; Vitzthum & Homberg, 1998; Kurylas et al., 2005; Homberg, Vitzthum, Müller, & Binkle, 1999; Beetz, el Jundi, Heinze, & Homberg, 2015). Hofer et al. (2005) demonstrated the presence of orcokinin in the locust central complex but did not identify the types of immunolabeled neurons. As a basis for future functional studies, the present study provides details on the identity and organization of orcokinin-immunolabeled neurons of the locust central complex. Double label experiments, in addition, compare the pattern of orcokinin labeling with GABA-, serotonin-, and allatostatin immunostaining.

2 | MATERIALS AND METHODS

2.1 | Animals

Experiments were performed on adult male and female desert locusts, S. gregaria. Animals were raised under crowded conditions at 27°C, about 60% relative humidity, and a 12:12 LD photoperiod at the University of Marburg. Only sexually mature individuals, at least 1 week after their final molt, were used for experiments. All animal procedures were in compliance with the guidelines of the European Union (Directive 2010/63/EU) and the German Animal Welfare Act.

2.2 | Orcokinin immunolabeling

Immunocytochemical staining for orcokinins was performed on vibratome sections using the peroxidase-antiperoxidase (PAP) technique (Sternberger, 1979). Animals were anesthetized by cooling to 4°C or on ice. Brains were dissected and fixed for 4 h or overnight in
a solution containing 4% paraformaldehyde in sodium phosphate buffer (0.1 mol l⁻¹, pH 7.4). Following rinses in phosphate buffer brains were embedded in gelatin/albumin as described by Hofer et al. (2005) and sectioned in frontal or sagittal plane with a vibrating-blade microtome (Leica Microsystems, Wetzlar, Germany) into 30- or 40-μm slices. The sections were rinsed in saline-substituted Tris buffer (SST; 0.1 mol l⁻¹ Tris–HCl/0.3 mol l⁻¹ NaCl, pH 7.4) containing 0.1% Triton X-100 (TrX) and processed further following the PAP technique (Sternberger, 1979). After preincubation in 5% normal goat serum (NGS; Dianova, Hamburg, Germany) in SST with 0.5% TrX (1 h, room temperature), the sections were incubated overnight at room temperature with primary polyclonal rabbit-antiserum against Asn¹³-orcokinin (Bungart, Dircksen, & Keller, 1994, characterized for insects in Hofer et al., 2005, see also below), diluted at 1:6000 to 1:12,000 in SST containing 0.5% TrX and 2% NGS. The secondary antiserum, goat anti-rabbit (1:40; RRID: AB_261363) and the PAP complex (rabbit PAP, 1:300; RRID: AB_2315056) were diluted in SST, 0.5% TrX and 2% NGS and incubated for 1 h at room temperature each. Following thorough rinses, the sections were treated with 3,3'-diaminobenzidine tetrahydrochloride (0.33 mg/ml, Sigma Aldrich) and 0.05% H₂O₂ in sodium phosphate buffer (0.1 mol l⁻¹, pH 7.4) for 15–30 min. After staining, the sections were rinsed in sodium phosphate buffer, dehydrated, cleared, and mounted in Entellan (Merck, Darmstadt, Germany) on chrome alum/gelatin-coated slides.

### 2.3 Double label experiments

All double labeling of orcokinins and γ-aminobutyric acid (GABA), dissected brains were immersed in fixative solution containing 4% formaldehyde and 0.5% glutaraldehyde in sodium phosphate buffer (NaPi, 0.1 mol l⁻¹, pH 7.4) for 2 h at 4°C and for an additional hour at room temperature. Brains were rinsed 4 x 15 min in NaPi, embedded in gelatin/albumin, fixed overnight in 8% formaldehyde in NaPi, and sectioned into 40-μm slices as described above. Following rinses in SST with 0.1% TrX, sections were incubated in 0.1% TrX and 10 mg/ml sodium borohydride (NaBH₄) in NaPi to reduce background autofluorescence (Clancy & Cauller, 1998). Sections were rinsed 3 x 5 min and 3 x 10 min and were then preincubated for 1 h in 10% NGS in SST with 0.5% TrX. Sections were incubated for 1 day with the primary antibodies, rabbit anti-Asn¹³-orcokinin diluted 1:6000, and guinea pig anti-GABA, diluted 1:1000 in SST containing 0.5% TrX and 1% NGS. Following thorough rinses, the sections were incubated for 1 h with the secondary antibodies, Cy2-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, cat# 111225-003; RRID: AB_2307385), and Cy3-conjugated goat anti-guinea pig IgG (Jackson ImmunoResearch, cat# 106165-003, RRID: AB_2337423) each diluted 1:300 in SST containing 0.5% TrX and 1% NGS. Sections were rinsed 3 x 15 min in SST with 0.1% TrX, mounted on chrome alum/gelatin-coated glass slides and left to dry for at least 2 h. Sections were dehydrated, cleared, and embedded in Entellan (Merck, Darmstadt, Germany).

For double labeling of orcokinin and serotonin, and orcokinin and Dip-allatostatin, brains were fixed overnight in 4% paraformaldehyde in NaPi (0.1 mol l⁻¹, pH 7.4). After rinsing, brains were embedded in gelatin/albumin and sectioned in frontal plane into 40-μm slices as described above. For orcokinin-serotonin double labeling, sections were preincubated in 5% normal donkey serum (NDS; Jackson ImmunoResearch, cat# 017000-121; RRID: AB_2337258) for 2 h at room temperature. Sections were incubated overnight with the primary antibodies, anti-Asn¹³-orcokinin, diluted 1:3000, and anti-5HT, diluted 1:7500 in SST containing 0.1% TrX and 2% NDS. Following rinses sections were incubated for 1 h at room temperature with the secondary antibodies, Cy3-conjugated donkey anti-goat IgG (Jackson ImmunoResearch, cat# 705165-003; RRID: AB_2340411) and Cy5-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, cat# 711175-152; RRID: AB_2340607) in SST containing 0.5% TrX and 2% NDS. For orcokinin/Dip-allatostatin double labeling, sections were preincubated for 1 h at room temperature in 5% NDS in SST with 0.5% TrX, followed by overnight incubation with the first primary antibody, anti-Dip-allatostatin, diluted 1:12,000 in SST containing 0.5% TrX and 2% NDS. Following rinses binding sites of the Dip-allatostatin antiserum were masked by incubation with unconjugated goat anti-rabbit Fab fragments (1:50; Jackson ImmunoResearch, cat# 111007-003; RRID: AB_2337925) and 2% NDS for 1 h at room temperature. Sections were incubated with the second secondary antibody, Cy3-conjugated donkey anti-goat IgG, diluted 1:300, and 1% NDS for 1 h. Several rinses were followed by incubation with the second primary antiserum, anti-Asn¹³-orcokinin, diluted 1:6000 and 2% NDS in SST overnight at room temperature. Following thorough rinses, the sections were incubated with the second secondary antibody, Cy2-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, cat# 711225-152; RRID: AB_2340612), diluted 1:300, and 1% NDS in SST for 1 h at room temperature. Embedding followed the protocol described above.

For double labeling of orcokinins and glutamic acid decarboxylase (GAD), dissected brains were fixed overnight in Zamboni’s fixative (4% paraformaldehyde and 7.5% picric acid in phosphate buffer (0.1 mol l⁻¹, pH 7.4)) at 4°C. Following rinses, 40-μm sections were obtained as described above. Sections were rinsed 3 x 15 min in SST containing 0.1% TrX. For unmasking of antigens, sections were successively dehydrated and rehydrated in an ascending ethanol series (50%, 70%, 90%, 95%, 100%, 5 min each) and a descending ethanol series, respectively. After preincubation with 5% NDS in SST containing 0.5% TrX, sections were incubated overnight with the first primary antibody, anti-Asn¹³-orcokinin, diluted 1:4000, and 1% NDS in SST containing 0.5% TrX. After several rinses they were incubated with the first secondary antibody, Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, cat# 711165-152; RRID: AB_2307443), diluted 1:300, and 1% NDS for 1.5 h. Following rinses, sections were incubated with unconjugated goat anti-rabbit Fab fragments, diluted 1:50, and 1% NDS overnight at room temperature. Sections were rinsed 4 x 15 min and incubated with the second primary antibody, anti-GAD, diluted 1:1000 and 1% NDS in SST containing 0.5% TrX for at least 18 h at room temperature. After rinses, sections
were incubated with the second secondary antibody, Cy2-conjugated
 donkey anti-rabbit IgG, diluted 1:300, and 1% NDS in SST for 1.5 h.
Embedding followed the protocol described above.

2.4 Antibody characterization

The polyclonal antiserum against orcokinin (RRID: AB_2315017) was
raised in rabbit against Asn13-orcokinin from the crayfish Orconectes
limosus (Bungart et al., 1994). Specificity of the antiserum was
demonstrated by testing high performance liquid chromatography (HPLC)
fractions of O. limosus ventral nerve cords with an enzyme-linked
immunosorbent assay (ELISA). Tests of C- and N-terminal analogs of
Asn13-orcokinin in ELISA showed that the antiserum is directed
against the C-terminal region of the peptide (Bungart et al., 1994). On
locust brain sections, liquid phase preadsorption of the diluted antisera-
rum with 1 nmol l⁻¹ Asn13-orcokinin abolished all immunostaining in S.
gregaria brain sections (Hofer et al., 2005). Sequence comparison rev-
ealed a high degree of conservation between S. gregaria orcokinin A6,
A7, and A8 and Asn13-orcokinin with 10 amino acids being identical
(Figure 1), suggesting that these peptides are primarily detected by
the antiserum. These three peptides most likely represent the three
immunoreactive HPLC-fractions identified previously in S. gregaria
brain extracts, one of which was even ESI-Q-TOF sequenced (Hofer
et al., 2005) and identical to Scg OK-A8 (see below).

The antiserum against GAD (RRID: AB_477019, Sigma-Aldrich,
St. Louis, MO, cat# G5163) was raised in rabbit against a synthetic
peptide, corresponding to the C-terminal region of both the 65- and
the 67-kDa isoforms of human GAD, coupled to keyhole limpet hemo-
cyanin (KLH) with glutaraldehyde. In Locusta migratoria the staining
pattern of anti-GAD matched with that reported for GABA, and West-
ern blot analysis revealed that the antibody labeled a double band
close to 50 kDa (Stern, 2009), which is in the range of molecular
weights for the two GAD subunits reported in S. gregaria (Stern, 2009;
Stapleton, Tyrer, Goosey, & Cooper, 1989).

The antiserum against GABA (RRID: AB_2314455, Protos
Biotech, New York, NY, cat# NT 108) was raised in guinea pig against
GABA linked to KLH with glutaraldehyde. On cockroach brain
sections, immunostaining was completely abolished following
preadsorption of the diluted antiserum with 10 µmol l⁻¹ GABA-conju-
gate (Schedzzielorz & Stengl, 2014). The staining pattern obtained
with this antiserum on locust brain sections was identical to that
reported with the GAD- and GABA antisera characterized by
Homberg et al. (1999).

The anti-5-HT antibody (RRID: AB_572262, ImmunoStar, cat# 20079)
was raised in goat against serotonin coupled to bovine serum
albumin (BSA) with paraformaldehyde. Immunostaining with this anti-
serum on locust brain sections was indistinguishable from the pattern
of immunolabeling using a different 5HT antiserum (Homberg, 1991).
Labeling was completely eliminated by preadsorption of the diluted

![FIGURE 1](attachment:image.jpg)

**FIGURE 1** (a) Sequence alignment of the orcokinin A prepropeptides from S. gregaria and L. migratoria. (b) Sequence alignment of orcokinin A neuropeptides from S. gregaria and comparison with crayfish Asn13-orcokinin (OrlN13OK, the antiserum epitope). Note that only Scg OK-A6, Scg OK-A7, and Scg OK-A8 are true orcokinins highly similar to Asn13-orcokinin (right panel). Amino acids are highlighted as: black identity, gray close chemical similarity, blue monobasic or dibasic cleavage sites flanking the putatively bioactive peptides (green); pink the only single amino acid exchange occurring in the derived peptide Scg OK-A5. Scg OK-A sequence from Genbank GHHP01003912.1, Lom OK-A sequence according to Veenstra (2014) [Color figure can be viewed at wileyonlinelibrary.com]
antiserum with 100 μg/ml serotonin/BSA conjugate (ImmunoStar product information sheet).

The antiserum against Dip-allatostatin (Jena Bioscience, cat# ABD-062; RRID: AB_2313971) was raised in rabbit against the Diploptera punctata allatostatin I coupled to bovine thyroglobulin using glutaraldehyde (Vitzthum, Homberg, & Agricola, 1996). Non-competitive ELISA revealed no cross-reactivity of the antiserum with corazonin, CCAP, FMRFamide, leucomyosuppressin, locustatachykinin II, perisulfakinin, and proctolin. Preadsorption of the diluted antiserum against Dip-allatostatin I with 10 μmol l⁻¹ of the respective antigen abolished all immunostaining in brain sections of S. gregaria (Vitzthum et al., 1996).

2.5 | Sequence analysis

The S. gregaria orcinokin A transcript (GenBank: GHHP01003912.1, Konopová, Buchberger, & Crisp, 2020) was identified based on the available sequence from L. migratoria (Veenstra, 2014) by using NCBIs BLAST (NCBI Resource Coordinators, 2016) (tblastn against transcriptome shotgun assembly [TSA] archives). The signal peptide was predicted by SignalIP (Petersen, Brunak, von Heijne, & Nielsen, 2011).

2.6 | Image acquisition and reconstructions

Images from PAP-labeled brain sections were captured by a digital camera (ProgRes C12plus, Jenoptik) mounted on a transmission light microscope (Axioskop, Zeiss, Oberkochen, Germany). Images were optimized in contrast and brightness using Photoshop CC 2017 (Adobe Systems, San Jose, CA; RRID:SCR_014235). Immunolabeled neurons were traced from serial PAP-labeled sections through a camera lucida attachment on a compound microscope (Leitz, Wetzlar, Germany). Drawings were digitized with a scanner (CanoScan 9000F Mark II, Canon, Tokyo, Japan). Images and 2D-reconstructions were assembled in CorelDRAW X8 software (Ottawa, Ontario, Canada; RRID:SCR_014235). The terminology of brain areas follows the nomenclature of von Hadedel, Althaus, Häger, and Homberg (2018), the terminology of neuronal cell types is based on Heinze and Homberg (2008), and von Hadedel et al. (2020). Positional information in all figures is given with regard to the animal’s body axis.

Images from fluorescence-labeled brain sections were obtained using a confocal laser scanning microscope (Leica, TCS SP5, Leica Microsystems, Wetzlar, Germany) with a 20x (HC PL APO 20x/0.75 Imm Corr CS2) or 40x immersion objective (HCX PL APO 40x/1.25 Oil). The fluorophores were excited using an argon laser (488 nm) for Cy2, a diode pumped solid state laser (561 nm) for Cy3 and a helium neon laser (633 nm) for Cy5. To avoid cross talk of overlapping emission spectra all specimens were scanned sequentially. Scanning frequency was set to 400 Hz and pinhole size was 1 airy unit. The resolution was 1024 x 1024 pixels per image and the z-step size varied from 0.5–1 μm. Primary processing of image stacks was done in Amira 5.6 (ThermoFisher Scientific, Waltham, MA; RRID:SCR_007353), whereas final figure panels were created in Affinity Photo and Affinity Designer (Serif, Nottingham, UK; RRID:SCR_016951).

3 | RESULTS

3.1 | Analysis of orcinokin A transcript sequences

The orcinokin A transcript of S. gregaria (GenBank GHHP01003912) is 785 bp long and encodes for a 167 residue prepropeptide with a 21 amino acid 5’ signal peptide (Figure 1(a)). It contains eight active neuropeptides ranging from 11 to 14 amino acids, of which only Scg OK-A6, A7, and A8 share residues 1–10 with Asn¹³-orcinokin from O. limosus (Figure 1(b); Tanaka, 2016). Comparison to the orcinokin A transcript of L. migratoria (Veenstra, 2014; Hou et al., 2015) revealed only 10 amino acid exchanges, of which only one is within an active neuropeptide (N→S in OK-A5).

3.2 | Identification of orcinokin-immunolabeled cell types

Analysis of orcinokin immunostaining in the central complex is based on series of PAP-labeled brain sections from 23 sexually mature locusts (11 males and 12 females). Five particularly well labeled brains were used for neuron reconstructions and images. No differences in staining pattern were observed between males and females. The central complex of the desert locust and the adjacent lateral complex are innervated by at least 100 orcinokin-immunolabeled neurons with cell bodies distributed in seven distinct groups or clusters (Figure 2). As reported but not yet fully identified by Hofer et al. (2005) these neurons densely innervate all substructures of the central complex as well as the lateral complex (Figure 3). Tracing fiber profiles through a camera lucida attachment on a transmission light microscope allowed us to partially reconstruct most immunolabeled cell types and identify them based on characteristics in projection pattern.

3.3 | Immunolabeled tangential neurons

The central body is innervated by at least five types of tangential neuron with cell bodies in three out of the seven cell clusters (Figure 2), a cluster in the superior lateral protocerebrum (SLP), a cluster near the lateral accessory lobe (LAL), and a third cluster near a small area posterior from the antennal lobe, the vest (VES). Three neurons of the SLP cluster, ventro-lateral from the mushroom-body calyx were immunolabeled (Figure 4). Their cell body fibers project medially, pass by the pedunculus anteriorly and give rise to side branches in the superior medial/intermediate protocerebrum that could not be traced further owing to dense immunostaining of other neurons (Figure 4(a)). Their main fibers approach the upper division of the central body (CUB) through the anterior bundles, two neurites in close proximity...
and the third one, further apart (Figure 4(a)). Fibers cross the brain midline along the anterior surface of the CBU, giving off beaded side branches that project dorsally into layer I of the CBU (Figure 4(c)). Contralaterally, the main neurites enter dorsal aspects of the lateral accessory lobe, but could not be traced any further. Judged from their pattern of arborizations, the neurons are TUSLP neurons, most closely resembling type TUSLP2 traced earlier by von Hadeln et al. (2020). A number of additional neurons enter the CBU through the anterior bundles, but those neurons showed much weaker immunostaining and could therefore not be reconstructed.

Two types of neuron have cell bodies in the VES cluster, one type innervating the CBU and the second type, the lower division of the central body (CBL). About nine cell bodies in the VES cluster give rise to fibers that project through isthmus tract 4 (von Hadeln et al., 2020) in a wide arc posteriorly bypassing the antennal lobe, then enter the lateral complex (Figure 5(a)). Eight neurites remain fasciculated and pass through the lateral complex toward the central body without side branches (Figure 5(a)). Near the lateral tip of the CBU, small side branches emerge and extend dorsally or anterior medially, but could not be traced over long distances (arrows in Figure 5(a)). The main neurites pass along the posterior face of the central body, dorsal from the noduli, and give off side branches into the CBU. Based on cell body position, fiber course, and branch points laterally from the CBU, these neurons are type TUVES4 neurons. Single cell dye injection showed that TUVES4 neurons have dendritic ramification in the crepine and superior intermediate protocerebrum and innervate layer Ia of the CBU and the anterior lip (fig. 7(f) in von Hadeln et al., 2020). In contrast to the single-cell data, the orcokinin-labeled neurons appear to innervate layer Ila of the CBU (Figure 3(b)). From the common fascicle of neurites above the noduli, side branches extend antero-ventrally toward the border between the CBL and CBU and from here continue into layer Ila. One neuron of the VES cluster is a TL1 neuron. Its cell bodies in the VES cluster give rise to fibers that project through isthmus tract 4 (von Hadeln et al., 2020) in a wide arc posteriorly bypassing the antennal lobe, then enter the lateral complex (Figure 5(a)).
body fiber gradually separates from the fascicle of TUVES4 neurons and takes a slightly different course through the lateral complex (Figure 5(a)). Near the lateral bulb, it gives rise to several major side branches (Figure 5(c)). The main fiber continues via isthmus tract 1 along the posterior face of the CBL, giving off side branches into the CBL. The uniformly dense beaded staining throughout the CBL

**FIGURE 3** Orcokinin immunolabeling in the locust central body. (a) Frontal section through the central body and lateral complex. Layers I and II of the upper division of the central body (CBU) are densely supplied by immunoreactive processes. Immunostained fibers in layer II have a fan-shaped appearance. In the lower division of the central body (CBL) the uppermost layer 1 shows strong immunostaining while all other layers are uniformly supplied by beaded processes. Dorsal aspects of the lateral complex (LX) are more densely supplied by immunostained arborizations than ventral parts. Arrowheads point to somata of TL4 tangential neurons of the CBL and TUJc,2 tangential neurons of the CBU with neurites passing through the LX. (b) Sagittal section through the central body slightly off the brain midline. Vertical arrow indicates plane of section in (a). Layers I–III of the CBU, the CBL and the lower unit of the noduli (NOL) are immunostained. Major fiber bundles that innervate the central body are visible in the posterior chiasma (PCH), dorsally from the upper units of the noduli (NOU; double arrowhead), ventrally from the CBL (asterisk) and at the anterior face of the CBU (arrowhead). a, anterior. Scale bars = 100 μm in (a); 50 μm in (b)

**FIGURE 4** Orcokinin-immunolabeled TUSLP neurons innervating layer I of the upper division of the central body (CBU). (a) Frontal reconstruction of three bilateral pairs of immunostained TUSLP neurons. The neurons have cell bodies ventral lateral from the calyces (CA) of the mushroom body and first ramifications in the superior intermediate protocerebrum (SIP). Axonal fibers project via the anterior bundle along the frontal surface of the CBU, giving off side branches into layer I of the CBU and toward the lateral accessory lobes. (b) Frontal section showing the cell bodies of the three TUSLP neurons (arrowheads). (c) Frontal section through layer I of the CBU and anterior lip (ALI) region. Axonal fibers of TUSLP neurons pass through the anterior bundle (AB) and along the anterior face of the CBU. Side branches extend into layer I of the CBU. Scale bars = 100 μm [Color figure can be viewed at wileyonlinelibrary.com]
largely originates from the immunolabeled pair of TL1 neurons (Figures 3(a) and 5(b)).

About 21 bilateral cell bodies (19–23, n = 6) are immunostained in the LAL cluster. These neurons are of two types, one type, TL4 neurons, innervates the CBL, and the second type, likely TULAL2, innervates the CBU (Figure 6(a)). Together with a small group of commissural neurons of the lateral accessory lobe, they share a similar domain of innervation in the lateral accessory lobe and wedge of the brain (Figure 6(a,e); see fig. 6(c–f) in von Hadeln et al., 2020). From the cell bodies in the LAL cluster primary neurites pass toward the central body (Figures 3(a) and 6(a,b)). Laterally, but especially dorsally from the medial bulb, side branches extend posterior-laterally (Figure 6(c)) and invade a strip of neuropil in the dorsal part of the lower shell of the lateral accessory lobe. This neuropil is one of the brain areas most densely supplied by orcokinin-immunolabeled processes (Figure 6(d)). The main fibers of the immunostained neurons continue toward the brain midline along the posterior ventral face of the CBL. At the brain midline, fibers turn upward. Most processes give rise to dense immunostaining in the uppermost layer 1 of the CBL, characteristic of TL4 neurons (Figures 3(a) and 6(a)). Others continue in a fan-like manner into the CBU and innervate layer IIb with varicose terminals, typical for TU_LAL2 neurons (Figures 3(a) and 6(a)). Along their course through the CBU, fibers fasciculate in a criss-crossing manner. The strip of neuropil in the lower shell of the lateral accessory lobe is, in addition, innervated by four pairs of commissural neurons of the lateral accessory lobe commissure with cell bodies in the cell cortex anterior to the crepine that connect the two neuropils of each side (CLAL in Figure 6(a)). Most processes, presumably from TL4 neurons, terminate at the posterior lateral margin of the lateral accessory lobe, but at least five major fibers and their ramifications continue further laterally and posteriorly into the wedge (Figure 6(a,e)). This is characteristic for TU_LAL2 neurons (von Hadeln et al., 2020). A small area in the lateral complex adjacent to the medial bulb shows dense orcokinin-immunolabeling (asterisk in Figure 6(c)). This has led us in an earlier overview on orcokinin-immunostaining (Hofer et al., 2005) to conclude that TL2 neurons with ramifications in the medial bulb are orcokinin-immunoreactive. However, closer inspection showed no immunolabeled neurites connecting this area to other brain areas, clearly not with the CBL. This suggests that substances immunologically related to true orcokinins are highly confined to terminals in this area.

The protocerebral bridge shows dense beaded staining originating from two types of tangential neuron. At least four neurons per hemisphere connect the posterior optic tubercle and protocerebral bridge,
FIGURE 6  Legend on next page.
indicating that these neurons are TB1- or TB2 types of tangential neuron of the protocerebral bridge (von Hadeln et al., 2020). The posterior optic tubercles are innervated uniformly with fine beaded processes (Figure 7(d)). Cell bodies of the neurons are posterior, but in close proximity to the tubercle-protocerebral bridge tract (double arrowheads in Figure 7(a)). Two to four neurons connect the posterior optic tubercle (POTU) to the PB. Their neurites cross the brain midline in the upper part of the PB, characteristic for TB1- and TB2 neurons. Their cell bodies are posterior from the POTU-PB tract (double arrowheads). (b–d) Frontal sections illustrating parts of the immunolabeled neurons. (b) Section at the level of the posterior slope (PS), showing two cell bodies of TB6 neurons (arrowheads) with wide dendritic ramifications in the PS (arrows). (c) Immunostaining in the PB is of beaded appearance. Arrows point to neurites of TB6 neurons along the ventral edge of the PB. (d) Immunostaining in the POTU is relatively weak. Scale bars = 100 µm in (a,c,d); 50 µm in (b)

**FIGURE 7** Orcokinin immunolabeling of tangential neurons of the protocerebral bridge (PB). (a) Frontal reconstruction of immunostained neurons. Two types of neuron invade the PB. Three neurons with cell bodies lateral or dorso-lateral from the PB (arrowheads) have large diameter neurites extending into the posterior slope (PS). Their axonal fibers pass along the ventral edge of the PB (arrows) characteristic for TB6 neurons (see fig. 4(a) in von Hadeln et al., 2020). Two to four neurons connect the posterior optic tubercle (POTU) to the PB. Their neurites cross the brain midline in the upper part of the PB, characteristic for TB1- and TB2 neurons. Their cell bodies are posterior from the POTU-PB tract (double arrowheads). (b–d) Frontal sections illustrating parts of the immunolabeled neurons. (b) Section at the level of the posterior slope (PS), showing two cell bodies of TB6 neurons (arrowheads) with wide dendritic ramifications in the PS (arrows). (c) Immunostaining in the PB is of beaded appearance. Arrows point to neurites of TB6 neurons along the ventral edge of the PB. (d) Immunostaining in the POTU is relatively weak. Scale bars = 100 µm in (a,c,d); 50 µm in (b)

**FIGURE 6** Orcokinin-immunolabeled tangential neurons with somata in the LAL cluster. (a) Ensemble reconstruction of immunostained TL4 neurons, TUlAL2 neurons, and commissural neurons (CLAL) of the lateral accessory lobes (LAL). About 22 bilateral pairs of neurons with cell bodies between the LAL and the antennal lobe send neurites into the LAL. Together with four bilateral pairs of commissural neurons of the LALs they give rise to dense innervation of a strip of neuropil in dorsal parts of the lower shell of the LAL that extends laterally beyond the LAL into deeper brain areas. The cell bodies of CLAL neurons are located in the anterior protocerebrum near the crepine and have been drawn separately (red dashed arrows). Their main fibers connect the densely innervated areas via the LAL commissure (arrowheads). Axonal fibers of TUlAL2 and TL4 neurons enter the central body at the midline below the CBL and give rise to fan-like innervations of layer 1 of the CBL (TL4 neurons) and layer IIb of the CBU (TUlAL2 neurons; see Figure 3a). (b) Frontal section through the LAL illustrating the position of TL4- and TUlAL2 cell bodies in the LAL cluster (asterisks) and their primary neurites projecting to the central body via isthmus tract 2. (c) At a slightly more posterior level side branches from the TL4- and TUlAL2 neurons join immunolabeled fibers of the LAL commissure (arrows, LALC) running below the medial accessory lobe (MAL). Asterisk indicates an ovoid area of immunolabeled terminals whose processes could not be traced to other brain areas. (d) Further posteriorly the three immunolabeled cell types densely innervate a dorsal strip of neuropil in the lower shell of the lateral accessory lobe (LLAL, asterisks). Arrows point to a fiber fascicle of TUlVES4 neurons, neurontes to the neurite of the labeled TL1 neuron (see Figure 5). (e) Deeper into the brain, the dense innervation area continues into outer aspects of the wedge (WED, asterisk). AL, antennal lobe; AMMC, antennal mechanosensory and motor center; GC, great commissure; ICL, inferior clamp; MALT, medial antennal lobe tract; PLP, posterior lateral protocerebrum; VES, vest. Scale bars = 100 µm in (a,b,d,e); 50 µm in (c) [Color figure can be viewed at wileyonlinelibrary.com]
Figure 7(a,b)). The neurons do not innervate the posterior optic tubercle but instead send large-size fibers into the posterior slope (Figure 7(b)). These neurites were only weakly immunolabeled, suggesting that they are largely dendritic, and could therefore be reconstructed only incompletely (Figure 7(a)). Staining becomes more intense, as fibers of these neurons enter the protocerebral bridge. Main processes of these neurons project along the ventral face of the bridge (arrows in Figure 7(a)) and innervate both hemispheres of the bridge. Their strongly immunolabeled processes in the bridge contribute substantially to the beaded appearance of immunolabeling throughout the bridge. This pattern of arborization indicated that these labeled neurons are TB6 neurons (von Hadeln et al., 2020).

3.4 Immunolabeled columnar neurons

In addition to tangential neurons, a system of columnar neurons exhibits orcokinin immunolabeling. In contrast to the identified tangential neurons, however, immunolabeling of the columnar neurons is much weaker, suggesting that they contain lower levels of orcokinin peptides (Figure 8(b)). Twenty to 22 immunoreactive cell bodies in the posterior pars intercerebralis send neurites into the protocerebral bridge, where they intermingle with processes from tangential neurons. In some cases, however, cell body fibers could be traced to neuronal processes in the posterior chiasma (Figure 8(a)). At the ventral side of the protocerebral bridge 2–3 fibers could be traced in the w-, x-, y-, and z-bundles to the central body. The x- and y-tracts usually had three processes, the w-tracts usually only two (Figure 8(a,b)). The number of labeled fibers in the four bundles corresponded to the number of cell bodies above the bridge. Owing to dense immunostaining, neurites could not be traced as they pass through the central body. Posterior from the central body, however, fibers entered the lower units of the noduli and here gave rise to uniform dense immunostaining (Figure 8(a,c)). Judged from these terminals, the immunolabeled neurons are CL2 neurons which connect single slices of the protocerebral bridge to slices in the lower division of the central body and lower units of the noduli (Heinze & Homberg, 2008; Müller, Homberg, & Kühn, 1997). Interestingly, the nodular innervations showed some variability regarding the innervated volume within the nodulus, including slight asymmetry in the invaded areas in the right and left nodulus (Figure 8(a,c–e)).

**FIGURE 8** Orcokinin-immunolabeled columnar neurons of the central complex. (a) Partial frontal reconstruction of immunolabeled columnar neurons. Twenty-two cell bodies above the protocerebral bridge (PB) send cell body fibers into the PB. Their main fibers project from the PB via the w-, x-, y-, and z-bundles to the central body, where their neurites could not be traced owing to dense immunostaining of other neural systems. Terminal ramifications are present in the lower units of the noduli (NO, arrowheads), suggesting that CL2 neurons are labeled. (b) Frontal section at the level of the posterior chiasma (PCH), showing labeled neurites of columnar neurons between the PB and the central body. (c–e) Frontal sections illustrating dense orcokinin-immunofluorescent staining in the lower units of the noduli (NOL). The innervated volumes within the noduli show some variation between specimens including slight asymmetries, most obvious in (d). The upper units of the noduli (NOU) are free of immunostaining. (a,b,e) Female, and (c,d) male locusts. CBL, lower division of the central body; CBU, upper division of the central body. Scale bars = 100 μm in (a,b); 20 μm in (c–e) [Color figure can be viewed at wileyonlinelibrary.com]
Double label experiments

Double label experiments were performed on two brains each analyzed for colocalization of GABA and orcokinin, GAD and orcokinin, and Dip-allatostatin I and orcokinin, and six brains for analyzing colocalization of serotonin and GABA. Large numbers of tangential neurons of the LAL cluster are immunoreactive for GABA and the GABA-synthesizing enzyme GAD (Homberg et al., 1999; von Hadeln et al., 2020). These include TL2, TL3, and TL4 neurons of the CBL and TULAL1 and TULAL2 neurons of the CBU (Homberg et al., 1999; von Hadeln et al., 2020). Double immunofluorescent labeling for GABA and orcokinin, as well as for GAD and orcokinin showed that all orcokinin-immunoreactive neurons of the LAL cluster exhibit colocalized GABA- and GAD staining (Figure 9). Accordingly, layer 1 of the CBL exhibits prominent double labeling resulting from TL4 neurons and likewise, layer IIb of the CBU (Figure 9(a–d)) confirming that some orcokinin-immunoreactive neurons of the LAL cluster are of type TULAL2. In contrast, no double staining was found in layer lb of the CBU which indicates that TULAL1 neurons do not exhibit orcokinin immunostaining (Figure 9(a)). Likewise, immunostaining for GABA/GAD and orcokinin was not colocalized in layers 2–6 of the CBL, confirming that different cell types are labeled in these layers, TL1 neurons exhibiting orcokinin immunostaining and TL2 and TL3 neurons showing GABA- and GAD immunolabeling.

Serotonin immunostaining of the locust central complex is prominent in the protocerebral bridge. Some of these neurons are columnar neurons with ramifications in the noduli of the central complex (Homberg, 1991). Although both neuropils are also prominently innervated by orcokinin-immunostained neurons, double serotonin-/orcokinin immunofluorescence did not reveal colocalization of both substances in central-complex neurons (Figure 10). This indicates that the protocerebral bridge is innervated by distinct sets of serotonin- and orcokinin-immunolabeled neurons (Figure 10(d–f)). Staining of the noduli supports this conclusion. While layer II of the upper units of
the noduli shows dense serotonin immunostaining, likely resulting from CPU4b neurons (Heinze & Homberg, 2008), the lower units are orcokinin-immunostained, which is characteristic for CL2 neurons (Figure 10(a–c)).

Neurons of the protocerebral bridge, CBU and CBL are, furthermore, immunoreactive for the neuropeptide Dip-allatostatin I (Vitzthum et al., 1996). Among these are TB1/TB2 neurons of the protocerebral bridge, TUVES4 and TUAL2 neurons of the CBU, and a...
FIGURE 11  Double immunofluorescent staining for orcokinin (green) and Dip-allatostatin (Dip-Ast; magenta) in the central complex. (a–c) Colocalization of orcokinin and Dip-Ast occurs in all processes of TUVES4 neurons in layer IIa and TULAL2 neurons in layer IIb of the CBU. (d–f) Close inspection shows that Dip-Ast immunostaining in layer 1 of the CBL is colocalized with orcokinin labeling (arrowheads), indicating that a few TL4 neurons express both peptides. (g–i) In the lateral accessory lobe cluster, about 50% of orcokinin-immunolabeled neurons (11–13 somata) are also immunoreactive for allatostatin A. (j–l) In the vest cell cluster, double labeling was found in about nine cell bodies, but whether all of these are TUVES4 neurons could not be determined. (m–o) Orcokinin and Dip-Ast are not colocalized in the protocerebral bridge (PB). AL, antennal lobe; LAL, lateral accessory lobe. Scale bars = 100 μm in (a) (applies to a–c); 30 μm in (d) (applies to d–f); 50 μm in (g) (applies to g–i); 50 μm in (m) (applies to m–o); 40 μm in (j) (applies to j–l) [Color figure can be viewed at wileyonlinelibrary.com]
few TL4 neurons of the CBL (Vitzthum et al., 1996; von Hadeln et al., 2020). Double labeling for orcokinin and Dip-allatostatin I revealed colocalization of both peptides in partly overlapping sets of TU_{VES4} neurons, innervating layer IIa, and TU_{LAS2} neurons with terminals in layer IIb of the CBU (Figure 11(a–f)). Colocalization of sparse Dip-allatostatin A labeling with anti-orcokinin in layer 1 of the CBL indicates that a few TL4 neurons of the CBL (Figure 11(d–f)) also contain both peptides. No colocalization was found in neurons of the protocerebral bridge (Figure 11(m–o)).

4 | DISCUSSION

Neuropeptides of the orcokinin A family are present in about 50 bilateral pairs of interneurons innervating the locust central complex. Anatomical tracings and comparison with catalogs of neuronal cell types (Heinze & Homberg, 2008; Müller et al., 1997; von Hadeln et al., 2020) allowed us to identify the immunostained neurons as one type of columnar neuron (CL2), two types of tangential neuron of the CBL (TL1 and TL4), three types of tangential neuron of the CBU (TU_{SLP}, TU_{LAS2}, and TU_{VES4}), and at least two types of tangential neuron of the protocerebral bridge (TB1/TB2 and TB6). Of these, CL2-, TL1-, TL4-, and TB1/2 neurons are involved in sky compass signaling in the locust brain (Heinze & Homberg, 2009; Pfeiffer, Pfeiffer, & Homberg, 2018). Double label experiments revealed colocalization of orcokinin A peptides with GABA and Dip-allatostatin I in certain tangential neurons of the central body. Most of these cell types appear to have morphological counterparts in the brain of the fly Drosophila (Franconville, Beron, & Jayaraman, 2018; Omoto et al., 2018; von Hadeln et al., 2020; Young & Armstrong, 2010), including CL2 neurons (PEN cells in the fly), TL1 neurons (likely homologous to ExR4), TL4 neurons (likely homologous to R1 neurons of the ellipsoid body), TU_{SLP} neurons (ExF2 neurons in Drosophila), and TB1 and 2 neurons (probably homologous to A7 neurons). Of these, at least PEN and A7 neurons are, likewise, involved in head-direction coding, but none of those cell types apparently contain orcokinin-related peptides (Chen et al., 2015).

4.1 | Specificity of immunostaining

Analysis of the orcokinin A transcript predicts eight peptides in S. gregaria, termed Scg OK-A1 to A8. Except for a single amino acid exchange in Scg OK-A5, these peptides are identical to Lom OK-A8 from the migratory locust Locusta migratoria (Veenstra, 2014; Hou et al., 2015). Three of these peptides, Scg OK-A6, A7, and A8 are real orcokinis highly similar to Asn^{13}-orcokinin from the crayfish, used to produce the antiserum (Bungart et al., 1994). They are, therefore, most likely the true orcokinin members in the Scg OK-A transcript that are detected by the antiserum as clearly evidenced by their earlier unambiguous identification by HPLC/immunodot assays and mass spectrometric sequencing in brain extracts of S. gregaria (Hofer et al., 2005). Immunostaining was strong in all identified cell types except CL2 columnar neurons which exhibited relatively weak staining. Whether this is a consequence of low levels or of differential expression of distinct orcokinin members of OK-A peptides in these neurons remains to be investigated. Complete lack of labeling by the Asn^{13}-orcokinin antiserum in the brain of the honeybee, fruit fly and sphinx moth (Hofer et al., 2005) suggests that the antiserum is preferentially directed against the C-terminal region of Asn^{13}-orcokinin which shares only residues 1–6 with the orcokinin A-type peptide from Drosophila and residues 1–8 with two orcokinin A-type peptides from the silk moth Bombyx mori (Chen et al., 2015; Yamanaka et al., 2011).

4.2 | Neurochemical organization of the central complex

The large number of different cell types of the central complex is reflected by a similarly large variety of neuroactive substances mapped in this brain area in various insect species (Pfeiffer & Homberg, 2014). Among these, neuropeptides are a particular prominent class (Nässel & Homberg, 2006). In a previous study, the distribution of orcokinin-related peptides has been mapped in the brains of the silverfish, the Madeira cockroach, and two locust species (Hofer et al., 2005). In all of these species, immunolabeling was prominent in the central complex, but except for some cell types in the locust, the immunolabeled neurons were not identified morphologically. In the fly Drosophila and Bombyx mori larvae, orcokinin-related peptides were detected in brain neurons using an N-terminal specific antiserum, but no labeling was reported in the central complex (Chen et al. 2015; Yamanaka et al., 2011).

The detailed analysis of immunostained cell types of the locust central complex extends and supports the earlier description of orcokinin distribution in the locust brain by Hofer et al. (2005). Immunostaining of TL2 neurons with ramifications in the medial bulb (previously termed medial olive) reported by Hofer et al. (2005), however, was not confirmed. Hofer et al. (2005) interpreted an ovoid structure immunostained ventro-laterally from the central body (asterisk in Figure 6(b)) as the median olive which is densely innervated by GABA-immunoreactive TL2 neurons (Hofer et al., 1999; Müller et al., 1997). Close inspection revealed that this area is distinct from but immediately adjacent to the medial bulb (= median olive). Lack of distinct orcokinin immunolabeling in layers 4 and 5 of the lower division of the central body, which would be typical for TL2 neurons, further supports the notion that TL2 neurons are not orcokinin-immunopositive.

Comparison with an extensive catalog of neuronal cell types innervating the locust central complex (Heinze & Homberg, 2008; von Hadeln et al., 2020) allowed us to identify the immunolabeled neurons based on partial reconstructions. An interesting mismatch with our catalog of cell morphologies was found in the TU_{VES4} neurons. Whereas dye injected TU_{VES4} neurons found so far innervated layer...
la of the CBU (fig. 7(f) in von Hadeln et al., 2020), the orcokinin-immunolabeled neurons had terminal arborizations in layer Ila as has been reported for Dip-allatostatin-immunolabeled TUVES4 neurons (Figure 11(a–c); Vitzthum et al., 1996). As shown for different members of the TL2-, TL3-, and TUSLP tangential cell types, different TUVES4 neurons, therefore, appear to target different central-body layers but have dendritic inputs in the same areas outside the central complex. The reconstructions further show that different cell types of the central complex may occupy similar cell body positions and are thus likely to share the same lineage. This applies to the TUVES4 and TL4 neurons in the LAL cluster and the TUVES4 and TL1 neurons of the VES cluster of cell bodies.

Three orcokinin-labeled cell types, TULAL2 neurons, TL4 neurons, and the commissural neurons of the lateral accessory lobes, termed CLAL neurons, share the same innervation domain outside the central complex in a small dorsal strip of the lateral accessory lobes (Figure 6). These neurons might, therefore, receive similar synaptic input. TL4 neurons are sensitive to visual stimuli, especially to the azimuth of an unpolarized sun-like stimulus (Pegel et al., 2018) suggesting that TL4 neurons participate in the sky compass network of the central complex. Unfortunately, no physiological data exist for the TULAL2 and CLAL neurons. A previously characterized polarization-sensitive neuron connecting the lateral complexes of both brain hemispheres (Heinze & Homberg, 2009) is clearly different from the CLAL neurons stained here.

Owing to dense immunostaining by other neural systems, the trajectories of the columnar neurons could only be traced through the posterior chiasma, but not through the CBU and CBL. Prominent immunostaining of the lower units of the noduli, however, strongly suggests that the labeled neurons are CL2 neurons, the only type of columnar neurons known to innervate the lower units of the noduli (Heinze & Homberg, 2008). Interestingly, 20–22 CL2 neurons were labeled and thus more neurons than the number of columns (16) in the protocerebral bridge. Especially the x- and y-bundles contained three instead of two neurites of CL2 neurons suggesting that the corresponding columns in the protocerebral bridge and CBL are innervated by two instead of one immunoreactive CL2 neuron. CL2 neurons are sensitive to the angle of polarized light and are, therefore, part of the sky compass network in the locust brain (Heinze & Homberg, 2009). In Drosophila, PEN neurons, the equivalents of locust CL2 neurons, mediate a shift in activity peaks in the protocerebral bridge during side turns of the fly and thus contribute to update the fly’s heading representation in the central complex during turns (Turner-Evans et al., 2017). In Drosophila, two types of PEN neurons exist, termed PEN_a (or PEN1) and PEN_b (or PEN2) which differ in their activity profiles during angular turns (Green et al., 2017). PEN_a neurons, determined from a single female fly, consist of 20 individual neurons with two neurons having axonal fibers in the w- and z-bundles, respectively, and three neurons with axons in the x- and y-bundles. PEN_b neurons consist of 22 neurons, three neurons with neurites in the w-, x-, and y-bundles and two neurons with fibers in the z-bundles (Scheffer et al., 2020). These data illustrate striking similarities in the numerical differences of CL2/PEN neurons across the CX columns between the fly and locust. The functional consequences of the increased supply of intermediate columns by CL2/PEN neurons remains to be uncovered.

4.3 | Functional implications

Across insects as diverse as locusts, butterflies, beetles, bees, and flies, the central complex has been implicated in functions related to spatial orientation (Honkanen et al., 2019; Pfeiffer & Homberg, 2014). These include topographic representations of azimuthal space, specifically sky compass signals in the columnar organization of the central complex, spatial learning and memory, and the control of heading directions in walking and flight (Dacke & el Jundi, 2018; Heinze & Homberg, 2007; Martin, Guo, Mu, Harley, & Ritzmann, 2015; Ofstad, Züker, & Reiser, 2011; Seelig & Jayaraman, 2015).

Corresponding with the large number of different cell types composing the central complex (el Jundi, Warrant, Pfeiffer, & Dacke, 2018; Heinze, Florman, Asokaraj, el Jundi, & Reppert, 2013; Heinze & Homberg, 2008; Hensgen, England, Homberg, & Pfeiffer, 2020; von Hadeln et al., 2020; Wolff, Iyer, & Rubin, 2015; Wolff & Rubin, 2018), a wide variety of neuroactive substances have been detected in the central complex. These include all known classical transmitters in insects, biogenic amines, nitric oxide, and a large variety of neuropeptides (Pfeiffer & Homberg, 2014). In Drosophila, peptide products of eight neuropeptide genes have been found in different layers of the central complex (Kahsai & Winther, 2011), and in the locust allatostatin A, allatotropin, crustacean cardioactive peptide, leucokinin, tachykinin, FMRFamide, SiFamide, EFamide, and as shown here, orcokinins, have been mapped to specific central-complex cell types (Dircksen & Homberg, 1995; Gellerer et al., 2015; Pfeiffer & Homberg, 2014; Veenstra & Šimko, 2020). The functional role of these peptides has been investigated for tachykinin, small neuropeptide F, and allatostatin in Drosophila. Here, RNAi knockdown of tachykinin in tangential neurons of the CBU led to increased avoidance of a central zone in an arena, while tachykinin depletion in pontine neurons increased the number of activity-rest phases (Kahsai, Martin, & Winther, 2010). Knockdown of small neuropeptide F expression in tangential neurons of the CBU had effects on walking distance and speed (Kahsai et al., 2010). Finally, disruption of allatostatin A gene expression in tangential cells of the CBU resulted in reduced sleep time, suggesting that allatostatin A released from these neurons has sleep promoting effects (Donlea et al., 2018). While no data exist for orcokinin A-type and all other peptides mapped in the central complex of the locust, these studies show that identified neuropeptides in central-complex neurons are involved in fine-tuning of activity levels and various aspects of spatial orientation behavior that have just begun to be explored.

4.4 | Colocalization of neuroactive substances

Colocalization of neuroactive substances occurs widely in neurons of the central complex. In the locust, subsets of GABA-immunolabeled neurons were identified that express allatostatin A, allatotropin, crustacean cardioactive peptide, tachykinin, and small neuropeptide F (Kahsai & Winther, 2011). In Drosophila, colocalization of neuroactive substances occurs widely in neurons of the central complex (Pfeiffer & Homberg, 2014). For example, CL2/PEN neurons are known to innervate the lower units of the noduli (Heinze & Homberg, 2009) is clearly different from the CLAL neurons stained here.
neurons are also stained with antisera against FMRFamide and orcokinin (TL4 neurons: Homberg et al., 1999; this study), locustatrypakinin (TL2 neurons: Vitzthum et al., 1996) and Dip-allatostatin I and orcokinin (TU$_{AL}$ neurons: Homberg et al., 1999, this study). In Drosophila, colocalization of GABA with FMRFamide in R neurons (equivalent to TL neurons) has, likewise, been reported (Kahsai & Winther, 2011). In addition, colocalization of neuropeptides with other peptides, serotonin or octopamine has been shown in locust TB neurons (Beetz et al., 2015; Vitzthum & Homberg, 1998), columnar neurons of the CBL (Vitzthum & Homberg, 1998) and CBU (Vitzthum et al., 1996), and, as shown here, in TU$_{AL}$ tangential neurons of the CBU, which may contain GABA, orcokinins, and allatostatin A, and in TU$_{VEX4}$ neurons. This extensive colocalization of neuroactive substances further increases the ways by which modulation of the neuronal circuitries in the central complex can occur. A number of functional studies have already pointed out that neural activity and signaling in the central complex is highly dependent on the behavioral state of the animal (Heinze & Homberg, 2009; Rosner, Pegel, & Homberg, 2019; Weir, Schnell, & Dickinson, 2014) and, in addition, is involved in circadian control of locomotor and sleep–wake cycles (Donlea et al., 2018; Liang et al., 2019; Liu, Liu, Tabuchi, & Wu, 2016). This requires considerable modulation of neural circuits at various levels of the central complex, part of which as pointed out above apparently involves a rich variety of neuropeptides including orcokinins.

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CONFLICT OF INTEREST
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The data that support the findings of this study are available from the corresponding author upon reasonable request.
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