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RESEARCH ARTICLE

Neural activity in the central complex of the cockroach brain is linked to turning behaviors

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SUMMARY

An animal moving through complex terrain must consider sensory cues around it and alter its movements accordingly. In the arthropod brain, the central complex (CC) receives highly preprocessed sensory information and sends outputs to premotor regions, suggesting that it may play a role in the central control of oriented locomotion. We performed tetrode recordings within the CC in cockroaches walking on an air-suspended ball to examine the role of the CC in turning behaviors. When a rod was placed near the cockroach's head, the cockroach touched the rod repeatedly with one or both antennae before locomotion was initiated. Some CC units responded to self-generated antennal contact with the object, but at lower levels compared with externally imposed antennal stimulation. The neural activity of other CC units responded to locomotion. We found that some CC units showed discrete firing fields corresponding to specific locomotion states. We also found that changes in firing rate of some CC units preceded changes in turning speed in one direction but not the other. Furthermore, such biased units were located in the side of the brain ipsilateral to the direction of the turning speed they could predict. Moreover, electrical stimulation of the CC elicited or modified locomotion, and the direction of some evoked locomotion could be predicted by the response property of locomotion-predictive units near the stimulation site. Therefore, our results suggest that, at the population level, asymmetrical activity in the CC precedes and influences turning behavior.

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INTRODUCTION

Animals must use sensory cues to navigate through complex terrain in order to avoid predators and seek out water, food, shelters and mates. These actions require that leg movements be redirected as the animal encounters objects in its environment. Direct control of leg movements resides in the local control circuits of the thoracic ganglia in arthropods or spinal cords of vertebrates (Deliagina et al., 1999). In insects, these circuits include central pattern generators and local reflexes that produce basic leg cycles, and then change to produce turns or adjust posture (Büschges et al., 2008; Mentel et al., 2008; Gruhn et al., 2009; Hellekes et al., 2012; Mu and Ritzmann, 2005; Ritzmann and Büschges, 2007). In order to respond to barriers properly, objects must be detected and evaluated by sensory structures that largely reside on the animal's head (Harley et al., 2009; Okada and Toh, 2000; Schütz and Dürr, 2011). The sensory signals detected by structures such as mechanoreceptors on antennae and by eyes are processed within primary sensory regions of the brain and then must alter descending commands that act upon local control circuits in the thoracic ganglia. Considerable evidence now implicates the midline brain neuropils, collectively referred to as the central complex (CC) (Strausfeld, 1999; Strausfeld, 2012), in this sensorimotor role (Bender et al., 2010; Huber, 1960; Strauss and Heisenberg, 1993; Strauss, 2002; Huston and Jayaraman, 2011).

The CC is a group of neuropils located on the midline of the protocerebrum in all insects (Strausfeld, 1999; Strausfeld, 2012). It includes the fan-shaped body (FB), ellipsoid body (EB), paired nodules and the protocerebral bridge (PB). Some laboratories refer to the FB as the upper division of the central body and the EB as the lower division of the central body. The cellular makeup of the CC has been extensively documented in locusts (Homberg, 2008). Tangential neurons with dendrites that spread horizontally across layers of CC neuropils provide the CC with inputs from the protocerebrum (Müller et al., 1997; Heinze and Homberg, 2008). Columnar neurons with arborizations in small domains of CC neuropils divide the PB, FB and EB into 8 or 16 columns and are the major output elements of the CC (Müller et al., 1997). They send connections to the lateral accessory lobes (LAL), a premotor region that mediates leg movements by descending interneurons to the thoracic ganglion (Homberg, 2008).

Previous studies have shown that many CC neurons respond to various sensory stimuli of different modalities. In cockroaches, extracellular recordings showed that CC units are involved in the integration of visual and antennal information (Ritzmann et al., 2008). Intracellular recordings in locusts, crickets and monarch butterflies showed that CC neurons are tuned to specific E-vector angles of polarized light (Vitzthum et al., 2002; Heinze and Homberg, 2007; Sakura et al., 2008; Heinze and Reppert, 2011). In flies, CC neurons that respond to moving visual stimuli and mechanical stimuli were identified (Phillips-Portillo, 2012).

Previous studies have also linked the CC to motor actions. Huber showed that stimulation within the CC enhances locomotor activity (Huber, 1960). More recently, electrophysiology studies showed that the neural activity of some CC units is correlated with, and often

precedes, changes in stepping frequency (Bender et al., 2010). In that study, stimulation through the same electrodes that recorded neurons whose activity is correlated with step frequency could also increase step rate. Genetic manipulations in flies revealed that the CC is required for the fine tuning and maintenance of locomotion (Martin et al., 1999; Strauss, 2002; Kahsai et al., 2010). In grasshoppers, injection of cholinergic agonists into the CC can induce long-lasting stridulation very similar to that which occurs naturally (Heinrich et al., 2001). Furthermore, behavioral studies have shown that the CC is involved in generating asymmetrical leg movements and, thus, influencing goal-directed locomotion. Flies with a defective CC failed to correct their walking path, resulting in severe circling behavior even in the presence of attractive cues (Strauss and Heisenberg, 1993; Strauss, 2002). Discrete electrolytic lesions in cockroaches revealed site-specific deficits in a range of turning and climbing behaviors (Harley and Ritzmann, 2010).

The behavioral and neurobiological data described above suggest that biased sensory information results in asymmetrical activity within the CC, eventually leading to asymmetrical leg movements associated with turning. However, no physiological data about CC neural activity that could be linked to turning have been documented so far. Here, we examined the relationship between CC neural activity and turning behavior in tethered walking cockroaches. Cockroaches, *Blaberus discoidalis*, were implanted with a pair of wire-bundle tetrodes in the CC, then transferred onto an air-supported Styrofoam ball. Locomotion was initiated either spontaneously or by the cockroach touching a rod. Spike times, ball movements and antennal movements were synchronized and recorded simultaneously for off-line analysis. Our results showed that CC units are tuned to the turning and forward walking speed of self-motion. Furthermore, we found that these units are located in the CC in a spatially biased manner such that a distinct and asymmetrical pattern of CC neural activity is generated in anticipation of a turn. Moreover, stimulating specific areas of the CC can elicit locomotion in different directions. Our stimulation results are consistent with the prediction based on the response properties and locations of locomotion-sensitive units that an asymmetrical population code within the CC influences turning movements following antennal contact.

MATERIALS AND METHODS

Animals

Adult cockroaches (*B. discoidalis*, Audinet-Serville 1839) from a laboratory colony were used in all experiments. Cockroaches were housed together in 5 gallon (~20 l) plastic bins, and given food and water *ad libitum*. The animals were kept on a 12 h/12 h light/dark cycle at 27°C. Only healthy animals with intact antennae were chosen for experiments.

Animal preparations and recording

The insect was first anesthetized with ice. After it stopped moving, its wings were removed and a flexible plastic tether was glued to its pronotum. The animal was then restrained vertically against a flat cork surface with large saddle pins that did not penetrate any part of the animal. The preparation was transferred into a plastic container and ice was placed around the animal to minimize blood flow and body movements, which could interfere with wire implantation. A plastic collar was positioned at the neck to support the head and dental wax was placed around the head to stabilize it. A small window between the ocelli was cut into the cuticle and removed over the brain. Connective tissues and fat were carefully removed to expose the brain. A small amount of cockroach saline was placed in the head capsule to cover the brain tissue.

A pair of wire-bundle tetrodes was used for recording. Each tetrode consisted of four, 12 μm nichrome wires (Kanthal RO-800, Sandvik Heating Technology, Hallstahammar, Sweden) twisted together. Tetrode wires were connected to an adaptor and secured in a Delrin and epoxy package. Before each experiment, the tip of each tetrode was cut, polished and plated with copper such that it had a regular arrow shape and a starting impedance of between 0.5 and 1.5 MΩ.

With the brain exposed, the two tetrodes were inserted into the brain with two micromanipulators and their adaptor was mounted in the headstage of a Neuralynx Cheetah (Bozeman, MT, USA) digital interface. A separate braid of three larger diameter (56 mm) insulated copper wires was inserted into the head capsule anterior to the brain to serve as a reference/ground electrode. The two tetrodes and the reference wires were then anchored in place with tiny pieces of acetate and the head capsule was covered and sealed with small amounts of melted dental wax. Next, the constraints were carefully removed and the animal was transferred onto an air-supported Styrofoam ball. Its tether was attached to a positioning rod. The plastic mount allowed the insect to bounce in the vertical plane, thus allowing the animal to walk on the ball with a normal posture. At least 60 min were provided for the animal to recover from the ice anesthesia. Preparations that exhibited abnormal leg and antennal movements were excluded. All experiments lasted between 2 and 4 h, depending on the quality of the neural recordings and the preparation. An experiment was terminated if the animal became inactive, such that each animal was as likely to move at the beginning of the experiment as at the end of the experiment.

All experiments were performed under infrared light illumination. During each experiment, a servo-motor was used to position a cylindrical rod (7 mm in diameter and 30 mm in height) near the animal's head at varying locations (Fig. 1A). The animal was allowed to use its antennae to explore the rod and initiate locomotion accordingly. The rod was retracted from the animal after 30 s and there was a 3 min interval between trials. Recordings were also made when spontaneous locomotion was initiated without any sensory cues. In some experiments, we also stimulated the antennae externally using a servo-motor or a soft brush for comparison with active antennal contact with the rod.

Video was captured at 60 frames s^{-1} with a Photron camera. The camera was positioned right above the animal's head so that antennal contact with the rod from all positions could be recorded without any blockage. Video analysis was performed manually using Photron's Viewer software.

A trackball assembly allowed us to track the movements of the air-supported Styrofoam ball system and, thereby, reconstruct each cockroach's locomotion trajectories. To construct the trackball, a Styrofoam ball (15.24 cm in diameter) was placed into a half-sphere ladle drilled with four evenly spaced holes. The ladle was coated with casting epoxy (BC7062, BCC Products, Franklin, IN, USA) so that the ball could be tightly fitted in. The ladle was mounted on a small cylinder through which a constant air flow was passed to lift the ball and allow it to rotate freely. Rotations along the forward–backward and left–right axes were detected using an optical mouse sensor (MX 518, Logitech, www.logitech.com) positioned behind the ball and recorded using a customized Matlab program.

The data from the Neuralynx system were saved directly to a PC. For each electrode, the collected data included voltage waveforms and time stamps that marked the point in time where each action

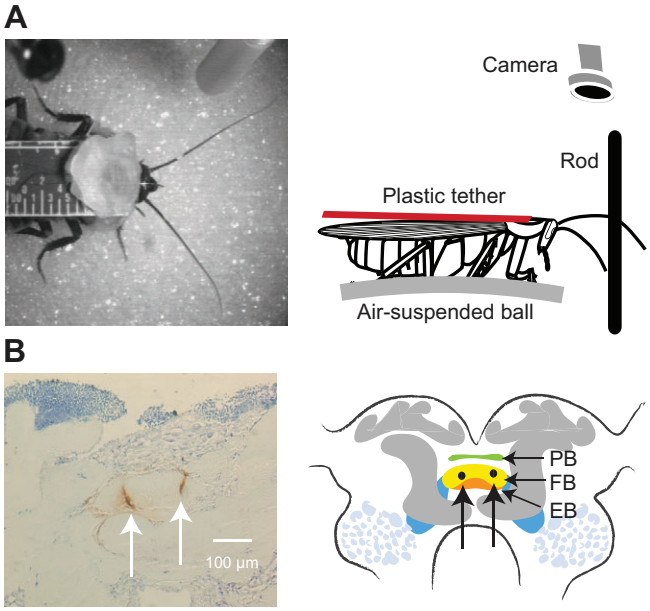

Fig. 1. Multi-unit recording in tethered walking cockroaches. (A) Left: dorsal view of the preparation set-up. The cockroach, implanted with two tetrode wires in the brain, is presented with a rod near its head while walking on an air-supported ball. Brain recordings and high-speed video were made simultaneously. Right: a diagram of the preparation. (B) A section of the brain of preparation no. 9, showing two brown copper-deposition sites in the fan-shaped body (FB). PB, protocerebral bridge; EB, ellipsoid body.

potential that exceeded a pre-set threshold occurred within a given data file. The data also included synchronization pulses to link the Neuralynx time with coincident high-speed digital video recording and ball-movement recording.

Spike-sorting analysis

Data from each tetrode were sorted off-line following procedures that have been described in detail elsewhere (Daly et al., 2004; Bender et al., 2010). Automated clustering of each tetrode was performed with the program KlustaKwik (version 1.5; K. Harris, Rutgers University) and then imported into MClust (version 3.5; A. D. Redish, University of Minnesota) for further classification and refinement. All similar initial clusters were merged and individual units were determined by comparing variables such as energy, peak height, time to peak and principal components across all four channels. We checked the baseline activity for all units very carefully. For each unit, if the baseline activity shifted dramatically during the experiment, data after the baseline activity was shifted were deleted. Additional information is available in the Appendix.

Histology

At the end of each experiment, a 5 mA, 5 ms DC current was applied between one of the tetrode wires from each bundle and the reference electrode in order to deposit copper at the recording sites. The brain was removed and placed in a diluted ammonium sulfide/saline solution for 15 min to precipitate the copper. The brain was then fixed with alcohol/acetic acid/formalin, dehydrated in an alcohol series, permeabilized with xylene, embedded in Paraplast and sectioned at 12 mm. Sections were run through Timm's sulfide–silver intensification and then fixed, dehydrated and covered for imaging. Concentrated brownish deposits occurring in several adjacent serial sections were identified as the tetrode locations (Fig. 1B). All recording sites within the CC were in the FB, and data from recording sites outside the CC were discarded.

RESULTS

We recorded the neural activity of 56 units from the CCs in 21 preparations. For 20 of those preparations (54 units), the cockroach's movement information was also collected. We first examined how neural activity changes between standing still and locomotion evoked by self-generated antennal contact. We binned each experiment into non-overlapping 2 s windows, calculated the mean firing rate for each data point, and categorized them according to whether the animal was standing still or walking at that time period. We then compared the firing rates between the two groups. The firing rate of 47 units (87%) was increased during locomotion (*t*-test, $P<0.01$), and four units (7%) showed decreased firing rates during locomotion (*t*-test, $P<0.01$). Only three units (6%) failed to alter their firing rates between locomotion and standing still.

Directional bias

Simple comparison of neural activity between standing still and locomotion cannot reveal how units responded to locomotion as a function of walking direction and speed. For instance, while some units increased activity uniformly in response to locomotion in either direction, others responded to locomotion in a biased manner, favoring one direction over the other (Fig. 2). For the individual walking bouts in Fig. 2, the changes in activity of a sorted unit with time are shown as changes in color from blue (low) to red (high). The bouts are arranged from top to bottom according to the insect's

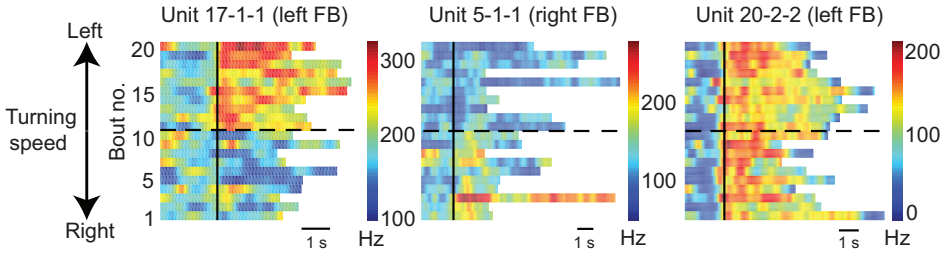

Fig. 2. Central complex (CC) units responded to locomotion in a directionally biased manner. Raster plots of 20 bouts of locomotion for three units. Each row is one bout and the color indicates the firing rate. For each graph, the solid black line indicates the start of each bout. Bouts of left turning are above the dashed black line and bouts of right turning are below it. For bouts of left turning, the higher the bout number, the higher the average turning speed. For bouts of right turning, the lower the bout number, the higher the average turning speed. Note the changes of firing rate after locomotion start as a function of locomotion direction. Individual units are named according to preparation, tetrode and unit numbers (e.g. 'unit 1-2-3' indicates preparation 1, tetrode 2, unit 3).

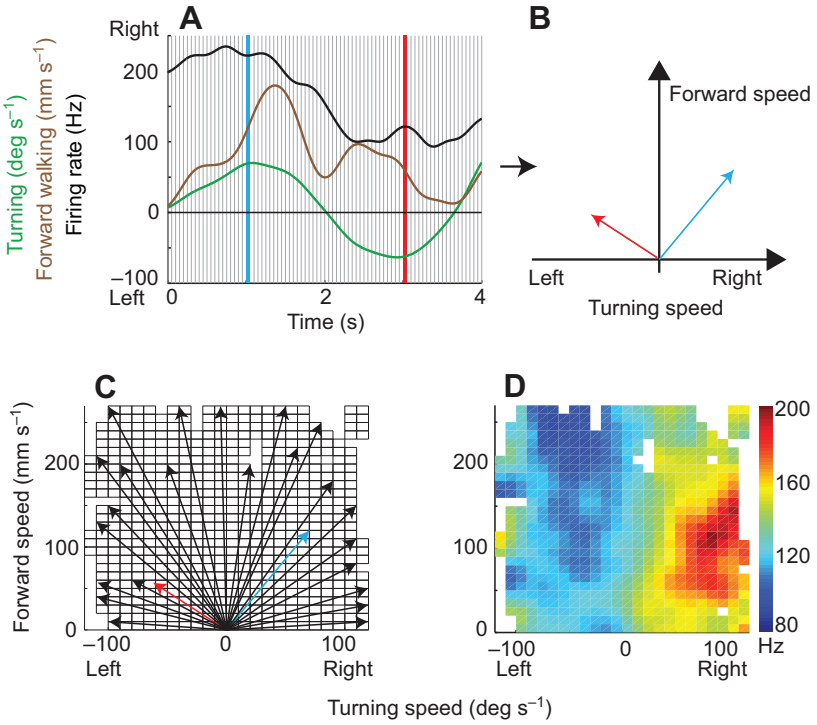

Fig. 3. Methodological concept for generating firing rate maps. (A) For every recording session, forward and turning speed as well as spike times of each unit were smoothed using a Gaussian kernel with a standard deviation of 150 ms. Each recording session was divided into non-overlapping 50 ms long sections (between individual gray lines). (B) For each divided section, a velocity vector was generated by averaging forward and turning speed within that period. Firing rate for each velocity vector was also calculated. The blue and red vectors were obtained from the blue and red lines, respectively, in A. (C) All velocity vectors were binned into a forward walking speed *versus* turning speed graph (10 mm s^{-1} for forward walking speed and 10 deg s^{-1} for turning speed). Only some of the vectors, including the two vectors in B, are shown here. (D) A firing rate map was generated by overlaying the averaged firing rate for each bin, obtained by averaging all the firing rates whose corresponding velocity vectors fell into that bin.

left–right turning tendency of each bout as indicated by the directional speed of the ball. Three categories of units are depicted; all three increased their activity after making antennal contact (black vertical line). Unit 17-1-1, which was recorded in the left FB, only showed increases in firing rates in bouts that were associated with left turns (bouts above the dashed line). Unit 5-1-1, which was recorded in the right FB, only increased activity in association with right turns (bouts below the dashed line). In contrast, Unit 20-2-2, which was also recorded in the left FB, increased activity regardless of the direction of movement.

Activity tuned to locomotion state (forward *versus* turning movements)

In order to further examine the tuning of individual units to the animal's locomotion state (i.e. speed and direction), we constructed firing rate maps based on forward walking speed and turning speed for locomotion initiated by the insect's own antennal contact with the rod (Fig. 3). These graphs remove the time domain from the data and place the focus on how firing rate changes as the animal moves forward or turns. Here, firing rate is related to the vector formed by simultaneous consideration of forward and left–right ball movement, i.e. the actual direction that the insect would have moved in had it not been tethered.

To generate these graphs, forward and turning speed as well as spike times of each unit were first smoothed using a Gaussian kernel with a standard deviation of 150 ms (Fig. 3A). For every non-overlapping 50 ms (gray lines in Fig. 3A), a velocity vector was generated by averaging forward walking speed and turning speed during that period. Two such vectors are shown in Fig. 3B. The blue vector shows the relationship in the bin marked by a blue line in Fig. 3A and the red vector shows the relationship for the bin marked with a red line in Fig. 3A. We also calculated the firing rate for each velocity vector (black curve in the Fig. 3A). All velocity vectors for each preparation were binned (in 10 mm s^{-1} for forward walking speed and 10 deg s^{-1} for turning speed) (Fig. 3C). We subsequently calculated the firing rate of each bin by averaging all the firing rates whose corresponding velocity vectors fell into that bin (square at the tip of the vector). A firing rate map was then generated by overlaying the firing rate for each bin (Fig. 3D). That is, we determined the average firing rate for all bins and expressed it as a color code in the final graph. Empty squares represent regions of the graph where no vector terminated. Each firing rate map was finally smoothed using a Gaussian average over the 2×2 bins surrounding each bin.

For many CC units, increased firing was restricted to specific locomotion states, such as left or right turning irrespective of forward walking speed (e.g. Fig. 4A,E), or forward walking irrespective of turning speed (e.g. Fig. 4C). In others, increased firing was further restricted to specific turn directions and walking speeds (e.g. Fig. 4B,D).

In order to quantify how sharply CC units were tuned to different locomotion states, we generated shuffled firing rate maps for each non-smoothed firing rate map by randomly assigning each bin to another previously occupied position (Fig. 5A). We then smoothed the shuffled maps (Fig. 5B). Thus, compared with its original firing rate map, the shape of each shuffled map was retained but the positional information was lost. One-thousand permutations were made for each unit. We measured the dispersion of the firing field as the mean distance between the 10% of pixels that had the highest firing rate for each firing rate map and its shuffled maps. If the dispersion of a unit's firing rate map is smaller than the majority of the dispersion of shuffled maps, it indicates that the firing field of this unit is restricted to some specific locomotion state. The firing rate dispersion of 15 units (28%) was less than the 99th percentile of the dispersion distribution generated from shuffled maps (Fig. 5C) (Z-score between 2.33 and 6.53, $P<0.01$).

Antenna-triggered *versus* spontaneous movement

To determine the role of tactile information from antennae in shaping the tuning of units to locomotion, we also constructed firing rate maps for spontaneous locomotion in the absence of a rod for 17 units in seven preparations. The speed range for spontaneous locomotion was expanded compared with that of antenna-guided

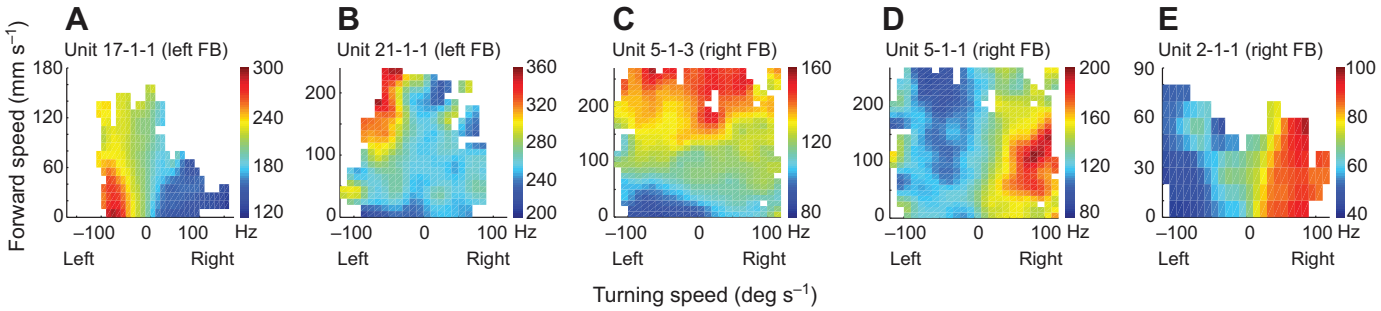

Fig. 4. CC units are tuned to self-motion. Firing rate maps for locomotion initiated by antennal contact with the rod for representative CC units. The *x*-axis is the turning speed and the *y*-axis is the forward walking speed. Positive turning speed indicates right turning and negative turning speed indicates left turning. CC units showed discrete locomotion-related firing fields, such as left turning irrespective of forward walking speed (A, Z=3.59, P<0.01), forward walking to the left (B, Z=4.08, P<0.01), forward walking irrespective of turning speed (C, Z=5.73, P<0.01), forward walking to the right (D, Z=6.00, P<0.01), and right turning irrespective of forward walking speed (E, Z=2.33, P<0.01).

locomotion for all animals. Nonetheless, all four units that had discrete firing fields for antenna-guided locomotion also exhibited similar firing fields for spontaneous locomotion (Z-score between 3.58 and 5.80, P<0.01) (Fig. 6A). Four other units showed significant firing fields for spontaneous locomotion but not for antenna-guided locomotion (Z-score between 2.83 and 4.62, P<0.01). This could be due to the expansion of the speed range such that a more comprehensive firing rate map was obtained. We therefore shrank the firing rate map for spontaneous locomotion to the size of that for antenna-guided locomotion. Three out of the four units no longer showed significant firing fields under these conditions (Fig. 6B). The other one unit still showed significant firing fields (Z-score=2.97, P<0.01) but the pattern of the firing rate maps was very similar between spontaneous and antenna-guided locomotion (Fig. 6C). Moreover, pair-wise correlation of each occupied bin between the two maps showed that they were highly correlated for all the eight units (r-value between 0.52 and 0.94). This suggests that the tuning of these units to self-motion does not depend on sensory cues.

Correlation of activity in individual bouts with direction of movement

While firing rate maps can be used to examine the tuning of individual units to self-motion, the dimension of time is lost in such analysis. Previous studies in cockroaches have shown that the neural activity of some CC units is correlated with stepping frequency on a moment-to-moment basis (Bender et al., 2010). We therefore examined the timing relationship between firing rate and locomotion changes within individual bouts of locomotion. A bout was defined as continuous locomotion if it contained no stops exceeding 500 ms. Each bout was at least 2 s in length and was separated by at least 1 s from the previous one. Instantaneous forward walking speed, turning speed and firing rate were calculated using a Gaussian kernel

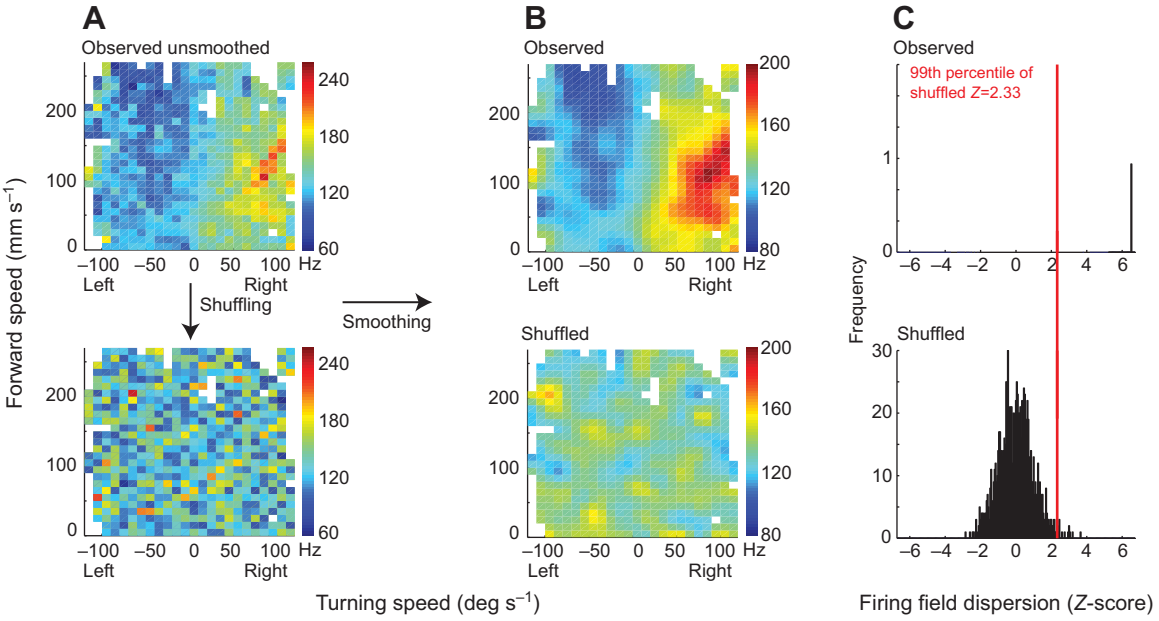

Fig. 5. Methodological concept for determining the tuning of the units to self-motion. (A) To quantify how sharply individual CC units were tuned to different locomotion states, unsmoothed shuffled firing rate maps were generated by assigning each bin of the unsmoothed original firing rate map to another previously occupied position. One-thousand permutations were made for each unit, but only one shuffled map is shown here. (B) Both the shuffled and original maps were then smoothed. (C) A unit was determined as tuning to some specific locomotion state if the dispersion of its firing rate map was smaller than that of most shuffled maps generated from the original map. Dispersion was defined as the mean distance between the 10% of pixels that had the highest firing rate for each map. Dispersion values were then transferred to Z-scores. The significance level was set as the Z-score of the 99th percentile of the shuffled data: Z-score=[dispersion–mean(dispersion)]/s.d.(dispersion).

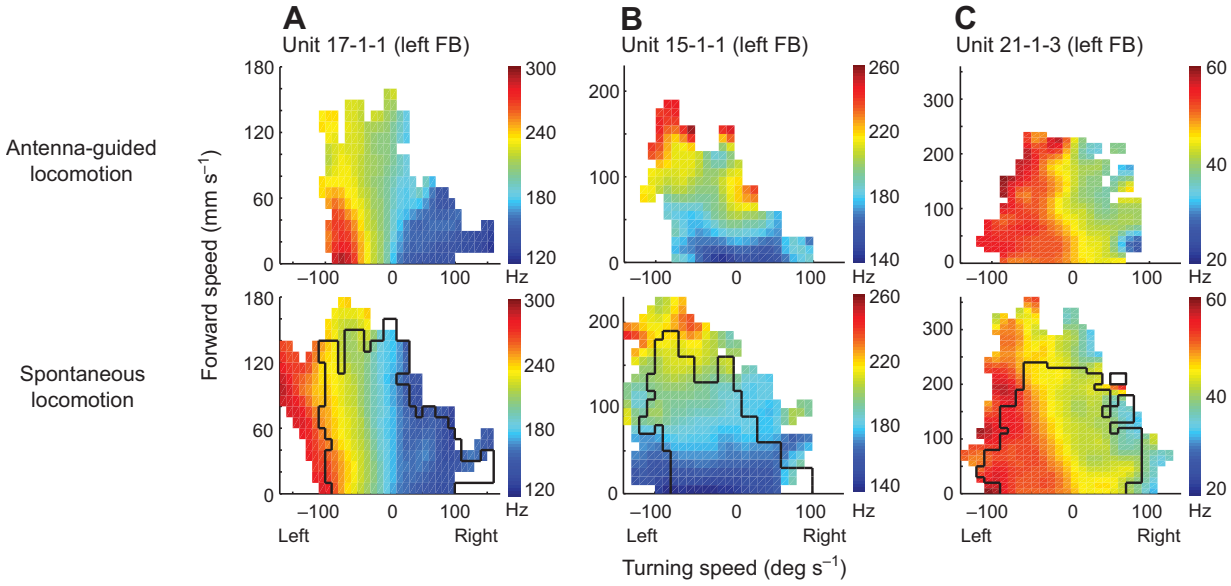

Fig. 6. The tuning of CC units to self-motion does not depend on sensory cues. (A) Unit 17-1-1 showed tuning to left turning for both antenna-guided and spontaneous locomotion (Z=3.59, 3.53, P<0.01) and the firing rate maps for the two were similar (r=0.94). The black line indicates the speed range for antenna-guided locomotion. (B) For unit 15-1-1, although the two maps were similar to each other (r=0.89), tuning to forward walking only occurred for spontaneous locomotion (Z=4.62, P<0.01). However, if the firing rate map for spontaneous locomotion was confined to the speed range for antenna-guided locomotion (inside the black line), the firing field was no longer statistically significant. (C) Unit 21-1-3 showed significant tuning to left turning irrespective of forward walking speed only for spontaneous locomotion under both full and restricted speed range situations (Z=4.11, 2.97, P<0.01). Nonetheless, the pattern of the two maps was similar (r=0.81).

with a standard deviation of 150 ms. A substantial fraction of CC units fired in accordance with at least one of the three locomotion parameters, right turning speed, left turning speed and forward walking speed, with some time offset (Fig. 7A–C, top graphs). For example, unit 17-1-1 (Fig. 7A), which was recorded in the left FB, showed a positive correlation with left turning speed but not with forward walking speed; this unit also showed a negative correlation with right turning speed. In contrast, unit 5-1-1 (recorded in the right FB) (Fig. 7B) showed a positive correlation with right turning speed but not with left turning or forward walking speed. Finally, unit 5-1-3 (also recorded in the right FB) (Fig. 7C) showed no correlation with either direction of turning but showed a correlation with forward walking speed.

In order to determine whether changes in neural activity preceded or followed locomotion changes, we shifted the timing of instantaneous firing rate either forward or backward, and re-calculated the correlation coefficient (ρ) between the forward walking or turning speed curve and each shifted firing rate curve. If maximum ρ is obtained when the firing rate curve is shifted forward [i.e. neural signal delay (δ)>0], it indicates that changes in firing rate precede locomotion changes. In contrast, if maximum ρ is obtained when the firing rate curve is shifted backward (i.e. δ<0), it indicates that changes in firing rate follow locomotion changes. We calculated ρ as a function of δ for every bout and averaged it according to locomotion parameter type for each unit (1425 total bouts and at least four bouts for each group from each preparation) (Fig. 7A–C, bottom graphs). We found that the neural activities of 35 units (65%) were correlated with at least one of the three locomotion parameters with a maximum ρ of at least 0.4 for all bouts. Of these 35 units, changes in firing rate of 32 units preceded locomotion changes, with a mean best delay of 0.53 s. Changes in firing rate of only three units followed locomotion changes, with a mean best delay of −0.19 s (Fig. 7D). Notably, the best delay of three units exceeded 1 s. As cockroaches are fast moving animals, such long best delays might be of little value in terms of predicting or guiding locomotion changes. Two possibilities may explain the biological relevance of those long best delays.

First, the ball has inertia and it is more difficult for cockroaches to walk on a ball than on a floor. As a result, when walking on a ball, motor neurons need to be recruited at a higher level in terms of number and activity level, which would take a longer time. If the animal was not healthy, this effect would be exaggerated, resulting in very long best delays. Second, the increase in firing rate that correlated with locomotion changes 1 s later could be readiness discharge, which has been reported in other invertebrates (Kagaya and Takahata, 2011). However, readiness discharge is transient and the fact that the increase in firing rate did correlate with locomotion changes suggests that the first speculation might be more plausible.

Units correlated with locomotion changes were located in the FB, a subdivision of the CC, in a spatial manner (Fig. 7E,F). A larger fraction of units recorded in the lateral FB showed correlation with locomotion changes compared with units in the middle FB (left FB: 64%, 16 units, N=25; right FB: 76%, 13 units, N=17; middle FB: 50%, six units, N=12). All the six units whose activity was correlated with left turning speed but not with right turning speed were clustered in the left FB. No unit whose activity was correlated with only right turning speed, not left turning speed, was clustered in the left FB. In contrast, 10 out of 11 units whose activity was correlated with right turning speed but not with left turning speed were clustered in the right FB. No unit whose activity was correlated with only left turning speed, not right turning speed, was clustered in the right FB. Only one unit recorded in the middle FB showed asymmetric activity in anticipation of a turn. Of the 18 non-biased units, seven units were correlated with only forward walking speed, three units were correlated with turning speed of either direction, and eight units were correlated with both forward walking and turning speed.

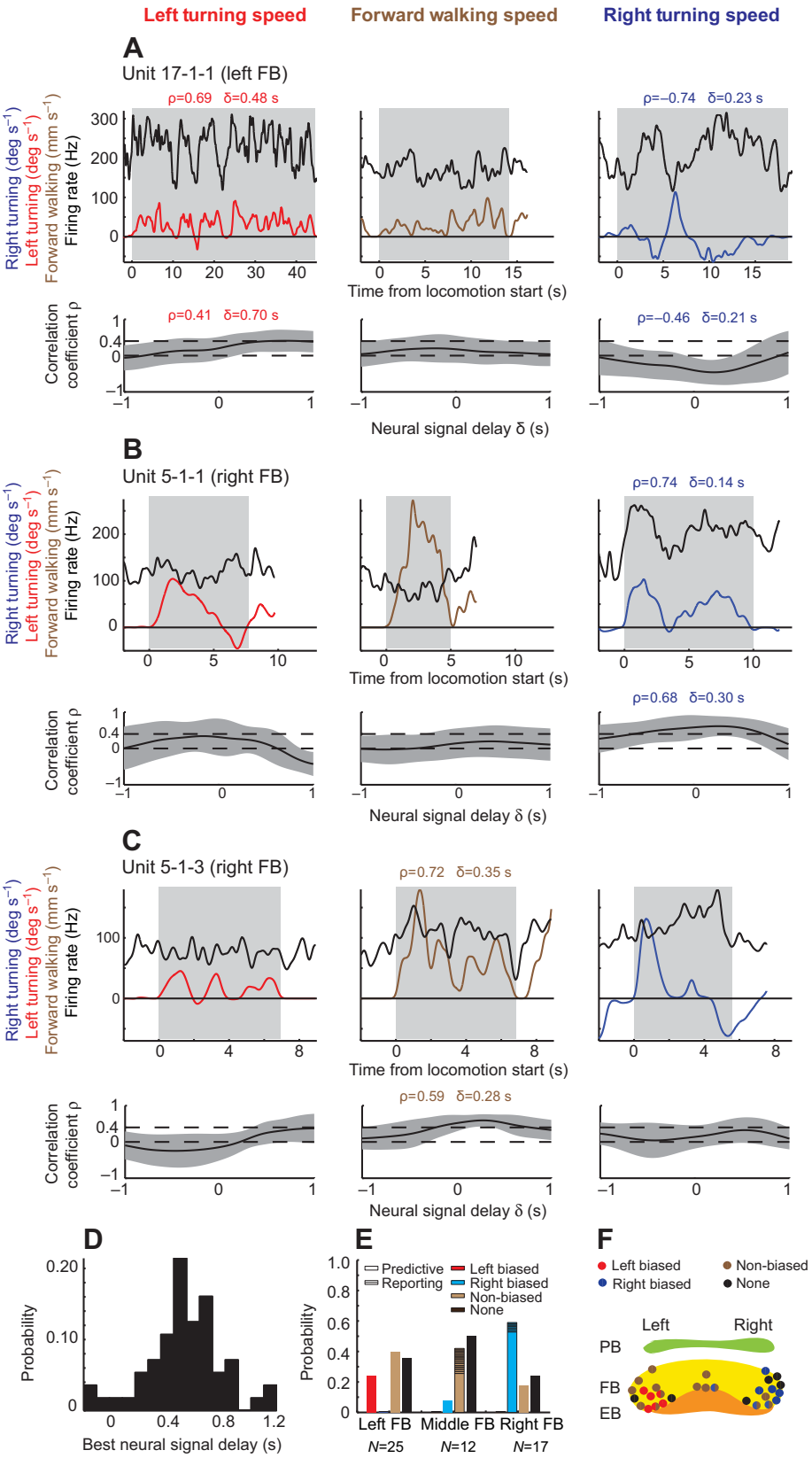

Fig. 7. Changes in firing rate of some CC units can predict locomotion changes in a biased manner. (A–C) Top: the instantaneous firing rate is plotted together with the instantaneous left turning, forward walking and right turning speed for individual bouts (gray boxes). The firing rate (black) was shifted to the right by the neural signal delay δ (–1 to 1 s with a step of 0.01 s) and cross-correlated with the locomotion speed (red, brown and blue). A maximum (or minimum) correlation coefficient ρ was then found between the two lines. Bottom: ρ as a function of δ for all bouts of each locomotion parameter (i.e. left and right turning speed and forward walking speed) for each unit. The black line and gray shaded area indicate the mean and s.d. envelope, respectively. A peak ρ at δ>0 indicates changes in firing rate precede locomotion changes and a peak ρ at δ<0 indicates changes in firing rate follow locomotion changes. Note, unit 17-1-1 and unit 5-1-1 showed asymmetrical activity in anticipation of a turn (i.e. only correlated with left and right turning speed, respectively). Changes in firing rate of unit 5-1-3 only preceded and correlated with changes in forward walking speed. (D) Histogram of δ when a maximum ρ of at least 0.4 was obtained including all three locomotion parameters for all units. (E) Bar graphs of the probability of units with different locomotion parameters. Units were categorized according to their locations. 'Left biased' and 'Right biased' indicate units whose activity was correlated with only left and right turning speed, respectively, but not with the turning speed of the other direction. 'Non-biased' indicates units whose activity was correlated with forward walking speed and/or turning speed of both directions. 'None' indicates units whose activity was not correlated with any of the three parameters. Filled bars indicate the percentage of locomotion-predictive units (i.e. δ>0) and filled bars with horizontal lines indicate the percentage of locomotion-following units (i.e. δ<0). Note, for units in the lateral FB, many can be predictive of only ipsilateral turns but none can be predictive of only contralateral turns. (F) The locations of all 29 recording sites where the animal's movement information was collected and analyzed. Each dot is one recoding site. If at least one unit whose activity was correlated with locomotion changes was recorded from that site, the dot was coded with the corresponding color(s) (red, brown and blue). Black dots indicate that no units whose activity was correlated with locomotion changes were recorded from that site.

Evoked movement

Our recording data show strong evidence for spatial correlations between neural activity and changes in movement. Can activation in the same regions evoke changes in movement that are consistent with these relationships? For 15 preparations, we injected electrical currents through one of the tetrode wires after the recording. In all, we stimulated at 24 recording sites. Current was injected for 2 s at 5–10 μA, pulsed at 100 Hz with a 5% duty cycle for each recording site. Stimulation evoked straight walking (0 or near 0 turning speed), turning (0 or near 0 forward walking speed) or antennal movements

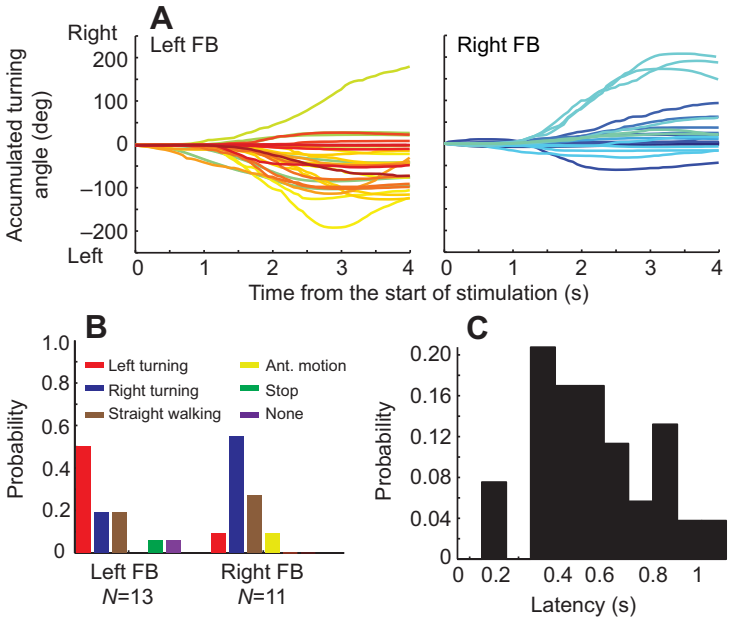

Fig. 8. The pattern of the evoked locomotion through electrical stimulation is consistent with the distribution of directionally biased locomotion-predictive units. (A) Raw traces of the angles turned after the start of stimulation at 21 recording sites through which locomotion was evoked (*N*=11, left FB; *N*=10, right FB). Each color represents data from one recording site. Note the difference in turning direction between stimulation from the left and right FB. (B) Bar graphs of the probability of behaviors evoked by electrical stimulation through 24 recoding sites. Stimulation was categorized according to the tetrode location. Note stimulation evoked much more turning to the ipsilateral side than to the contralateral side. Ant., antennal. (C) Histogram of the latency between stimulation onset and locomotion start.

at 22 recording sites (92%) and caused locomotion to cease at one recording site (4%). There was only one recording site (4%) where stimulation failed to generate a response. Of the 23 recording sites where stimulation evoked some sort of behavior, only one type of behavior was evoked at 21 sites (91%). If stimulation evoked more than one type of behavior, all types of evoked behaviors were counted as the stimulation outcome. The pattern of evoked locomotion was quite different between stimulation in the left FB and stimulation in the right FB (Fig. 8A,B). Stimulation in the left FB evoked left turning at eight recording sites (62%, *N*=13) and right turning at only three recording sites (23%). In contrast, stimulation in the right FB evoked right turning at six recording sites (55%, *N*=11) and left turning at only one recording site (9%). Such bias is consistent with the uneven distribution of directionally biased locomotion-predictive units that we found. Furthermore, at eight out of the 10 recording sites (80%) where directionally biased units were found, stimulation evoked turning in the direction preferred by these units (i.e. regions with left-biased units evoked left turns and *vice versa*). Stimulation at the other two recording sites (20%) evoked straight walking. The mean delay between stimulation and locomotion initiation was 0.56 s, roughly the same as the mean best delay for all locomotion-predictive units (Fig. 8C). Other behavioral changes that were noted in only a few cases involved cessation of leg movement or antennal movement.

Self-generated antennal stimulation *versus* imposed stimulation

Previous reports on antennal stimulation leading to activation of CC units relied on imposed movements of the antennae using a servo-driven or hand-held stimulator (Ritzmann et al., 2008; Bender et al., 2010). In the experiments reported here, antennal stimulation occurred as a result of the insect's own antennal movements to contact a rod near its head. In several systems, there are distinct differences between responses resulting from self-generated *versus* imposed stimulation (Staudacher et al., 2005; Szwed et al., 2003). We therefore compared the responses of CC units with these two types of antennal stimulation.

Antennal contacts were made either by the animal moving its antennae to the rod (insect generated) or by a gentle touch of either of the antennae using either a brush or servo-controlled stimulator (imposed stimulus). We compared the number of spikes within a time window of 100–150 ms after each antennal contact with an identical period before the contact. Only three out of 56 units (5%) responded to self-generated antennal contact (paired *t*-test, $P<0.01$) and none of them responded to locomotion. The response was at a much lower level compared with externally imposed antennal stimulation (*t*-test, $P<0.01$) (Fig. 9A). Among these three units, the response of two (located in the middle FB) did not differ between right and left antenna or between locomotion and standing still (two-way ANOVA, $P>0.05$). The third unit (located in the left FB) only responded to contact with the left antenna (paired *t*-test, $P<0.01$) and the response decreased after the animal started walking (*t*-test, $P<0.01$) (Fig. 9B). This bias to weaker responses during walking is consistent with previous reports (Bender et al., 2010). In contrast, 25 out of 32 units (78%) responded to imposed antennal stimulation.

While the number of units responding to self-generated stimulation is much lower than that to imposed stimulation, the fact that some do respond indicates that such actions can and do activate a subset of CC units. This relationship could represent a sparse response pattern that would provide more spatial detail than was indicated in previous studies. More will be said about this in our Discussion.

DISCUSSION

Spatially organized control of turning in the CC

Our results support the idea that the CC supervises locomotion (Strausfeld, 1999, Ritzmann et al., 2012). We constructed firing rate maps based on animals' continuously changing speed and direction and showed that many of the CC units that we recorded are tuned to certain forward walking and turning speeds. Such tuning does not depend on how locomotion was initiated. Temporal analysis revealed that changes in firing rate of a large proportion of the CC units we recorded precede changes in locomotion. These results are consistent with previous correlations that were found between CC unit activity and changes in step frequency (Bender et al., 2010). Interestingly, in our study, many locomotion-predictive units were only correlated with turning in one direction but not the other. Furthermore, these biased locomotion-predictive units were located

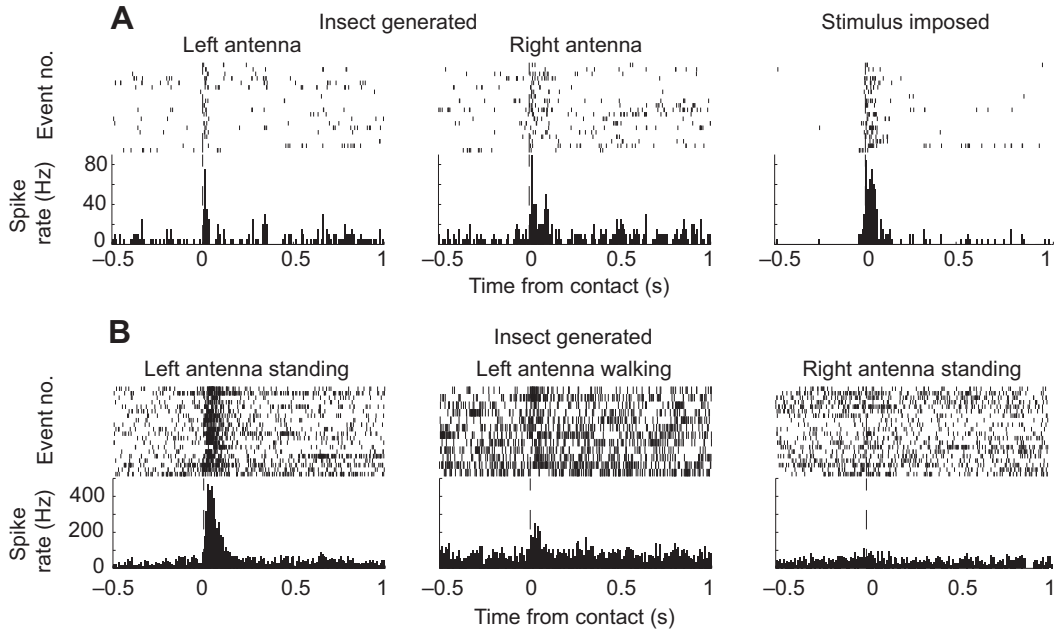

Fig. 9. The response of CC units to antennal contact is contact type, antenna and animal state dependent. The raster plot on the top of each display shows 12–20 trials aligned with the antennal contacts (time 0). Below each raster plot is a peristimulus time histogram of all the trials. (A) Unit 1-1-1 responded to self-generated antennal contact from both antennae (paired *t*-test, $P<0.01$), but at a much lower level compared with externally imposed antennal stimulation (*t*-test, $P<0.01$). (B) Unit 12-1-5 only responded to self-generated left antennal contact (paired *t*-test, $P<0.01$) and the response was diminished after the animal started walking (*t*-test, $P<0.01$).

in the FB in a spatially biased manner. Units whose activity was only correlated with one side of turning were only found on the ipisilateral side of the FB. Moreover, the directional bias of movements evoked by stimulating the lateral FB is consistent with the spatial bias of locomotion-predictive units. Again, these evoked changes are consistent with effects found in stepping frequency (Bender et al., 2010). Taken as a whole, these studies strongly support the notion of a sensorimotor role for neural circuits in the CC. More specifically, our current results suggest that at the population level, asymmetrical activity in the CC precedes and influences turning behavior such that the animal turns to the side that has a higher activity level in the CC.

Such ‘right–left bargaining’ has been suggested as one of the general functions of the CC (Heisenberg, 1994). Several previous studies have also supported this notion. In the mutant fly line C31, the FB and EB were partially interrupted along the midline of the brain (Strauss, 2002). When a C31-defective brain controlled a unilaterally defective body, the flies continually turned towards the defective side, where the strides were shorter than on the intact side, even in the presence of attractive cues. In contrast, when an intact brain controlled a unilaterally defective body, the flies were able to walk straight. This suggests that an intact brain is both necessary and sufficient to control or correct asymmetrical movements. In cockroaches, discrete electrolytic lesions on the midline of the EB and FB altered climbing but had little or no effect on turning behaviors (Harley and Ritzmann, 2010). However, lesions in the lateral FB led to turning deficits. This lesion study reported that turning deficits were only found when the direction of turning was opposite to the side of the brain where the CC was lesioned. For instance, lesion on the right side led to a failure to turn to the left or errors in left turns but had no effect on right turning. This is exactly the opposite of our study, in which directionally biased units were clustered in the ipisilateral side of the FB and stimulating the lateral FB led to turning of the same side. Further study will be needed to resolve this difference but there are several possible explanations. For example, it is possible that histological sections in one of the studies were reversed at some point. These mistakes can occur at various points in the procedure. As our data were opposite to the previously reported results, we were particularly careful to avoid such reversals. However, a re-examination of the sections used by Harley and Ritzmann (Harley and Ritzmann, 2010) could not eliminate the possibility that a reversal occurred in those data (C. M. Harley, personal communication). As the ganglia that were studied were all completely sectioned, it is impossible to resolve this issue with the original data at this time.

A complete resolution of this issue is beyond the scope of this paper. However, we have performed lesions in two cockroaches and the effects were consistent with the notion that histological sections were reversed in the earlier study. In one preparation, stimulating the right FB evoked right turning. However, after lesioning the right FB by applying 15 μA current for 15 s through one of the tetrode wires, the animal failed to turn to the right, resulting in severe circling behavior to the left. In the other preparation where the tetrode was located in the left FB, stimulation evoked left turning and lesion resulted in circling behavior to the right, i.e. failure to turn left. With these new data, it seems that the explanation that the brain sections were reversed in the previous study is more plausible. However, more work needs to be done before this discrepancy can be fully resolved. It should be noted that even if such an error occurred, it does not in any way detract from the primary finding of the original study, that lesions in the lateral regions of the FB were particularly effective in altering turning behavior, while midline lesions were not. This conclusion is consistent with our findings.

If future studies support the conclusion that the direction of turns that were affected by lesions in the original study is correct (lesions

primarily altered turns in directions contralateral to the side of the lesion), the discrepancy with our data could be due to sign changes in the neural circuitry or more global effects of the population of neurons that impact the turn decision. It is possible that in the lateral FB, inhibitory neurons innervate excitatory neurons that guide turning behaviors. Our neural recordings and stimulation were more prone to capture and impact these excitatory neurons. Alternatively, discrete electric lesions could have affected inhibitory neurons, leading to hyperactivity of the ipisilateral side of the FB, which in turn caused turning deficits to the contralateral side. Nonetheless, both studies suggest asymmetrical activity between lateral regions of the left and right FB plays a role in controlling asymmetrical movements in cockroaches.

Imposed *versus* self-generated antennal stimulation

While most of the units that we recorded responded to imposed antennal stimulation, very few responded to self-generated antennal contact. Previous studies have shown that upon antennal contact with a vertical rod, tethered cockroaches were able to orient themselves towards the object (Okada and Toh, 2000). This suggests that cockroaches are capable of coding the antennal contact angle and distance. Given the fact that there are some CC units that did respond to self-generated antennal contact, it is possible that, similar to the maplike representation of celestial E-vector orientations in the CC (Heinze and Homberg, 2007), a neural polar coordinate system encoding antennal contact angle and distance also exists in the CC. Under such a system, individual neurons would be tuned to antennal contact of a specific angle and distance from the antennal base.

This may explain the discrepancy in the number of units that responded to imposed antennal stimulation and self-generated antennal contact. The strong stimulus generated by an imposed antennal stimulation device might override biases in the system and cause most neurons to fire. Alternatively, a weaker stimulus such as that associated with self-generated antennal contact would only activate neurons tuned to this specific stimulus. Indeed, recordings in the CC in response to imposed antennal stimulation indicated that most units are velocity or acceleration sensitive (Ritzmann et al., 2008). Because we only categorized antennal contacts according to which antenna made the contact and whether the animal was walking or standing still because of the resolution of our tracking system, such a response could be masked by this overgeneralization. A careful and thorough experimental design may be able to test this hypothesis in the future.

Interactions with thoracic neurons

How might the asymmetrical activity recorded in the CC lead to asymmetrical leg movements associated with turning? Based on the organization that has been described for the locust polarized light system (Homberg, 2008), the asymmetrical activity in the CC would be expected to project to the LAL. Activity in descending neurons would be altered by CC activity as they pass through the LAL. These neurons would then project to thoracic ganglia where they impact local control circuits that provide direct control of leg movements (Büschges, 2005; Büschges et al., 2008).

One way in which the CC-altered descending commands could affect local control is through changes in local reflexes. In stick insects, inter-joint leg reflexes serve to influence the timing of joint cycles, thereby creating coordinated leg movements (Hess and Büschges, 1997; Akay and Büschges, 2006). During turning and backward walking movements, the signs of these reflexes can change resulting in altered joint coordination in specific legs (Akay et al., 2007; Hellekes et al., 2012). In cockroaches, elimination of descending activity through bilateral lesion of cervical connectives can reverse the local chordontonal organ reflexes (Mu and Ritzmann, 2008a). Taken together, these studies suggest that descending commands from the brain can alter leg coordination by changing a few crucial reflexes that start a cascade of changes in leg movements or posture (Mu and Ritzmann, 2008b), which in turn could lead to the transition from straight walking to turning. Ongoing studies that incorporate brain stimulation and EMG recordings will be able to directly examine the relationship between CC activity and local leg reflexes, but this was beyond the scope of the present study. Nonetheless, our results represent a crucial step in linking neural activity in the CC with turning behaviors and may ultimately lead to important insights into how adaptive locomotor behavior is controlled by higher brain centers.

APPENDIX

Tetrode data analysis

Each electrode within a tetrode bundle records action potentials from multiple neural sources. In order to correlate specific electrical impulses to the activity of single neurons, we followed spike-sorting procedures laid out in detail elsewhere (Daly et al., 2004; Bender et al., 2010). We used the MClust toolbox for MATLAB (version 3.5; authors A. D. Redish et al., University of Minnesota). Initial, automated clustering was performed by the program KlustaKwik (version 1.5, author K. Harris, Rutgers University) and then imported into MClust for further refinement and analysis. By sequentially superimposing spike waveforms from pairs of initial clusters, we determined which should be combined into a single cluster and which are more likely to comprise their own clusters. An example of the spike waveforms viewed in MClust is shown in supplementary material Fig. S1A. In this case, three distinct patterns of spike shapes can be seen. Unit 6-1-1 has a larger peak on electrode 3 and smaller peaks on the remaining three electrodes. Unit 6-1-2 has similarly sized spikes on electrodes 1, 2 and 3, while those on electrode 4 are smaller. Unit 6-1-3 has a larger peak on electrode 4 and smaller peaks on the remaining three electrodes.

In the end, all similar initial clusters were merged, and the quality of the resulting clusters was examined by plotting different combinations of electrodes and shape metrics (including energy, peak height, time to peak and principal components) in 3-dimensions. Supplementary material Fig. S1B shows two different 3-dimensional views of the relationships between the waveform energy recorded on three of the four channels of tetrode 6-1.

Three metrics can be used to determine the quality of the resulting clusters. For every cluster, the isolation distance (ID) is the ratio of the cluster's standard deviation to the distance from extra-cluster spikes. High values of ID typically indicate that spikes from a different neuron are incorrectly included in a cluster. The ID values for the clusters in supplementary material Fig. S1 signify around a 1% rate of spikes being incorrectly included. Furthermore, the distribution of interspike intervals (ISIs) may also provide information about the quality of the resulting clusters (supplementary material Fig. S1C). Very short ISIs (<1–2 ms) are unlikely to occur in a single unit because of its refractory period, so an ISI histogram with a substantial number of occurrences at small ISIs suggests that multiple neurons may be included within a cluster. Finally, the L_{ratio} is a measure of how close to the center of a given cluster are spikes from other clusters and indicates the chance that spikes from a single neuron are incorrectly excluded from a cluster. Our L_{ratio} values suggest that perhaps 10% of the spikes in each of our clusters may have been incorrectly excluded.

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