Descending neurons coordinate anterior grooming behavior in *Drosophila*

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Summary

The brain coordinates the movements that constitute behavior, but how descending neurons convey the myriad of commands required to activate the motor neurons of the limbs in the right order and combinations to produce those movements is not well understood. For anterior grooming behavior in the fly, we show that its component head sweeps and leg rubs can be initiated separately, or as a set, by different descending neurons. Head sweeps and leg rubs are mutually exclusive movements of the front legs that normally alternate, and we show that circuits in the ventral nerve cord as well as in the brain can resolve competing commands. Finally, the left and right legs must work together to remove debris. The coordination for leg rubs can be achieved by unilateral activation of a single descending neuron, while a similar manipulation of a different descending neuron decouples the legs to produce single-sided head sweeps. Taken together, these results demonstrate that distinct descending neurons orchestrate the complex alternation between the movements that make up anterior grooming.

Introduction

Complex behaviors are assembled from simpler movements executed in sequence[1–8]. How the central nervous system controls each component and coordinates their combination is not well understood. Fly grooming behavior is an advantageous system to investigate the problem of sequential motor control[3]. When a fly is covered in dust, it cleans each body part in an anterior to posterior progression. Flies usually clean their heads with bilateral symmetric movements of the front legs that we called "head sweeps." Sometimes a head sweep covers most of the head, but these movements can also target different areas more specifically, such as the eye, antenna, proboscis, and the dorsal or ventral head regions. After sweeping the head once or several times, the fly then rubs its front legs together to remove the accumulated dust. Head sweeps and rubs both use the front legs and so are mutually exclusive. A fly usually alternates between them, and together they constitute anterior grooming. Neurons in both the brain and ventral nerve cord contribute to these movements, but how the alternation is achieved is unknown. Here, we identify different descending neurons connecting the brain to the ventral nervous system whose optogenetic activation evokes head sweeps, front leg rubbing, or both. Mapping the neural circuits that connect to these descending neurons illuminates how these movements are coordinated.

Fly behavior is controlled by neurons in the brain and the ventral nerve cord (VNC). Circuits in the brain integrate sensory information and send commands to start or stop actions, while circuits in the nerve cord include the central pattern generators and motor neurons that produce rhythmic limb movements[9,10]. These two parts of the nervous system are connected by ascending and descending neurons (DNs) that travel through the neck connective. These DNs serve as an information bottleneck: 1100 DNs connect ~120,000 neurons in the brain to ~30,000 neurons in the VNC[11]. New genetic reagents available to target different sets of DNs[12,13] make them logical candidates to investigate to better understand principles of sequential motor program selection.

Different sensory cues can elicit similar behaviors. Previous work has shown that activation of head and eye mechanosensory bristles, or the chordotonal neurons in the antenna, can each induce anterior grooming,[14,15] but how these sensory experiences are translated into appropriate motor responses by descending neurons is not known.

Descending neurons may convey commands, initiating whole behavioral programs executed by circuits in the VNC (the "pull-cord toy" model). Theoretically, this control mechanism could elicit a rapid, reliable, but stereotypical response, using a minimal number of neurons. For example, in fly courtship, activation of a single descending neuron aSP22 can trigger a sequence of male behaviors by a ramp-to-threshold mechanism[16]. Alternatively, parallel DNs may each control part of a behavior, and the timing or combination of their activity may be required for coordinated motor output (the "marionette" model). This organization can produce more flexible behavior responding to context or state[17,18]. In vertebrates, different corticospinal neurons were reported to control distinct sets of muscles to orchestrate goal-directed forelimb reach[19]. These different control mechanisms may co-exist, creating partially redundant ways to achieve a behavioral outcome. Examples of multiple control modes occur in courtship, locomotion, and escape behaviors[16,20–22].

Here, we investigated how descending neurons control anterior grooming. We identified three distinct groups of DNs with different effects. Optogenetic activation of DNg12 induced two subroutines of anterior grooming, front leg rubbing alternating with head sweeps directed toward the ventral surfaces of the head. In contrast, activation of DNg11 caused only front leg rubbing, while activation of aDN evoked only head sweeps targeting the antenna. This demonstrates that the descending control for each anterior grooming module is different. Some descending neurons are dedicated to a specific action, while others can mediate several sequential movements, suggesting both "marionette" and "pull-cord toy" models co-exist in anterior grooming regulation.

We also investigated how conflicting descending signals compete for behavior selection. The competition between ventral head sweeps and front leg rubbing may be resolved in VNC since both subroutines can be induced by the same descending neurons. Conflicts between antenna grooming and front leg rubbing can also be resolved in the VNC, since simultaneous optogenetic activation of their separate command neurons, DNg11 and aDN, results in alternation between the two movements. In addition, we found that the brain may also play a role. Using the FlyWire platform to reconstruct neurons in a whole-brain electron microscope volume[23,24], we identified a novel inhibitory circuit connecting head sensory neurons to DNg11s that command front leg rubbing. Different types of sensory neurons may contribute to the selection of appropriate motor subroutines, indicating that decisions between actions can also be made in the brain.

Lastly, we explored the contribution of the brain to neural control of leg coordination. Both head sweeps and front leg rubbing typically employ both front legs working together, but how the limbs are coordinated is not known. Activating DNs on only one side of the body had different effects: unilateral activation of DNg12 or aDN1 induced single-legged head sweeps, while unilateral activation of DNg11 induced both legs to perform front leg rubbing. We identify a neural circuit in the brain that indirectly connects the left and right DNg11s, suggesting how unilateral activation can result in bilateral execution.

In summary, our results show that multiple descending neurons organize different aspects of anterior grooming with potential redundancies, and that both the brain and VNC participate in coordinating the alternation of front leg rubbing and head sweeps.

Results

Anterior grooming consists of alternating head sweeps and front leg rubs

When covered with dust, flies clean their heads by alternation between head sweeps and front leg rubs (Figure 1A). They spend approximately equal amounts of time performing each type of movement (Figure 1B). Undusted flies groom much less, but they also perform these movements in equal proportions (Figures 1C and 1D). In both conditions, the amount of front leg rubs and head sweeps are closely correlated in time, supporting the observation that these anterior grooming movements are strongly coupled.

Although flies usually perform the two movements together, they can do isolated head sweeps or front leg rubbing as well. To quantify anterior grooming structure, we recorded video of fly behavior and analyzed their movements using the Automatic Behavior Recognition System (ABRS)[25]. The behavior records or ethograms of many flies can be compared to extract patterns. Real time ethograms were discretized by consolidating consecutive video frames where the same movement occurs (Figures S1A and S1B)[26]. We then calculated the percentage of time flies spend alternating between head sweeps and front leg rubbing ("coupled") as opposed to performing only one type of front leg movement ("isolated"). As an alternative metric, we measured the transition probabilities between the two movements. In both dust-induced and spontaneous grooming, front leg rubbing and head sweeps are coupled over 90% of the time, and more than 90% of the transitions from one anterior grooming action are to the other (Figure 1E).

Different descending neurons can command head sweeps and/or front leg rubbing

There are approximately 1100 descending neurons (DNs) that connect the brain to the ventral nerve cord. Using optogenetic activation, we screened a collection of genetic reagents that target different populations of DNs[12] to identify neurons capable of commanding specific grooming movements. We found different groups of DNs that can induce alternation of two anterior grooming motifs, or front leg rubbing or head sweeps alone.

Optogenetic activation of DNg12 with csChrimson[27] induced both head sweeps and front leg rubbing. Head sweeps are more common, but around 50% of induced anterior grooming movements are in alternation (coupled), reminiscent of dust-induced grooming (Figures 1F, I, J and S1O). The transition probability between the two actions is also relatively high: over 70% of transitions from front leg rubbing are to head sweeps, while over 45% of transitions from head sweeps are to front leg rubbing. Silencing DNg12 (and some additional neurons) did not affect dust induced grooming as measured by our automatic detection assay (data not shown). DNg12 dendrites arborize extensively in the gnathal ganglion (GNG) and their axons project toward the ipsilateral T1 ganglion in the VNC, the neuropil associated with the front legs. Thus, DNg12 are command-like descending neurons capable of inducing multiple actions within the anterior grooming program.

We also found two groups of DNs that control anterior grooming actions independently. Optogenetic activation of DNg11 induces front leg rubbing in undusted flies (Figures 1G, 1I, S1P). Here, front leg rubbing is decoupled from the head sweeps: over 80% of the front leg rubbing bouts are isolated, and less than 20% of instances of front leg rubbing are followed by head sweeps (Figure 1J). DNg11 is a bilateral group of DNs with cell bodies in the ventral posterior brain, and dendrites in the superior posterior slope (SPS) and GNG. The DNg11 neurons show large axons descending to the T1, T2 and T3 ganglia in the VNC, but they also extend neurites that synapse in contralateral brain regions (Figures 1G, S1C, 5A). We used four different genetic combinations to target DNg11 (*ss02391, ss01579, ss01550, ss02617*), and all four showed isolated front leg rubbing upon optogenetic activation (Figures S1C-I). Considering both anatomical sparseness and phenotype strength, we selected the split-GAL4 combination *ss01550* to represent DNg11 in the following experiments. Optogenetic inhibition of DNg11 in dusted flies reduced but did not eliminate front leg rubbing (Figures S1J-L); head sweeps and leg

rubbing remained coupled (Figures S1M and S1N). These results indicate that DNg11 acts as command-like descending neurons for front leg rubbing.

aDN have been reported to be command-like DNs in antenna grooming[28]. We confirmed that aDN activation causes head sweeps exclusively: only 2% of these head sweeps bouts are followed by front leg rubbing (Figures 1H-J, S1Q). Evidence that DNg11 activation evokes front leg rubbing while aDN induces head sweeps demonstrates that these two motor programs are separable at the level of DN commands.

Flies can target head sweeps to clean specific parts of the head such as the eye, antenna, proboscis, and the dorsal or ventral surface. To further understand the role of individual descending neurons in anterior grooming, we employed a fly-on-a-ball preparation to distinguish differences between head sweeps evoked by DNg12 or aDN. With higher resolution recordings, it is clear that DNg12 and aDN induce different kinds of head sweeps. The activation of DNg12 induced ventral head sweeps with front leg rubbing, while aDN mainly induced antenna grooming, consistent with previous reports (Figure 1K, Videos S1 to S3). In the analyses that follow, we consider all the targeted head cleaning movements as head sweeps (see Methods). Our results suggest that for anterior grooming, both "pull-cord toy" and "marionette" control models exist, with different descending neurons initiating distinct subroutines of anterior grooming (Figure 1L). Targeted head sweeps are controlled by different descending neurons, and front leg rubbing can be evoked either independently or collectively with head sweeps.

Conflicting anterior grooming commands can be resolved in VNC

When a fly is completely covered in dust, it experiences competing drives to perform all the grooming movements, but it only executes one at a time. Where are these choices made? Activation of DNg12 initiates both ventral head grooming and front leg rubbing. DNg12 has major outputs in the VNC, indicating that action selection can be accomplished there.

When antenna grooming and front leg rubbing are induced in parallel by activation of different DNs, flies alternate between the two actions, indicating that they are still able to resolve the competing drives. If the choice between front leg rubbing and head sweeps is usually made in the brain and conveyed to the body by activating only one of the command streams, co-activation of DNs should result in flies that freeze or attempt both behaviors at once. Instead, activating both DNg11 and aDN caused alternation between head sweeps and front leg rubs (Figure 2), demonstrating that mutually exclusive motor actions can still be achieved in spite of competing descending commands. Apparently, neurons in VNC can resolve conflicting descending drives in both behavioral control systems.

An additional test of conflict resolution was conducted by activating DNg11 in dust-covered flies. Consistent with previous results, the amount of time spending on front leg rubbing increased (Figure S2). However, the grooming structure remains stable, with alternation between front leg rubbing and head sweeps. These results also suggest that neurons in VNC can prevent flies from attempting to perform both actions at the same time.

A brain circuit that can select antenna grooming (head sweeps) over front leg rubbing

While neurons in the VNC may be capable of enforcing action selection among grooming movements, anatomical evidence indicates that circuits in the brain may also be involved. We identified the DNg11 descending neurons in the electron microscopy datasets (EM) covering the *Drosophila* brain using the FlyWire interface[23,24] (Figure 3A) and traced their pre and post-synaptic partners. We found three DNg11 neurons in the right hemisphere (DNg11-R) and four in the left (DNg11-L); they show very similar morphology (Figure S3A). The light confocal preparations showed that most flies have 3 DNg11 on each side, consistent with a previous report[12]. As the DNg11-4 displayed a smaller arbor than other members, we propose that DNg11-4 is the result of a developmental anomaly in this individual and do not include it in further analysis.

To identify the neurons that synapse onto DNg11, we focused on their dendritic regions in the posterior slope. Using the automatic synapse detection algorithms[29] confirmed by manual annotation, we reconstructed over 100 hundred neurons synapsing onto DNg11 neurons on each side. We then applied two criteria to select candidate pre-synaptic neurons with the strongest input: a neuron must make connections to all DNg11 on the same side and the synapse number onto each DNg11 must be larger than five. How synapse number correlates with strength is still an open question, but we adopted the threshold of five synapses based on a previous study[29]. We call these pre-synaptic neurons "preferred partners" (Figure 3B). We identified 11 neurons pre-synaptic to DNg1l_R neurons and 17 upstream of DNg11-L neurons that meet these stringent criteria. Most of the candidates are contralateral homologs, further supporting the hypothesis that they are part of functional circuits (Table S1). The DNg11 appear to receive synaptic input from the visual system, the contralateral superior posterior slope (SPS), and the antenna-mechanosensory and motor center (AMMC), suggesting that DNg11 may integrate diverse signals to initiate and adjust front leg rubbing behavior. The complete list of upstream partners, with nomenclature and synapse numbers, is presented in Figure S3B and S3C, but here we describe the projection pattern of one neuron we name JO-in.

JO-in is upstream of DNg11-L neurons and connected by 133 synapses; the contralateral homolog is connected to DNg11-R neurons by 107 synapses. JO-in was noticeable for its looping commissural projection and a large cluster of dendrites in the region of the AMMC, the area to which the chordotonal neurons of the Johnston's organ project (Figures 3C and S4A). Of the >1300 synaptic contacts onto JO-in, more than 400 come from JO chordotonal neurons (JOs, Figures 3K and S4M). JOs detect diverse types of mechanical forces and send their projections into discrete zones in the AMMC[30]. EM reconstruction showed that the majority of JOs that connect to JO-in belong to the JO-E subtype, which are thought to detect static deflection of the antenna[31,32]. Since the JO-E neurons were previously shown to connect directly or indirectly to aDN and their activation induces antennal grooming[14,28] (Figures S4B), we were puzzled to find them upstream of the DNg11 neurons that induce the competing behavior of front leg rubbing.

The conundrum was resolved by determining that the JO-in neurons are likely to be inhibitory. To test JO-in function, we made two different splitGAL4 combinations, *JO-in1* and *JO-in2* to target these neurons (Figures 3D and S4C). Behavioral experiments suggest that the JO-ins can inhibit DNg11: optogenetic activation of JO-in neurons in dusted flies suppresses front leg rubbing but not head sweeps (Figures 3E, 3F, S4D, S4E). No significant behavioral change in front leg rubbing or head sweeps were detected when the JO-in neurons were silenced (Figure

S4F-I), indicating potential redundancy. We show that JO-in neurons are glutamatergic because they can be co-labeled with glutamatergic reporter lines but not with cholinergic or GABAergic ones (Figures 3G and S4J). Although glutamate is typically an excitatory neurotransmitter at the neuromuscular junction in flies, it can also be inhibitory in CNS, acting mainly through the glutamate-gated chloride channel (GluClα) [33–37]. We hypothesized that DNg11 should express GluClα if the glutamatergic JO-ins inhibit them, and indeed, a protein trap reporter for GluClα co-localized with DNg11, labeling their soma and the dendrites where JO-ins connect (Figures 3H, S4K, S4L). Furthermore, front leg rubbing was increased in dusted flies when GluClα expression in DNg11 is reduced using RNAi (Figures 3I and 3J). These results suggest that JO-ins likely inhibit DNg11 through GluClα.

Interestingly, the change in front leg rubbing that occurs when GluClα expression in DNg11 is knocked down *is greater than* when JO-in is activated, suggesting that other inhibitory glutamatergic neurons may also impinge on DNg11 to limit front leg rubbing, perhaps from other head sweep promoting circuits.

In summary, we identified a neural circuit in the brain (Figures 3K and S4M) that helps arbitrate the choice between head sweeps and leg rubs. Activation of sensory neurons in the JO can induce antenna grooming through aDN[28] and inhibit front leg rubbing through JO-in inhibitory connections onto DNg11. Conflicting drives can potentially be resolved in the brain as well.

Left and right leg movements can be decoupled for head sweeps but not for leg rubs

Head sweeps in response to dust usually involve the two front legs moving symmetrically, but we can decouple the legs and induce one-legged head sweeps by activating head sweeping related DNs on only one side (Figure 4).

The split GAL4 line *ss02608* targeting DNg12 that we initially identified in our screen also labels several additional neural classes, including leg mechanosensory neurons and mushroom body neurons (Figures S5A and S5B). We used a recombinase-based strategy that generates expression in random subsets of the split GAL4 pattern to determine the contribution of DNg12 more precisely. Clones that include only mushroom body neurons did not induce the head sweeps (Figure S5C). When both left and right DNg12 neurons were labeled, bilateral head sweeps were induced (Figure 1J), but when only DNg12-Rs were activated, right-leg head sweeps occurred, and when DNg12-Ls were activated, left-leg head sweeps occurred (Figures 4A, 4B, S5D). Since aDN and DNg12 control different kinds of head sweeps, we tested whether left and right coordination can also be decoupled with unilateral activation of aDN. Here, we used aDN1, a subset of aDN, as an example. aDN1 dendrites arborize extensively in the AMMC and GNG, and their axons project to the ipsilateral T1 ganglion, but their cell bodies are located on the contralateral side of the brain. Like DNg12, activation of aDN1-L initiated left-leg head sweeps, whereas activation of aDN1-R initiated right-leg head sweeps (Figures 4C, 4D, S5E). Although single side head sweeps are rare in normal fly grooming, they can be induced by unilateral activation of DNg12 or aDN1 (Figure 4E).

Head sweeps remove debris when dust is detected by activation of local sensory neurons. Usually ipsilateral mechanical stimulation evokes the ipsilateral leg scratching[38]. A previous

study reported that aDN1 co-localizes with the JO projection in AMMC, suggesting aDN1 receive sensory information from ipsilateral JOs[28]. Mechanosensory bristles neurons (MBSN) are the main sensory inputs that induce grooming[14,15], and MBSN axons from the head terminate in the GNG, where DNg12 dendrites are located. Reconstitution of split GFP (GRASP) shows contact between MBSN and DNg12 in the GNG (Figure 4F). Anatomical analysis showed that the MBSNs on the head project ipsilaterally to the gnathal ganglia[14,15]. Taken together, our results suggest that left and right head sweeps can be decoupled, and DNg12 receive sensory information from ipsilateral MBSNs directly.

Next, we investigated left and right coordination in front leg rubbing induced by DNg11 activation. Taking advantage of mosaic strategies, we were able to express csChrimson on a single side (11 right, 3 left; Figure 5A). Unlike head sweeps, unilateral activation of DNg11 triggers front leg rubbing with both legs (Figures 5B and S5F).

We sought a circuit-level explanation for this coordination between the left and right legs. In addition to the axons that descend into the VNC, DNg11 neurons have an additional axonal projection to the contralateral brain hemisphere. The left and right DNg11 neurons do not connect to each other directly, but we examined DNg11's post-synaptic partners using the electron microscopy data. We used DNg11-L1 and DNg11-L2, the neurons with the most complete axonal reconstructions, for our analysis, and identified 8 neurons downstream of DNg11-L and 10 downstream of DNg11-R with the same criteria for "preferred partners" described above; many of these seem to be contralateral homologs (Figures 5C, Table S1). A full description of these post-synaptic partners is presented in Figures S3D and S3E, but we focused on one neuron downstream of DNg11-R that we call LNR1 because it connects to another neuron, LNR2, which we had previously identified as *pre-synaptic* to DNg11-L (Figure 5D). This forms an indirect bridge connecting DNg11-R and DNg11-L. DNg11-Rs and LNR1 are connected by 211 synapses, LNR1 and LNR2 are connected by 44 synapses, and LNR2 and DNg11-L are connected by 94 synapses (Figures 5E and 5F). The reciprocal circuit from DNg11-L to DNg11-R was also found and shows similar synaptic contact numbers (Figures S5G and S5H). The commissural circuit connecting the left and right DNg11 neurons can potentially explain how unilateral activation of DNg11-R results in the bilateral leg rubbing behavior we observe.

Discussion

Execution of complex behavior requires coordinating motor programs[39]. Bundling activation of motor neurons in synergy to produce a limb movement, or combining subroutines together to achieve a goal-directed behavior, is one organizational strategy for reliably and efficiently producing a behavior. We describe this coordinated control of multiple elements as the "pull-cord toy" model because activating one command elicits a whole program of actions. An alternative mode of organization can be described by analogy to the marionette: separate command strings control each subroutine, and the different commands work together to produce the final movement program, the way strings to each limb can be moved in concert to make the puppet dance. This alternative control mechanism might permit greater flexibility, adjusting the

behavioral program to different needs or conditions by varying the ratio or timing of component actions.

We find evidence for both “pull cord toy” and “marionette” control modes in anterior grooming at the level of the descending neurons. Anterior grooming typically involves alternation of targeted head sweeps and front leg rubbing to remove debris. We can artificially induce these two subroutines in alternation by optogenetically activating one class of descending neurons, DNg12, for the “pull cord toy”, but we can induce them separately as well, by activating different descending neurons (DNg11 for front leg rubbing or aDN for antenna grooming) for the “marionette” (Figure 6).

Why would the fly need both control modes? Perhaps distinct circuits provide protective redundancy for an important behavior. The limited reduction in front leg rubbing that occurs when we inactivate DNg11 supports this. Silencing DNg12 also has only a small effect on grooming in our experimental assay, so we cannot definitively answer why the fly needs both control mechanisms. Perhaps stereotyped or flexible coordination is optimal under different circumstance, such as when dust must be removed quickly or when only some parts of the body are dirty. Global head sweeps and leg rubs might clear parasites or food particles, while targeted antennal grooming might be a better choice if this sensory apparatus is displaced by wind. While this is speculation, the fly does display multiple control modes for other complex behaviors. The Moonwalker Descending Neurons command backward locomotion, while a distributed rather than centralized control pattern is seen downstream[17]. Escape behavior, for example, can be induced by rapidly-expanding visual stimuli that trigger the giant fiber and immediate launch, or by slower expansions that act through a different circuit to initiate a motor program producing a more controlled, directional takeoff[22]. It may be an advantage to have both stereotyped and flexible modes of control for redundancy and for deployment in different circumstances.

There may be selection bias that leads to experimental identification of “pull-cord toy” mechanisms more frequently than components of a “marionette” model. Thermo- and optogenetic screens have revealed command-like neurons capable of inducing courtship, aggression, feeding, oviposition, grooming, and escape, but our own experience and the published literature suggests that neurons whose activation induce coherent behaviors - either recognizable natural movements or whole motor programs with sequential subroutines - are relatively rare[13,40–42] When they do occur, these phenotypes are striking and immediately recognizable by human observers. In future, we may begin to identify neurons that induce specific movements or smaller subroutines as new automatic behavior recognition and limb tracking pose identification software becomes more commonly employed in screens.

Defining the neural circuits that command and control motor sequences is challenging. Here we screened descending neurons as the bottleneck in the sensorimotor circuits governing fly grooming behavior and used those neurons as the starting point to map circuits. While our competitive activation experiments show that conflicting descending commands can be resolved in the ventral nerve cord for both pull-cord toy and marionette models, the downstream circuits that enforce mutual exclusivity, produce rhythmic alternation, and ultimately engage the motor neurons remain to be mapped.

While the conflict can be resolved in VNC, it is possible that descending neurons normally fire in alternation in response to decisions made in the brain. Our work here focused on the circuits in the brain, the pre-synaptic inputs to the DNs. We identified sensory inputs, as well as a myriad of other connected partners. Tracing neurons with the most synaptic contacts and those that have clear homologs on both sides of the brain led us to map circuits connecting sensory neurons to descending neurons. In both vertebrate and invertebrate systems, theoretical and computational models of action selection have emphasized that inhibitory neurons are often important to resolve a single outcome from many possible alternatives[39,43–46]. In our study, we identified a feedforward inhibitory mechanism for selecting antenna grooming over front leg rubbing in the brain: sensory neurons in the antenna (JO) activate the aDN command neuron to induce antenna grooming and simultaneously inhibit the DNg11 command neuron to prevent front leg rubbing.

Left and right coordination is essential for bilateral limb movements, such as locomotion - or grooming[47–53]. Central pattern generating circuits (CPGs) in the insect ventral nerve cord or vertebrate spinal cord can produce rhythmic movements, and connections between them are key for coordinated movements. Our study shows that anterior grooming is a useful model to address left and right limb coordination. Head sweeps requires the front legs to move in synchrony, while front leg rubbing requires them to move against each other, out of phase. While much of the commissural communication may occur in the VNC, the results of our mosaic activation experiments show that descending neurons can play a role as well. Ipsilateral activation of DNg12 or aDN1 results in single-leg head sweeps, while ipsilateral activation of DNg11 induces leg rubbing in which both the left and right leg participate. Using the EM data, we identified an indirect commissural connection between the DNg11 neurons in the brain that can explain this obligatory coordination.

We noticed that ipsilateral head sweeps induced by DNg12 were eventually followed by symmetric head sweeps (Figure 5) and speculate that this is caused by sensory feedback from the head or leg, or commissural circuits connecting CPGs in the VNC, revealing another redundant control mechanism. While VNC circuits may also contribute to coordinating the out-of-phase leg rubbing movements, we show the participation of the brain, where indirect commissural connections allow unilateral activation of DNg11 to induce bilateral front leg rubbing.

Our results show that descending neurons can evoke either specific grooming movements or combinations, and that circuits in the brain or VNC can resolve conflicting commands. Understanding the timing of neural activity in these DNs relative to each other and to different grooming movements, and mapping the way they mesh with the motor neurons, will be the next steps in deciphering a more complete picture of the competitive and redundant neural architecture controlling anterior grooming behavior.

Acknowledgements

For EM data analysis, we thank FlyWire community, especially Claire E. McKellar, Kale Patrick Willie, Ryan Willie, Austin Burke, Sarah Morejohn, Emil Kind, Dustin Garner and the labs of Mathias Wernet and Sung Soo Kim for to permission to use neuronal edits. We also thanks members of the University of Cambridge Drosophila Connectomics Group (supported by

Wellcome Collaborative Award 203261/Z/16/Z) including Imaan Tamimi, Yijie Yin, Joseph Hsu, Katharina Eichler and Gregory Jefferis for additional tracing. For fly strains, we thank Kenta Asahina, Gerald M. Rubin, and Adam Claridge-Chang. We thank Andrew Seeds and Steffi Hampel for productive discussions. We thank the Simpson lab, especially Primoz Ravbar (ABRS support), Preston Terle (dissection), Angel Okoro (manual scoring), Durafshan Sakeena Syed and Sharon Tamir (data contributions for Figure S1 J-N). The study is supported by NSF CAREER: Descending neural control of a motor sequence in Drosophila, 1943276.

Author Contributions

L.G. and J.H.S. conceptualized the study; L.G. and N.Z. performed experiments and analyzed data; L.G. and J.H.S. wrote the manuscript; J.H.S. acquired the funding and supervised the study.

Declaration of Interests

The authors declare no competing interests

Figure Legends

Figure 1. Head sweeps and front leg rubbing normally occur together but can be induced either separately or collectively by activation of different descending neurons.

(A) Diagram of alternation between head sweeps and front leg rubbing.
(B-C) Progression of head sweeps and front leg rubbing in dusted or undusted flies ($n \geq 44$). Behavioral probabilities are calculated every 33s. Each data point is the average among all individuals. The shaded region shows standard error of mean.
(D) Probability of head sweeps or front leg rubbing in dusted and undusted flies.
(E) Anterior grooming structure in dusted or undusted flies. Left, the probability of coupled or isolated anterior grooming; right, the transition probability between head sweeps and front leg rubbing.
(F-H) Grooming response of undusted flies upon activation of DNg12(F), DNg11(G) and aDN (H). Left, expression pattern of descending neurons (green) in central nervous system (CNS, magenta). Scale bars,100 μm. Right, progression of head sweeps and front leg rubbing. Optogenetic stimulation was given between 60 and 120s, indicated by red line. Behavioral probabilities were calculated every 10s ($n \geq 9$).
(I) Head sweeps and front leg rubbing probability upon activation of DNg12, DNg11 or aDN.
(J) Anterior grooming structure upon activation of DNg12, DNg11 or aDN. Left, coupled or isolated anterior grooming probability; right, head sweeps and front leg rubbing transition probability.
(L) The probability of different anterior grooming modules evoked upon activation of DNg12 or aDN.
(M) Diagram of two descending control models for anterior grooming. See also **Figure S1 and Videos S1-S3**.
Wilcoxon rank-sum test were used. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.
Note that the full genotypes for each panel are included in the Methods table.

Figure 2. Competition between head sweeps and front leg rubbing commands can be resolved in VNC.

(A) Expression pattern (green) of DNg11 (white arrowheads) and aDN (yellow arrowheads) in the CNS (magenta). Scale bars, 100 μm.
(B) Example ethograms of the behavioral response of undusted flies upon activation of DNg11, aDN, or both DNg11and aDN.
(C) Progression of head sweeps and front leg rubbing upon DNg11 and aDN activation (n=13).
(D) Anterior grooming structure upon dust or DNg11 and aDN activation. Left, coupled or isolated anterior grooming probability; right, head sweeps and front leg rubbing transition probabilities. See also **Figures S1 and S2**.

Figure 3. A brain circuit that can select head sweeps over front leg rubbing.

(A) Reconstruction of DNg11 from EM data. Left, the skeletons of DNg11 neurons. Arrowheads show where the axons leave the brain to project to VNC. Right, one example of post-synaptic sites and pre-synaptic sites of DNg11.
(B) Venn diagrams of presynaptic "preferred partners" (with synaptic connections >5) for each DNg11-L (left) and DNg11-R (right).
(C) A neural circuit identified from EM dataset connecting from JOs-R to DNg11-L via JO-in-R.
(D) The skeleton of JO-in from EM data and the expression pattern (green) in the CNS (magenta) of *JO-in1 spGal4*, (*R49E07-AD* and *VT014567-DBD)*.
(E) Progression of head sweeps and front leg rubbing upon JO-in activation in dusted flies (n ≥15).
(F) Head sweeps or front leg rubbing probability in dusted flies upon JO-in activation.
(G) Double-labeling of JO-in (magenta) with glutamatergic neurons (green).
(H) Double-labeling of DNg11(magenta) with GluClα (green).
(I) Progression of head sweeps and front leg rubbing in DNg11 GluClα-RNAi dusted flies (n ≥14).
(J) Head sweeps or front leg rubbing probability in DNg11 GluClα-RNAi dusted flies.
(K) Summarized inhibitory circuit from JOs to DNg11 via JO-in. Numbers represent the synapse counts. See also **Figure S3, S4** and **Table S1**.
Wilcoxon rank-sum test were used. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Scale bars, 100 **μ**m unless otherwise indicated on the figure.

Figure 4. Left and right leg movements can be decoupled for head sweeps by unilateral activation of DNs.

(A-D) Grooming response to the activation of DNg12-L (A), DNg12-R (B), aDN1-L (C) and aDN1-R (D). Left, expression pattern (green); right, progression of each anterior grooming actions in optogenetic activated flies (n≥ 9).
(E) The probability of bilateral or unilateral head sweeps evoked upon unilateral activation of DNg12 or aDN1, compared to dust-induced head sweeps.
(F) GRASP between mechanosensory bristle neurons (MBSNs; *R38B08-lexA)* and DNg12 (*ss02608 spGal4)*. Scale bars, 100 **μ**m. See also **Figure S5**.

Figure 5. Left and right leg movements can be coordinated by commissural connections between left and right DNg11

(A) Unilateral expression of DNg11(green) in the CNS (magenta).
(B) Front leg rubbing response to unilateral activation of DNg11 in undusted flies, including progression and probability (n=14, 11 right, 3 left).
(C) Venn diagrams of postsynaptic "preferred partners" (synaptic connections >5) for each DNg11-L (left) and DNg11-R (right).
(D) The skeletons of LNR1 (left) and LNR2 (right) from EM data.
(E) A neural circuit identified from EM data connecting DNg11-R and DNg11-L via LNR1 and LNR2.

(F) Summarized circuit connecting left and right DNg11. Numbers represent the synapse counts. See also **Figure S5 and Table S1**.
Wilcoxon rank-sum test were used. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Scale bars, 100 **μ**m.

Figure 6. Schematic of neural circuits orchestrating anterior grooming

Based on light and electron microscopy, we propose distinct neural circuits to control antenna grooming (aDN) and front leg rubbing (DNg11), while a separate circuit (DNg12) controls the coupled alternation of ventral head grooming and front leg rubbing. Both the brain and VNC prevent head sweeps and front leg rubbing from being executed at once.

STAR METHODS

Detailed methods include the following:

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STAR METHODS

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Julie H. Simpson (jhsimpson@ucsb.edu).

Materials Availability

This study did not generate new unique reagents.

Data and code availability

- EM data are publicly available as of the date of publication. Segment IDs and access link are listed in the Table S1. Behavioral data, representative videos, and example photomicrographs are included as part of the manuscript submission.
- All original code has been deposited at GitHub, page associated with ABRS.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Fly husbandry

Flies were raised on common cornmeal food at 25°C and 50% relative humidity on a 12hr light/dark cycle. The behavioral experiments were performed in male flies aged 3-8 days. For optogenetic experiments, male adults were collected on normal food shortly after eclosion, starved for 12h and transferred to the retinal food 3-5 days prior to testing. Retinal food contains

0.4mM all-trans-retinal. The flies are kept in dark until testing. For unilateral activation experiments, crosses were set at 25°C: flies were tested individually and retrieved for anatomical characterization.

Detailed fly genotypes in each figure are listed below.

Figure	Genotype
Figure 1	*CantonS*
	DNg12: *VT025739-p65ADZ/Otd-nls::FLP; VT025999-ZpGDBD/ 20XUAS-FRT>STOP>FRT-CsChrimson-mVenus*
	Control_DNg12: *VT025739-p65ADZ/Otd-nls::FLP; VT025999-ZpGDBD/ 20XUAS-FRT>STOP>FRT-CsChrimson-mVenus*
	DNg11: *20XUAS-ChrimsonR-mCherry/ Y; VT037583-p65ADZ/+; VT025598-ZpGDBD/+*
	aDN: *20XUAS-ChrimsonR-mCherry/ Y; ;R18C11Gal4/+*
	Control_DNg11, aDN: *20XUAS-ChrimsonR-mCherry/ Y; BPp65ADZp/+; BPZpGDBD/+*
Figure 2	DNg11: *20XUAS-ChrimsonR-mCherry/ Y; VT037583-p65ADZ/+; VT025598-ZpGDBD/+*
	aDN: *20XUAS-ChrimsonR-mCherry/ Y; ;R18C11Gal4/+*
	DNg11+aDN: *20XUAS-ChrimsonR-mCherry/ Y; VT037583-p65ADZ/+; VT025598-ZpGDBD/ R18C11Gal4*
Figures 3 and S4	JOs: *20XUAS-CsChrimson-mVenus/Y; R61D08AD/+; R27H08-DBD/+*
	Control>cschrimson: *20XUAS-CsChrimson-mVenus/ Y; BPp65ADZp/+; BPZpGDBD/+*
	JO-in1>cschrimson: *20XUAS-CsChrimson-mVenus/ Y; R49E07ADZp/+; VT014567DBD/+*
	JO-in2>cschrimson: *20XUAS-CsChrimson-mVenus/ Y; R49E07ADZp/+; VT050229DBD/+*
	Glutamatergic neurons double labeling: LexAop2-syn21-opGCaMP6s,10XUAS-Syn21-Chrimson 8B-tdTom3.1; Vglut LexA#5.1/ R49E07ADZp; VT050229DBD/+
	Cholinergic neurons double labeling: LexAop2-mCD8::GFP, UAS-mCD8::RFP; +/ R49E07ADZp; VT050229DBD/chat-lexA
	GABAergic neurons double labeling: LexAop2-mCD8::GFP, UAS-mCD8::RFP; +/ R49E07ADZp; VT050229DBD/GAD1-lexA
	GluClα double labeling: 20XUAS-ChrimsonR-mCherry/ Y; VT037583-p65ADZ/+; VT025598-ZpGDBD/ GluClα MI02890-GFSTF.2
	Control for GluClα double labeling: 20XUAS-ChrimsonR-mCherry/ Y; VT037583-p65ADZ/+; VT025598-ZpGDBD/ TM6B (from the same cross as GluClα double labeling)
	Control>UAS-GluClα-RNAi: UAS-Dicer2/ Y; BPp65ADZp/UAS-GluClα-RNAi; BPZpGDBD/+
	DNg11>UAS-GluClα-RNAi: UAS-Dicer2/ Y; VT037583-p65ADZ /UAS- GluClα-RNAi; VT025598-ZpGDBD /+
Figure 4	DNg12_control, DNg12_left, DNg12_right: *VT025739-p65ADZ/Otd-nls::FLP; VT025999-ZpGDBD/ 20XUAS-FRT>STOP>FRT-CsChrimson-mVenus*
	Unilateral aDN1: *R57C10-Flp2::PEST/Y; R18C11AD /+; R71D01DBD / 20XUAS-FRT>STOP>FRT-CsChrimson-mVenus*
	GRASP: VT*025739-p65ADZ/ R38B08-lexA; VT025999-ZpGDBD/ UAS-CD4-spGFP1-10, lexAop-CD4-spGFP11*

Figure 5	Unilateral DNg11: *R57C10-Flp2::PEST/Y; VT037583-p65ADZ/+; VT025598-ZpGDBD/ 20XUAS-FRT>STOP>FRT-CsChrimson-mVenus*
Figure S1 and S2	ss02391: *20XUAS-CsChrimson-mVenus/Y; 81C11-p65ADZp/+; 66B05-ZpGDBD/+*
	ss01579: *20XUAS-CsChrimson-mVenus/Y; VT037583-p65ADZ/+; 81C11-ZpGDBD/+*
	ss01550 or DNg11>csChrimson: *20XUAS-CsChrimson-mVenus/Y*; *VT037583-p65ADZ/+; VT025598-ZpGDBD/+*
	ss02617: *20XUAS-CsChrimson-mVenus/Y*; *VT037574-p65ADZ/+; VT025598-ZpGDBD/+*
	Control> csChrimson: *20XUAS-CsChrimson-mVenus/ Y; BPp65ADZp/+; BPZpGDBD/+*
	Control> GtAcr1: *BPp65ADZp/+; BPZpGDBD/UAS-GtAcr1*
	DNg11> GtAcr1: *VT037583-p65ADZ/+; VT025598-ZpGDBD/ UAS-GtAcr1*

Method Details

Fly dusting

Fly dusting was performed as described in Seeds et al.[42] and Zhang et al.[14]. Briefly, dusting experiments were done in a WS-6 Mystaire downflow hood. Male flies were anesthetized on ice and gently transferred to the 4 middle holes of a transfer chamber. Each hole contained one fly, and flies rested at least 15 mins before testing. Before use, dust was sifted twice using the mesh from the transferring and recording chamber. Dust was placed in 4 middle wells of the dusting chamber (~5mg/each). Flies were transferred to the dusting chamber, shaken, tapped to remove excess dust, and then transferred to the recording chamber. 30Hz videos were recorded by a DALSA Falcon2 color 4M camera and grooming movements were scored by ABRS[25].

Optogenetic experiments

For undusted flies, males were anesthetized on ice and rested in the recording chamber for at least 15 mins before testing. Custom-made LED panels (LXM2-PD01-0050, 625nm) were used for light activation from below. The light intensity was 3.4 mW/cm^2 except for the DNg12 (1.7 mW/cm^2), with 20Hz 20% duty cycle. For dusted flies, flies were dusted by the procedure described above. The light intensity used in the most activation experiments was 3.4 mW/cm^2 except for the *JO-in1* (13.6 mW/cm^2), *JO-in2* (1.7mW/ cm^2). For inactivation experiments, custom-made LED panels (LXM2-PD01-0050, 525nm) were used with 3.12 mW/cm^2 light intensity. 30Hz videos were recorded by a FLIR Blackfly S USB 3 camera. Grooming movements were manually annotated by Vcode or automatically detected by ABRS[25]. Different head sweeps are hard to be characterized with this resolution by either eye or ABRS. As we are interested in the alternation between head sweeps and front leg rubbing, different head sweeps are not differentiated in these experiments.

Fly-on-a-ball experiments

Individual male flies were anesthetized by cooling and then tethered to a size 1 insect pin with UV-cured glue. Air flow (500-600mL/min) passing though the water was used to support the 10mm diameter foam ball (LAST-A-FOAM FR-7120 material). A fiber-launched LED (CLED_635) with custom-made collimator (Doric Lenses) were used to target light to the fly head. Because it is hard to measure the illumination area, LED light power rather than intensity was used. For aDN activation, 40uW constant red light was used in all experiments. For DNg12 activation, 80uW 20Hz 20% duty cycle red light was used in all experiments. As it is hard to obtain the bilateral labelling of DNg12 in the clonal experiments, all the DNg12 flies used in this experiment are DNg12-R, which has DNg12 expressed in only right side. The activation of original line (ss02608, bilateral DNg12+ leg mechanosensory bristle neurons) triggered the flies to clean the same head region as DNg12-R did (data not shown). The detailed experimental set up was described by Zhang et al.[14]. 100Hz videos were recorded with a FLIR Blackfly S BFS-U3-13Y3M-C camera and manually annotated in VCode. Head sweeps directed to different parts of the head were manually coded from videos of the fly-on-a-ball experiments.

Immunofluorescence and confocal imaging

For antibody staining, adult males were fixed rotating with 4% paraformaldehyde at room temperature for 2 hours. Samples were dissected in 1xPBS, and then washed 3 times for 1 min each with 1xPBST (1xPBS and 1% Triton X-100). CNS tissues were blocked with 5% normal goat serum (NGS) in PBST for 30mins at room temperature. Primary antibody staining was performed overnight at 4°C on a nutator in 300ul 1xPBST with following dilutions: 5% NGS, rabbit polyclonal anti GFP (Invitrogen, 1:1000), mouse monoclonal anti brp (DSHB nc82, 1:200). Samples were then washed 4 times with 1xPBST for 15mins each. Secondary antibody staining was performed at room temperature on a nutator in dark. CNS were incubated in 300ul 1xPBST with secondary antibodies with following dilutions: 5% NGS, goat anti-rabbit Alexa Flour 488 (Invitrogen, 1:500), goat anti-mouse Alexa Flour 568 (Invitrogen, 1:200). Samples were washed again with 1xPBST 4 times for 15 mins each and mounted in VectaShield.
For endogenous GFP analysis in GRASP, adult males were rinsed in 100% ethanol and dissected in 1xPBS. Tissues were washed 3 times in 1xPBS for 1min each and then mounted in VectaShield. All confocal images were taken on a Zeiss LSM710 microscope using sequential scanning mode and processed by Image J.

EM Reconstruction

Neuron skeletons were reconstructed in a serial section transmission electron microscope volume of an adult female brain by both auto-segmentation and manual proofreading in FlyWire (https://flywire.ai). Neuroanatomical landmarks from the confocal images such as position, backbone orientation, and the projection boundaries were used to search for the potential candidates for DNg11. After tracing one DNg11, we tried to find all the sister cells by searching within the neurite bundle. Three DNg11 were found in the right hemisphere and four in the left. DNg11-Rs and DNg11-L1 cells were traced and proof-read to near-completeness. Due to some missing or damaged sections in the EM dataset, DNg11-L2 projections in left and right hemisphere were traced separately and DNg11-L3 projections in ipsilateral hemisphere were

traced but not the ones in the contralateral hemisphere. Since DNg11-4 has a smaller arbor and most flies have 3 DNg11 on each side from light confocal data, we did not include this member in our further analysis. The FlyWire segments IDs for these neurons are listed in Table S1.

Synapses were annotated manually based on the criteria for a chemical synapse: an active zone with vesicles, synaptic clefts, and T bars. Both upstream and downstream partners of DNg11 were identified and proof-read in FlyWire. The connections were further confirmed by automatic synapse detection[29]. All the figures used the automatic synapse detection results to avoid human bias. We set strict criteria to pick the "preferred partners" for both upstream and downstream: a neuron must make connections to all DNg11 on one side, and the number of synapses onto each must be larger than 5, in accordance with previous studies[29,60]. Preferred partners are named by their connections and synapse number as a proxy estimate for connection strength. Genetic reagents to target JO-in were identified by manually searching through Janelia Neuprint (https://neuprint.janelia.org). EM data was plotted with the Natverse R package[61].

QUANTIFICATION AND STATISTICAL ANALYSIS

Data analysis was performed in MATLAB and R. Mann-Whitney U test was used to evaluate significance. For progression analysis, behavioral probabilities were calculated every 33s (video length ≥ 20min), or 10s (video length ≤ 4min). For anterior grooming structure, the real time ethograms were processed to generate discrete-bout ethograms, and each bout was labelled by its action and duration for further analysis. If front leg rubbing is followed by head grooming or vice versa, the bout is considered *coupled* anterior grooming. If not, the bout belongs to the *isolated* anterior grooming category. Transition probability was analyzed based on the Markov chain model, which states that the transition from one behavioral state to another depends only on the current state. The detailed structure analysis was described by Mueller et al.[26]. Data was plotted with ggplot2 R package.

Video S1 Activation of DNg12-R induced ventral head sweeps and front leg rubbing. Related to Figure 1.
Video S2 Activation of aDN induced antennal grooming. Related to Figure 1.
Video S3 Activation of DNg11 induced front leg rubbing. Related to Figure 1.

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