

1 **GABAergic inhibition of leg motoneurons is required for**
2 **normal walking behavior in freely moving *Drosophila***

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25 ABSTRACT

26 **Walking is a complex rhythmic locomotor behaviour generated by**
27 **sequential and periodical contraction of muscles essential for**
28 **coordinated control of movements of legs and leg joints. Studies of**
29 **walking in vertebrates and invertebrates have revealed that**
30 **premotor neural circuitry generates a basic rhythmic pattern that is**
31 **sculpted by sensory feedback and ultimately controls the amplitude**
32 **and phase of the motor output to leg muscles. However, the identity**
33 **and functional roles of the premotor interneurons that directly**
34 **control leg motoneuron activity are poorly understood. Here we take**
35 **advantage of the powerful genetic methodology available in**
36 ***Drosophila* to investigate the role of premotor inhibition in walking**
37 **by genetically suppressing inhibitory input to leg motoneurons. For**
38 **this, we have developed a novel algorithm for automated analysis of**
39 **leg motion to characterize the walking parameters of wildtype flies**
40 **from high speed video recordings. Further, we use genetic reagents**
41 **for targeted RNAi knockdown of inhibitory neurotransmitter**
42 **receptors in leg motoneurons together with quantitative analysis of**
43 **resulting changes in leg movement parameters in freely walking**
44 ***Drosophila*. Our findings indicate that targeted down regulation of**
45 **GABA_A receptor Rdl in leg motoneurons results in a dramatic**
46 **reduction of walking speed and step-length without the loss of**
47 **general leg coordination during locomotion. Genetically restricting**
48 **the knockdown to the adult stage and subsets of motoneurons yields**
49 **qualitatively identical results. Taken together, these findings identify**
50 **GABAergic premotor inhibition of motoneurons as an important**
51 **determinant of correctly coordinated leg movements and speed of**
52 **walking in freely behaving *Drosophila*.**

53 **KEY WORDS**

54 Walking, Pre-motor inhibition, interneurons, leg motoneurons, Speed

55 **SIGNIFICANCE STATEMENT**

56 **Inhibition is an important feature of neuronal circuit and in walking**
57 **it aids in controlling coordinated movement of legs, leg segments and**
58 **leg joints. Recent studies in *Drosophila* reports the role of premotor**
59 **inhibitory interneurons in regulation of larval locomotion. However,**
60 **in adult walking the identity and functional role of premotor**
61 **interneurons is less understood. Here, we use genetic methods for**
62 **targeted knockdown of inhibitory neurotransmitter receptor in leg**
63 **motoneurons that results in slower walking speed and defects in**
64 **walking parameters combined with novel method we have developed**
65 **for quantitative analysis of the fly leg movement and the observed**
66 **changes in walking parameters. Our results indicate that GABAergic**
67 **pre-motor inhibition to leg motoneurons is required to control the**
68 **normal walking behaviour in adult *Drosophila*.**

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71 INTRODUCTION

72 Walking is a complex rhythmic locomotor behavior that requires the
73 coordinated control of movements among legs, leg segments and leg
74 joints (1–5). While the complete neural circuitry of the motor control
75 networks that orchestrate this control is not known in any animal,
76 significant progress has been made in understanding the general
77 organization of the underlying networks. Studies of reduced preparations
78 in vertebrates have revealed that premotor interneuronal circuits present
79 in the spinal cord are capable of generating patterned rhythmic locomotor
80 activity and distributing this activity to motoneurons (6–8). Furthermore,
81 in several cases the function of identified spinal interneuron types in this
82 type of fictive locomotion has been elucidated (9, 10). However, the
83 complexity and the difficulty of genetic intervention in vertebrate models
84 have made the cellular identification and functional analysis of these
85 premotor control circuits difficult.

86 Invertebrate models have reduced complexity and for some of them, such
87 as *Drosophila*, genetic reagents that enable visualization and perturbation
88 of neural circuitry are available (11–13). In *Drosophila*, the neural
89 connectivity of leg motoneurons, their structural organization and their
90 relationship with leg muscles has been well established (14–17).
91 Moreover, extensive neurogenetic analyses of walking behavior in
92 *Drosophila* have shown that higher brain centers such as central complex,
93 the ellipsoid body and the mushroom bodies affect higher order
94 phenomenon such as the drive to walk, ability to maintain a fixed course
95 or goal directed locomotion (18,19,21). However, these central brain
96 structures are entirely dispensable for the more mundane aspect of
97 walking – maintaining all the moving parts in harmony with each other.

98 The observation that headless flies can walk, demonstrates that the higher
99 order brain regions has less contribution for coordination of leg motion
100 and that the minimal circuitry required for the generation of rhythmic
101 walking behavior resides in the ventral nerve cord (22,23). Thus, as in
102 other insects, premotor neural circuitry in the ventral nerve cord is
103 thought to generate a basic rhythm, which is sculpted by bilateral and
104 intersegmental coordination processes; modified by sensory feedback
105 such that it ultimately controls the magnitude and timing of patterned
106 motoneuronal output to leg muscles as the animal walks (20,24–26).

107 Inhibitory interactions are important features of the neural circuitry that
108 generate walking (27-29). Inhibition is involved in controlling flexor-
109 extensor alternation, in bilateral and intersegmental coordination as well
110 as in propagation and termination of motoneuron activity as shown in
111 vertebrates (9, 30, 31). In *Drosophila*, recent studies on larval stages have
112 identified two types of inhibitory premotor interneurons that are involved
113 in controlling the motor activity required for larval crawling (32, 33).
114 Moreover, also in larvae, segmentally repeated GABAergic interneurons
115 have been identified and implicated in the control of the peristaltic wave
116 of activity that underlies larval crawling (34). However, in adult flies the
117 identity and functional circuit features of the premotor interneurons that
118 control leg motoneuron activity are poorly understood and virtually
119 nothing is known about a role of inhibitory premotor interneurons in
120 walking behavior.

121 Here we investigate the role of premotor inhibition in walking by
122 selectively suppressing GABAergic input to leg motoneurons. For this,
123 we first develop a novel algorithm for the automated analysis of leg
124 movements in order to characterize the walking parameters of wild type
125 flies from high speed video recordings. Using genetic reagents that allow

126 selective labeling of leg motoneurons together with targeted RNAi
127 knockdown of neurotransmitter receptors, we then interrogate the nature
128 of their pre-motor neuronal input during walking by analyzing the
129 walking parameters. Our findings indicate that knocking-down the
130 expression of GABA_A receptor Rdl (Resistance to dieldrin) results in
131 dramatically reduced walking speed as well as reduced step-length and
132 failure to achieve sustained leg extensions during locomotion. Genetically
133 restricting the knockdown to the adult stage or to subsets of motoneurons
134 gives qualitatively identical results - slower walking speeds, shortened
135 step lengths without a general loss of coordination. Altogether, these
136 findings identify premotor inhibition of motoneurons as an important
137 determinant of leg movement and resulting speed in freely behaving
138 *Drosophila*.

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142 **RESULTS**

143

144 **Recording and analysis of leg movements in freely walking flies**

145 As a prerequisite for investigating whether pre-motor inhibitory inputs
146 are required for appropriate walking behavior in *Drosophila*, we
147 developed an automated high-speed video recording and analysis
148 technique. This technique made it possible to record the protraction
149 (swing) and retraction (stance) phases of leg motion at high resolution for
150 each leg in freely walking flies (**see methods**). For a detailed
151 characterization of these leg movement phases, an auto-detection of leg
152 movements from video frames was carried out followed by the
153 quantitative analysis of leg movements from the extracted leg-tip
154 coordinate data.

155 To auto-detect the movement of all six legs in their entirety given the
156 video frame of a fly, a novel algorithm was developed using an open
157 source image analysis software package - Fiji (2013 lifeline version). In
158 this algorithm, light intensity thresholding was used to obtain a binary
159 image of the fly and its contour was auto-selected as a full ROI (region of
160 interest). Subsequently, the full ROI was adjusted such that legs were
161 uncovered (torso ROI) and individual leg ROIs are computed by pixel-
162 wise logical XOR between the full ROI and the torso ROI (Fig. 2A).
163 (Alternatively, in simplified terms, this algorithm can be described
164 according to binary morphological operations of erode and dilate as
165 follows: erode the binary mask of the fly n times such that only the
166 partially eroded torso remains, dilate the binary mask of the torso n times
167 in order to restore the size of the torso, compute a third binary mask by
168 performing a pixel-wise XOR operation between the full fly binary mask
169 and the torso binary mask, obtain the binary mask of all six legs without
170 the torso). After the leg's ROIs were isolated, the leg-tip coordinates
171 were extracted for further analysis.

172

173 A temporal projection of the leg-tip coordinates resulted in an image that
174 consisted of six distinct clusters, which allowed efficient isolation and
175 assignment of each leg-tip coordinate to the respective hemithoracic
176 segment (Fig. 2B). Each leg-tip coordinate was a 3-dimensional entity
177 comprising x , y and frame number values. From these values, two distinct
178 phases of leg motion could be identified, namely the protraction (swing)
179 phase in which the leg moves in a posterior to anterior direction and the
180 retraction (stance) phase in which the leg moves in an anterior to
181 posterior direction (2C). During the protraction (swing) phase the leg is
182 raised from the substrate and is moving in a posterior to anterior direction
183 relative to the body; during the retraction (stance) phase the leg is in

184 contact with the substrate and is moving in an anterior to posterior
185 direction relative to the body. The dynamics of swing/stance events for
186 each of the six legs time was represented in a diagram similar to the “gait
187 diagram” traditionally used to visualize walking gaits (Fig. 2D). To
188 indicate the degree of coordination between legs, concurrency scores
189 were calculated and depicted in the concurrency score pie chart (Fig. 2E).

190

191 **Characterization of swing and stance phases in freely walking wild** 192 **type flies**

193

194 In an initial baseline behavioral analysis of freely walking flies we used
195 this technique to analyze the relationship between walking speed and leg
196 protraction (swing) and retraction (stance) phase characteristics in the
197 wild type. For this, we acquired approximately 39 video recordings of
198 flies walking freely in an arena. These video recordings were grouped
199 into two classes with respect to walking speed. These classes are fast
200 (21.0 mm/s to 34.0 mm/s) and slow (7.0 mm/s to 20.0 mm/s) see
201 **supplementary movie 1**.

202 Analysis of recorded leg movements indicated that faster walking flies
203 protracted their legs farther (increased swing amplitude) than slower
204 walking flies (Fig. 3A). Similarly, faster walking flies retracted their legs
205 farther (increased stance amplitude) when compared to slower walking
206 flies (Fig. 3C). The largest difference in the length of protraction between
207 the fastest walking group and the slowest walking group was ~300um;
208 this difference amounts to about one third of the average body length of
209 the animal. Faster walking flies completed their leg protractions more
210 rapidly (decreased swing duration) than the slower walking groups (Fig.
211 3B). Faster walking flies also completed their leg retractions more rapidly
212 (decreased stance duration) than the slower walking groups (Fig. 3D).

213 The largest difference in the duration of retraction (stance duration)
214 between the fastest and the slowest walking group was ~30ms. The
215 fastest walking animals thus complete each metachronal wave in ~33%
216 less time than the slowest walking animal. We also observed that the
217 concurrency state proportions change with respect to walking speed.
218 Concurrency state 3 corresponding to a tripod gait is proportionately
219 more prevalent for faster walking flies than for slower walking flies (Fig.
220 3E). Overall these results quantify the way in which wild type flies alter
221 the length and duration of leg swing and stance phases as they modulate
222 their walking speed.

223

224 **Targeted knockdown of Rdl provides evidence for GABAergic pre-** 225 **motor inhibitory input to leg motoneurons during walking**

226

227 To determine if pre-motor inhibition of leg motoneurons might be
228 important for correct walking behavior, we conducted a screen for
229 walking deficits caused by targeted knockdown of neurotransmitter
230 receptors. For this, UAS-RNAi constructs for receptors of the
231 neurotransmitters GABA, glutamate and acetylcholine were used together
232 with the OK371Gal4 driver, which targets UAS transgene expression to
233 glutamatergic neurons and hence labels all leg motoneurons (Fig. 4A).
234 Expression of OK371Gal4 is controlled by the enhancer of the
235 *Drosophila* vesicular glutamate transporter gene, VGLUT; (35).

236

237 Initial screening for walking deficits in these RNAi knockdown
238 experiments was carried out using a soot assay of leg footprints (36, 17).
239 In this prescreen, the targeted knockdown of the GABA_A receptor Rdl
240 (Resistance to dieldrin) resulted in a prominent walking phenotype

241 characterized by shorter step length as compared to controls (Fig. 4B).
242 For a more extensive characterization of the walking behavior deficits
243 that resulted from this targeted Rdl knockdown, we used our automated
244 video analysis to characterize the relationship between walking speed and
245 leg swing and stance phase characteristics in a quantitative manner.
246 Analysis of the video data revealed that the Rdl knockdown flies had a
247 markedly reduced walking speed as compared to controls (Fig. 4C) see
248 **supplementary movie 2**. Moreover, the amplitude of the leg swing phase
249 and consequently that of leg stance phase was markedly shorter as
250 compared to controls (Fig. 4D and 4E). Swing phase duration was
251 significantly higher than controls (Fig. 4F) but there was no change in the
252 duration of stance phases (Fig. 4G). The concurrency state 3, in which
253 three legs swing nearly simultaneously, was proportionally reduced as
254 compared to controls (Fig. 4H). Shorter average swing amplitudes in
255 every metachronal walking wave markedly reduced the reach of the
256 animal, thereby reducing the walking speed. (Data shown here is for T2
257 leg segment as a representative of other leg segments)

258

259 Given that Rdl is an ionotropic inhibitory neurotransmitter receptor, these
260 results suggest that normal, wild type-like walking behavior requires
261 inhibitory pre-motor input to leg motoneurons mediated by GABA acting
262 through the Rdl receptor.

263

264 **Adult-specific targeted Rdl knockdown in leg motoneurons also** 265 **results in abnormal walking behavior**

266

267 Suppression of inhibitory pre-motor input by constitutive knockdown of
268 Rdl throughout all developmental stages could potentially impact the

269 formation of the walking circuit. To rule out the possibility that
270 developmental effects were not responsible for the observed walking
271 phenotypes, we refined our analysis by performing adult-specific
272 knockdown of Rdl in leg motor neurons. For this we used the temperature
273 sensitive Gal4 repressor, Gal80ts, together with OK371Gal4 to limit the
274 effects of the targeted knockdown to adult stages. Flies were grown at
275 18°C and shifted to 29°C post eclosion (Fig. 5A; Gal80ts is active at
276 18°C and is inactive at 29°C).

277 Soot assay prints reveal that adult-specific targeted Rdl knockdown also
278 caused walking behavior phenotypes characterized by shorter step length
279 as compared to controls (Fig. 5B). More detailed quantitative
280 characterization of the walking phenotypes using video analysis showed
281 that these flies had a markedly reduced walking speed as compared to
282 controls (Fig. 5C) see [supplementary movie 3](#). Moreover, the amplitude
283 of the leg swing phase and consequently that of leg stance phase was
284 markedly shorter as compared to controls (Fig. 5D and 5E) and the
285 durations of swing and stance phases were significantly higher than in
286 controls (Fig. 5F and 5G). The concurrency state 3, in which three legs
287 swing together (tripod gait), was proportionally reduced as compared to
288 controls (Fig. 5H). (Data shown here is for T2 leg segment as a
289 representative of other leg segments)

290

291 These results indicate that abnormal walking behavior also results if
292 GABAergic inhibitory pre-motor input to leg motoneurons acting through
293 the Rdl receptor is impaired specifically in mature adult stages

294

295 **Leg motoneuron subset-specific knockdown of Rdl also results in**
296 **abnormal walking behavior**

297 In the above-mentioned experiments, the inhibitory GABA_A receptor Rdl
298 was down regulated in all of the fly's motoneurons by the OK371 Gal4
299 driver. To determine if targeted knockdown of Rdl limited to only a
300 subset of the leg motoneurons might also result in aberrant walking
301 behavior, we took advantage of a generated VGN 1-Intron Reg-3 Gal4
302 driver. This driver targets reporter gene expression to only a small subset
303 of leg motoneurons that innervate the trdm, fedm, tidm, and tadm
304 muscles of the leg (Fig 6A).

305 Soot assay print assays reveal that motoneuron subset-specific targeted
306 Rdl knockdown also caused walking behavior phenotypes (Fig 6B).
307 Detailed quantitative characterization of these walking phenotypes using
308 video analysis showed that the flies had reduced walking speeds (Fig 6C)
309 see [supplementary movie 4](#), and the amplitude of leg stance was
310 markedly shorter; but no change in swing amplitude was observed (Fig.
311 6D-E). The durations of leg swing and stance phases were significantly
312 different as compared to wildtype controls (Fig. 6F-G). The concurrency
313 state 3, in which three legs protract nearly simultaneously, was
314 proportionally reduced as compared to controls (Fig. 6H). The magnitude
315 of the walking deficits caused by VGN 1-Intron Reg-3 Gal4 targeted Rdl
316 knockdown in leg motoneuron subsets was generally smaller than those
317 observed in OK371Gal4 targeted knockdown of Rdl in all leg
318 motoneurons. (Data shown here is for T2 leg segment as a representative
319 of other leg segments)

320 Nevertheless, these findings suggest that the suppression of premotor
321 inhibitory input, even if it is limited to a small subset of leg motoneurons,
322 is sufficient for perturbation of normal walking behavior.

323

324 **Analysis of Rdl knockdown in a Tsh-Gal80 background confirms leg**
325 **motoneurons as sites of premotor inhibition**

326

327 The behavioral phenotypes caused by OK371Gal4 targeted knockdown
328 of Rdl indicate that reduced GABAergic inhibition in the neurons
329 genetically accessed by this Gal4 driver (“OK371 neurons”), results in
330 walking behavior deficits. Given that the leg motoneurons are prominent
331 among the OK371 neurons, it seems likely that impairment of GABA-
332 ergic inhibition of these motoneurons is responsible for the observed
333 walking defects. However, the full complement of OK371 neurons as
334 visualized by using OK371Gal4 to drive a UAS-GFP reporter includes
335 numerous interneurons in the central brain and optic lobes, in addition to
336 the leg motoneurons (Fig. 7A). Might impairment in the GABA-ergic
337 inhibition of these central brain interneurons contribute to the walking
338 defects observed in the OK371 targeted knockdown experiments?

339 To investigate this, we repeated the OK371Gal4 driven Rdl knockdown
340 experiments in a Tsh-Gal80 background. Tsh-Gal80 does not affect the
341 Gal4/UAS system in the central brain (37). However, it inhibits the
342 Gal4/UAS system in all of the neurons of VNC, including motoneurons,
343 as can be seen by using OK371Gal4 to drive a UAS-GFP reporter in a
344 Tsh-Gal80 background (Fig 7B).

345

346 Soot print assays reveal that, the walking pattern observed for the OK371
347 targeted Rdl knockdown animals in a Tsh-Gal80 background corresponds
348 to that of wild type controls (Figure 7D); no walking deficits were
349 apparent in the soot print assay. Quantitative characterization of these
350 walking phenotypes using video analysis also showed that walking
351 speeds (Fig 7E) see **supplementary movie 5**, leg swing and stance

352 phases in these flies were not significantly different as compared to
353 wildtype controls (Fig.7F-I). Thus, no effects on walking were observed
354 when OK371 targeted knockdown is limited to neurons in the central
355 brain. (Data shown here is for T2 leg segment as a representative of other
356 leg segments)

357 This finding strongly supports the notion that the behavioral phenotypes
358 observed in OK371-Gal4 driven Rdl knockdown experiments is
359 specifically due to the reduction of GABAergic inhibition in leg
360 motoneurons and not in brain interneurons.

361

362 **DISCUSSION**

363

364 In this report, we investigate the role of inhibitory premotor input to leg
365 motoneurons in the walking behavior of freely moving *Drosophila* by
366 suppression of GABAergic inhibitory input to leg motoneurons. Our
367 findings indicate that the reduction of inhibitory GABAergic input to leg
368 motoneurons caused by targeted Rdl down regulation has marked effects
369 on walking behavior. Thus, walking speed was markedly slower as
370 compared to controls and, correlated with this, the amplitudes of the leg
371 protraction (swing) and retraction (stance) phases were significantly
372 smaller and the durations of protraction (swing) and retraction (stance)
373 phases were significantly higher than in controls. Moreover, the
374 concurrency state, in which three legs swing together in a tripod gait, was
375 proportionally reduced as compared to controls. These prominent effects
376 on walking parameters are similar regardless of whether Rdl down
377 regulation occurs throughout development or whether it is restricted to
378 the adult stage. Taken together, these findings reveal a prominent albeit
379 highly specific role of GABAergic premotor inhibitory input to leg
380 motoneurons in the control of normal walking behavior.

381 The insight into the role of premotor inhibition in walking control
382 reported here is the result of two key experimental methodologies.
383 The first is the highly specific genetic access to identified neuronal
384 populations that can now be attained in the *Drosophila* model system.
385 This is made possible through remarkable targeted expression systems,
386 which together with the availability of libraries of genetically encoded
387 drivers and reporters for molecular manipulation, make it possible to
388 selectively up or down regulate gene expression in highly specific
389 neuronal populations in intact and freely behaving animals (11,13,38). In
390 this study, we have used the Gal4/UAS expression system to achieve
391 targeted genetic access to leg motoneurons and downregulate GABAergic
392 premotor input to these motoneurons by Rdl RNAi expression. In
393 addition we have utilized different forms of the Gal80 repressor to limit
394 Gal4/UAS targeted expression to adult stages or to specific regions of the
395 central nervous system and, hence, refine the spatiotemporal specificity
396 of the resulting genetic access to motoneurons. Given the wealth of Gal4
397 drivers and UAS-RNAi reporter currently available, it will be possible to
398 use similar transgenic technology to manipulate other inhibitory and
399 excitatory neurotransmitter receptors in future studies of interneuronal
400 components of the walking circuitry.

401 The second key methods is the development of an advanced automated
402 high-speed video recording and analysis technique that makes it possible
403 to record the protraction (swing) and retraction (stance) phases at high
404 spatiotemporal resolution for each leg in freely walking flies. With this
405 technique, a quantitative assessment of leg motion parameters in freely
406 walking animals can be carried out which can reveal subtle differences in
407 amplitude and phase of movements of individual legs. This quantitative
408 assessment has been critical for uncovering the role of premotor

409 inhibition in walking behavior. Indeed, since the overall leg coordination
410 during walking is unaffected by the reduction of GABAergic input to leg
411 motoneurons, a more conventional qualitative behavioral analysis is
412 unlikely to discern differences in walking between experimental and
413 control animals. Recently, comparable high resolution recording and
414 analysis methods have been used to quantify leg movement parameters in
415 freely walking flies (24,25,39). These studies have provided important
416 information on walking speed, interleg coordination and other locomotor
417 parameters and have also documented a role of sensory proprioceptive
418 input to step precision during walking. The fact that these methods for
419 high resolution recording and analysis are currently available for studying
420 leg movement parameters in freely walking flies should accelerate our
421 understanding of walking behavior and of the neuronal circuitry involved
422 in its control in *Drosophila*.

423 Given the prominent role of inhibitory premotor input to leg motoneurons
424 reported here, it will now be important to identify and genetically access
425 the premotor interneurons that provide this inhibitory input. While, there
426 is currently little information on the identity of the premotor interneurons
427 that control the activity of leg motoneurons in adult flies, insight into
428 inhibitory premotor interneurons has recently been obtained in larval
429 stages. Thus, in *Drosophila* larva, a set of inhibitory local interneurons
430 termed PMSI neurons have been identified, which control the speed of
431 axial locomotion by limiting the burst duration of motoneurons involved
432 in peristaltic locomotion (32). Moreover, a second set of inhibitory
433 premotor interneurons called GVLI neurons have been reported which
434 may be part of a feedback inhibition system involved in terminating each
435 of the waves of motor activity that underlie larval peristalsis (33). Finally,
436 a pair of segmentally repeated GABAergic interneurons termed GDL

437 neurons have been identified which are necessary for the coordinated
438 propagation of peristaltic motor waves during both forward and backward
439 crawling movements of larvae (34). Whether or not these inhibitory
440 premotor interneurons persist into the adult stage and act in the control of
441 walking behavior is not known.

442 In general terms there are fundamental similarities in the principle
443 mechanisms of locomotion in insects and vertebrates (e.g. 3). These
444 mechanistic similarities might also reflect similar motor circuit
445 properties. For example, much like the PMSI neurons in *Drosophila*,
446 which control the speed of locomotion by limiting motoneuron burst
447 duration, the premotor V1 spinal interneurons in mammals are involved
448 in the regulation of leg motoneuron burst and step cycle duration and thus
449 is also likely control the speed of walking movements (40). Hence, a
450 characterization of the behavioral effects of inhibitory input to leg
451 motoneurons in *Drosophila*, notably in freely walking flies, is likely to
452 provide useful comparative information for understanding the functional
453 role, and possibly the evolutionary origin, of premotor inhibition in
454 vertebrate locomotory circuitry.

455

456

457 **MATERIAL AND METHODS**

458 **Fly strains used:**

459 OK371-Gal4, UAS-dicer; OK371-Gal4, TubGal80ts, UAS-dicer;

460 OK371 Gal4, UAS-mCD8GFP; VGN 1-Intron Reg-3 Gal4.

461 UAS-Rdl i8-10G RNAi from Ron Davis (41); UAS-Trip RNAi – 31622
462 (Bloomington). Tsh-Gal80,UAS-eGFp(37) ; Tsh-Gal80, UAS-Rdl RNAi.

463

464

465 **Generation of VGN 1-Intron Reg-3 Gal4 transgenic flies**

466 DVGlut regulatory region of a 640 bp, corresponding to genome
467 coordinates 2L:2403206-2403845 (release 6.16) was PCR amplified from
468 wild type *Drosophila melanogaster* canton-s and cloned into restriction
469 endonuclease enzyme sites, EcoR1 and BamH1 of pPTGal-attB vector
470 (42) to generate VGN- Intron region3 Gal4.

471

472 **Behavioural analysis procedure:**

473

474 **Soot assay procedure:**

475 Soot assay analysis was done as previously described (17)

476

477 **Video recording procedure:**

478 Recording walking behavior of freely moving flies: In order to record leg
479 movements of freely walking flies at high spatio-temporal resolution,
480 flies were placed in a transparent plexiglass arena and their locomotor
481 behavior was recorded by an overhead video camera at 200 frames/s (Fig.
482 1A). The arena had a square-shaped flat central bottom surrounded by two
483 opposing inclined surfaces and had Teflon coated walls to discourage
484 climbing. Single fly with wings clipped were introduced into the arena
485 and preferentially walked across the flat part of its bottom. The recording
486 video camera was fitted with a lens of 10x magnification. Illumination
487 was from below resulting in high contrast images (Fig 1B). An
488 automated recognition procedure was employed to extract the leg
489 movements of the freely walking fly from the recorded video images;
490 only linear walking sequences were selected. For this, thresholding based
491 on light intensity differences was used to demarcate the fly torso,
492 watershed filtering was employed to delimit head, thorax and abdomen
493 and computation of a body axis orientation vector based on these three
494 body parts was carried out (Fig. 1C). Subsequently, based on the dynamic

495 changes of this vector, the fly's motion was transformed from the
496 camera's frame of reference to an inertial reference frame (fly's frame of
497 reference) (Fig. 1D). This provided a stable image of the fly suitable for
498 auto-detection and analysis of leg movements. 3-4 day old flies starved
499 for 2-3 hours were used for recording.

500

501 **Immunohistochemistry and Confocal Microscopy:**

502

503 Adult brain and thoracic ganglia were dissected in phosphate buffer
504 saline (pH 7.8) (PBS) and fixed in 4% buffered formaldehyde for 45 min
505 at 4°C. Staining procedures was performed as previously described (17).
506 Imaging of adult brain and thoracic ganglia were performed using
507 Olympus FV 1000 confocal microscope at 1 micron intervals. The
508 imported Z stack images were processed using FIJI and Adobe photoshop
509 for further adjustments in brightness and contrast.

510

511 **Antibodies used:**

512 **Primary antibodies:** Chicken pAb a-GFP (1:1000, Abcam), Rabbit (Rb)
513 a-GFP (1:4000, Abcam), mouse (ms) a-Neuroglian (BP104, 1:40;DSHB).

514 **Secondary antibodies:** Alexa fluor-488 and 647 (1:400) from Invitrogen
515 were used in all staining procedures.

516

517

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527

528

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674 **FIGURE LEGENDS:**

675 **Figure 1: Automated recording of walking behaviour of freely moving flies.** (A)
676 Leg movements of freely walking flies were recorded with a 200fps camera; flies
677 were placed in the rectangular shaped arena illuminated from below with LED light
678 source. (B) Dorsal view of the fly captured at high resolution. (C) To obtain linear
679 walking sequences, each frame is thresholded such that only the torso is visible. A
680 watershed filter cuts the torso into three sections - the head, the thorax and the
681 abdomen. The centroids of the head and the body define the orientation vector.
682 Enforcing a maximum angular deviation of 10° as a chief criterion, contiguous frames
683 that constitute a linear walking bout are selected. (D) A minimum bounding box that
684 encloses the fly body is used as a mask to crop each frame and rotational
685 transformation is applied so that the fly's linear motion occurs along the positive
686 direction of the new X-axis. The centroid of the fly was computed in each frame and
687 the origin of the new coordinated axis was shifted to centroid. This transforms the
688 non-inertial frame of reference to inertial frame of reference such that the fly appears
689 to be walking on a treadmill.

690 **Figure 2: Automated detection of leg movements in freely walking flies.** (A) Fly
691 body contour is auto-selected and shrunk by 5px. The contour selection is grown back
692 by 5px as a result of which only the torso gets selected, leaving the legs out. The fly
693 contour selection and the fly torso selection are combined through a XOR function.
694 This gives a composite selection containing all six legs. (B) Temporal Z-projection of
695 leg-tip trajectories allows rapid extraction of individual leg-tips by drawing region of
696 interest selections around six clusters. The temporally color-coded heat-map is a
697 record of the trajectory of the leg-tip during the entire walking bout and aids in
698 demarcating adjacent legs. The leg-tips and the body centroid are annotated in every
699 frame of the video in order to aid in assessing the quality of their detection. (C) Swing
700 phase and stance phase classification is performed based on the sign of change in the
701 X co-ordinate of the leg tip with respect to time. A positive change implies swing
702 phase of the leg while negative change implies stance phase of the leg. A swinging
703 leg was annotated by overlaying a green filled circle on its tip; a stancing leg was
704 annotated by overlaying a red filled circle on its tip. No leg was annotated if it was
705 stationary (D) The swing, stance or steady phase of each of the six legs with respect to
706 time is represented as series of colored ticks in a gait diagram. A representative gait
707 diagram is shown in which each tick is 5ms in duration; red tick implies swing,
708 turquoise tick implies stance and white tick implies steady phase. (E) Three legs
709 swinging concurrently defines a concurrency state of 3, two legs swinging
710 concurrently defines a concurrency state of 2, one leg swinging alone defines a
711 concurrency state of 1, while state 0 implies no leg was swinging.

712 **Figure 3: Relationship between walking speed and leg phase characteristics in**
713 **freely walking flies.** Leg movement parameters are quantified and classified into two
714 speed intervals; slow (7.0-21.0 mm/s) and fast (21.0-34.0) mm/s.) (A) Swing
715 amplitude is the average displacement of the leg-tip during swing phase as calculated
716 in the fly's inertial reference frame; it increases as the walking speed increases. (B)
717 Swing duration is the time of the swing phase; it decreases as the walking speed
718 increases. (C) Stance amplitude is the average displacement of the leg-tip during
719 stance phase as calculated in the fly's inertial reference frame; it increases as the
720 walking speed increases. (D) Stance duration is the time stance phase; it decreases as
721 the walking speed increases. (E) Concurrency state proportions to change with respect
722 to walking speed. State 3 is proportionately higher at faster walking speeds but lower
723 at slower walking speeds. N=39 videos. All the bar represents mean \pm SEM (***: P
724 \leq 0.001; **: P \leq 0.01; *: P \leq 0.05.). Student's t-test was performed for all
725 statistical analysis.

726 **Figure 4: Targeted knockdown of Rdl provides evidence for GABAergic**
727 **inhibitory input to motoneurons in walking flies.** (A) The OK371-GAL4 driver
728 targets UASmCD8GFP expression to leg motoneurons. Schematic representation of
729 motoneuron cell bodies localized in the ventral nerve cord (right). Enlarged T2
730 hemineuromere region showing GFP marked motor neuron cell bodies and dendritic
731 arborization (left). Orange circled region indicates neuropil. (B) Rdl receptor

732 knockdown using OK371 GAL4 in all leg motoneurons results in shorter step length
733 as revealed by the soot imprints. (C) Rdl receptor knockdown using OK371 GAL4 in
734 all leg motoneurons results in significant decrease in walking speed. In this and all
735 subsequent figures left bar indicates control, right bar indicates knockdown. D,E,F)
736 Rdl receptor knockdown using OK371 GAL4 in all leg motoneurons results in
737 reduced amplitudes of swing and stance and increased swing duration. g) Stance
738 duration is unaffected. H) Concurrency state 3 is significantly decreased by Rdl
739 receptor knockdown. Left circle control, right circle knockdown. Controls N=76,
740 Experiment N=68. All the bar represents mean \pm SEM (***: $P \leq 0.001$; **: $P \leq$
741 0.01 ; *: $P \leq 0.05$). Student's t-test was performed for all statistical analysis.

742 **Figure 5: Adult-specific Rdl knockdown in leg motoneurons results in abnormal**
743 **walking behaviour.** (A) For adult specific knockdown of Rdl receptor, the GAL4
744 repressor Gal80ts was used to limit the effects to adult stages. For this flies were
745 grown at 18° C and shifted to 29° C post eclosion. (B) Adult-specific Rdl receptor
746 knockdown using OK371 GAL4 in all leg motoneurons results in shortened step
747 length as shown in soot print images (C) Rdl receptor knockdown flies show slower
748 walking speed. In this and all subsequent figures left bar indicates control, right bar
749 indicates knockdown. (D,E) Amplitudes of swing and stance are shorter in Rdl
750 knockdown flies as compared to controls.(F,G) Swing and stance duration are
751 increased longer in Rdl knockdown flies as compared to controls. (H) Concurrency
752 state 3 is significantly reduced in Rdl receptor knockdown flies. Left circle control,
753 right circle knockdown. Controls N=108, Experiment N=77. All the bar represents
754 mean \pm SEM (***: $P \leq 0.001$; **: $P \leq 0.01$; *: $P \leq 0.05$). Student's t-test was
755 performed for all statistical analysis.

756 **Figure 6: Subset specific knockdown of Rdl in leg motoneurons results in**
757 **deficits in walking behaviour.** A) Innervation patterns of leg motoneurons labeled
758 by VGN 1-Intron Reg-3 GAL4 driving UAS-GFP (Scale bar 50 μ m). Motoneuron
759 axons innervating most of the depressor muscles (trdm- trochanter depressor muscle,
760 fedm-femur depressor muscle, tidm-tibia depressor muscle, tadm-tarsal depressor
761 muscle) are GFP labelled. B) Motoneuron subset specific Rdl receptor knockdown
762 results in shortened step lengths as shown in the soot prints. C) Rdl knockdown in
763 subsets of leg motoneurons results in reduced walking speed. In this and all
764 subsequent figures left bar indicates control, right bar indicates knockdown. D,E) Rdl
765 knockdown in subsets of leg motoneurons results in decreased stance amplitude;
766 swing amplitude is not affected. F,G) Rdl knockdown in subsets of leg motoneurons
767 results in longer swing duration and stance duration. H) Concurrency state 3 is
768 significantly reduced in Rdl receptor knockdown flies. Left circle control, right circle
769 knockdown. Controls N=48, Experiment N=45. All the bar represents mean \pm SEM
770 (***: $P \leq 0.001$; **: $P \leq 0.01$; *: $P \leq 0.05$). Student's t-test was performed for all
771 statistical analysis.

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773 **Figure7: Analysis of Rdl knockdown in Tsh-Gal80 background confirms leg**
774 **motoneurons as sites of premotor inhibition.** (A) OK371 Gal4 driven UAS-eGFP,
775 which is expressed in motoneurons, is also expressed in numerous interneurons of the
776 central brain and optic lobe (Scale bar 100 μ m) (B) OK371 Gal4 driven UAS-eGFP
777 expression labels motoneurons in VNC. (Scale bar 50 μ m) (C) Tsh-Gal80 inhibits the
778 OK371Gal4 UAS-eGFP expression system in all VNC neurons including
779 motoneurons. N=7. (Scale bar 50 μ m) (D) Behavioural analysis of OK371 driven Rdl
780 Knockdown in a Tsh-Gal80 background results in normal walking behaviour as
781 revealed in soot print images. (E) Rdl knockdown on walking behaviour in Tsh-Gal80
782 background shows no change in walking speed as compared to controls. In this and all
783 subsequent figures left bar indicates control, right bar indicates knockdown. (F-I)
784 Amplitudes and durations of swing and stance are not affected in Rdl receptor
785 knockdown animals using OK371 GAL4 in Tsh-Gal80 as compared to wildtype
786 controls. Controls N=30, Experiment N=30. All the bar represents mean \pm SEM (***:
787 P \leq 0.001; **: P \leq 0.01; *: P \leq 0.05.). Student's t-test was performed for all
788 statistical analysis.

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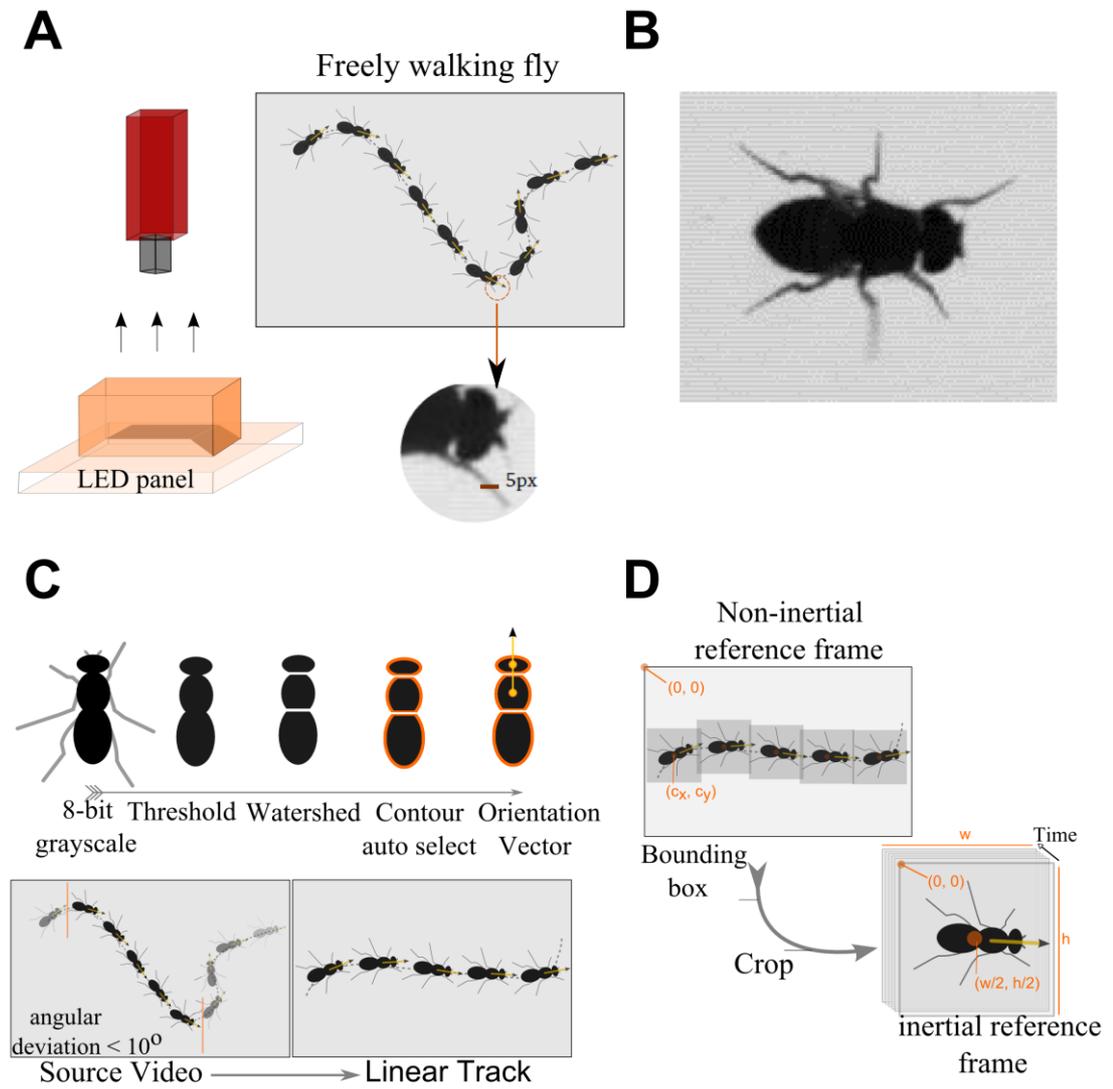
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806 **FIGURE 1: Automated recording of walking behaviour of freely moving flies.**



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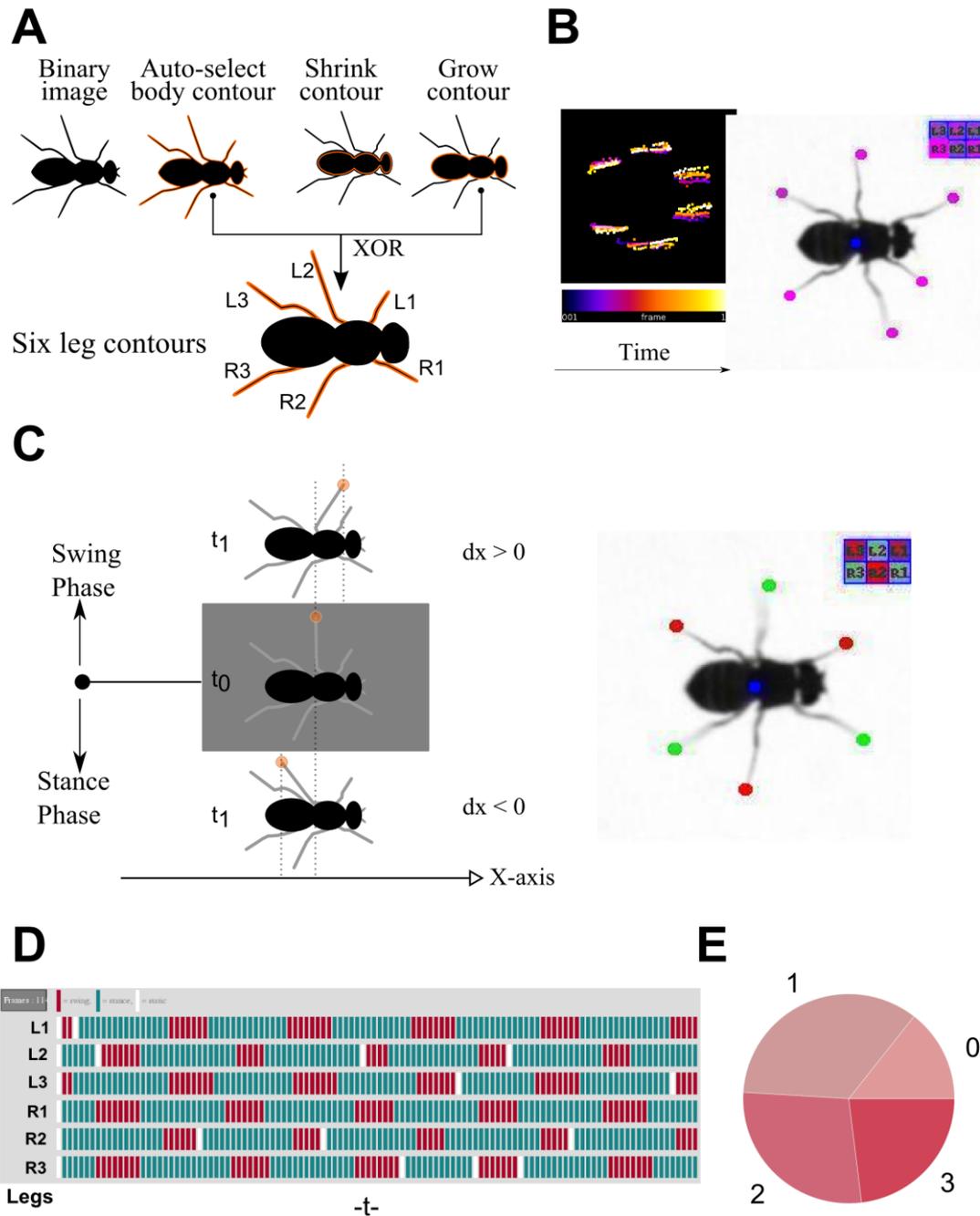
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812 **FIGURE 2: Automated detection of leg movements in freely walking flies.**



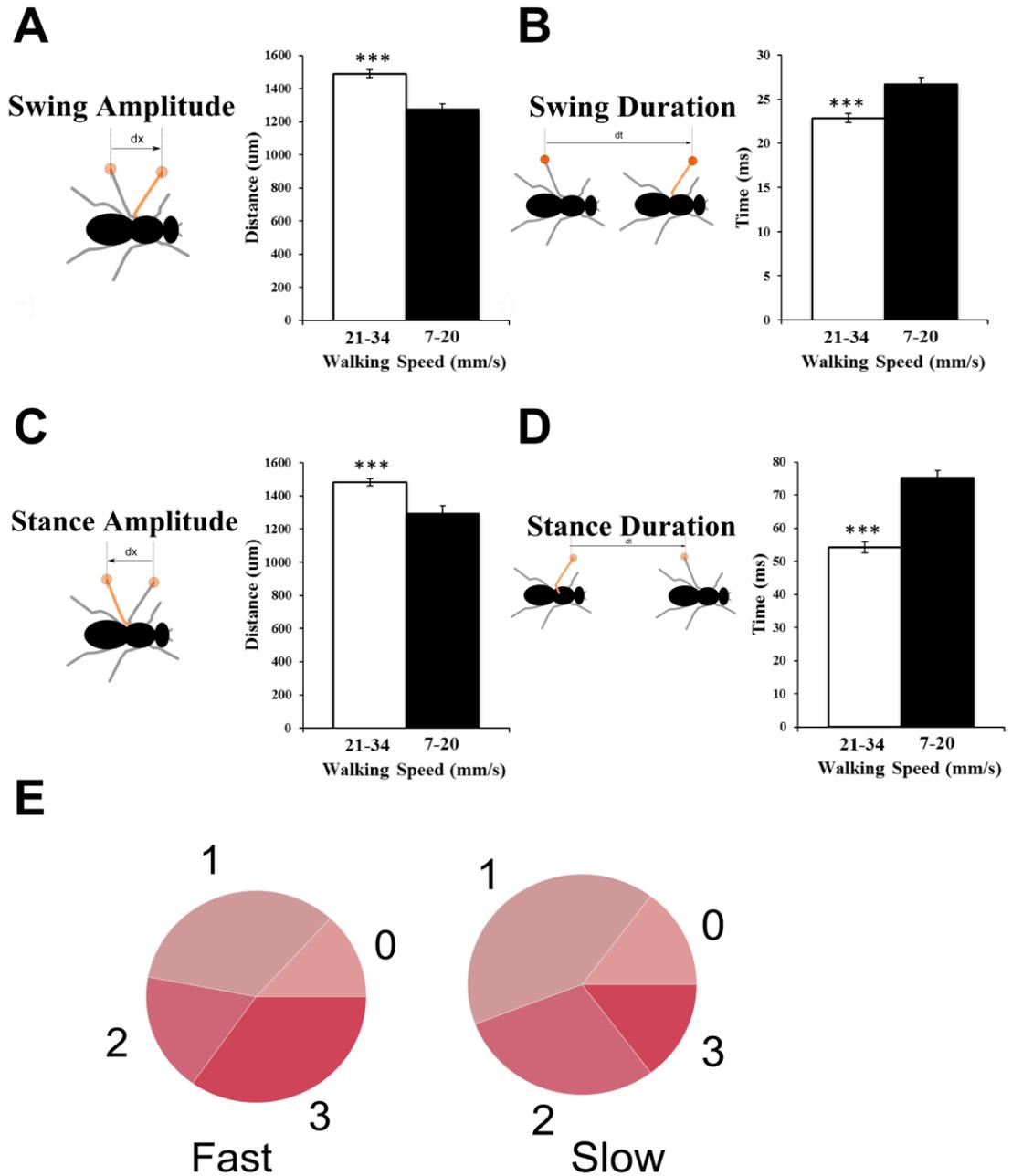
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817 **FIGURE 3: Relationship between walking speed and leg phase characteristics in**
818 **freely walking flies**

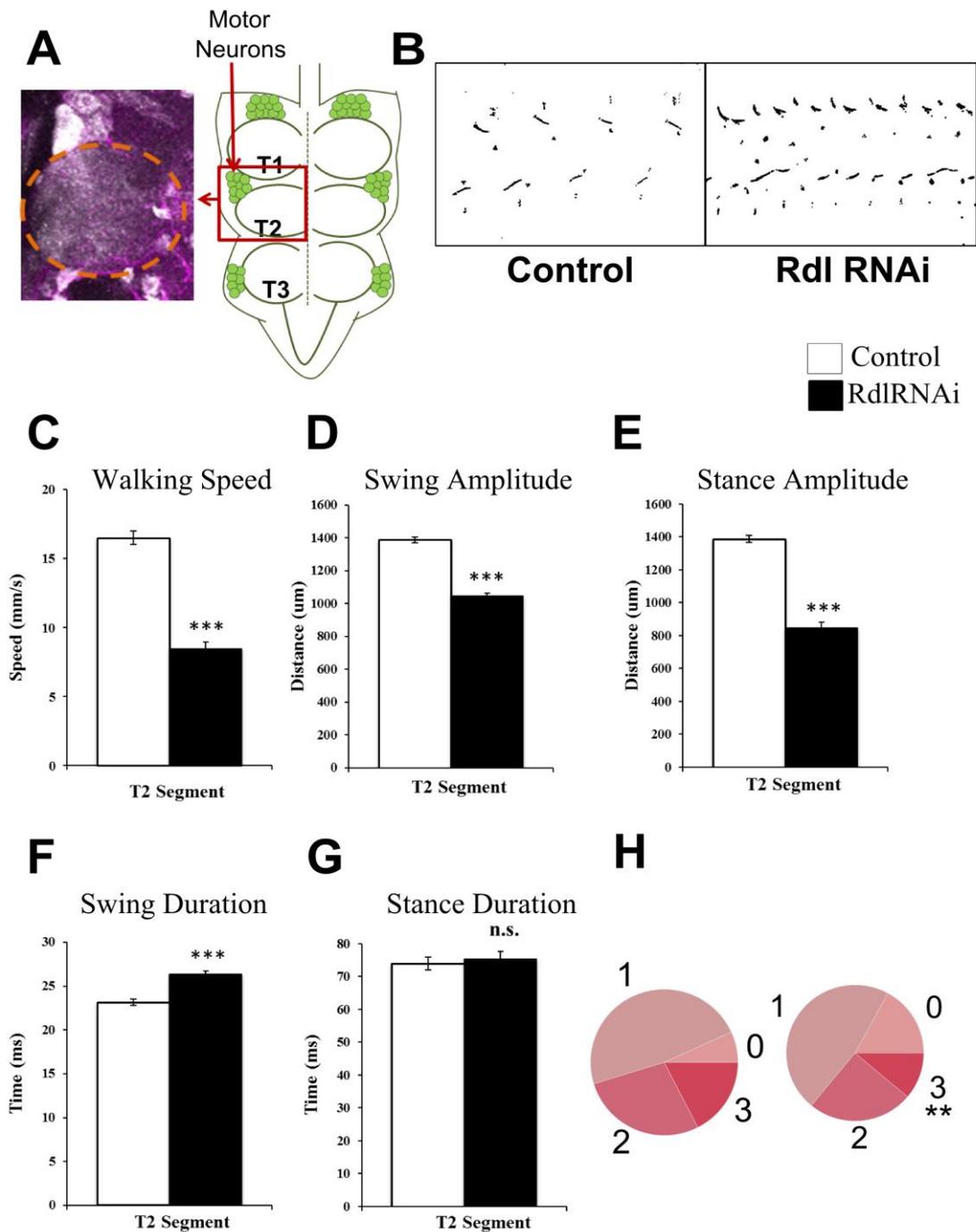


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822 **FIGURE 4: Targeted knockdown of Rdl provides evidence for GABAergic**
823 **inhibitory input to motoneurons in walking flies.**



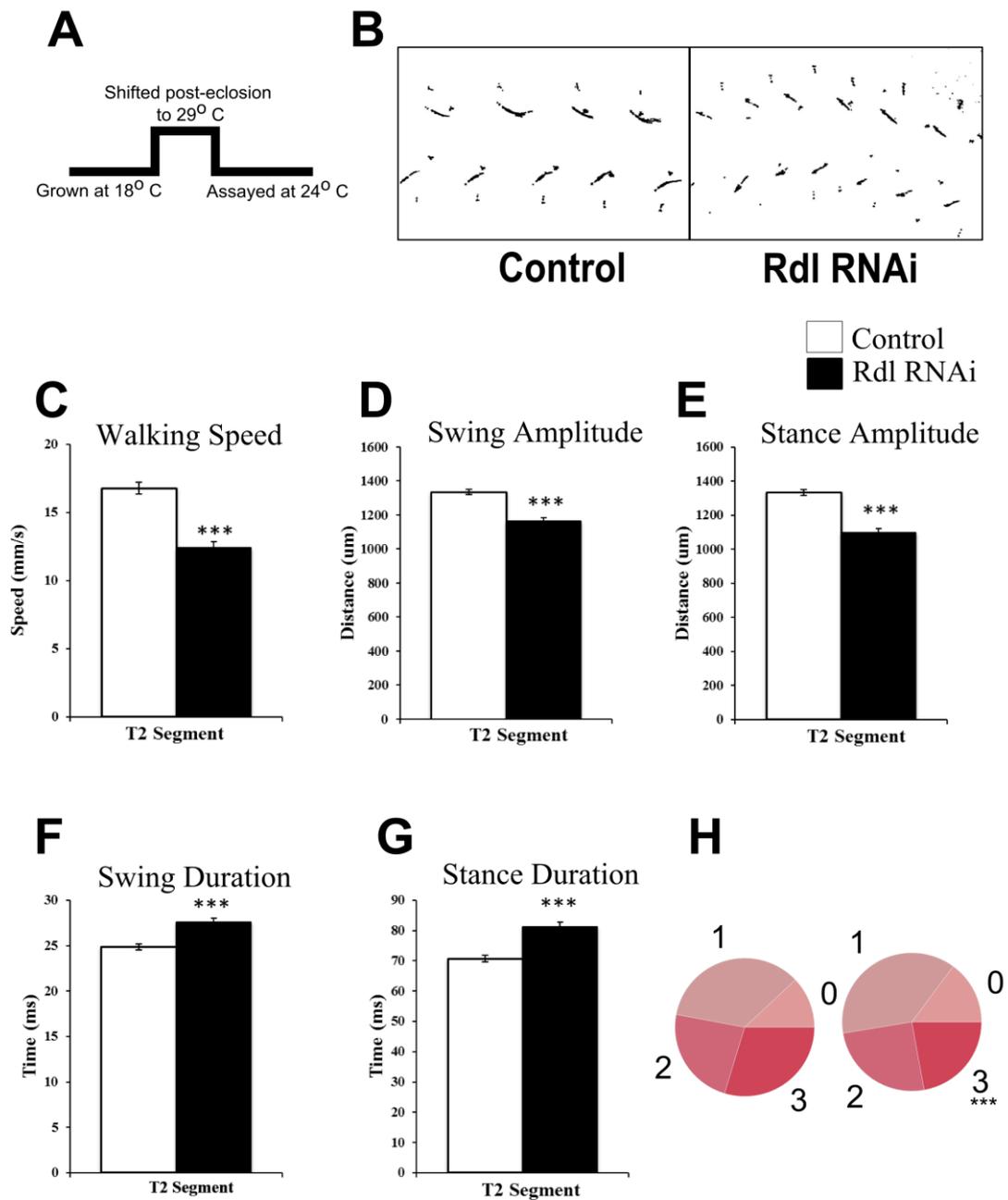
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827 **FIGURE 5: Adult-specific Rdl knockdown in leg motoneurons results in**
828 **abnormal walking behaviour**

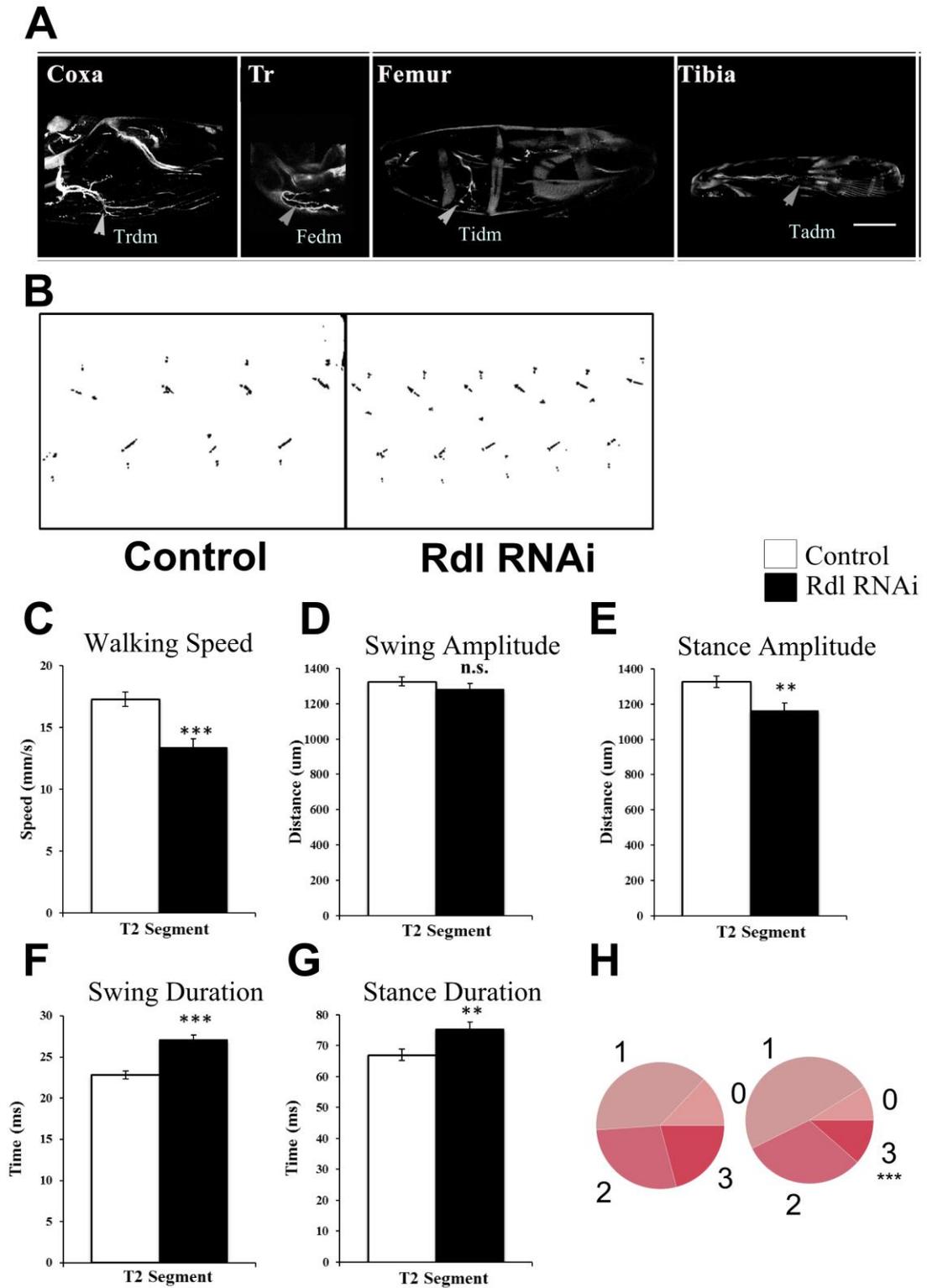
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832 **FIGURE 6: Subset specific knockdown of Rdl in leg motoneurons results in**
833 **deficits in walking behaviour**

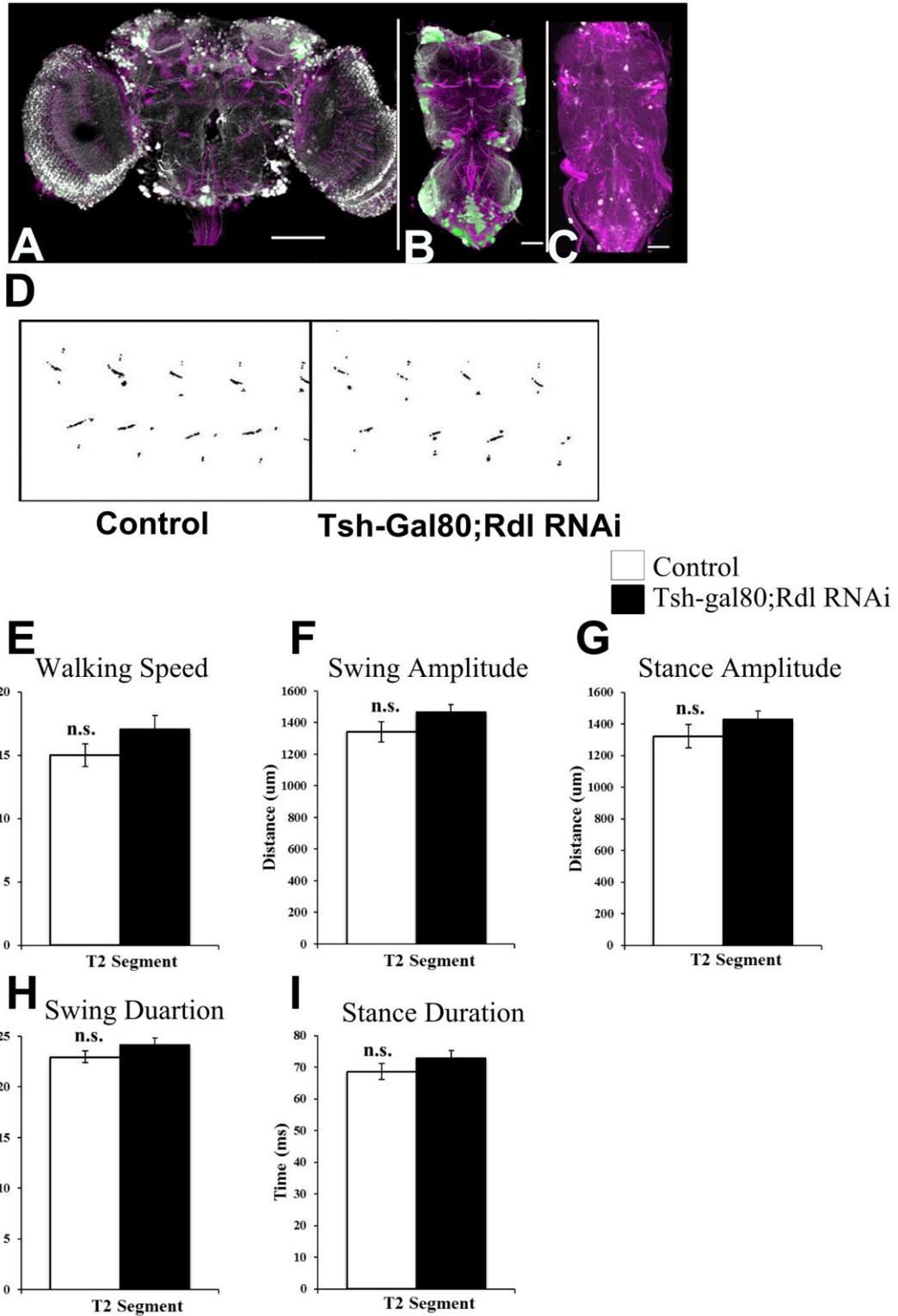


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837 **FIGURE 7: Analysis of Rdl knockdown in Tsh-Gal80 background confirms leg**
838 **motoneurons as sites of premotor inhibition**



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