Neuromuscular control of a single twitch muscle in wild type and mutant Drosophila, measured with an ergometer

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Abstract

How do deficits in neuronal growth, aging or synaptic function affect the final, mechanical output of a single muscle twitch? We address this *in vivo* (indeed *in situ*) with a novel ergometer that records the output of a large specialised muscle, the *Drosophila* jump muscle. Here, we describe in detail the ergometer, its construction and use. We evaluated the ergometer by showing that adult fly jump muscle output varies little between 3 hours and 7 days; but newly eclosed flies produce only 65%. In a mutant with little octopamine (*Tbh*), jump muscle performance is reduced by 28%. The initial responses of synaptic growth mutants (*highwire* and *spinster*) do not differ from wild type, as expected on the homeostatic hypothesis. However, responses in *highwire* mutations gradually decline following repeated stimuli, suggesting physiological as well as anatomical abnormalities. We conclude that the assay is robust, sensitive and reliable with a good throughput.

Introduction

Jumping is the first crucial stage of fly escape behaviour. As the neurons of the neural circuit that produces this fixed action pattern are easy to record from, this system has been extensively studied for nearly 30 years (King and Wyman 1980; Tanouye and Wyman 1980) and recently reviewed (Allen et al. 2006). The cell bodies of the paired giant descending neurons (GDNs) are located in the brain, with axons projecting to the mesothoracic neuromere (Fig. 1). There they synapse with the large motoneuron innervating the TDT (tergal depressor of the trochanter, also known as TTM, tergo-trochanteral muscle). The GDNs also synapse with a local interneuron, the PSI (Peripherally Synapsing Interneuron), which in turn synapses with the 5 motoneurons for the IFM (indirect flight muscles). This pathway can be activated by a supra-threshold electrical stimulus to the head, eliciting an action potential in the GDN, which in turn evokes action potentials in the TDT motoneuron and PSI interneuron; the PSI in turn excites the motoneurons supplying the IFM. Thus the first muscle to be activated is the TDT, which runs between the top section of the leg (the trochanter) and the cuticle of the lateral dorsal thorax. As it contracts, it both extends the middle (mesothoracic) leg and elevates the wings. It also stretches (and so activates) the IFMs and flight begins. Although the GDN $\rightarrow$ motoneuron $\rightarrow$ muscle circuit is completely developed by the time that the fly emerges from the pupa (Allen et al. 1998; Phelan et al. 1996), flies never jump until the cuticle has tanned, about 1 hour after emergence (Hammond and O'Shea 2007c).

Despite the thorough neural analysis, only recently (Zumstein et al. 2004) have recordings of the final, i.e. mechanical, output of the system been published. Our first approach, described in that paper, measured the force produced isometrically by the TDT. This showed that the peak force produced by one muscle (100 μN) was about 10 times the

weight of the fly (so together left and right TDTs generate 20 times). While the force produced by any muscle will be less if it contracts, we calculated from the maximum distance travelled in jumping that the force should be about 135 μN per leg, and concluded that energy storage in elastic elements was unlikely to be a major part of the escape strategy of *Drosophila*. This is a major difference from most of the specialised jumping insects that have been examined (fleas (Bennet-Clark and Lucey 1967), locusts (Bennet-Clark 1975), froghoppers (Burrows 2003)), where the high-performance jump is powered by stored elastic energy. Fruit flies do not need to store a large quantity of energy because they only need to jump off the substrate and start flying. We calculate from the kinetics of their take-off that, without any input from the wings, a fly jumping at 45 ° would reach a peak height of 20 mm and this corresponds with the height reached by clipped wing flies in high speed video records (Card and Dickinson 2008).

While the isometric assay helped us to understand the mechanics of take-off, it also gave us the opportunity to examine the physiological consequences of nerve and muscle mutations which are a key feature of *Drosophila* biology. Although we found a reduction in force in flies which did not synthesise any octopamine (Zumstein et al., 2004), the isometric technique was not very suitable for the high throughput required for comparing several genotypes, and so we have now developed a less time consuming, more robust, and simpler ergometric assay (Elliott et al. 2007b). In this higher-throughput assay, the fly is securely glued by its dorsal thorax to a rigid support mounted over a platform at the end of a flexible optical fibre. “Jumping” is elicited by stimulating electrodes, one in each eye, and the movements of the leg against the platform transduced optically and recorded on computer.

Although the mechanical characteristics of the system mean that we cannot measure the

force or energy output exactly, we nonetheless obtain a quantitative index of the work done by the jump muscle. Since the TDT is much larger than any other muscle that moves the legs, and as it contracts in response to a single action potential in the motoneuron, our assay provides a direct measure of the performance of a single twitch muscle *in vivo* - indeed *in situ*. Our aim here is to provide a detailed description of the technology, its use in studies of the maturation of the jump and of the physiological impact of mutations affecting the neural, as well as the muscular, system to demonstrate the sensitivity, reliability and potential throughput of the ergometer.

Experimental

How did we build the apparatus? Our ergometer (Fig. 2) was constructed so that the fly remains fixed while the platform moves. The platform (5 mm x 5 mm) was centred 7.5 mm from the end of a flexible light pipe (RS Components Ltd, Corby, UK part number 435-8202) which had been shortened to 160 mm by removing the distal end with wire cutters. The light pipe was clamped onto a MM3 micromanipulator next to the integral red light-emitting diode. The red light diverges from the truncated end of the light pipe onto a quadrant photodiode (RS part 652-027), mounted 0.5-1mm from the end of the light pipe. Initially the micromanipulator is adjusted so that the light beam is centred on the diode. This illuminates all sectors equally. When the fly “jumps” the platform and light pipe move and so the intensity changes on all 4 quadrants of the diode. The 4 outputs are amplified with 4 current-to-voltage convertors and the outputs digitised onto a PC. Comparison of the outputs of all 4 quadrants is performed using DasyLab (measX GmbH & Co. KG, Moenchengladbach, Germany) or Labview (National Instruments, Newbury, Berkshire, UK) and is sufficient to determine both the vertical and horizontal displacement of the platform (Fig. 2B). Calibration curves (Fig. 2C) show that the displacement in both

vertical and horizontal directions is linear up to 500 µm. We find that illuminating the apparatus with a fibre optic source, with the tip placed behind the quadrant photodetector, provides sufficient illumination to watch the fly without affecting the sensitivity of the system.

Copies of our worksheets and supporting software may be freely downloaded from http://biolpc22.york.ac.uk/drosophila/jumping/download/.

How do we stimulate the flies? The flies are anaesthetised with carbon dioxide, and glued by the dorsal surface of their thoraces to tungsten pins on the ends of wooden cocktail sticks. In early experiments we used a rubber solution manufactured by Cow Gum, but this product was discontinued and we now use Marabu Fixo Gum (Tamm, Germany). We usually glue all but one leg upwards, onto the thorax and tungsten pin. In some experiments, we increase our throughput (and the signal/noise ratio) by leaving all the legs free. After allowing 20-30 minutes to recover from CO_2 anaesthesia, the cocktail stick with fly is mounted in a second MM3 micromanipulator, horizontally above the platform, and lowered under visual control. We found previously (Elliott et al. 2007a) that the distance that the fly was positioned above the platform had a major influence on the ergometer's output. To standardise the distance, we watch as the fly is lowered, and fix the manipulator when the fly moves its legs away from the body, reaches out and places its legs on the platform. We check that the fly is holding its legs with their mesothoracic femurs horizontal. Once settled, a pair of sharpened tungsten stimulating electrodes are brought up to dimple the eyes and tapped gently to make good electrical contact. Stimuli of 28 V and 1 ms duration are normally employed to activate the escape circuit.

In this setting, flies are usually quite active, often moving their legs about, for example grooming. We therefore normally apply the stimuli when the fly is quiescent, and the leg(s) are on the platform. Some flies begin oviposition movements, and in these cases we

check that the abdomen is not touching the platform when the stimulation is applied.

How do we compute the output of the muscle? Each stimulus is followed by an initial downwards movement of the platform (Fig. 3). Normally this is accompanied by a horizontal deflection, either backwards or forwards. The initial response is followed by a damped resonant oscillation at 14 Hz, as the flexible light pipe acts both as a spring and a mass. This resonance means that the light pipe cannot move fast enough for us to determine the time course of the force produced by the fly, which lasts for less than 30 ms. Since the efficiency of energy transfer drops off rapidly above a resonance, the energy transferred to the beam is a small fraction of the work done by the fly, and so we have not attempted to calculate the actual work done by the fly in the jump. However, for each fly we determine the magnitude and direction of the initial displacement of the light pipe, and these provide an index of the work done by the fly.

To find magnitude and direction, the traces (Fig. 3) are replayed in Dasyview (custom software). The changes in vertical and horizontal traces are imported into Excel and the magnitude of the total displacement calculated using Pythagoras' theorem. The direction of the movement is determined using trigonometry.

How sensitive is the ergometer? In all experiments, we use female flies, because they are larger than the males. With just one mesothoracic leg on the platform, the mean peak beam deflection for wild type flies (Canton-S, CS) is 110 ± 34 μm (mean ± SD, max = 220 μm; N = 100), well above the unfiltered noise, normally 4.0 μm (Fig. 3). The noise can be reduced by a digital filter (integrated in Dasyview), so that the much smaller responses produced in some mutants can still be resolved.

How repeatable are the ergometer data? As shown in Fig. 3A and Fig. 4A, successive stimuli are usually reliable replicates of each other, but this is not always the case (Fig. 3B). Where

there is variability in the traces, the largest response is usually from the first stimulus. Therefore, analysing the response to the first 4 stimuli is sufficient to find the maximal response; increasing the number of stimuli to 10 or 15 does not statistically increase the size of the maximum response finally observed in that fly.

However, we find habituation of the response in the mutant *highwire* (Wan et al. 2000b), which has overgrown synapses at the 3rd instar larva neuromuscular junction. Our initial analysis of the TDT morphology indicates that the *highwire* mutant has more branches than control line (Fig. 4D). On the ergometer, the size of response in *highwire* homozygotes or transheterozygotes is initially the same as the controls, but declines with repeated stimulation, reaching ~60% of the initial response after 6 stimuli (Fig. 4 B). This reduction is significant (for the transheterozygote after only 3 stimuli). In neither of the heterozygotes was a significant decline in performance measured.

How sensitive is the assay to the age of the fly? We have tested our standard laboratory wild-type (CS) and w^- (white-eyed flies, w^{1118}) flies from emergence to 1 week old. Fig. 4C shows that the jump performance increases over the first 3 hours and then remains stable. With newly emerged flies, the average of the maximum beam deflection is 82 ± 3.2 (mean ± SE) µm, but at 3 hours old, flies produce 123 ± 4.3 µm. This increase is significant (ANOVA, $F_{5\ df} = 6.7$ $P < 0.001$) but there is no significant change between 3 hours and 7 days in either of these lines (ANOVA, $P = 0.349$). While we cannot exclude an increase in the work done by the muscle contraction, we expect that the gradual increase in beam deflection is most likely to be due to the stiffening of the thorax as the cuticle tans. There is no significant difference in the jump performance of CS and w^- flies, (ANOVA, $F_{1\ df} = 3.5$, $P = 0.06$) despite recent a report suggesting w^- is a poor model for jumping behaviour (Hammond and O'Shea 2007b). We find that flies kept in petri dishes filled with agar to

within 2 mm of the lid do not jump and fly away promptly after the removal of the lid, even when they have been kept in the petri dish for up to 1 week. Nonetheless, their performance on the ergometer is identical to those kept in vials where visual inputs and movement are not restricted (Fig 4C). Furthermore, jump performance does not improve with repeated stimuli in flies kept in this restricted environment (Fig 4A).

What about the six-leg assay? We have also tested the ergometer with flies in which all six legs were kept free. When on the platform, these flies attempt to walk, and groom, sometimes lifting their mesothoracic legs up, which severely reduces the size of the response. Successive stimuli produce more variable responses than in the 1 leg assay, and visual control of the stimulation is essential to ensure that the stimuli are delivered when both mesothoracic legs are on the platform. When comparing different experimental runs, we therefore take the maximum deflection achieved by each fly as the raw datum. Not only is this preparation quicker to set up, but the responses generated by flies with all 6 legs on the platform are larger than when only one leg is free, giving a better signal / noise ratio.

To test the effectiveness of our new assay, we have used one of the same fly lines that we used in our isometric assay (Zumstein et al., 2004), the null *M18* mutation in the *tbh* (tyrosine beta-hydroxylase) gene (Monastirioti et al. 1996). This mutation blocks the synthesis of octopamine, and these flies show elevated levels of tyramine. We find that the *M18* homozygotes produce significantly less output (145 ± 17 µm, mean ± SE) than the *M18* / + heterozygotes (201 ± 16 µm, Student's t-test, t = 2.42, 29 df, P = 0.022) and the other 6 control genotypes tested (Fig. 5A).

In some situations the *M18* mutation rescues a mutation in a potassium channel gene, *shaker* (Rivlin, pers. comm.). The maximum beam deflection achieved by flies expressing

sh^5 allele is larger than the controls (251 ± 20 μm; mean ± SE) as expected from the reduction in transient inward potassium current (Haugland and Wu 1990). The output of the homozygote *M18 – sh*^{5} double mutant (201 ± 17) is intermediate between the *M18* and *sh*^{5} lines as might be expected for an additive effect, rather than a rescue.

We have also examined a number of *spinster* mutations (Sweeney and Davis 2002c), in which neuronal growth and excitatory junction potential (EJP) are known to be affected at the larval neuromuscular junction. We find no difference in the maximal jump response between any of the genotypes tested (Fig 5B).

What is the throughput of the system? A key motivation for developing the apparatus is to facilitate the quantitative comparison of genotypes. A number of students have learnt quickly to use the apparatus and then consistently achieved the following timings. Anesthetising and mounting a group of 10-15 flies takes about 30 minutes, followed by 20 minutes recovery. Each fly then takes 2-3 minutes to assay, followed by a similar time for initial data analysis; a total time of about 3 hours for this sized group of flies. We have seen above that we can achieve significance for a difference of 25% with two groups of 10-15 flies, and so the comparison of genotypes is possible over a short time frame.

Conclusion

We have demonstrated that this equipment provides a high throughput system to detect changes in jumping performance due to changes in neurally expressed mutations.

A neuromodulatory mutation, *Tbh* mutation, resulting in a 10x increase in tyramine and reduction to nearly zero octopamine (Monastirioti et al., 1996) production by midline (VUM) neurons reduces the jump muscle output by 28%. This is a similar change to the one we recorded when the muscle contracted isometrically, 45%, (Zumstein et al., 2004) and the reduction in the maximum distance jumped (42%).

We find no difference in the jump performance of CS and w^- lines in this assay, though doubts had been raised recently about differences in the escape behaviour of these two lines (Hammond and O'Shea 2007a). It is likely that the variation in visual systems may be responsible for the different probabilities of activation of the giant fiber output circuit. The electrical stimulation we employ and the consequent jump response does not seem to be affected by the white mutation.

Two overgrowth mutants, *highwire* (Wan et al. 2000a) and *spinster* (Sweeney and Davis 2002b) were compared with wild type lines. The *spinster* mutants have jumping outputs indistinguishable from wild type. This suggests that these mutants compensate homeostatically for the increased neuronal endings so that the muscle response is maintained the same. This might be achieved by reducing the excitatory junction potential (EJP) as at the larval neuromuscular junction or by changes to the calcium influx to the myofibrils.

Our data shows that the apparatus can detect changes in habituation (or presumably facilitation) in the response to successive stimuli. With the *highwire* lines, the response decremented with repeated stimulation. Unlike the situation with the 3rd instar wandering larva, where the neuromuscular junction is responding to growth and developmental control, the adult TDT and its motoneuron are faced with a static set point.

The ergometer also gives new insights into development and aging. We find from 3 hours – 7 days, the jumping performance is consistent and that delaying the time at which flies initiate jumping voluntarily, by keeping the flies in a restricted environment, does not affect the time course of development of the neuromuscular performance.

Thus our ergometer technology is able to analyse a range of mutations affecting the

nervous system, including transmitter synthesis, neuronal growth and transmitter release at both central and peripheral synapses as well as muscle performance e.g. the degeneration of the myofibrils (Elliott et al., 2007b).

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Figure Legends

Fig 1. The organisation the escape jumping pathway: stimulation of the CNS by electrodes implanted in the head leads to an action potential in the GDN (giant descending neuron) which runs from the dorsal CNS to the mesothoracic neuromere of the VNC (ventral nerve cord). There is excites the motoneurons (mn) that supply the thoracic muscles. The motoneuron that innervates the TDT (tergal depressor of trochanter, also known as the tergotrochanteral muscle) is the first motoneuron to be excited. This is achieved though a mixed chemical-electrical synapse. Other motoneurons innervating the IFM (indirect flight muscles) are activated after a short delay. Contraction of the TDT pivots the trochanter (tc), extending the femur of the mesothoracic leg and propels the fly off the substrate.

Fig. 2. Construction of the ergometer. A. A small platform is glued to the end of the flexible light pipe. The fly is suspended over the light pipe, so that it can “jump” and move the beam downwards and horizontally. The movement of the light pipe is transduced by projecting the beam onto the surface of a quadrant photodiode. B. Movement of the beam of light changes the intensity recorded on each segment of the photodiode. Initially, the beam is centered on all the segments. If the fly moves the beam down and back, the intensity of the beam decreases on the anterior 2 quadrants, and increases on the rear 2. Further, the intensity increases on the lower two segments. Subtracting the left and right halves gives the horizontal displacement, while subtracting the top and bottom halves gives the vertical signal. C. The calibration curve shows the relationship between the position of the platform and the calculated output of the photodiode circuit. Blue diamonds: platform moved vertically; pink squares: platform moved horizontally. The horizontal data is offset 100 μV vertically to avoid overlap. Note that the output is linear for beam displacements up to 500 μm in both directions, with the same slope. Parts A and

B of the figure reused (with permission) from (Elliott et al., 2007b).

Fig. 3. Ergometer responses to jumping with a single mesothoracic leg free on the platform. A. Overlay of 9 stimuli and responses of the middle, mesothoracic leg showing a consistent response. B. Overlay of 6 stimuli and responses showing a variable response, with some jumps propelling the fly forwards (downwards movement of the horizontal trace) and others back. A small stimulus artifact occurs in both A and B. The black line with arrowheads indicates the time of peak response.

Fig. 4. A. In wild type flies, repeated stimulation produces consistent responses. Mean ± SE response of CS flies to repeated stimuli (1.6s separation). We tested newly emerged flies, and 1-day old flies which were placed into conventional vials after emergence, or placed in well-filled petri dishes to restrict jumping possibilities B. Adaptation in *highwire* : in older flies (11-17 days) the *highwire* jump response habituates to repeated stimuli (interval 1.6s) unlike the heterozygote control flies. The first two responses are not significantly different between controls and *highwire* lines, but from the third response *highwire* responses are significantly less than control flies. Although neither of the control lines show a statistically significant decline in response with repeated stimuli, *highwire* $^{ND8/ND8}$ responded significantly less ($P < 0.05$) than their starting values after 6 stimuli. The response of *highwire* $^{ND8\,/\,ND9}$ was significantly reduced from its starting value by the third response, and remained less ($P < 0.05$ at each point). The *highwire* ^{ND8} and ^{ND9} mutations were outcrossed to FM6 balancer chromosomes and then recrossed or backcrossed. 8 flies of each genotype sampled, mean (± SE) of maximum for each fly. C. Ergometer output of developing flies. The output of the jump muscle is already apparent 20 minutes after emergence, but increases over the first 3 hours (ANOVA, $F_{5\ df} = 6.7$ $P < 0.001$). After 3 hours, there is no significant change in output (ANOVA, $P = 0.349$). Flies taken from CS

and *w*$^{-}$ (*w*^{1118}) lines show no differences (ANOVA $F_{1df} = 2.4$, P = 0.11). Flies placed directly after emergence in petri dishes had restricted opportunities for jumping for 1 week but were not different from their siblings raised in conventional vials. In these experiments, vials were checked at 15 minute intervals for newly emerged flies. Data show mean (± SE) of maximum response in each fly. D. Innervation of the tergal depressor of trochanter (TDT) shown by low power confocal micrographs in control (*Mhc-weeP26-GFP*) and *highwire* $^{ND8 / ND9}$ preparations. The nerve branches extensively across the muscle as shown by the staining with antibody 22C10. The distance from the nerve entry to the first primary dorsal branch (see black lines[red in online color version]) is significantly less in *highwire* than in the control (78 ± 14 μm v 139 ± 13, mean ± SE; Student's t-test, 9 df, P= 0.03). A: anterior, D: dorsal, P: posterior, V: ventral. All ergometer data from right mesothoracic legs.

Fig. 5. Ergometer responses of flies with mutations affecting the neuromuscular system. . A. A null mutation (M18) in the *Tbh* (tyramine beta-hydroxylase) gene significantly (P = 0.02) reduces jump output in the homozygote compared to the heterozygote. The dotted line indicates the mean level achieved in 6 different control lines. *shaker*^{5} (*sh*^{5}) allele increases the jump performance (P = 0.44). The double mutant M18 *sh*^{5} has a jump performance intermediate between M18 and *sh*^{5}. Each bar is derived from 16 flies using all 6 legs on the platform. B. *spinster* mutations have no significant effect on the TDT neuromuscular response. Fly stocks as in (Sweeney and Davis 2002a). Heterozygotes were taken from *spin*^{4} / *CyO-GFP* line (marked *spin*^{4} / +), transheterozygotes from a cross between *spin*^{4} / *CyO-GFP* and *spin*^{5} / *CyO-GFP* and rescues from the *spin*^{4} / *CyO-GFP;UAS-spin/TM6B* x *spin*^{5}*/CyO-GFP*;*spin-GAL4/TM6B* cross. At least 9 flies in each sample, except for *spin*^{4}*/spin*^{5} where only 3 flies were found. All flies aged 3-9 days, with 6 legs resting on

the platform.

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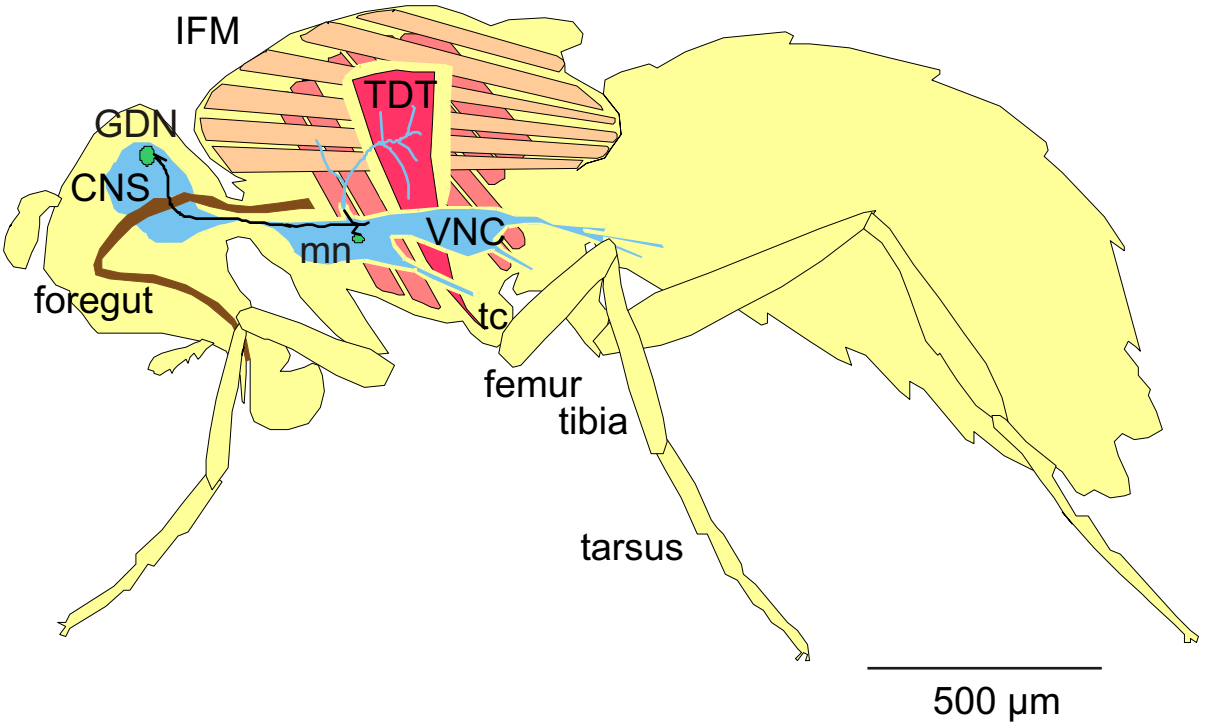
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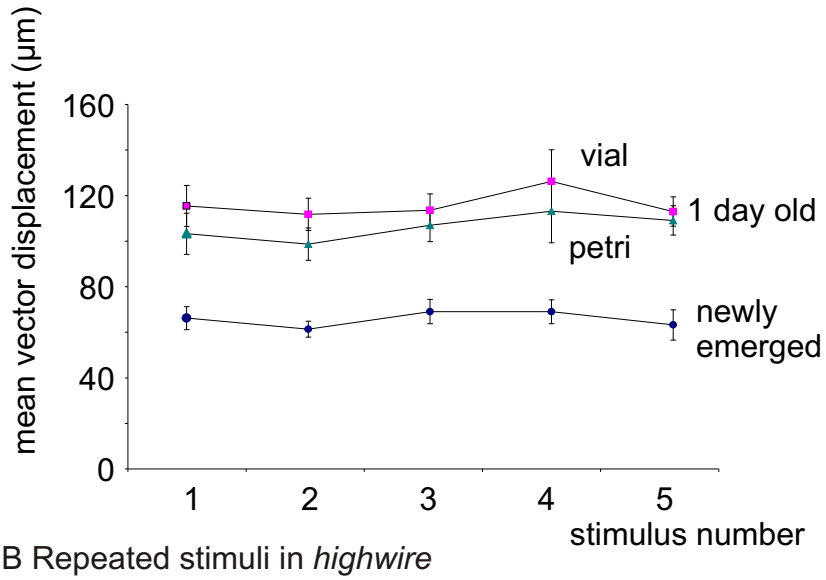
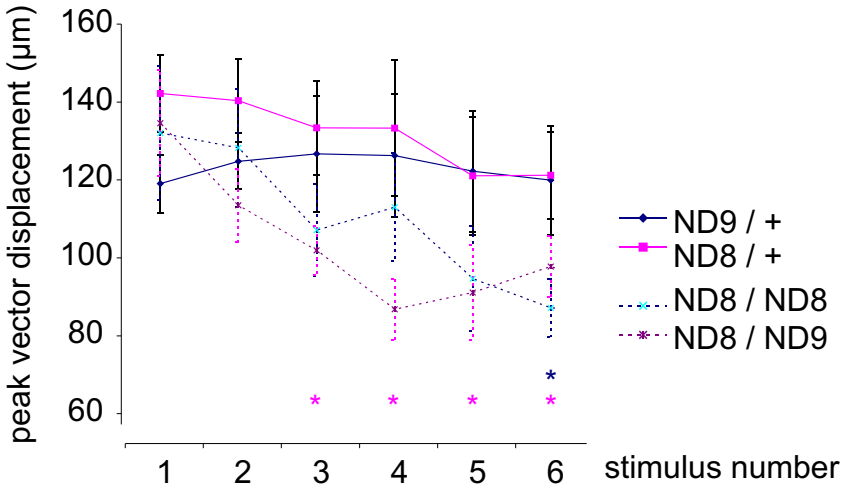
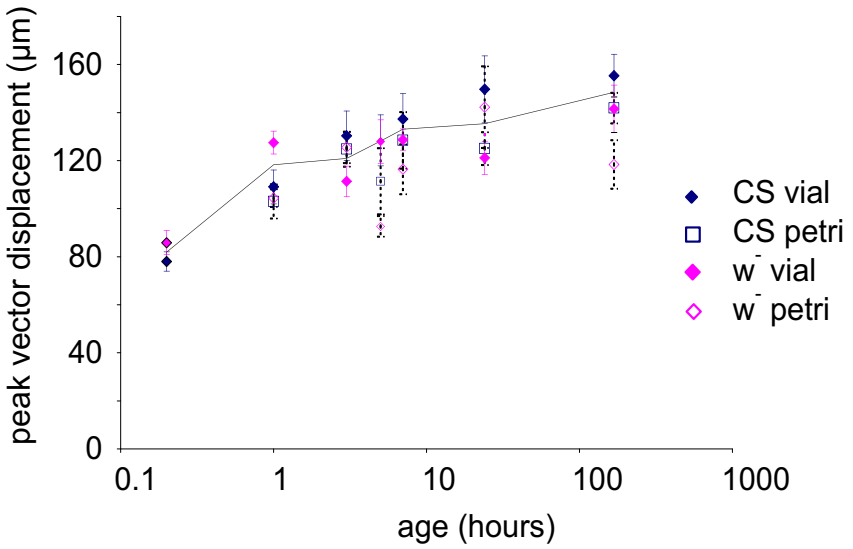
A Repeated stimuli in CS



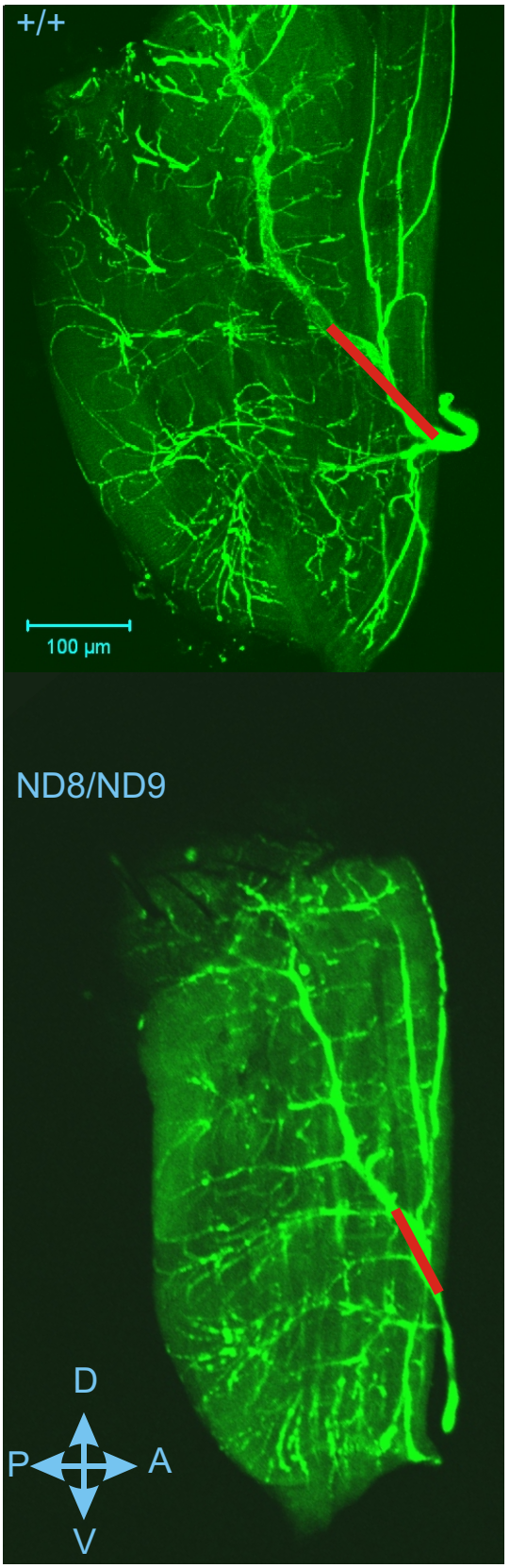
B Repeated stimuli in *highwire*

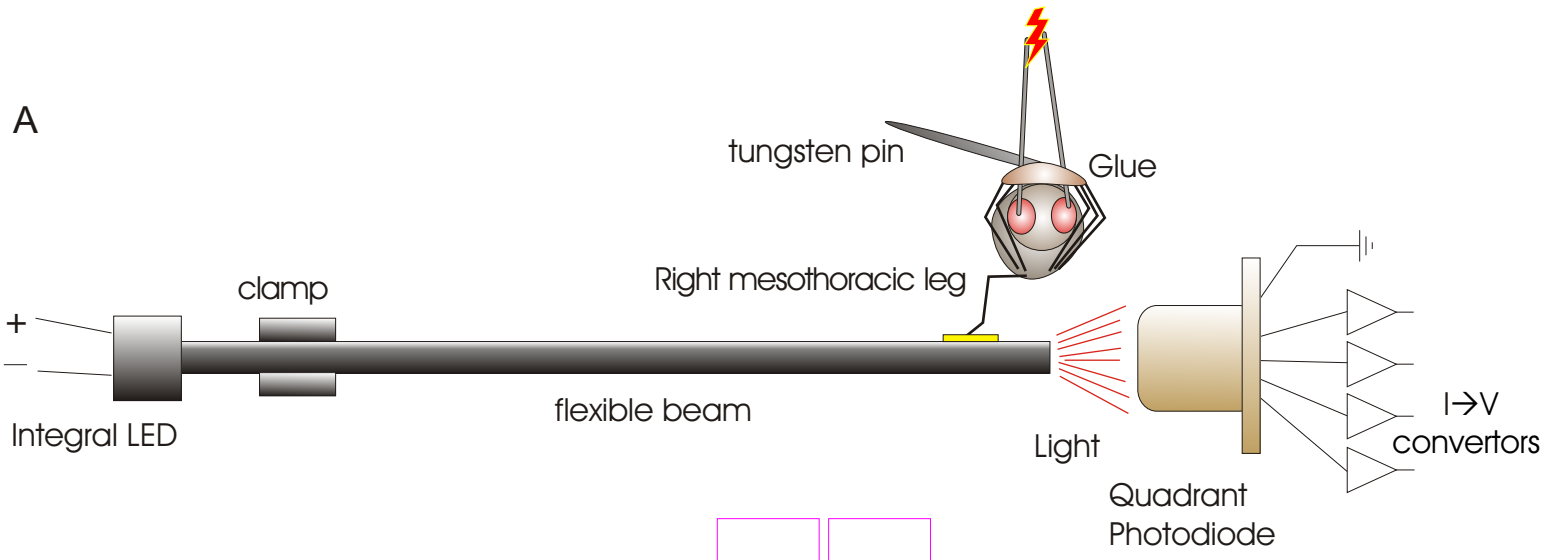


C Development with age



D Innervation of TDT

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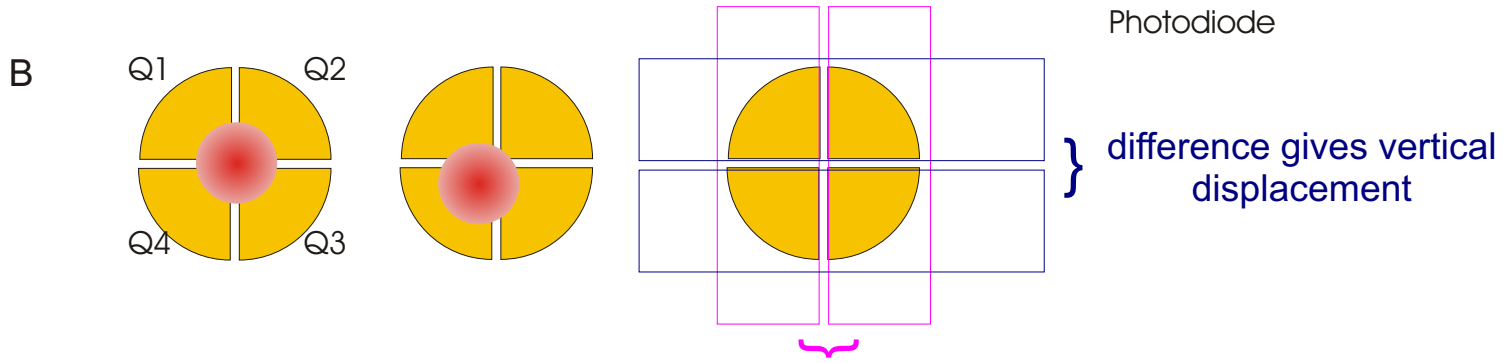
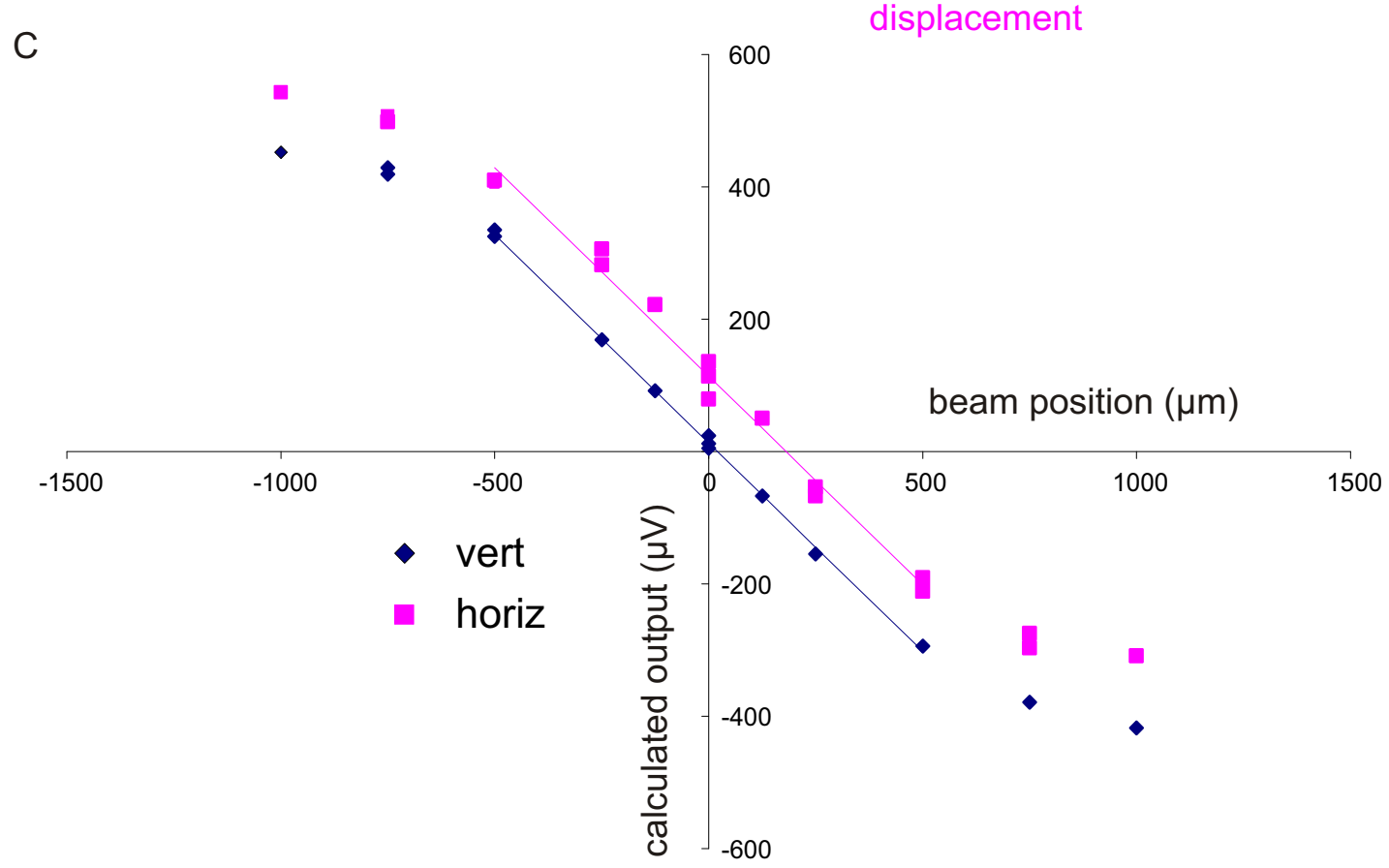
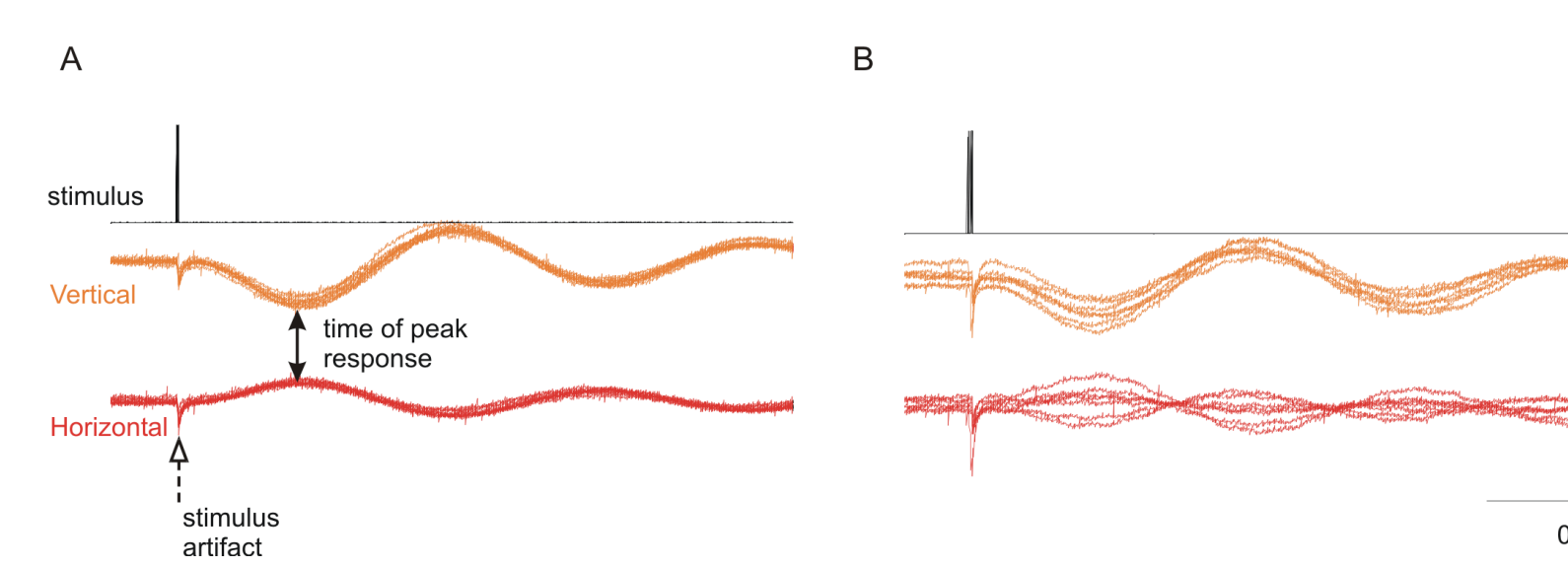
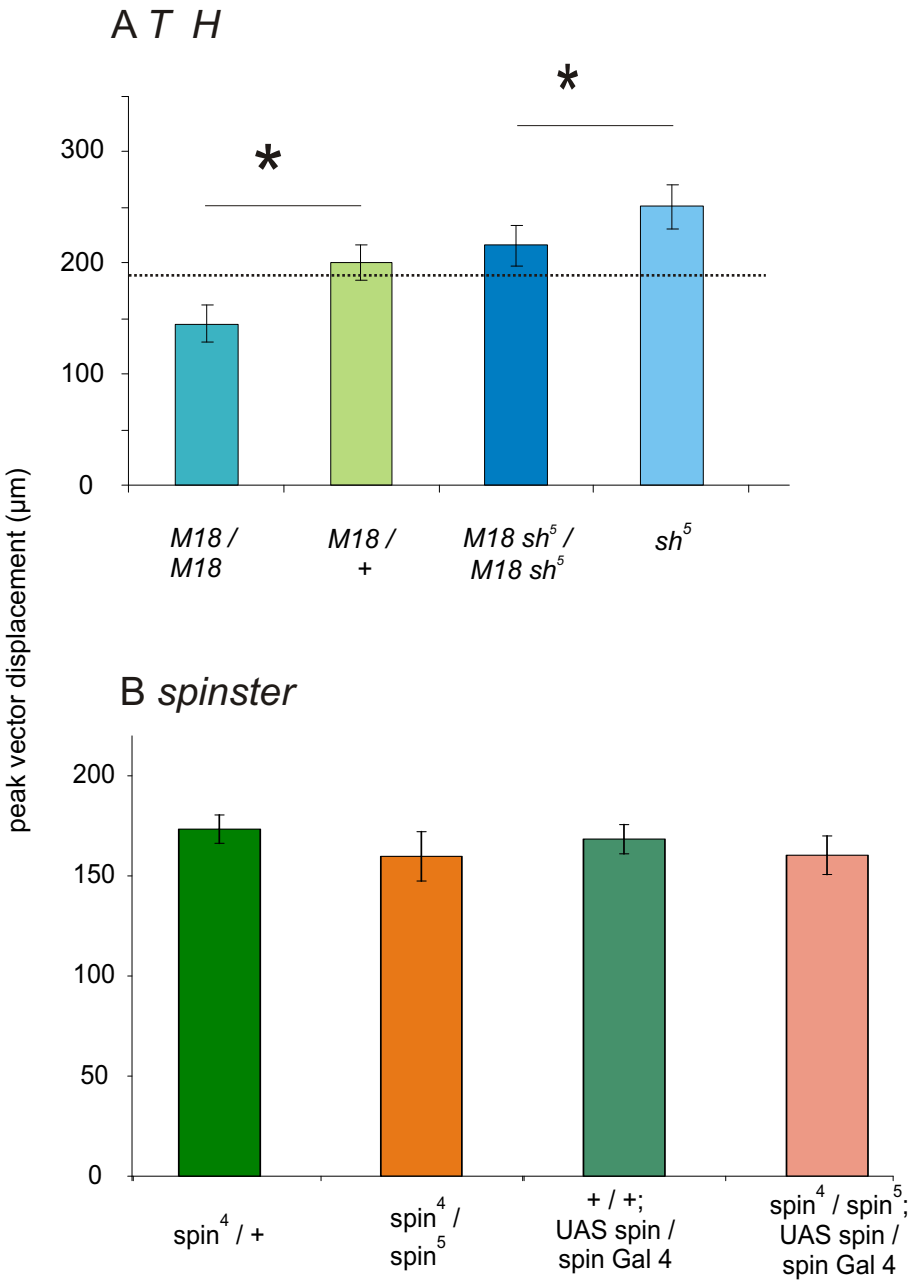
tungsten pin
Glue
Right mesothoracic leg
clamp
+
—
Integral LED
flexible beam
Light
I→V
convertors
Quadrant
Photodiode


B
Q1
Q2
Q4
Q3
difference gives vertical
displacement
difference gives horizontal
displacement


C
600
400
200
-200
-400
-600
-1500
-1000
-500
0
500
1000
1500
beam position (μm)
calculated output (μV)
vert
horiz


A
stimulus
Vertical
time of peak
response
Horizontal
stimulus
artifact
B


A T H
peak vector displacement (µm)
300
200
100
0
*
*
M18 / M18
M18 / +
M18 sh5 / M18 sh5
sh5
B spinster
200
150
100
50
0
spin4 / +
spin4 / spin5
+ / +; UAS spin / spin Gal 4
spin4 / spin5; UAS spin / spin Gal 4