Video Article

Studying the Neural Basis of Adaptive Locomotor Behavior in Insects

Matthias Gruhn[1], Philipp Rosenbaum[1], Hans-Peter Bollhagen[1], Ansgar Bueschges[1]

[1]Zoological Institute, University of Cologne

Correspondence to: Matthias Gruhn at mgruhn@uni-koeln.de

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Abstract

Studying the neural basis of walking behavior, one often faces the problem that it is hard to separate the neuronally produced stepping output from those leg movements that result from passive forces and interactions with other legs through the common contact with the substrate. If we want to understand, which part of a given movement is produced by nervous system motor output, kinematic analysis of stepping movements, therefore, needs to be complemented with electrophysiological recordings of motor activity. The recording of neuronal or muscular activity in a behaving animal is often limited by the electrophysiological equipment which can constrain the animal in its ability to move with as many degrees of freedom as possible. This can either be avoided by using implantable electrodes and then having the animal move on a long tether (i.e. Clarac *et al.*, 1987; Duch & Pflüger, 1995; Böhm *et al.*, 1997; Gruhn & Rathmayer, 2002) or by transmitting the data using telemetric devices (Kutsch et al, 1993; Fischer *et al.*, 1996; Tsuchida *et al.* 2004; Hama *et al.*, 2007; Wang *et al.*, 2008). Both of these elegant methods, which are successfully used in larger arthropods, often prove difficult to apply in smaller walking insects which either easily get entangled in the long tether or are hindered by the weight of the telemetric device and its batteries. In addition, in all these cases, it is still impossible to distinguish between the purely neuronal basis of locomotion and the effects exerted by mechanical coupling between the walking legs through the substrate. One solution for this problem is to conduct the experiments in a tethered animal that is free to walk in place and that is locally suspended, for example over a slippery surface, which effectively removes most ground contact mechanics. This has been used to study escape responses (Camhi and Nolen, 1981; Camhi and Levy, 1988), turning (Tryba and Ritzman, 2000a,b; Gruhn *et al.*, 2009a), backward walking (Graham and Epstein, 1985) or changes in velocity (Gruhn *et al.*, 2009b) and it allows the experimenter easily to combine intra- and extracellular physiology with kinematic analyses (Gruhn *et al.*, 2006).

We use a slippery surface setup to investigate the timing of leg muscles in the behaving stick insect with respect to touch-down and lift-off under different behavioral paradigms such as straight forward and curved walking in intact and reduced preparations.

Video Link

The video component of this article can be found at https://www.jove.com/video/2629/

Protocol

1. The Walking Surface

The black walking surface is made up of two nickel coated brass plates permanently joined side by side and electrically insulated from one another by a 2mm wide strip of 2-component epoxy glue (UHU plus, UHU GmbH, Germany) directly underneath the longitudinal axis of the animal. They produce a total surface area of 13.5x15.5cm (Figure 1). The separation of the half-planes for left and right legs allows independent tarsal contact monitoring for a single leg on each side.

1. The plate is lubricated with a glycerin/water mix at a ratio of 95 parts glycerin to 5 parts saturated NaCl-solution to convey slipperiness and electrical conductivity; the viscosity is approximately 430cStokes (Shankar and Kumar, 1994).
2. The mixture is applied directly onto the surface and is dispersed with a piece of soft tissue to assure an overall thickness of 0.1-0.2mm.

2. Optical Stimulation Setup

1. Walking episodes are elicited using two projectors as described by Scharstein (1989) to project a striped pattern onto two round, ground glass screens (diameter 130mm, Marata screens, Linos Photonics, Göttingen, Germany) that are positioned at a distance of approx. 70mm from the eyes, and at an angle of 45° at the left and right front end of the plate (Fig.1). The wave length of the striped pattern is kept constant at λ=21°. The contrast frequency of the moving stimuli can be varied between 0.35, 0.72, 1.07 and 1.49Hz. Since the reaction of the animals depends only on the contrast frequency ω/λ (ω = angular velocity, λ = pattern wave length) if the pattern contrast is high enough (Fermi and Reichhardt, 1963), we did not vary λ throughout the experiments. The luminance of the bright stripes on the side of the animal is approximately 15cd/m2 and the contrast 0.8. The compound eye of the stick insect (Carausius morosus) has 24 ommatidia in the dorso-ventral axis (Friza, 1928) and a few more in the rostro-caudal axis. As the divergence angle of the stick insect eye is on average 6.2° (Jander

and Volk-Henrichs, 1970) our pattern wavelength was approximately 4 times the angle of the ommatidia. The experiments are set up in a darkened Faraday cage and performed in a darkened room at 20-22°C.
2. Forward walking is induced by a progressive pattern on both screens where the stripes on each screen move outward, or a single black beam on white background placed between the screens
3. Turning is induced by stripes moving into the same direction on both screens. We have not found a correlation between the speed of the moving stripes and the angle of the turn. Once luminance of the striped pattern is set to induce walking, it usually does not require further adjustment, but it can be adjusted for different animals according to need by the voltage of the halogen lamps in the projectors.

3. Preparing the Experimental Animal

1. Female adult stick insects (Carausius morosus) are glued (dental cement, ProTempII, 3M ESPE, Seefeld, Germany) ventral side down onto a thin balsa stick (3x5x100mm, WxHxL), which is inserted in a brass tube that has multiple connectors. The animal is held down for approximately a minute until the glue is at least partly hardened.
2. The head and legs protrude from the front and side of the dowel to allow their free movement. The area of the coxae of all legs as well as the major part of the abdomen has to be left free of glue.
3. Insect head, thorax, and the distal ends of each femur and tibia are marked with fluorescent pigments (Dr. Kremer Farbmühle, Aichstetten, Germany) mixed with dental cement for later video tracking.

4. Placement of EMG Electrodes

Muscle activity of different leg muscles such as, in this example, the protractor and retractor coxae muscles, which are located in the thorax and cause protraction (forward movement) and retraction (backward movement) of the leg, is recorded by means of EMG electrodes implanted into the thorax. All EMG recordings are differentially amplified. The signal was amplified 100x in a preamplifier (electronics workshop, Zoological Institute, Cologne), band-pass filtered, (100Hz-2000Hz), further amplified (10x), and imported through an AD converter into spike2 (Vers.5.05, CED, Cambridge, UK).

1. The electrodes are made from coated copper wires (Elektrisola, Eckernhagen, Germany; 47µm outer diameter). For one electrode, two wires are first smeared with glue, then, the two sticky wires are twisted around each other
2. At one end the twisted wire ends are soldered to a mini-plug. At the other end the wires are cut to provide clean ends without insulation for the insertion into the animal.
3. The EMG electrodes are plugged into the small connector at the end of the brass tube.
4. A minuten pin is used to punch small holes into the cuticle
5. The cut ends of the electrode are then separated and inserted into a muscle at approximately 1mm distance from each other, which, in our experience, gives a good signal to noise ratio. Care should be taken not to insert the electrodes too deep into the muscle in order to avoid its damage. Figure 2 shows the location of the electrodes in the thorax of the animal. Figure 3 shows an animal with the electrode wires in place.
6. We use a small drop of histoacrylic glue (3M Vetbond, St.Paul, MN, USA) to hold the wires in place.
7. A ground wire is inserted into the abdomen.

5. Recording of the Tarsal Contact

Tarsal contact is always recorded together with EMG traces. Since the slippery surface is split into two halves, a maximum of two contralateral tarsal contacts can be registered at the same time. For this purpose, the leg is used as a "switch" to close a circuit between the plate and the amplifier. Current can be applied to the plates separately through two sockets at the base of each plate. We can generate two square wave signals with 2-4mV amplitudes with a pulse generator (Model MS501, electronics workshop, Zoological Institute, Cologne) that are phase-delayed by 90°. The signals are attenuated by 60dB (/1000) and applied to the two halves of the slippery surface. In this experiment we only use one of the two signals. It is simultaneously fed into a lock-in-amplifier as a reference signal. The 2-phase lock-in-amplifier (electronics workshop, Zoological Institute, Cologne) selectively amplifies only signals of the same frequency and phase as the provided reference signal. A second channel only detects the 90° phase delayed signal. Noise signals at frequencies other than the reference frequency are rejected and do not affect the measurement. The amplifier output signal is fed into an AD converter (Micro 1401k II, CED, Cambridge, UK) and digitalized using Spike2. Between touch-down and lift-off of the tarsus onto and from the slippery surface, current flows from the plate through tarsus and tibia into the copper wire (see below: 5.3-5.8). The chosen amplifier has a high input resistance (1MΩ) and the signal voltage is very small in order to avoid affecting the walking behavior of the animal. The current passing through tarsus and tibia is between 2 and 4nA. The signal lead time during touch-down, between the entry into the lubricant and the surface contact is less than 1ms. The contact signal transition during lift-off is less steep and more delayed. This is due to the delayed tearing of the lubricant from the tarsus by a capillary rise effect and due to movement of the leg without complete lift-off in swing phase.

1. The brass tube with the balsa stick to which the animal is glued is inserted into a micromanipulator to allow height adjustment of the animal at between 7 and 12mm above the slippery surface, which corresponds to the height during free walking.
2. In order to measure current flow whenever the insect tarsus of the monitored leg is on the ground, a wire (47µm diameter) is cut into approximately 15cm long pieces.
3. The insulation of a copper is removed by burning it away with alcohol.
4. A loop is formed with one end of the wire and slid over the tarsus of the animal
5. The loop is tied at the distal end of the tibia to provide good contact between animal and wire.
6. The wire is then fixed on the thorax of the animal with a drop of two-component glue (ProTempII, see above), and connected to the differential amplifier through an alligator clamp.
7. To improve conductivity of the wire at its ends, a drop of electrode cream is applied (Marquette Hellige, Freiburg, Germany) on the areas of contact.

8. The signal strength is then tested on the monitor. Due to the low-pass filter properties of the lock-in-amplifier (10ms time constant) and the gradual lift-off/touch-down of the tarsus, the signal is not an exact square signal. One therefore has to set suitable thresholds close to the transition point to determine the exact timing of tarsal contact and manually check each event determined by the digitalization software. Based on the tarsal contact, we defined the time when the tarsus of a given leg was on the ground as stance and the time when it was in the air as swing phase.
9. The trigger signal for the camera is recorded simultaneously with the EMG trace and the tarsal contact signal, and allows a precise frame by frame correlation of behavior and recorded traces.

6. Optical Recording of Leg Movements for Digital Analysis

The stepping frequency of stick insects on slippery surfaces can reach 3-4Hz (Graham and Cruse, 1981).

1. Before recording walking sequences, a mirror is placed at a 45°angle behind the animal to have an additional visual monitor for touch-down. The camera is externally triggered and pictures are fed into a PC through a FireWire interface.
2. We record walking sequences from above with a high speed video camera (Marlin F-033C, Allied Vision Technologies, Stadtroda, Germany) at 100fps to analyze the leg movements digitally, and correlate them with EMG activity and tarsal contact.
3. During the recording of walking sequences, the animal is illuminated with blue LED arrays (30-50V DC, luminance 24cd, Electronics Workshop, Zoological Institute, University of Cologne), which are adjusted in intensity to illuminate the fluorescent markers. According to the literature (Schlegtendal, 1934), stick insects do not possess color vision and we have not observed an adverse effect of the UV light illumination on the turning response. A yellow filter in front of the camera lens suppresses the short wavelength of the activation light and gives a higher contrast for the video recordings.
4. The video files can be analyzed using motion tracking software (WINanalyze, Vers.1.9, Mikromak service, Berlin, Germany).

7. Recording of Walking Sequences

1. As a control, first a walking sequence with an intact animal is recorded.
2. The velocity of the striped pattern is set, and pattern and the video recording are started approximately at the same time.
3. If the animal does not start locomotion spontaneously, it can be stimulated at the abdomen with a brush (Bässler and Wegener, 1983), or by a puff of air to the antennae. In this case, animals touched on the left side of the abdomen tend to turn left and vice versa. The striped pattern is kept moving until the animal stops walking or until after 3 minutes of continuous recordings.
4. In order to test how far the muscular activity in one leg is dependent on sensory input from neighboring legs, the animal is induced to autotomize its legs. The site of autotomy closes up naturally within seconds. An example of an animal with autotomized front and hind legs is shown in Figure 3.
5. For example, for experiments with animals having only one middle leg, we induce autotomy of the pro- and metathoracic legs by pinching the proximal femur with a pair of forceps (Schmidt and Grund, 2003) or cut the legs at the level of the coxae after recording from the intact animal. After that a minimum of 30min for recovery time is given to the animal.
6. Then we record new walking sequences as before. In reduced preparations, we often need to induce walking using paint brush strokes.

8. Representative Results:

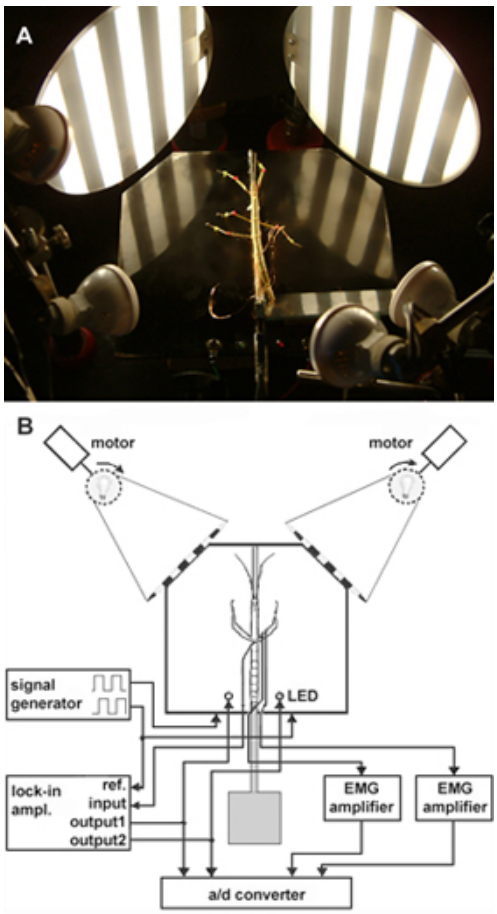

Figure 1. Experimental setup; A: photograph of a stick insect tethered above the slippery surface, marked with fluorescent pigments for leg tracking and wired for EMG recording and tarsal contact measurement. B: wiring diagram for dual leg tarsal contact recording during walking on the slippery surface.

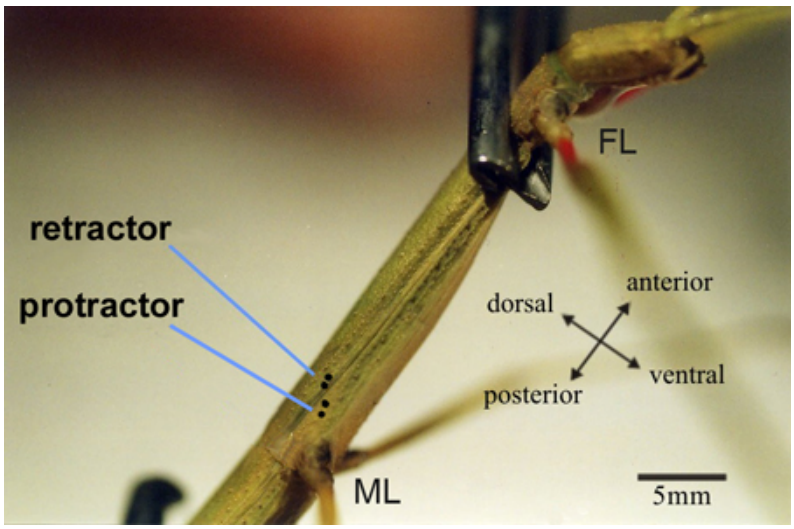

Figure 2. Positions for the placement of EMG electrodes in the protractor and retractor muscles in the thorax of the stick insect middle leg. ML: middle leg; FL: front leg

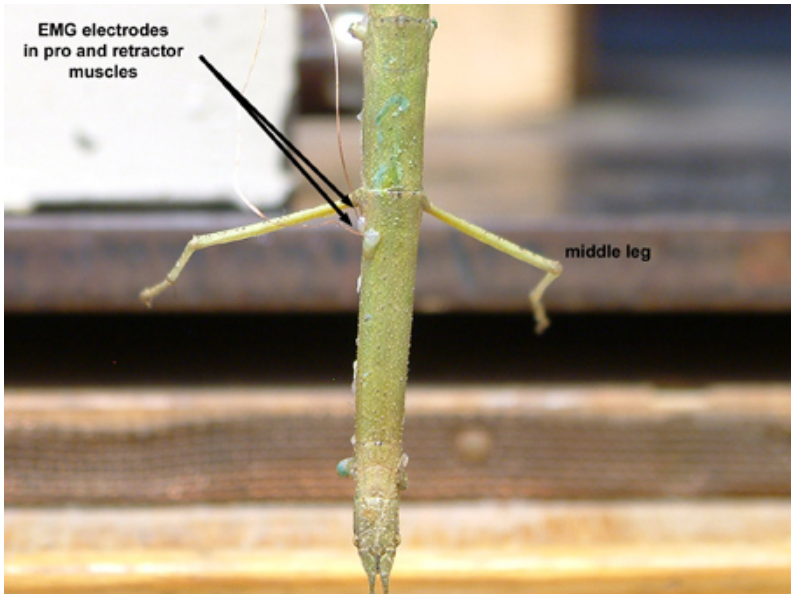

Figure 3. Stick insect with EMG wires in the pro- and retractor muscles of the middle leg in place; front and hind legs have been removed to study the effect of the presence of neighboring legs on the kinematics and muscle activity of the middle legs in the reduced preparation.

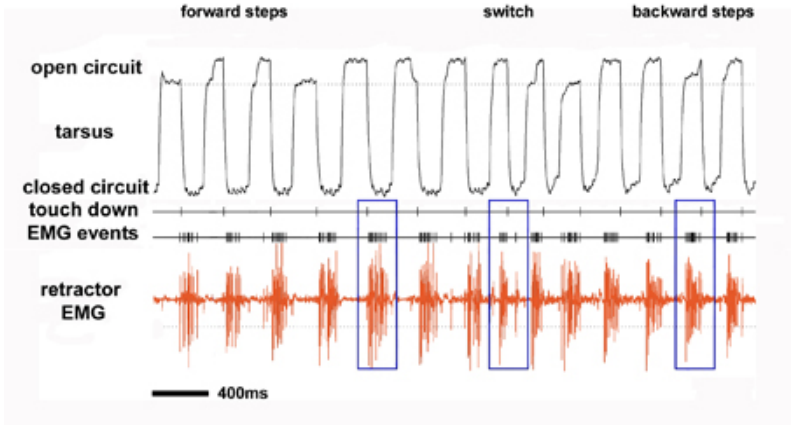

Figure 4. Example trace of retractor EMG activity with reference signal of tarsal contact in an inside middle leg during turning. At the beginning of the trace (first blue box) the retractor is active during stance, while the leg has ground contact ("closed circuit"), indicating that the leg makes forward steps; after a short switch (second blue box), the retractor is active during swing, while the leg is in the air ("open circuit", third blue box), showing the occurrence of backwards directed steps.

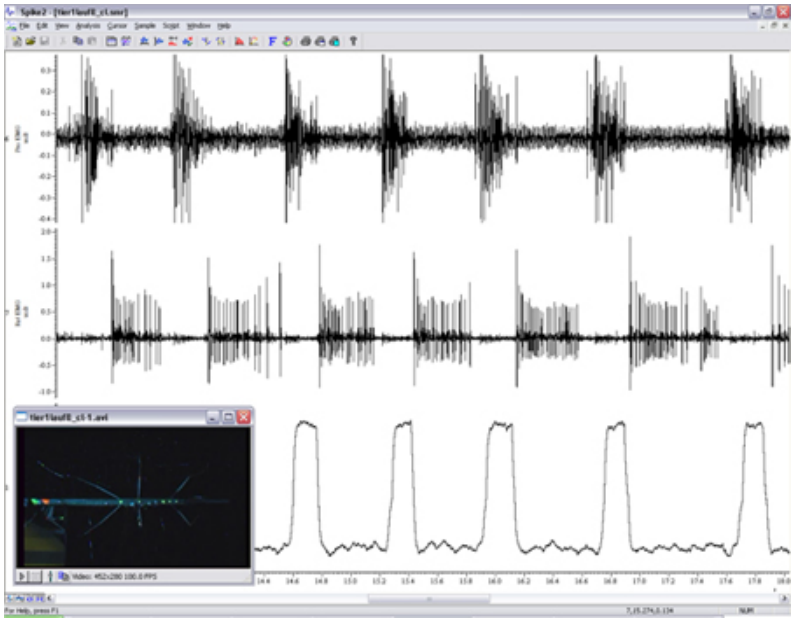

Figure 5. Screen shot of a recording from the protractor (top trace) and retractor (middle trace) muscles and the simultaneously recorded tarsal contact trace (bottom trace). Note the alternating activity in the EMG traces which corresponds to the activity of the two muscles in the step cycle, that is protractor activity just before and during swing, when the tarsal contact trace signals loss of ground contact, and retractor activity after and during touch-down of the tarsus, when leg is in stance. The insert shows the matching video file corresponding to this recording that is used to verify the behavior of the animal.

Discussion

We have described a setup that allows the optically induced generation of turning behavior and permits to a large degree the uncoupling of neuronally generated walking activity from the passive effects caused by the mechanical displacement of the other walking limbs on the ground. Potential information flow between the legs through the nervous system about ground reaction forces or tarsal contact, on the other hand, is still possible and allows the experimenter to study the influence of such information in the reduced preparation. Major advantages of the slippery surface setup include that the animals show a very high tendency to walk, and contrary to walking or stepping on a treadwheel, the animal can perform swing and stance phase movements in all the directions of natural walking. In addition, the degrees of freedom for all the legs allow the animal to perform curve walking whether it is an intact or semi-intact preparation. Because the legs cannot be passively moved simply by the forward movement of the animal or the movement of the substrate underneath, every movement reflects the motor output of that leg (Cruse, 1976; Graham and Wendler, 1981). The setup is highly suitable to investigate the neuronal basis of adaptive behaviors such as turning or forward vs. backward walking, because one can combine electrophysiological recordings of motor activity with the analysis of limb movement kinematics.

We used the stick insect's optomotor response to elicit walking. The responses of the animals to the rotating stripe pattern show their readiness to perform curve walking while tethered over the slippery surface. Most surprisingly for us, single legs in single-leg preparations qualitatively show the same moving pattern as in the intact animal. We thus have reason to believe that the control of curve walking can function largely without coordinating sensory input from neighboring legs. It will be important to test in further experiments whether the activity of the motor neurons of the removed legs is also influenced by the optomotor pattern. The setup can easily be modified to allow the study of other tasks such as straight forward and backward walking by placing a single stripe in front of the animal or gently pulling the antennae.

The precise measurement of ground contact allows us to correlate muscle activity and leg position. The high time resolution of this electric contact signal is better than 1ms and leads us to a new insight into the timing of the switch from swing to stance phase. The resolution is worse for the stance to swing transition due to the delay in shearing of the conducting lubricant and a lack of need for a complete lift-off during protraction on the slippery surface. Nevertheless, the knowledge on the precise swing to stance transition is a particularly useful first step if we want to understand the mechanisms that control muscle timing and the coordinated activities of leg muscles in different behavioral contexts (see also: Büschges *et al.*, 2008; Büschges & Gruhn 2008).

As an example, we used the retractor and protractor coxae muscle of the middle leg and precisely correlated its activity with the switching from swing to stance phase while we simultaneously monitored the behavioral context in which the leg was used. For this purpose, we induced walking and recorded the muscle activity continuously. A given leg can be an inside or an outside leg, depending on the turning direction. In the stepping middle leg, acting as an inside leg in the functional sense, it can be observed that retractor and protractor muscles can both work as functional stance muscles because the leg intermittently produces backward steps in addition to forward directed steps (see Fig.4).

The electromyograms (EMGs) from both muscles were rectified and normalized to the time of touch-down and the latency of the first muscle spikes was calculated. Interestingly, the latencies of both muscles with respect to lift-off and touch-down depend on the function of the muscle as respective swing or stance muscle (see Fig.4) and not on the muscle itself, and show only minor alterations in the timing of activity onset. Most explanations for the change of state from swing to stance assume that sensory signals of tarsal contact trigger the start of stance. The

interesting question of how the short latencies between touch-down and muscle activation in the stick insect are brought about and on what sensory information they depend can now be addressed with the modified setup.

In summary, we show a slippery surface setup that allows us to reliably elicit straight and curve walking in stationary stick insects. Kinematics, muscle activity and the timing of tarsal touch-down and lift-off can be monitored and correlated in the two different behavioral contexts at the same time. This gives us an excellent tool to study the detailed connection between muscle activity and behavioral context for a single leg as well as in the intact animal and the underlying mechanisms.

Disclosures

No conflicts of interest declared.

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