

## RESEARCH ARTICLE

# No functionally relevant mechanical effects of epimuscular myofascial connections between rat ankle plantar flexors

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## ABSTRACT

Triceps surae muscles are mechanically connected by the shared Achilles tendon and by epimuscular myofascial connections. We aimed to assess the effects of proximal lengthening of gastrocnemius and plantaris muscles, imposed by changes in knee angle, on the magnitude and direction of the 3D ankle moment exerted by the soleus muscle and the mechanical interaction between ankle plantar flexor muscles during co-activation of gastrocnemius muscle, in the rat ( $N=9$ ). Ankle angle was kept constant (90 deg), whereas knee angle was varied between 60 deg and 130 deg. At each knee angle, the soleus muscle was excited individually as well as simultaneously with the gastrocnemius muscle (SO+GA). The mathematical sum of individual soleus and gastrocnemius ankle moments was compared with the ankle moment exerted by SO+GA to assess nonlinear summation. Knee angle did not affect the magnitude of the soleus ankle moment ( $P=0.695$ ). Moment directions in the transverse ( $P=0.050$ ) and frontal ( $P=0.008$ ) planes were affected by knee angle, but dissection indicated that this was not caused by length changes of the two-joint synergistic muscles. Nonlinear summation was found in the magnitude ( $-1.4\pm 1.9\%$ , mean $\pm$ s.d.,  $P<0.001$ ) and in the frontal plane vector direction of the ankle moment ( $0.13\pm 0.23$  deg,  $P=0.003$ ); however, the extent did not change with knee angle. Although contraction of SO+GA increased the length of the Achilles tendon compared with its length at rest, this was not dependent on the knee angle ( $P=0.649$ ). Despite the fact that intermuscular force transmission per se cannot be excluded, we conclude that, *in vivo*, the mechanical effects of epimuscular myofascial connections between rat ankle plantar flexors are not functionally relevant.

**KEY WORDS:** Soleus, Gastrocnemius, Achilles tendon, 3D kinetics, Muscle relative position

## INTRODUCTION

In the past decade, the various pathways of force transmission from the muscle to the skeleton within the rat have been investigated in many studies. These studies have shown that, parallel to the path in which the force of a muscle is transmitted to the skeleton via its origin and insertion, connective tissue linkages located at the epimysium of a muscle transmit force to surrounding structures (Huijing, 2009; Maas and Sandercock, 2010). This so-called epimuscular myofascial force transmission suggests that muscles cannot be seen as independent actuators and that the classical view of a pure myotendinous transmission of skeletal muscle force may not be valid. However, in these studies, the muscular compartment

was disrupted and muscle lengths and relative positions were beyond physiological ranges.

More recently, the physiological importance of mechanical interaction between ankle flexor muscles has been subject of investigation. By using changes in knee angle to alter the muscle-tendon unit length of the gastrocnemius (GA) muscle exclusively, these studies imposed only GA muscle lengths and positions relative to the soleus (SO) muscle that can occur during normal movements. Studies on passive human triceps surae muscles using MRI or ultrasound imaging showed that passive knee extension (lengthening GA) caused shortening of SO muscle fibers (Tian et al., 2012), local strains in SO muscle (Huijing et al., 2011) and distal displacement of SO aponeurosis of insertion (Bojsen-Møller et al., 2010). While these studies did show intermuscular mechanical interaction for passive muscle conditions in humans, the mechanical effects in active muscle conditions were not investigated. In a recent study using cats, in which GA and plantaris (PL) muscles were inactive, the plantar-flexion moment exerted upon activation of SO was not significantly affected by knee angle (Maas and Sandercock, 2008). Thus, although epimuscular force transmission was found between passive triceps surae muscles in humans, in cats these connections did not affect the mechanical output of SO.

The different findings regarding mechanical interaction between species and muscle groups may have several explanations: (1) muscles in the anterior crural compartment, which were studied most frequently in the rat experiments, do not share an origin or insertion site, whereas triceps surae muscles merge distally into the common Achilles tendon. This additional pathway may offset epimuscular myofascial force transmission (Tijs et al., 2014); (2) the non-physiological conditions in the rat studies described above may have affected the extent of mechanical interaction; (3) co-activation might increase the stiffness of epimuscular connective tissue linkages and thereby enhance the magnitude of mechanical interaction (Maas and Sandercock, 2010). In the previous rat studies, all synergistic muscles within a compartment as well as some antagonistic muscles were excited simultaneously and maximally, whereas in the cat study, SO muscle was excited exclusively and partially. (4) In the cat study, the magnitude of the ankle moment was assessed only in the sagittal plane (Maas and Sandercock, 2008). Ankle plantar flexor muscles in cats and rats also exert ankle moments outside the sagittal plane (Lawrence and Nichols, 1999; Lawrence et al., 1993; Tijs et al., 2014). It is possible that the effects of mechanical interaction are insignificant in the sagittal plane, but larger in the other anatomical planes.

In the present study, the above issues will be addressed by investigating the mechanical effects of epimuscular myofascial connections during individual SO activation and during co-activation of rat plantar flexor muscles, using a 3D kinetic analysis. Knee extension was used to impose displacements of GA relative to SO and, hence, increase the stiffness of linkages

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**List of symbols and abbreviations**

EMG	electromyography
FES	functional electrical stimulation
GA	medial and lateral gastrocnemius muscles
$L_{AT}$	length of distal portion of the Achilles tendon
LG	lateral gastrocnemius muscle
$\%M_{nl}$	nonlinear magnitude summation relative to the mathematical sum of the ankle moments exerted by individual SO and GA muscles
MG	medial gastrocnemius muscle
PL	plantaris muscle
SO	soleus muscle
SO+GA	simultaneous excitation of soleus and gastrocnemius muscles

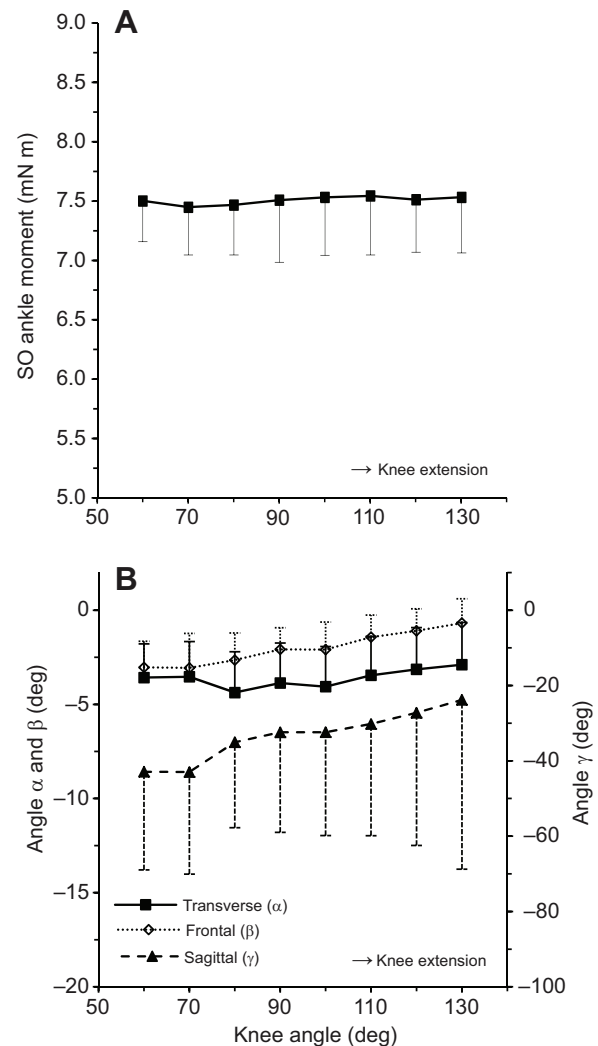
between these muscles. The extent of mechanical interaction is dependent on the stiffness of epimuscular myofascial connections, which is affected by the relative position of muscle bellies (Maas and Sandercock, 2010). If the stiffness is sufficient, we predict that increasing knee angle will affect the ankle moment exerted by SO. Specifically, we tested (1) the effects of knee angle on the 3D ankle moment exerted by SO muscle, both in magnitude and direction of its vector; (2) the effects of knee angle on mechanical interactions during co-activation of GA; (3) the effects of knee angle and muscle activation on the common Achilles tendon length; and (4) whether any of the effects found could be attributed to intermuscular connective tissue linkages.

**RESULTS****Effects of knee angle on 3D ankle moments of SO muscle**

Knee extension (i.e. proximal lengthening of GA and PL muscles, but not of SO) did not significantly affect ( $P=0.695$ ) the magnitude of the 3D active SO ankle moment (Fig. 1A; average across knee angles:  $7.5\pm 0.4$  mN m,  $N=9$ ). Knee angle did affect SO vector direction (Fig. 1B; see Materials and methods for definition of vector direction) in the transverse ( $\alpha$ , angle between vector and plantar-flexion moment axis) and frontal ( $\beta$ , angle between vector and plantar-flexion moment axis) planes but not in the sagittal plane ( $\gamma$ , angle between vector and eversion moment axis;  $P=0.141$ ). In the transverse plane, the angle of the vector ranged from  $\alpha=-4.4\pm 2.2$  deg at 80 deg knee angle to  $\alpha=-2.9\pm 2.2$  deg at 130 deg knee angle ( $P=0.050$ ). In the frontal plane, the angle of the vector ranged from  $\beta=-3.1\pm 1.8$  deg at 70 deg knee angle to  $\beta=-0.7\pm 1.3$  deg at 130 deg knee angle ( $P=0.008$ ).

When muscles were passive, knee extension increased ( $P=0.005$ ) the length of the distal portion of the Achilles tendon from  $6.62\pm 0.88$  mm at 60 deg to  $6.75\pm 0.85$  mm at 130 deg. The Achilles tendon length during SO activation (mean across knee angles:  $6.80\pm 0.82$  mm) was significantly larger compared with its length at rest ( $P<0.001$ ), but this was not dependent on the knee angle ( $P=0.159$ ).

After severing the proximal tendon of GA and PL muscles to eliminate effects of knee angle on proximal length changes of GA and PL (performed in a subset of the animals,  $N=5$ ), SO ankle moment magnitude ranged from  $7.3\pm 0.3$  mN m at 60 deg to  $6.9\pm 0.4$  mN m at 130 deg. This was, on average across knee angles, not different from the intact condition ( $P=0.120$ ) and no interaction effects were found between knee angle and dissection ( $P=0.149$ ). SO vector directions were also not affected by proximal GA and PL tenotomy. No main effects of dissection were found in the transverse ( $P=0.655$ ), frontal ( $P=0.443$ ) and sagittal ( $P=0.217$ ) planes and no



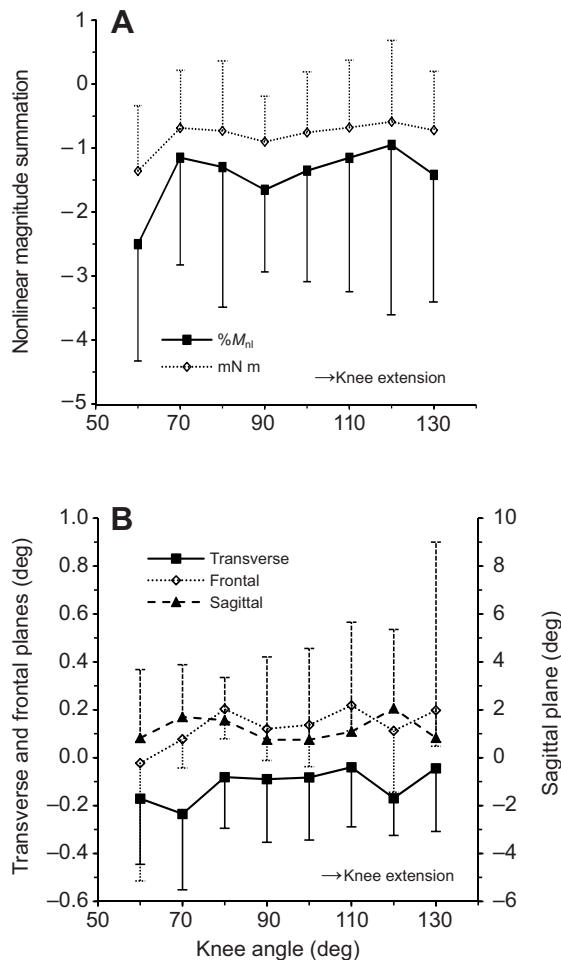
**Fig. 1. Effects of knee angle on ankle moment exerted by rat SO muscle.** (A) The magnitude of the active moment exerted by SO plotted as a function of knee angle. Ankle angle was kept constant at 90 deg. (B) Effects of knee angle on the SO vector direction in the transverse ( $\alpha$ ), frontal ( $\beta$ ) and sagittal ( $\gamma$ ) planes. Means $\pm$ s.d. are shown ( $N=9$ ).

interaction effects ( $P=0.166$ ,  $P=0.122$  and  $P=0.339$ , respectively) between knee angle and dissection were found. Thus, the effects of knee angle were similar for the intact and cut conditions.

To summarize, although a passive change in knee angle affected the Achilles tendon length, the length was not affected by knee angle during SO activation. Knee angle did not affect the magnitude of SO ankle moment, but it did affect the direction of SO vectors. However, these changes were not caused by mechanical interaction with the adjacent two-joint GA and PL muscles.

**Mechanical interaction during co-activation of GA**

Averaged across knee angles, the moment exerted by simultaneous excitation of SO and GA muscles (SO+GA) was less than the sum of the individual SO and GA ankle moments, resulting in a negative nonlinear magnitude moment summation of  $-0.8\pm 1.0$  mN m, that is  $-1.4\pm 1.9\%$  of the mathematical sum, which was significantly different from zero ( $P<0.001$ , Fig. 2A). No effects of knee angle on nonlinear magnitude summation were found ( $P=0.802$ ). Nonlinear direction summation (Fig. 2B) was found only in the frontal plane ( $P=0.003$ ): the vector during simultaneous excitation



**Fig. 2. Effects of knee angle on mechanical interaction between active SO and GA muscles.** Nonlinear magnitude summation (A) is plotted as function of knee angle, both in absolute values (mN m) and relative to the mathematical sum of the ankle moments exerted by individual SO and GA muscles ( $\%M_n$ ). Negative values indicate a lower ankle moment exerted on simultaneous excitation of SO+GA muscles than the mathematical sum of the ankle moments exerted by individual SO and GA muscles. (B) Nonlinear direction summation in the transverse, frontal and sagittal planes. Positive values indicate a vector direction more towards that of SO muscle than predicted based on the mathematical sum (=0 deg). Means $\pm$ s.d. are shown ( $N=9$ ).

of SO+GA was directed  $0.13\pm 0.23$  deg more towards the SO ankle moment vector than predicted based on the mathematical sum of individual SO and GA ankle moment vectors. Nonlinear direction summation in the transverse and sagittal planes was not significant ( $P=0.144$  and  $P=0.261$ , respectively). In none of the planes were any effects of knee angle found on the nonlinear direction summation (transverse:  $P=0.143$ ; frontal:  $P=0.338$ ; sagittal:  $P=0.804$ , respectively).

The length of the distal portion of the Achilles tendon during SO+GA activation was  $6.98\pm 0.79$  mm across all knee angles ( $P=0.649$ ), which was higher (by  $0.18\pm 0.08$  mm) compared with the length during SO activation only ( $P<0.001$ ). No interaction effect between knee angle and condition (SO only versus SO+GA) was found ( $P=0.231$ ).

Thus, the magnitude and the direction in the frontal plane of ankle moments exerted by triceps surae muscles do not sum in a perfectly linear manner. However, proximal lengthening of GA and PL muscles did not affect the summation of ankle moments exerted by SO and GA.

## DISCUSSION

This study is the first to investigate effects of knee angle on the magnitude and direction of the ankle joint moment exerted by SO and on the extent of mechanical interaction between ankle plantar flexor muscles during co-activation of GA in rats. We found that only the direction in the transverse and frontal planes of the ankle moment exerted by SO was affected by changes in knee angle. However, these effects were not caused by mechanical interaction with the adjacent two-joint GA and PL muscles. In addition, summation of the ankle moments exerted by SO and GA was nonlinear in both magnitude and direction, indicating mechanical interactions between these muscles. However, the extent of such mechanical interaction was limited and was not affected by the knee angle.

### Effects of knee angle on 3D ankle moments of SO muscle

Previous studies using rats have clearly shown that connective tissue linkages between synergistic muscles are capable of transmitting substantial forces (Huijing and Baan, 2001; Huijing et al., 2003; Maas et al., 2001, 2004, 2005). However, these studies involved several aspects that were non-physiological. Not only were the tendons detached from the skeleton and attached to force transducers, but also the imposed muscle lengths and relative positions were larger than those occurring during normal movement. In most cases, the length of a single muscle was changed distally up to 14 mm (Huijing, 2009), whereas during ankle joint movement, the length of its synergists would also be affected, thus yielding non-physiological relative positions for the muscle. In the present study, physiological muscle lengths and relative positions were obtained by changing the knee angle. During rat walking and swimming, knee angles between 50 deg and 125 deg have been reported (Gruner and Altman, 1980; Gruner et al., 1980; Schmidt and Fischer, 2011), although these angular displacements may be slightly overestimated because of skin movement artifacts (Bauman and Chang, 2010). This range would then correspond to a 6 mm maximum change in muscle-tendon unit length of the two-joint calf muscles (Johnson et al., 2008). The physiological importance is, therefore, still subject to debate (Herbert et al., 2008). A study using cats, in which SO muscle was not detached from the skeleton, showed that a change in knee angle did not significantly affect the plantar-flexion moment exerted by SO (Maas and Sandercock, 2008). It was concluded that SO is mechanically independent of passive synergistic muscles, when acting at physiological lengths and relative positions. Although force transmission per se cannot be excluded, our results on SO moment magnitude in rats show similar results, which indicate that these linkages do not affect mechanical output of SO at the joint level.

The direction of the SO moment vector in the transverse and frontal planes was significantly affected by knee angle. However, the extent of these changes was minimal. As similar effects were found after severing the origin of GA and PL, these changes cannot be attributed to changes in length and relative position of synergistic muscles proximally. Therefore, other factors explain the effects of knee angle on the direction of the SO moment. Displacement of the intact sciatic nerve with knee joint movements (Ellis et al., 2012) may have changed the force exerted by the neurovascular tract on SO. In addition, as the knee is not a perfect hinge joint, tibiofemoral contact points may change with knee angle. Both mechanisms could result in slight changes in the line of action of SO.

The triceps surae muscles are mechanically connected via the shared Achilles tendon and via epimuscular myofascial connections. In a recent study in humans (Tian et al., 2012), an increase ( $0.007$  mm deg $^{-1}$ ) in Achilles tendon length was reported



when the knee was extended by 60 deg with the ankle fixed at an included angle of 65 deg, which was accompanied by a decrease in distal SO fascicle length of  $0.06 \pm 0.06 \text{ mm deg}^{-1}$ . We also found that, with all muscles in a passive state, knee extension resulted in a length increase of the distal portion of the Achilles tendon. Although we did not assess changes in SO fascicle length, the present results confirm the possibility of mechanical interactions between triceps surae muscles mediated by the common Achilles tendon when passive. In contrast to the passive muscle condition, knee angle did not affect the length of the Achilles tendon during SO activation. Therefore, only epimuscular myofascial connections remained as a potential pathway for mechanical interaction. However, the active SO ankle moment was not affected by changes in length of its passive synergists. This indicates that these connections are not functionally relevant for the mechanical output of SO.

### Mechanical effects of epimuscular myofascial linkages during GA co-activation

In previous studies that assessed mechanical interactions between muscles in rats (Huijing, 2009), all synergistic muscles and also occasionally some antagonistic muscles were simultaneously activated. As hypothesized (fig. 4 in Maas and Sandercock, 2010), co-activation may increase the stiffness of epimuscular connective tissue linkages and thereby enhance mechanical interaction between muscles. We found significant but limited nonlinear moment summation, which was, as shown recently (Tijs et al., 2014), most likely caused by differences in Achilles tendon length during SO contraction and SO+GA contraction. However, the nonlinearity, as well as the Achilles tendon length during SO+GA contraction, was not dependent on the knee angle. Although we cannot rule out the possibility that co-contraction increased the stiffness of epimuscular myofascial connections, we can conclude that any effects did not alter the summation of SO and GA ankle moments.

Note that, whereas SO was excited maximally, GA was activated submaximally in this study. A study using electromyography (EMG) in rats (Roy et al., 1991) showed that, during treadmill walking at level surfaces, an increase in walking speed increases SO EMG activity only minimally, while EMG of MG increases substantially. During upslope walking (30%), SO EMG activity does not increase with walking speed. Similar data has been found in cats (Pierotti et al., 1989), indicating nearly maximal activation levels of SO muscle in combination with submaximal levels in MG muscle. The activation levels during locomotion are thus comparable with those of the present study. Increasing the mechanical demands can further increase the level of activation of GA muscles (Gregor et al., 2006; Maas et al., 2009). Maximal activation of GA may result not only in a larger increase in Achilles tendon length but also in an increased stiffening of the connective tissue linkages. In a recent study, which involved maximally activated rat ankle plantar flexor muscles, significant effects of lengthening GL and PL proximally (simulating knee extension) on the force exerted at the distal tendon of SO (a 10% increase) was found (Bernabei et al., 2015). The imposed length changes were within the range that occurs during normal movements. These results indicate significant mechanical interaction between SO and synergistic muscles at higher levels of co-activation.

### Functional relevance of mechanical interaction

In the present study, we found no evidence of mechanical interactions between ankle plantar flexor muscles mediated by

epimuscular myofascial connections. This appears to be in contrast to the study described above (Bernabei et al., 2015). In that study, however, the distal tendons were divided and cut from the skeleton, whereas they were kept intact in the present study. In an intact hindlimb, the direction of SO and GA moment vectors is dominated by the plantar-flexion moment. Consequently, these vectors are largely comparable (Tijs et al., 2014). We have found only small differences in internal rotation moment between these muscles in the rat (Tijs et al., 2014). Thus, even if substantial force is transmitted between SO and GA, the net result at the ankle joint would be limited. Hence, the functional consequences of mechanical interactions may not be mechanically relevant between triceps surae muscles. In contrast to triceps surae muscles, muscles within the rat anterior crural compartment do not share an insertion site and have distinctly different lines of action. While the tibialis anterior muscle exerts an inversion moment at the ankle, the extensor digitorum longus muscle exerts an eversion moment (Johnson et al., 2008). Therefore, the net effects of mechanical interaction at the joint level are expected to be higher.

The results of the present study are relevant for practical applications such as functional electrical stimulation (FES) to control limb movements, because such methods assume that muscles are fully independent. Endpoint forces were recently assessed for several combinations of rat hindlimb muscles (Jarc et al., 2013) to validate FES control. A close to linear relationship was found between the direction of the force exerted by two muscles and the sum of the directions exerted by each muscle individually. The results of the present study support that finding. We found that nonlinear direction summation is limited and is not caused by epimuscular myofascial force transmission between ankle plantar flexor muscles. However, triceps surae muscles are connected mechanically via the common Achilles tendon and are therefore not fully independent. Thus, small errors may occur when muscles that have a common tendon are controlled.

### Conclusions

We found that knee angle did not affect the magnitude of the ankle moment exerted by SO if it is excited alone. The direction in the transverse and frontal planes was affected, but to a minimal degree and was not caused by mechanical interactions between the active SO muscle and its passive synergists. This indicates that magnitude and direction of joint moments exerted by one-joint SO muscle is mainly dependent on the location of its origin and insertion, and not on length changes of nearby two-joint muscles. Although submaximal co-activation of the nearby muscles resulted in limited nonlinear summation, the extent of such mechanical interaction was not affected by the knee angle. Therefore, we conclude that *in vivo*, the mechanical effects of epimuscular myofascial connections between rat ankle plantar flexors are not functionally relevant.

### MATERIALS AND METHODS

#### Animals

Data were obtained from nine male Wistar rats (body mass:  $326.8 \pm 11.9 \text{ g}$ , mean  $\pm$  s.d.). All procedures were in agreement with the guidelines and regulations concerning animal welfare and experimentation set forth by Dutch law, and approved by the Committee on Ethics of Animal Experimentation at the VU University Amsterdam (Permit Number: FBW 11-02). At the end of the experiment, animals were killed with an overdose of intracardially injected pentobarbital sodium followed by a double-sided pneumothorax.

According to standard procedures in our laboratory (Maas et al., 2001), the rats were deeply anesthetized using intraperitoneally injected urethane

(initial dose 1.2 ml/100 g body mass, 12.5% urethane solution). Throughout the experiment, depth of anesthesia was tested by eliciting palpebral reflexes, ear withdrawal reflexes and hindlimb withdrawal reflexes. To suppress all of the above reflexes, supplemental doses (0.3–0.5 ml) of urethane were given as needed. To prevent hypothermia during surgery and data collection, the animals were placed on an electrical heating pad, maintaining core temperature at approximately 37°C.

### Surgery

The skin and biceps femoris muscle of the left hindlimb were removed. Medial and lateral malleoli and origin of medial and lateral collateral ligament were marked and used as axis of rotation of ankle and knee joints, respectively. The femur was exposed to allow attachment of a metal clamp and tissues between the malleoli and Achilles tendon were removed to secure the calcaneus to the set-up. The sciatic nerve was partly dissected free for placement of a cuff electrode. To prevent muscle excitation via spinal reflexes, the sciatic nerve was crushed by tying a knot proximal to the cuff electrode. All branches of the sciatic nerve distally to the cuff electrode were cut, except the branch innervating SO muscle (Maas and Sandercock, 2008; Tijs et al., 2014). Connective tissues between medial (MG) and lateral (LG) gastrocnemius muscles were thereby slightly disrupted. However, SO muscle and its epimuscular myofascial connections were not affected. MG and LG muscles consist of multiple compartments with multiple motor endplates (De Ruyter et al., 1995; Prodanov et al., 2005). To excite these muscles, bipolar intramuscular wire electrodes were inserted near the motor endplates located in the distal region of MG and the proximal region of LG. To allow measurement of length of the common Achilles tendon, a suture was placed on its posterior side at the point where the tendons of both muscles merge (~45% of the LG distal tendon length). During surgery and data collection, saline solution was applied frequently to prevent dehydration of nerves, muscles and connective tissues.

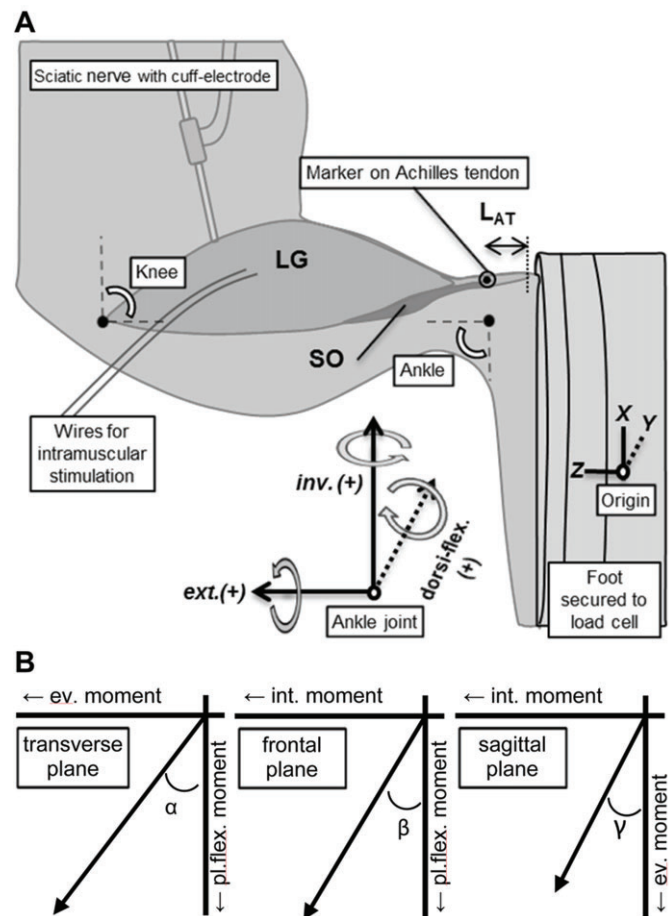
### Fixation in experimental apparatus

The left hindlimb was secured to the experimental set-up by clamping the femur and attaching the foot to a 6 degrees-of-freedom load cell (Mini40-E, ATI, Apex, NC, USA). For alignment of the ankle and knee joint with the set-up's rotational axes, knee and ankle joints were set to an included angle of 90 deg around the dorsi-flexion/plantar-flexion axis (Fig. 3A). The midpoint between both malleoli of the ankle was aligned with the origin of the *y*-axis of the load cell. In addition, the midpoint between the bilateral landmarks of the knee joint was aligned with the midpoint between both malleoli using a laser pointer. Finally, the *x*- and *z*-position of the medial anatomical landmarks of ankle and knee joint were aligned with the set-up's rotational axes and their position relative to the origin of the load cell was measured using rulers integrated in the set-up.

### Experimental protocol

SO muscle was excited maximally by supramaximal stimulation of the sciatic nerve (amplitude: 0.4–0.5 mA, frequency: 100 Hz, pulse width: 100  $\mu$ s) via the bipolar cuff electrode connected to a constant current source (Digitimer DS3, Digitimer Ltd., Hertfordshire, England). MG and LG muscles were excited simultaneously (amplitude: 0.7–2.0 mA, frequency: 100 Hz, pulse width: 100  $\mu$ s) via the intramuscular electrodes. As the threshold current near a motor endplate is substantially lower than that of muscle fibers (Mortimer and Bhadra, 2004), the probability of directly exciting surrounding muscle fibers was minimal. In addition, the electrodes were placed rather superficially within MG and LG muscle bellies, and submaximal stimulation was applied to make sure that only MG and LG muscle fibers were excited. As only one compartment of each MG and LG muscle was stimulated (De Ruyter et al., 1995; Prodanov et al., 2005), not all muscle fibers of these muscles were excited. A pilot experiment, in which excitation of LG and MG via nerve stimulation was compared with that via intramuscular stimulation, showed that approximately 20% of maximum active ankle moment exerted by MG and LG muscles fibers was exerted when stimulating intramuscularly (Tijs et al., 2014).

The extent of mechanical interactions between ankle plantar flexor muscles was assessed by imposing a wide range of knee angles while



**Fig. 3. Experimental set-up and vector direction definitions.** (A) Lateral view of the rat left hindlimb in the set-up. Ankle and knee joints were at an angle of 90 deg around the dorsi-flexion/plantar-flexion axis. The femur was fixed and the foot was attached to the load cell using custom-made clamps. Anatomical landmarks for ankle and knee joint were aligned with the rotational axes of the set-up. A marker was placed on the posterior side of the Achilles tendon, which was used to assess length of the distal part of the Achilles tendon ( $L_{AT}$ ). Positive ankle moments are inversion (inv.), dorsi-flexion (dorsi-flex.) and external rotation (ext.). SO, soleus; LG, lateral gastrocnemius. Dotted vertical line represents the insertion site of the Achilles tendon on the calcaneus. (B) Vector direction of the ankle moments in the separate planes. For each vector in the transverse and frontal planes, the angle relative to the plantar-flexion moment axis was calculated ( $\alpha$  and  $\beta$ , respectively). For each vector in the sagittal plane, the angle relative to the eversion moment axis was calculated ( $\gamma$ ). These angles were used as a measure of the vector direction (see Fig. 1B). ev., eversion; int., internal rotation; pl.flex., plantar-flexion.

keeping the ankle angle constant. Consequently, one-joint SO, spanning only the ankle joint, was kept at a constant muscle-tendon-unit length, while the length and relative position of two-joint synergistic muscles were changed. As a result, the stiffness of the connections between these muscles increases, which could enhance intermuscular mechanical interaction. This combination of joint angles was used for two experimental approaches. In the first approach, only SO muscle was activated and effects of changes in knee joint angle on magnitude and direction of the active ankle moment exerted by SO were assessed. In the second approach, SO and GA were excited first separately and then concurrently. As shown in a previous study (Tijs et al., 2014), the shared Achilles tendon may cause the joint moment exerted during simultaneous excitation of SO and GA (SO+GA) to be unequal to the sum of the joint moments exerted by each muscle individually (i.e. nonlinear summation). Effects of changes in knee joint angle on the extent of such nonlinear summation were assessed (see data analysis).

In the present study, the knee was rotated from 60 deg to 130 deg or vice versa in the sagittal plane (alternated between experiments) in steps of 10 deg, while the ankle angle was kept constant at 90 deg. At least part of this range (60–100 deg) has been observed during rat walking using X-ray video (Bauman and Chang, 2010; Schmidt and Fischer, 2011). For both joints, the angles around the two other axes were kept at 0 deg. The actual rotation was performed with maximal compliance in the joints, allowing the knee to rotate around its true axis as well as possible. This procedure was used to minimize effects of misalignment of the knee joint with the rotational axis of the set-up. When the knee was set to the designated angle, the component of the reaction force in the direction of the tibia (external rotation/internal rotation axis, see Fig. 3A) was kept constant (approximately 4N) to ensure similar compression of the lower hindlimb and minimal compliance in the ankle and knee joints. At each knee angle, two stimulation protocols were performed (Fig. 4). First, SO and GA muscles were stimulated separately (500 ms, Fig. 4A), to assess isometric ankle moments of each muscle individually. Second, isometric ankle moments were assessed during tetanic contractions of both SO and GA (Fig. 4B), but with trains of different length (i.e. GA was excited for 500 ms followed after 200 ms by SO for 300 ms). After each stimulation protocol, 2 min rest periods with the knee and ankle joints at 90 deg were allowed. The order of the stimulation protocols was alternated between animals.

In some experiments ( $N=5$ ), additional measurements were performed after severing the proximal tendon of GA and PL muscles to eliminate effects of knee angle on proximal length changes of GA and PL. After this surgical intervention, the effects of only knee angle on SO ankle moment were assessed. Video recordings (Panasonic HC-V720, 1080/50p, resolution 1 pixel  $\sim 0.03$  mm) were made to record the position of the Achilles tendon marker before and during isometric muscle contractions.

#### Data analysis

Forces and moments measured by the load cell were corrected for gravity caused by the masses of the load cell itself and the additional plates attached for foot fixation. Ankle moments were calculated around three axes [axis

perpendicular to the transverse plane: inversion(+)/eversion; axis perpendicular to the sagittal plane: plantar-flexion/dorsi-flexion(+); axis perpendicular to the frontal plane: external rotation(+)/internal rotation] using inverse static analysis (Tijs et al., 2014).

For each trial and around each axis, ankle moments were assessed before (passive) and during muscle contractions (total) by calculating the mean over 50 ms time windows (Fig. 4). Active ankle moments were calculated by subtracting the passive moment from the total moment at equal ankle and knee joint angles. Time windows 'b' and 'e' were used to assess active ankle moments for SO (b–a) and GA (e–c), respectively. Time window 'h' was used to assess the active ankle moments during simultaneous SO+GA excitation (h–f). In addition, the difference in active GA ankle moments between time window 'g' and 'd' (g–d) was calculated for each knee angle and around each axis (average across knee angles for eversion moment:  $-0.1 \pm 0.2$  mN m; plantar-flexion moment:  $-0.0 \pm 1.0$  mN m; internal rotation moment:  $0.0 \pm 0.3$  mN m). These values were subtracted from the active ankle moment exerted by SO+GA to exclude possible effects of initial differences in GA muscle excitation on nonlinear summation.

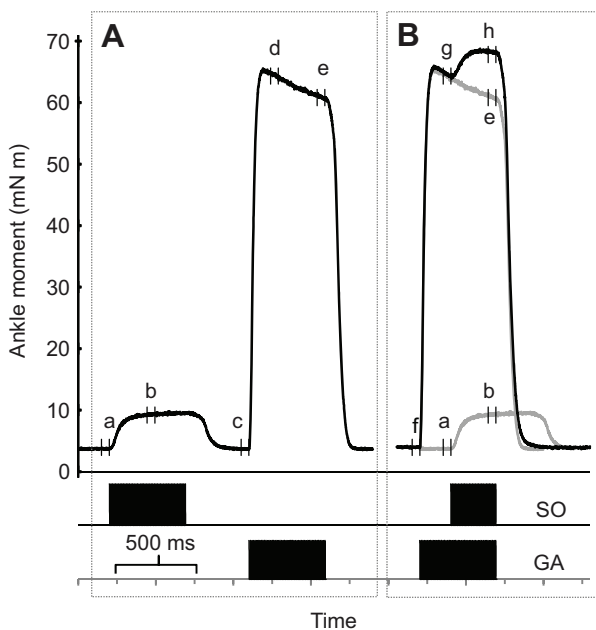
For the individual excitation of SO and GA, and for the simultaneous SO+GA excitation, the active ankle moments around each axis were used to calculate the magnitude of the 3D ankle moment vector and the direction of the projection of the 3D vector in the three anatomical planes. For each vector in the transverse and frontal planes, the angle relative to the plantar-flexion moment axis was calculated, whereas for each vector in the sagittal plane, the angle relative to the eversion moment axis was calculated (see  $\alpha$ ,  $\beta$ ,  $\gamma$  in Fig. 3B).

Nonlinear magnitude summation was assessed by subtracting the magnitude of SO and GA ankle moments from the magnitude of simultaneous SO+GA ankle moments and was normalized ( $\%M_{nl}$ ) relative to the magnitude of the mathematical sum of the ankle moments exerted on excitation of SO and GA individually. Nonlinear direction summation was assessed by first summing SO and GA ankle moment vectors, followed by calculating the angle between this resultant moment vector and the simultaneous SO+GA moment vector in the three anatomical planes.

The length of the distal portion of the Achilles tendon was assessed by calculating the distance from the Achilles tendon insertion to the tendon marker ( $L_{AT}$  in Fig. 3A). Achilles tendon lengthening as a result of SO and SO+GA contraction was calculated by subtracting the marker position before muscle contraction from the position during SO and SO+GA muscle contractions. All calculations were performed in MATLAB (R2014a, MathWorks, Natick, MA, USA).

#### Statistics

One-way repeated-measures ANOVA (SPSS Statistics 20, IBM Corporation, Armonk, NY, USA) with 'knee angle' as independent factor (8 levels) were used to test for effects of knee angle on (1) the magnitude of the active SO ankle moment and the orientation in the three anatomical planes; (2) the length of the distal portion of the Achilles tendon during rest, during SO activation and during SO+GA activation; and (3) the nonlinear magnitude summation and nonlinear direction summation in the three anatomical planes. A two-way repeated-measures ANOVA with 'knee angle' (8 levels) and 'condition' (2 levels: intact and GA and PL cut proximally) as independent factors was used to test if mechanical interaction was caused by proximal length changes of GA and PL muscles. A two-way repeated-measures ANOVA with 'knee angle' (8 levels) and 'muscle activation' (2 levels: SO and rest) as independent factors was used to test if the increase in Achilles tendon length by SO activation was dependent on knee angle. A two-way repeated-measures ANOVA with 'knee angle' (8 levels) and 'muscle activation' (2 levels: SO and SO+GA) as independent factors was used to test if the difference between Achilles tendon length during SO activation and during SO+GA activation was knee angle dependent. A one-sample *t*-test was used to test if the relative nonlinear magnitude summation ( $\%M_{nl}$ ) and nonlinear direction summation averaged across knee angles was significantly different to zero. To monitor any changes in SO and GA muscle conditions due to previous muscle contractions, paired *t*-tests were performed. Greenhouse Geisser correction was used when the assumption of sphericity was violated. Level of significance was set at  $P \leq 0.05$ .



**Fig. 4. Schematic of the two stimulation protocols.** In this example, ankle and knee joint were positioned at 90 deg. (A) Stimulation (500 ms) of SO and GA separately. (B) Simultaneous stimulation (solid black line) of GA (500 ms) and SO (300 ms). Gray lines represent the individual SO and GA ankle moments as shown in A, respectively. Ankle moments were assessed for several time windows (a–h) by calculating the mean for each 50 ms time windows (see section on data analysis in the Materials and methods). The black bars indicate the times during which SO and GA stimulation was applied.



**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

C.T. performed the experiments, data-analysis and wrote the draft article. C.T., J.H.v.d. and H.M. contributed to conception and design of the experiments as well as interpretation of the data and revising the article.

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