Role of muscle spindle feedback in regulating muscle activity strength during walking at different speed in mice.

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ABSTRACT

Terrestrial animals increase their walking speed by increasing the activity of the extensor muscles. However, the mechanism underlying this speed dependent amplitude modulation is achieved remain obscure. Previous studies have shown that group Ib afferent feedback from Golgi tendon organs that signal force is one of the major regulators of the strength of muscle activity during walking in cats and humans. In contrast, the contribution of group Ia/II afferent feedback from muscle spindle stretch receptors which signal angular displacement of leg joints is unclear. Some studies indicate that group II afferent feedback may be important for amplitude regulation in humans, but the role of muscle spindle feedback in regulation of muscle activity strength in quadrupedal animals is very poorly understood. To examine the role of feedback from muscle spindles, we combined *in vivo* electrophysiology and motion analysis with mouse genetics and gene delivery with adeno associated virus. We provide evidence that proprioceptive sensory feedback from muscle spindles is important for the regulation of the muscle activity strength and speed dependent amplitude modulation. Furthermore, our data suggest that feedback from the muscle spindles of the ankle extensor muscles, the triceps surae, are the main source for this mechanism. In contrast, muscle spindle feedback from the knee extensor muscles, the quadriceps femoris, has no influence on speed dependent amplitude modulation. We provide evidence that proprioceptive feedback from ankle extensor muscles is critical for regulating muscle activity strength as gait speed increases.

New & Noteworthy:

Animals upregulate the activity of extensor muscles to increase their walking speed, but the mechanism behind this is not known. Here we show that this speed dependent amplitude modulation requires proprioceptive sensory feedback from muscle spindles of ankle extensor muscle. In the absence of muscle spindle feedback, animals cannot walk at higher speeds as they can when muscle spindle feedback is present.

Keywords: Walking, mice, speed dependent amplitude modulation, muscle spindles, proprioception

INTRODUCTION

In terrestrial legged animals, the stereotyped and rhythmic leg movements (step cycle) during locomotion can be divided into two phases, swing and stance. The swing phase starts with lifting the foot off the ground and moving it towards the direction of the locomotion in the air and ends with placing the foot back on the ground. Once the foot is placed on the ground, the stance phase starts, in which the foot stays stationary while the body moves in the direction of locomotion. Thus, the limb carries the body weight and provides propulsion. In mammals, this leg movement is mainly achieved by the precisely patterned contraction pattern of multiple extensor and flexor muscles that move hip, knee, and the ankle joint (Engberg and Lundberg, 1969; Grillner, 1981; Rossignol, 1996; Akay et al., 2014). In a simplified version, extensor muscles are mostly activated during stance and flexor muscles during the swing phase. This coordinated action of muscles is in turn controlled by the patterned activity of pools of motor neurons (locomotor pattern) in the spinal cord. This locomotor pattern is known to be the result of the integrated function of a network of interconnected interneurons in the spinal cord (central pattern generator, CPG) and the sensory feedback from cutaneous and proprioceptive systems in the periphery (McCrea, 2001; Pearson, 2004; Rossignol et al., 2006).

Animals can move around their environment at different speeds to fulfill diverse purposes such as hunting, escape, migration, or foraging. When the locomotor speed is increased, the step cycle duration decreases. This is caused by a decrease in the stance phase duration, whereas the swing phase duration stays relatively constant (Grillner, 1981). Furthermore, electromyogram (EMG) activity recordings from flexor and

extensor muscles during different speeds revealed that with increasing speed, the EMG activity of mainly the extensor muscles increases accordingly (Walmsley et al., 1978; Pierotti et al., 1989; Roy et al., 1991; Prilutsky et al., 1994). However, the circuits that give rise to this speed dependent regulation of extensor activity are not understood. One possibility is that the speed dependent amplitude regulation is controlled by proprioceptive sensory feedback. Experiments with walking cats and humans suggest that proprioceptive sensory feedback regulates the activity strength of extensor muscles during stance phase (Sinkjaer *et al.*, 2000; Grey *et al.*, 2007; Donelan & Pearson, 2004a, 2004b; Donelan *et al.*, 2009). Experiments with human subjects suggest that extensor activity strength during walking is regulated by proprioceptive sensory feedback from the Golgi tendon organs (GTO) and from the muscle spindles (Yang et al., 1991; Sinkjaer et al., 2000; Grey et al., 2004, 2007; Mazzaro et al., 2005; af Klint et al., 2010). In contrast, experiments with cats suggest that the extensor activity during stance is regulated by the GTO feedback, but no evidence has been found regarding the feedback from muscle spindles (Donelan and Pearson, 2004a, 2004b; Donelan et al., 2009). In this article, we present evidence that proprioceptive sensory feedback from the muscle spindles regulates the EMG activity of extensor muscles during stance and this regulation is necessary for locomotion at higher speeds.

To address the role of proprioceptive sensory feedback from muscle spindles in the regulation of activity strength of extensor muscle during walking at different speeds, we recorded EMG activities of multiple muscles while mice walked on a treadmill at various speeds, as previously described (Akay et al., 2014). We hypothesize that "proprioceptive sensory feedback from muscle spindles is necessary for extensor

muscle EMG activity upregulation during waking at different speeds." To address the global role of muscle spindles in speed dependent amplitude modulation, we performed the same experiments with the *Egr3*$^{-/-}$ mice, in which muscle spindles fail to properly develop postnataly (Tourtellotte and Milbrandt, 1998; Oliveira Fernandes and Tourtellotte, 2015). Further, we developed a viral and mouse-genetics based strategy to acutely eliminated muscle spindles only from quadriceps femoris (*QF*, knee extensor muscles) or the triceps surae muscles (*TS*, ankle extensor muscles). In these experiments, data from the same animal obtained before and after the removal of the muscle spindles were compared to gain insight into the role of the muscle spindle feedback from specific muscles in the speed dependent amplitude modulation. Our data suggest that speed dependent amplitude modulation requires proprioceptive sensory feedback from the muscle spindles specifically from the TS but not from the QF muscle groups.

METHODS

The experiments were done on adult mice, ages ranging from 60 days to 90 days of either sex. None of the mice were trained prior to the experiments. All procedures were in accordance with the Canadian Council on Animal Care and were approved by the University Committee on Laboratory Animals at Dalhousie University.

Removal of muscle spindles: We used two methods to remove proprioceptive feedback from the muscle spindles. The first utilized the *Egr3* knock-out mice (*Egr3*$^{-/-}$) where all muscle spindles are ablated (Tourtellotte and Milbrandt, 1998; Chen et al., 2002). In

these mice, the muscle spindles fail to form properly during development while the proprioceptive feedback from the GTOs are left intact. The second method allowed us to acutely eliminate muscle spindle feedback from a subset of muscles (**Figure 1**). Here we used a mouse line that expresses the *cre*-recombinase under the control of the calcium binding protein parvalbumin (Pv) expression (*Pv::cre*) (Hippenmeyer et al., 2005). To conditionally and selectively ablate proprioceptors we generated an AAV serotype 9 encoding DTR-GFP fusion in a Flex-switch (AAV9-DTR-GFP) (Azim et al., 2014). When injected into the muscle of *Pv::cre* animals, the AAV infects sensory neurons and expresses DTR-GFP in Pv^{+} proprioceptors. We injected each one of the QF muscles or the TS muscles when the *Pv::cre* mice were 7-10 days old (P7-P10). As a control, AAV9 encoding only GFP was injected to the same muscle groups of the contralateral leg. After these AAV injections, *Pv::cre* mice were kept until adulthood when they underwent EMG implantation. EMG and kinematic data recordings were performed before and 5-15 days after intraperitoneal diphtheria toxin (DTX, 400 ng dissolved in sterile phosphate buffer) injection.

Electrode implantation surgeries: Each wild type, *Egr3*$^{-/-}$, or *Pv::cre* mouse injected with AAV9-DTR-GFP, received an electrode implantation surgery as previously described (Akay et al., 2014; Mayer and Akay, 2018). Briefly, the animals were anesthetized with isoflurane, ophthalmic eye ointment was applied to the eyes, and the skin of the mice was sterilized with three part skin scrub using hibitane, alcohol, and povidone-iodine. A set of six bipolar EMG electrodes were implanted in all experimental mice (Pearson et al., 2005; Akay et al., 2006) as the following: The neck region and the right hind leg was shaved. Small incisions were made to the neck region and in the leg above muscles.

The electrodes were drawn subcutaneously from the neck incision to the leg incisions and the head piece connector was stitched to the skin around the neck incision. The EMG recording electrodes were implanted into hip flexor (iliopsoas, Ip) and extensor (anterior biceps femoris, BF), knee flexor (semitendinosus, St) and extensor (vastus lateralis, VL), and ankle flexor (tibialis anterior, TA) and extensor (gastrocnemius, Gs). The incisions were closed, and buprenorphine (0.03 mg/kg) and ketoprofen (5 mg/Kg) were injected subcutaneously as analgesics. Additional buprenorphine injections were performed in 12 hour intervals for 48 hours. The anesthetic was discontinued and mice placed in a heated cage for 3 days and finally returned to their regular mouse rack. Food mash and hydrogel was provided for the first 3 days after the surgery. Handling of the mice was avoided until they were fully recovered. The first recording session was started no earlier than ten days after electrode implantation surgeries.

Behavioral recording sessions: Following the full recovery from electrode implantation surgeries, the behavioral recordings were performed as previously described (Pearson et al., 2005; Akay et al., 2006). Under brief anesthesia with isoflurane, custom made cone shaped reflective markers (1-2 mm diameter) were attached to the skin at the level of anterior tip of the iliac crest, hip, knee, ankle, the metatarsal phalangeal joint (MTP), and the tip of the fourth digit (toe). The anesthesia was discontinued and the mouse placed on the mouse treadmill (model 802; custom built in the workshop of the Zoological Institute, University of Cologne, Germany). The electrodes were connected to the amplifier (model 102, custom built in the workshop of the Zoological Institute, University of Cologne, Germany). We waited at least five minutes before the session started to allow the mice to fully recover from anesthesia. The mice started walking on

treadmill when the treadmill was turned on. The speed of the treadmill was changed starting from 0.2 m/s and increased up the 0.6m/s a speed at which all wild type mice could walk. The walking mouse was filmed from the sagittal plane with a high speed video camera (IL3, Fastec Imaging) at 250 frames per second, and video files stored at the computer for later motion analysis. The EMG data was stored separately on the computer by using the Digitizer (Power 1401, Cambridge Electronic Design, UK) combined with Spike 2 software (Version 8, Cambridge Electronic Design, UK). Only walking sequences where the mouse would walk stationary without drifting forward or backward indicating equal walking speed and treadmill speed were considered for data analysis. These recordings were performed once with each wild type and *Egr3*$^{-/-}$ mouse. In most of the experiments with the *Pv::cre* mice that received AAV9 injection, three sets of recordings at different days were performed before the DTX injection and three sets of recordings were performed after DTX injection. The three recordings before and after DTX injections were used to ensure stable EMG recordings over multiple days. Since *Pv is also expressed in some* extrafusal muscle fibers (Celio and Heizmann, 1982) and AAV9 is known to also infect extrafusal muscle fibers (Katwal et al., 2013) we did histological assessments to show in our experiments, DTX injection does not affect extrafusal muscle fibers (**Figure 2**; see discussion).

Immunohistology: After each experiment where muscle spindles afferents were removed acutely by AAV9 and DTX injections in *Pv::cre* mice, the efficiency of muscle spindle removal was assessed with immunohistology. After the last recording sessions following DTX injection, mice were euthanized with an intraperitoneal injection of pentobarbital sodium (40mg/Kg). After thoracotomy, the animals were perfused with

20mL of saline solution followed by 10mL of 4% paraformaldehyde (PFA) through the left cardiac ventricle. The Gs or the VL were dissected, then cryoprotected by immersion in 30% sucrose/PBS solution overnight at 4°C. The following day, the muscles were embedded in optimal cutting temperature compound (OCT) mounting medium, flash frozen on dry ice, and stored at -80ºC. Muscle tissue was sectioned longitudinally at 80 µm by using a cryostat (Leica CM3050 S) and the sections were placed on microscope slides. For immunofluorescence staining, the sections were washed in PBS to remove the OCT and incubated in blocking solution (PBS/1%BSA/0.3% triton) for one hour and latter incubated overnight with primary antibody (Rabbit anti-VGluT1 1:8000) (de Nooij et al., 2015). The next day, tissues received multiple washes in blocking solution and they were incubated overnight at room temperature with secondary antibody (goat anti-rabbit conjugated to Alexa-Fluor 488 1:500, Life technologies) in the blocking solution. The sections were washed with blocking solution, followed by a wash with PBS to remove the BSA. Finally, the microscope slides with the sections were coverslipped using mounting medium (Permafluor).

Data analysis: The kinematic parameters of walking were obtained from the video files using a custom made software written by Dr. Nicolas Stifani with ImageJ (KinemaJ) and R (KinemaR) (Bui et al., 2016; Fiander et al., 2017). The coordinates and the angular joint movements were then imported into the Spike2 files containing the EMG data using a custom written Spike2 script to analyze the kinematic and EMG data. All plots were created with the Excel 2016 software and statistical analysis with the data analysis package for Excel: the statistiXL (version 1.8). Student's t-test was used to compare

data between wild type and *Egr3*$^{-/-}$ mice, and the walking at 0.2 m/s and 0.4 m/s in wild type mice. Moreover, t-test for paired data to compare data before and after DTX injection in *Pv::cre* mice injected with AAV was used. The changes were considered statistically significant if $p<0.05$.

RESULTS

Speed dependent amplitude modulation in wild type mice:

To provide insights into the mechanisms of amplitude modulation, we first examined the muscle EMG activities of different muscles in wild type mice. None of the seven recorded wild type mice had any difficulty walking on the treadmill at speeds up to 0.6m/s (**Figure 3**). In **figure 3A**, kinematic and EMG recording data during three episodes of walking at 0.2 m/s, 0.4 m/s, and 0.6 m/s speed are illustrated including three, five, and seven swing phases (shaded backgrounds). As the walking speed increased, the step duration decreased (**Figure 3B**). The decrease in the step duration was largely the result of a decrease in the stance phase and to a lesser extend a change in the swing duration (**Figure 3B**). Note also that the EMG activity recorded from the biceps femoris (BF, hip extensor), vastus lateralis (VL, knee extensor), and the gastrocnemius (Gs, ankle extensor) increased with increased walking speed. The increase in the extensor muscle activity was also shown in pooled average traces of the rectified EMG traces from all muscle in **figure 4** showing minor increase in the flexor muscles (left traces) but major upregulation in the extensor muscles (right traces). This data suggest that during walking, the EMG activity of the extensor muscles are upregulated in a speed dependent manner.

The maximal EMG activities normalized to the EMG amplitude at 0.2 m/s walking speed in individual animals are plotted against walking speed to more easily visualize the speed dependent amplitude modulation (**Figure 5**). The EMG amplitude of all extensor muscles were upregulated depending on the speed of locomotion (Gs and VL $p<0.05$; BF $p<0.01$; after ANOVA). In contrast, the activity amplitude of the other flexor

muscles were not dependent on the walking speed, except for the TA muscle ($p<0.05$; after ANOVA).

Speed dependent amplitude modulation is compromised in Egr3$^{-/-}$ mice:

To address the role of proprioceptive sensory feedback from muscle spindles in the speed dependent modulation of muscle activity, we measured EMG signals from leg muscles during walking at different treadmill speeds in *Egr3$^{-/-}$* mice. In contrast to wild type mice, none of the seven *Egr3$^{-/-}$* mice could walk at speeds higher than 0.4 m/s and only five out of seven mice could reach the 0.4 m/s speed. The kinematic and EMG recording data during two episodes of walking at 0.2 m/s and 0.4 m/s speeds are illustrated in **figure 6A** that includes four and five swing phases (shaded backgrounds). As in wild type mice, the step duration decreased as the walking speed increased from 0.2 m/s to 0.4 m/s (**Figure 6B**). Furthermore, in the *Egr3$^{-/-}$* mice, the decrease in the step duration was the result of the decreasing stance duration with lesser contribution of a change in the swing duration similar to wild type animals (**Figure 6B**). Comparing the duration from *Egr3$^{-/-}$* mice and wild type mice revealed that the cycle periods and the swing durations were significantly shorter in *Egr3$^{-/-}$* mice then in wild type mice. Stance duration however, was not significantly different at 0.2 m/s but was significantly shorter at 0.4 m/s in *Egr3$^{-/-}$* mice compared to wild type ($p<0.05$). One possible explanation for the altered swing phase with minor change in stance phase is that the lack of muscle spindle feedback may be compensated by GTO signaling during stance (Akay et al., 2014). These data suggest that proprioceptive sensory feedback from the muscle spindles are important for regulating the temporal characteristics of leg movement during walking at different speeds.

Further examination of extensor muscle activity revealed that the strength of the EMG signal of each extensor muscle did not change considerably when *Egr3*$^{-/-}$ mice increased walking speed from 0.2 m/s to 0.4 m/s (**Figure 6A**). This missing upregulation of the extensor muscles is illustrated in **figure 7** where the pooled averages of the rectified EMG activities from flexor (left) and extensor (right) muscles during 0.2 m/s and 0.4 m/s are shown. Our data therefore suggest that in the absence of muscle spindles, mice are unable to reach walking speeds >0.4 ms. Furthermore when they do increase their walking speed there is no speed dependent amplitude modulation of extensor muscles.

Comparison of maximal EMG activities in wild type mice and *Egr3*$^{-/-}$ mice during walking at 0.2 m/s and 0.4 m/s revealed that the amplitude modulation in hip extensor muscle was reduced (**Figure 8**). When the overall increase in the amplitudes at 0.4 m/s were compared between *Egr3*$^{-/-}$ and wild type mice, only the amplitude modulation in BF activity was significantly different. When wild type mice walked at 0.4 m/s, the BF EMG amplitude increased on average 260% (±88 standard deviation). In *Egr3*$^{-/-}$, the EMG amplitude increase was 142% (±42), which was statistically smaller (p=0.045, Student's t-test, **Figure 8**). There was no significant differences in VL (wild type: 138% ±29; *Egr3*$^{-/-}$: 168 ±73; p=0.43) or Gs (wild type: 170% ±44; *Egr3*$^{-/-}$: 131 ±68; p=0.34). We could not detect any significant change in EMG amplitudes at 0.4 m/s in wild type versus *Egr3*$^{-/-}$ mice in any of the recorded flexor muscles (Ip: p=0.24; St: p=0.13; TA: p=0.44). These data suggest that in the 5/7 *Egr3*$^{-/-}$ mice that could walk at 0.4 m/s the increase in extensor EMG activity was compromised, but strong and consistent enough to be statistically significant only in the hip extensor muscle.

Our data suggest that the speed dependent increase of the hip extensor EMG activity requires proprioceptive sensory feedback from the muscle spindles. In the absence of feedback from muscle spindles, the hip extensor amplitude modulation is compromised, preventing the animal from reaching higher walking speeds.

Muscle spindle feedback specifically from the TS muscle group is particularly important in speed dependent amplitude modulation:

How is the information from the muscle spindle processed by the central nervous system to achieve the speed dependent amplitude modulation of the extensor muscle activities? We reasoned that one possibility may be that information signaling individual joint proprioception could affect the amplitude modulation of the extensor muscles of the same joints (local processing). Alternatively, the information from muscle spindles of multiple joints could be collectively processed in the central nervous system to regulate all or larger group of the extensor muscles (global processing). To differentiate between these two possibilities, we selectively removed feedback from either the ankle extensor TS muscles (including Gs muscles) or the knee extensor QF muscles (including VL muscle) and measured the amplitude modulation as described above.

If the information from the muscle spindles is processed locally, elimination of the muscle spindles only from the TS muscles should selectively affect speed dependent amplitude modulation only in Gs muscle, which is one the three TS muscles. To ensure that with this method we were effectively removing muscle spindle innervation, we counted all the afferent endings at the muscle spindles in the gastrocnemius muscle of the right leg and the left leg (**Figure 9A**). The left leg, received AAV injections that only express GFP and did not envelop gene that encodes the DTR (control leg), therefore no

ablation of nerve endings after the diphtheria toxin infection. The right leg was injected with AAV, enveloping the gene that encodes the DTR (experimental leg), that would affect the nerve endings after the DTX infection. After the final postDTX recording session was performed, we counted the VgluT1 positive afferent endings at muscle spindles and the GTOs in left (control) and right (experimental) legs. The results suggested that on average 55% (±23% standard deviation) of the muscle spindle afferents in the gastrocnemius muscle were eliminated after DTX injection, whereas GTOs were not affected (**Figure 9A**).

The averaged EMG traces from all recorded muscles before and after DTX injection to acute elimination of proprioceptive feedback from muscle spindles from only the TS muscles are illustrated in **figure 9B**. Note that in all muscles and at all speeds, pre-DTX (black) and the post-DTX (red) EMG traces overlapped except for the Gs and to a lesser extent VL and TA muscles. These three muscles showed reduced activity already at 0.2 m/s speed and the difference increased with increasing locomotor speed in Gs and VL, whereas the difference diminished in TA at 0.6 m/s. These observations were consistent when EMG traces from individual animals were investigated. That is, in seven out of nine Gs recordings and in five out of nine TA recordings, the speed dependent amplitude modulation was absent. Moreover, in VL muscle the amplitude modulation was present but less prominent in three out of four and absent in the one remaining recording. Our data suggest that acute elimination of muscle spindles from only TS muscles reduced amplitude modulation in two distal extensor muscles, the VL and Gs, and partly in one of the distal flexor muscle, the TA.

As in the **figures 5** and **8**, the maximum of rectified EMG activity in each step was averaged and normalized to the average amplitude of that value in 0.2 m/s and is plotted as a function of treadmill speed before and after the DTX injection (**Figure 10A**). The values at each speed were then compared before and after the DTX injection using paired t-test. Only the amplitude increase at Gs muscle at 0.6 m/s was significantly compromised after the removal of muscle spindle feedback from the TS muscles by DTX injection (preDTX: 229% ±156, postDTX: 175% ±125, p=0.018). There was no difference in the amplitude modulation of all other recorded muscles. Analysis of the cycle period, swing duration and stance duration revealed that cycle period and swing duration consistently decreased after removal of muscle spindle feedback from TS muscles, but changes in stance duration were limited to 0.2 m/s only (**Figure 10B**). This result suggests that muscle spindle feedback from the TS muscles mainly regulate the amplitude modulation selectively in the one TS muscle that was recorded here: the Gs muscle.

To address the question of whether or not the amplitude modulation was achieved by muscle spindles only from the TS group we performed another set of experiments where muscle spindle afferents only to the QF muscles were removed. As for the gastrocnemius muscle presented in **figure 9A**, we counted the afferent endings at muscle spindles and the GTOs. Similar to the GS muscles, we found a 73% (±10% standard deviation) reduction of muscle spindle afferents in the VL muscles after DTX injection, whereas the GTOs were not affected (**Figure 11A**).

The average EMG traces from the recorded muscles before (black) and after (red) DTX injection to remove muscle spindles only from QF muscles are illustrated in

figure 11B. After the elimination of the proprioceptive sensory feedback from muscle spindles of the QF muscles, no visual effect could be detected on the speed dependent amplitude in any of the recorded muscles. As for the Gs recordings, these observations were consistent when EMG traces from individual animals were investigated. That is, the speed dependent amplitude modulation was present in all recorded extensor muscles. This finding suggests that proprioceptive sensory feedback from the QF muscle spindles are not used to regulate the activity strength in any of the recorded muscles. To quantify this observation, we analyzed the maximum EMG activity in all muscles at different speeds before and after DTX injection (**Figure 12**). Note that the changes in amplitude modulation were not statistically significant in any of the recorded muscles (**Figure 12A**). Following the removal of muscle spindle feedback from the QF muscles, a consistent decrease was only detected in cycle period, whereas only minor change in swing duration limited to 0.2 m/s was observed (**Figure 12B**). No change could be detected in stance duration following DTX injection (**Figure 12B**). These data confirm that muscle spindle feedback from QF muscles is not used in speed dependent amplitude regulation.

DISCUSSION

Proprioceptive feedback has been known to play a major role in the regulation of muscle activity strength during walking in humans and cats. However the type of proprioceptive sensory feedback used for this amplitude control is somewhat controversial and how this proprioceptive information is processed is not known. We have shown that proprioceptive feedback from muscle spindles are important in

regulating the muscle activity strength during locomotion in mice. Furthermore, the amplitude modulation required muscle spindle feedback to regulate the extensor activity increase at higher speeds. Finally, we have shown that muscle spindle feedback from knee extensor muscles did not have an effect on amplitude modulation, whereas muscle spindle feedback from the ankle was important for the regulation of activity mainly in ankle extensor muscles.

Speed dependent amplitude modulation of extensor muscle activity requires feedback from muscle spindles:

Proprioceptive feedback is known to be important for regulating muscle activity strength during walking (Donelan and Pearson, 2004a; Pearson, 2004; Hiebert and Pearson, 2017). In humans, proprioceptive sensory feedback from the group Ib afferents that innervate Golgi tendon organs (GTOs) as well as the afferent fibers that innervate muscle spindles are both important for regulating muscle activity strength during walking (Yang et al., 1991; Sinkjaer et al., 2000; Grey et al., 2004, 2007; Mazzaro et al., 2005; af Klint et al., 2010). In contrast, in a quadrupedal animal, the cat, group Ib afferent feedback from the GTOs has been shown to be important for amplitude modulation (Donelan and Pearson, 2004a, 2004b; Donelan et al., 2009), but the contribution from muscle spindles has not been appreciated.

Our data provide evidence that muscle spindle feedback is important for regulating muscle contraction strength during walking in a quadrupedal animal. When wild type mice walk at different speeds, an increasing activity is observed in extensor muscles (**Figures 3-5**) as it has been described previously in cat (Walmsley et al., 1978; Smith et al., 1993; Prilutsky et al., 1994; Kaya et al., 2003). However, in a mutant

mouse model in which muscle spindle do not properly form, the *Egr3* knock out mice (*Egr3*$^{-/-}$), the mice no longer walk at speeds higher than 0.4 m/s (**Figure 6**) and the extensor amplitude modulation is compromised (**Figures 7-9**). Furthermore, the swing duration is significantly shorter at the measured speeds in *Egr3*$^{-/-}$ mice with minor changes in the stance duration leading to decreased cycle period (**Figure 6B**). These data suggest that muscle spindle feedback is necessary for both the increased speed of walking and the speed dependent amplitude modulation.

Since the *Egr3*$^{-/-}$ mice could not walk at 0.6 m/s, we measured muscle activity at different walking speeds in another model, where muscle spindles were removed only from subset of muscles in an acute way which resulted in a milder phenotype. Using this method that combines *Pv::cre* mice combined with gene delivery with AAV-9 and later DTX injection, we could successfully acutely eliminate muscle spindle afferents only in a subset of muscles while leaving the GTO afferents intact (**Figure 10**). This is an interesting observation given that the group Ib proprioceptive afferent fibers from the GTOs have also been shown to express Pv (de Nooij et al., 2015). So why then we did not observed significantly lower number of GTOs after DTX injection, even though the number of MS afferents were consistently decreased? One possible explanation is that the AAV9s were injected approximately in the center of the belly of the muscles. This could possibly avoid the infection of the Groub Ib afferents because the GTOs are typically located at the myotendinous junctions, further away from the injections site. Nevertheless, regardless of the explanation of why GTOs were not eliminated by the AAV9/DTX method, our data clearly suggest the number of GTOs after DTX injection remained unaffected.

With the AAV9/DTX method to acutely eliminate MS feedback, we have shown that acute elimination of muscle spindle feedback from only a subset of muscles does not affect the animals' ability to walk at higher speed, but compromises the amplitude modulation in the extensor muscles (**Figures 9-10**). Our data provide evidence that proprioceptive feedback from the muscle spindles is important for the regulation of muscle activity strength during walking. Previous findings with humans (Sinkjaer et al., 2000) that concluded that afferent feedback from the group II afferents from the muscle spindles and/ group Ib afferent from the GTOs are important for the regulation of muscle activity strength during walking. Our results provide evidence that muscle spindle feedback-dependent amplitude modulation is necessary for the animals to walk at higher speeds.

One concern with elimination of muscle spindle afferents with AAV9 injection into *Pv::cre* mice is the possibility of DTX also killing extrafusal muscle fiber that also express *Pv* (Celio and Heizmann, 1982). Moreover, it is known that AAV9 can infect extrafusal muscle fibers (Katwal et al., 2013). Therefore, it is conceivable to expect that in our experiments, DTX injections would have killed Pv expressing muscle fibers that could also compromise amplitude modulation measured in this article. To exclude this possibility, we have performed histological assessment to provide proof that extrafusal muscle fibers are not affected (**Figure 2**). We show that Pv expressing extrafusal muscle fibers do not present any sign of damage. Therefore, we are confident that the effect measured in the AAV9/DTX experiments are due to elimination of proprioceptive feedback from the muscle spindles.

Could the reduced activity modulation at higher speeds be an indirect effect produced by inability to achieve the faster speeds because of the ataxia previously observed in *Egr3*$^{-/-}$ mice (Akay et al., 2014; Takeoka et al., 2014) rather than a direct influence of spindle feedback on muscle activity? Our data suggest that compromised amplitude modulation in the absence of muscle spindle feedback is due to the elimination of direct influence of spindle feedback on muscle activity. Our observation is that amplitude modulation is compromised when muscle spindles are degenerated in only a small group of muscles with AAV9/DTX approach. None of these animals showed any sign of ataxia or had any difficulty to walk on the treadmill at higher speeds. The only changes we could detect after DTX injection was the speed dependent amplitude modulation mostly in the Gs muscle after muscle spindle feedback from the TS was significantly attenuated.

Sensory feedback from muscle spindles of only TS muscles regulate the strength of distal extensor muscle activity:

The results from the experiments in which muscle spindles were removed acutely and selectively from the TS or the QF muscle groups strongly suggest that proprioceptive feedback from only TS muscles are necessary for speed dependent amplitude modulation. Speed dependent amplitude modulation of extensor muscle activity was described in the past in rat (Hutchison et al., 1989; Roy et al., 1991) and cat (Walmsley et al., 1978; Smith et al., 1993; Prilutsky et al., 1994; Kaya et al., 2003) model systems, but the mechanism for this modulation was not understood. We have presented evidence that proprioceptive sensory feedback from muscle spindles in the ankle extensors, the TS muscle groups is necessary for the speed dependent amplitude

489 modulation of distal extensor muscles. Interestingly, our data also suggest that the
490 muscle spindles of the QF group, the knee extensor muscles, are not necessary for the
491 speed dependent amplitude modulation. These data, however, do not suggest that this
492 is the exclusive picture of amplitude control. Muscle spindles from other muscles that
493 were not infected with AAV in this project could have additional function and also QF
494 muscle spindles might have influence on other muscles not recorded here.
495 Nevertheless, the data presented suggest distinctive roles of muscle spindles in specific
496 muscles, such that TS, but not QF, controls the amplitude modulation in muscles
497 recorded here. This finding is in accordance with the previous finding that activation of
498 muscle afferents from the ankle extensor muscles, but not from the knee extensor
499 muscles strongly enhances ipsilateral extensor activity during fictive locomotion elicited
500 by electrical stimulation of the mesencephalic locomotor region (MLR) in decerebrated
501 cats (Guertin et al., 1995; McCrea, 2001). Our data provide evidence that proprioceptive
502 muscle spindle feedback, selectively from the ankle extensor muscles, regulate the
503 speed dependent amplitude modulation of the distal extensor muscle during walking.

504 In addition, when the muscle spindles were removed from the TS, the changes in
505 the swing and stance durations, and the cycle period during walking at different speeds
506 mimicked the results observed in *Egr3*$^{-/-}$ mice (**Figure 6B**). That is, the swing durations
507 significantly decreased following muscle spindle removal from the TS with significant
508 decrease in the stance duration only at 0.2 m/s, leading to a significant decrease in the
509 cycle period (**Figure 10B**). Interestingly, reduction of the muscle spindle afferents from
510 the QF resulted in much milder effect on swing duration and the cycle period and no
511 effect on the stance duration (**Figure 12B**). This result suggests that muscle spindles

from the TS have more prominent effect on step duration parameters than the muscle spindles from the QF muscles.

Why are the changes in amplitude modulation statistically significant only in BF when all muscle spindles are removed systemically as in *Egr3*$^{-/-}$ mice, but not different when muscle spindles are removed acutely only from a subset of muscles? There could be multiple reasons for this observation. First, when muscle spindles improperly form during development (Tourtellotte and Milbrandt, 1998; Tourtellotte et al., 2001; Oliveira Fernandes and Tourtellotte, 2015), there may be resultant compensatory changes in BF. Therefore, the acute elimination of muscle spindle feedback from a subset of muscles would have no effect on BF. Second, BF modulation might be controlled by muscle spindle feedback from different muscles that did not receive AAV injection in these studies. Third, all extensor muscles could contribute to the speed dependent modulation of BF activity and therefore elimination of feedback only from a small number of muscle does not cause a detectable effect. Our current data do not clearly differentiate between these possibilities.

Negative feedback from muscle spindles controls amplitude modulation:

Previous studies suggest that positive force feedback signals from the group Ib afferents from the GTOs are the major source of amplitude regulation during walking (Pearson and Collins, 1993; Gossard et al., 1994; Mccrea et al., 1995; Duysens et al., 2000). In addition, cutaneous afferents have also been shown to be important of the regulation of extensor muscle activity (Duysens and Stein, 1978; Duysens et al., 1996). In contrast, how feedback from the muscle spindles might contribute to amplitude modulation has been unclear. It has been shown that muscle spindle muscle afferents

(group Ia and II) from the triceps surae muscles and from vasti muscles (only known for group Ia; group II not known) are active during the early part of stance (Prochazka et al., 1989; Prochazka and Gorassini, 1998) at which ankle and knee joints flexes. In addition, there is an excitatory influence from the muscle spindle afferents from the TS and VL muscle groups on the extensor muscles (Guertin et al., 1995; Mccrea et al., 1995) indicating the existence of a negative feedback pathway (Pearson, 2004). Our data provide functional evidence of this negative angular displacement feedback during walking behavior.

Role of muscle spindle feedback during walking:

Locomotion is controlled by a network of interconnected spinal premotor interneurons and sensory feedback from the periphery (McCrea, 2001; Pearson, 2004; Rossignol et al., 2006). Previous data and our present data suggest that proprioceptive sensory feedback from the muscle spindles from leg muscles regulate three aspects of a step cycle. First, it has an important role in the phase transitions. That is, muscle spindle feedback signaling leg extension at the end of stance phase is an important signal to initiate swing phase (Grillner and Rossignol, 1978; Hiebert et al., 1996). Second, muscle spindle feedback is important in the regulation of the precise timing of the activity offset of flexor muscles and therefore important for the precise foot placement at the end of swing phase (Akay et al., 2014). Third, muscle spindle feedback from ankle extensors is important for the regulation of muscle activity strength during stance phase as our current data suggest. It is well established that ankle joint exerts a brief flexion movement at the beginning of the stance phase that causes the ankle extensor muscles to stretch activating the stretch sensitive muscle spindles

(Engberg and Lundberg, 1969). It has also been shown that the speed of this yield and the ankle muscle stretch increase with the walking speed (Prilutsky et al., 1994) that would result in higher muscle spindle signaling. The higher muscle spindle signaling would then in turn provide stronger excitatory drive to the distal extensor motor neurons to accommodate the speed dependent amplitude modulation of muscle activity.

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FIGURE LEGENDS:

Figure 1. Acute elimination of proprioceptive afferents from selected muscles. AAV was used to deliver a cre conditional (flexed) gene encoding the receptor for DTX into specific muscle of a *Pv::cre* mouse, where *Pv* is selectively expressed in proprioceptive afferents and some interneurons. **(A)** *Pv::cre* mice were injected at less than two weeks of age, infecting proprioceptive afferents and motor neurons innervating that muscle. Since motor neurons do not express *Pv*, the DTR gene was only expressed in proprioceptive afferents. Once the mice were older than 50 days, EMG electrodes were implanted into their muscles and control recordings were performed. Their locomotor pattern was recorded again 5-15 days after intraperitoneal (IP) DTX injection. The locomotor patterns recorded during pre-DTX and post-DTX sessions were compared. **(B)** Confocal images of proprioceptive afferents innervating muscle spindle (top) and a Golgi tendon organ (bottom) from a vastus lateralis muscle. Afferent fibers were labeled with antibody staining against VGluT1. Control muscle is from the left leg, which did not receive an AAV injection. Experimental muscle shown is from the right leg after AAV and DTR injection. Notice that the typical annulospiral structure is degraded to some punctuated structures in the experimental leg after DTX injection but the GTOs appear normal. Scale bars in all images indicate 50 µm.

Figure 2. No sign of muscle fiber degeneration in *Pv::cre* mice previously injected with AAV9 to deliver gene encoding DTX receptor after DTX injection. (A) Fluorescence microscope images of a cross-section through a Gs muscle previously injected with AAV9 to deliver *DTR-GFP* gene and after DTX injection. Parvalbumin

730 expressing fibers (red, in left image) have a very healthy appearance in toluidine blue
731 (TB) staining (right image). Occasional fibers with centralized nuclei were observed in
732 either Pv^+ (white arrow) and Pv^- (blue arrows) muscle fibers indicating that this feature
733 was independent of DTX effect. **(B)** Fluorescence images previously injected with AAV9
734 to deliver *DTR-GFP* gene and after DTX injection. The fibers that are expressing GFP
735 are Pv^+ fibers that were infected with AAV9 (asterisks in left image). The same fiber
736 successfully infected with AAV9 and express the delivered *DTR-GFP* gene show no
737 sign of any degeneration (asterisks in right image). Scale bars indicate 50 µm.

738

739

740 **Figure 3. Locomotor pattern during walking at different speeds in wild type mice.**
741 All wild type mice (N=7) were able to walk on the treadmill up the 0.6 m/s speed. **(A)**
742 Angular movement of hip, knee, and ankle joints, toe coordinates as toe height and
743 horizontal toe position relative to hip position (dashed horizontal line) (rel toe_X), and
744 EMG activities of the six recorded flexor and extensor muscles at three different speeds
745 are shown. Shaded background indicates swing phase. **(B)** Bar graphs illustrating the
746 mean and standard deviation step cycle periods, swing and stance durations at three
747 different walking speeds. Notice that all parameters decrease with the increasing
748 walking speed with a stronger effect in cycle period and stance duration than the swing
749 phase.

750

Figure 4. EMG activity in leg muscles increases at higher walking speeds. Average EMG activities (rectified and smoothened) from all flexor (left) and the extensor (right) muscles at different speeds (black: 0.2 m/s, yellow: 0.4 m/s, blue: 0.6 m/s) recorded in this paper indicate that the EMG activity of extensor muscles increases at higher speeds. On top of the EMG activities, the durations of stance and swing are shown by the bars indicating averages ± standard deviations.

Figure 5. Speed dependent amplitude modulation of extensor EMG activities in wild type mice. Maximal EMG activities (peak-to-peak amplitude, PTP ampl.) in all recorded flexor (left) and extensor (right) muscles normalized to the maximal activity at 0.2 m/s as a function of walking speed. Note that activity in extensor muscles increases with increasing speed, but flexor muscle activities remain relatively unchanged. Colors indicate individual animals (N=7).

Figure 6. Locomotor pattern during walking at different speeds in *Egr3*$^{-/-}$ mice. None of the *Egr3*$^{-/-}$ mice would walk at 0.6 m/s and only five of seven could walk at 0.4 m/s. **(A)** Hip, knee, and ankle joint movements, along with toe height and horizontal toe position relative to hip position (dashed horizontal line) (rel toe$_X$) and EMG activities of the six recorded flexor and extensor muscles at three different speeds are shown. Shaded background indicates swing phase. **(B)** All mean and standard deviations of step cycle periods, swing and stance durations at three different walking speeds in the *Egr3*$^{-/-}$ mice were smaller than the same parameters in wild type mice. ***: $p<0.001$, **:

773 p<0.01, *: p<0.05, and ns: not significant after Student's t-test to detect statistical
774 significance in differences between these parameters with the wild type parameters
775 shown in **figure 2**.

776 **Figure 7. EMG activity in extensor muscles does not increase as the walking**
777 **speed increases from 0.2 to 0.4 m/s in *Egr3*$^{-/-}$ mice.** Average EMG activities (rectified
778 and smoothened) from all flexor (left) and the extensor (right) muscles at different
779 speeds (black: 0.2 m/s and yellow: 0.4 m/s) indicate that the EMG activity of extensor
780 muscles increases at higher speeds. At the top of the EMG activities, the durations of
781 stance and swing are shown by the bars indicating averages ± standard deviations.

782

783 **Figure 8. Speed dependent amplitude modulation of extensor EMG activities is**
784 **compromised in *Egr3*$^{-/-}$ mice.** Maximal EMG activities (peak-to-peak amplitude, PTP
785 ampl.) in all recorded flexor (left) and extensor (right) muscles normalized to the
786 maximal activity at 0.2 m/s at 0.2 and 0.4 m/s walking speed in individual (color coded)
787 *Egr3*$^{-/-}$ mice (N=7). Based on the Student's t-test, only in BF muscle was the amplitude
788 modulation at 0.4 m/s sufficiently weaker in Egr3$^{-/-}$ mice compared to wild type mice to
789 reach statistical significance (p<0.05 after Student's t-test) for BF muscle. Each color
790 represents data from one animal.

791

792 **Figure 9. Acute elimination of the muscle spindle afferents only from TS muscle**
793 **group affects speed dependent amplitude modulation in distal extensor muscles.**
794 **(A)** Graphs illustrating that on average, 55% (±23% standard deviation) of afferent

endings (GrIa/II) at muscle spindles were degenerated in the gastrocnemius muscle ($p<0.01$ after paired t-test) whereas no difference was found in the number of afferents to GTOs (GrIb). Comparisons were made between the right legs (control) with the left legs (experimental). **(B)** Average EMG activities (rectified and smoothened) from all recorded muscles at different speeds recorded before (black) and after (red) the elimination of muscle spindles in TS muscle group through DTX injection. Note that the Gs EMG traces after removal of muscle spindles (red lines) in TS are much lower than before removal (black lines) at 0.4 and 0.6 m/s. In VL there is a milder change that is more obvious only at 0.6 m/s. On top of the EMG activities, the durations of stance and swing are indicated by the bars indicating averages ± standard deviations. Mean (±SD) of swing and stance durations are shown on top of each column for before (black) and after (red) muscle spindle elimination from the TS muscles DTX injection. Statistical significance of differences based on paired t-test is indicated as ***: $p<0.001$, **: $p<0.01$, or ns: not significant.

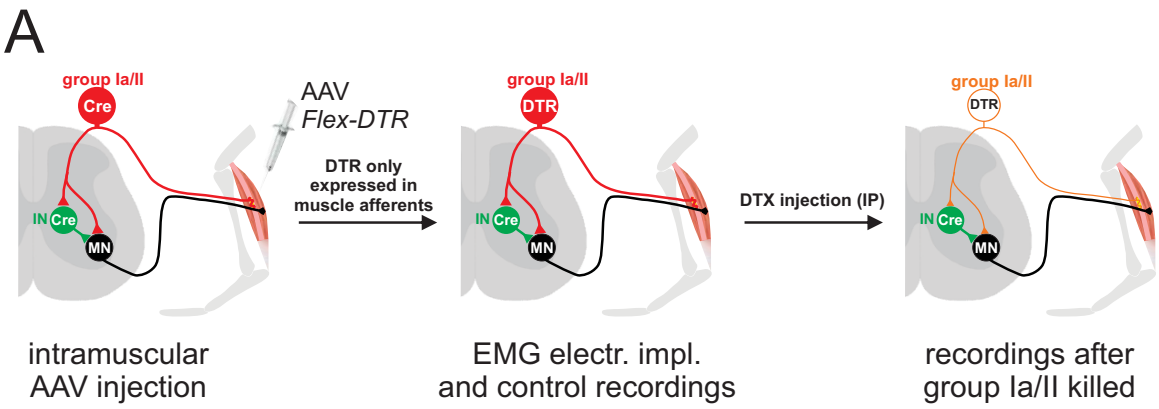
Figure 10. The decline of speed dependent amplitude modulation is only statistically significant in Gs muscle after acute removal of muscle spindle afferents from the TS muscles. (A) Maximal EMG activities (peak-to-peak amplitude, PTP ampl.) in all recorded flexor (left) and extensor (right) muscles normalized to the maximal activity at all three walking speeds in individual (color coded) *Pv::cre* mice in which TS muscle groups were infected with AAV before and after DTX injection are shown. Based on the paired t-test, the decrease of the amplitude modulation at 0.6 m/s was only statistically significant ($p<0.05$) for Gs muscle. **(B)** Mean and standard

deviations of step cycle periods, swing and stance durations at three different walking speeds before and after DTX injection indicates after DTX injection cycle period and swing durations decreased at all speeds and stance duration only decreased at 0.2 m/s. ***: $p<0.001$, **: $p<0.01$, and ns: not significant after paired t-test.

Figure 11. Acute elimination of the muscle spindle afferents only from QF muscle group does not cause a systematic change in speed-dependent amplitude modulation. (A) Graphs illustrating that on average, 73% (±10% standard deviation) of afferent endings at muscle spindles (GrIa/II) were degenerated in the vastus lateralis ($p<0.01$ after paired t-test), whereas no differences were found in the afferents to of the GTOs (GrIb). Comparisons were made between the right legs (control) with the left legs (experimental). **(B)** Average EMG activities (rectified and smoothened) from all recorded muscles at different speeds recorded before (black) and after (red) the elimination of muscle spindles in QF muscle group through DTX injection. Notice that all black and red EMG traces overlap at all speeds. On top of the EMG activities, the duration of stance and swing are indicated by the bars indicating averages ± standard deviations. **: $p<0.01$, and ns: not significant after paired t-test.

Figure 12. The decline of speed dependent amplitude modulation is not statistically significant in any muscle after acute removal of muscle spindle afferents from the QF muscles. (A) Maximal EMG activities (peak-to-peak amplitude, PTP ampl.) in all recorded flexor (left) and extensor (right) muscles normalized to the

maximal activity at all three walking speeds in individual (color coded) *Pv::cre* mice in which TS muscle groups were infected with AAV before and after DTX injection. Based on the paired t-test, amplitude modulation did not change significantly changed after DTX injection. **(B)** Mean and standard deviations of step cycle periods, swing and stance durations at three different walking speeds before and after DTX injection indicates after DTX injection cycle periods decreased at all speeds and swing duration only decreased at 0.2 m/s. No change could be detected in stance duration at any speed. **: $p<0.01$, *: $p<0.05$, and ns: not significant after paired t-test.



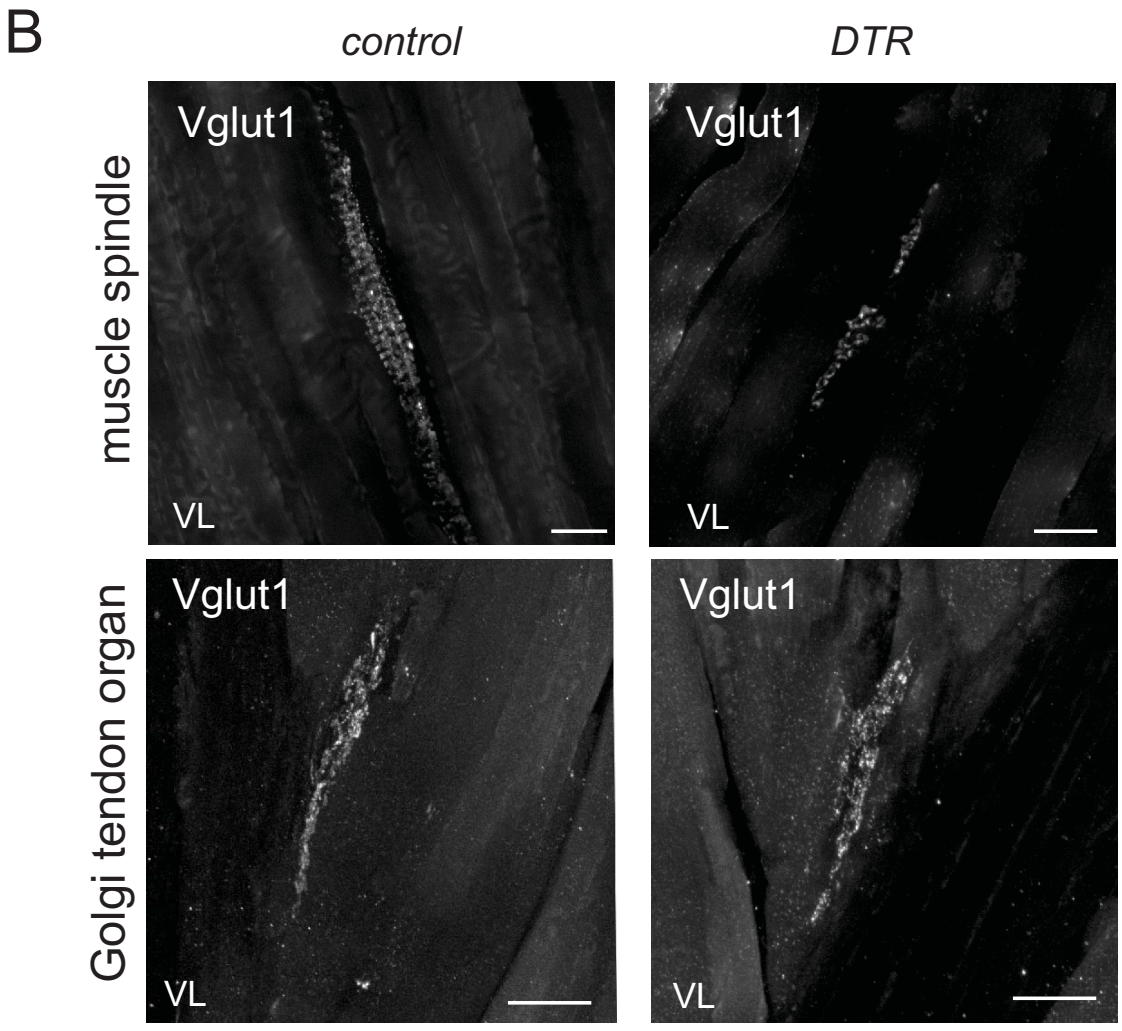
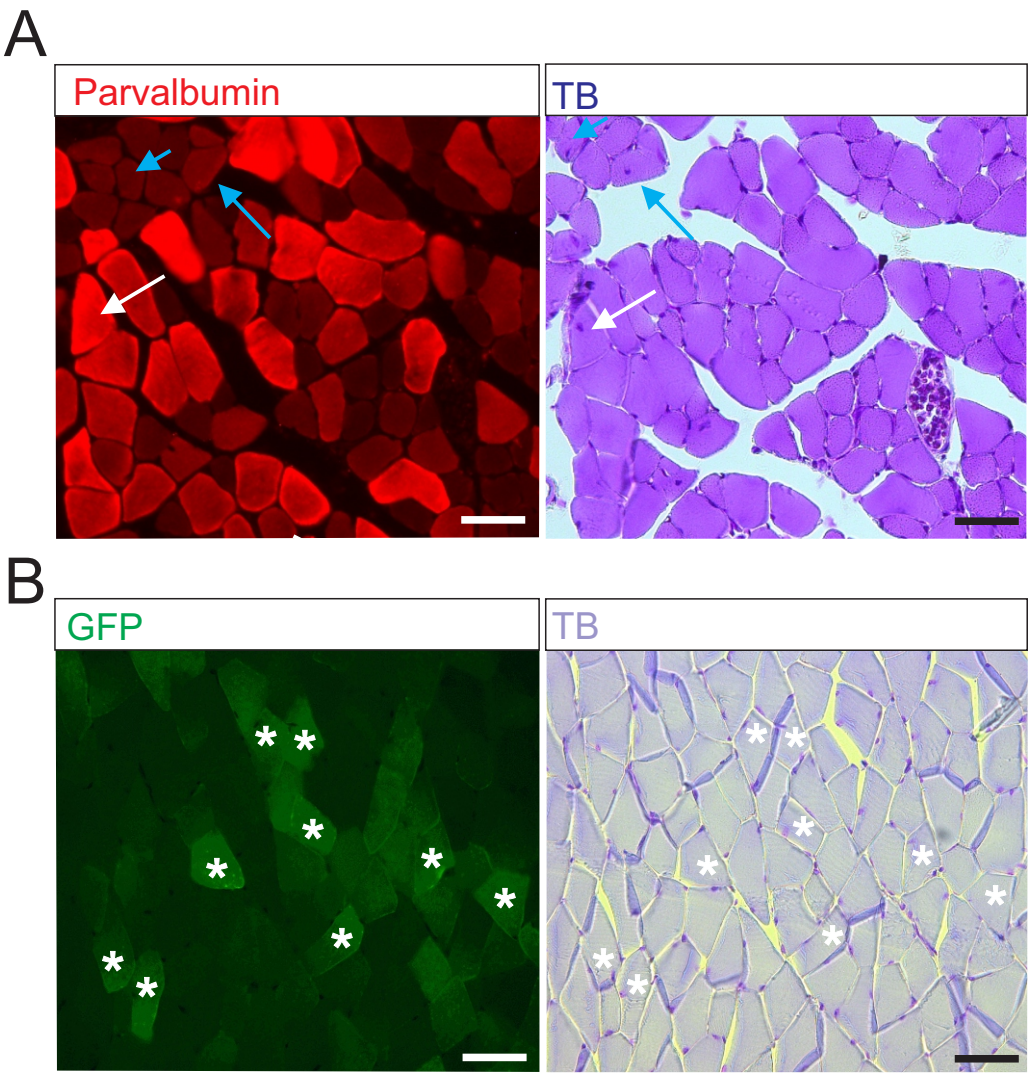


Figure 1



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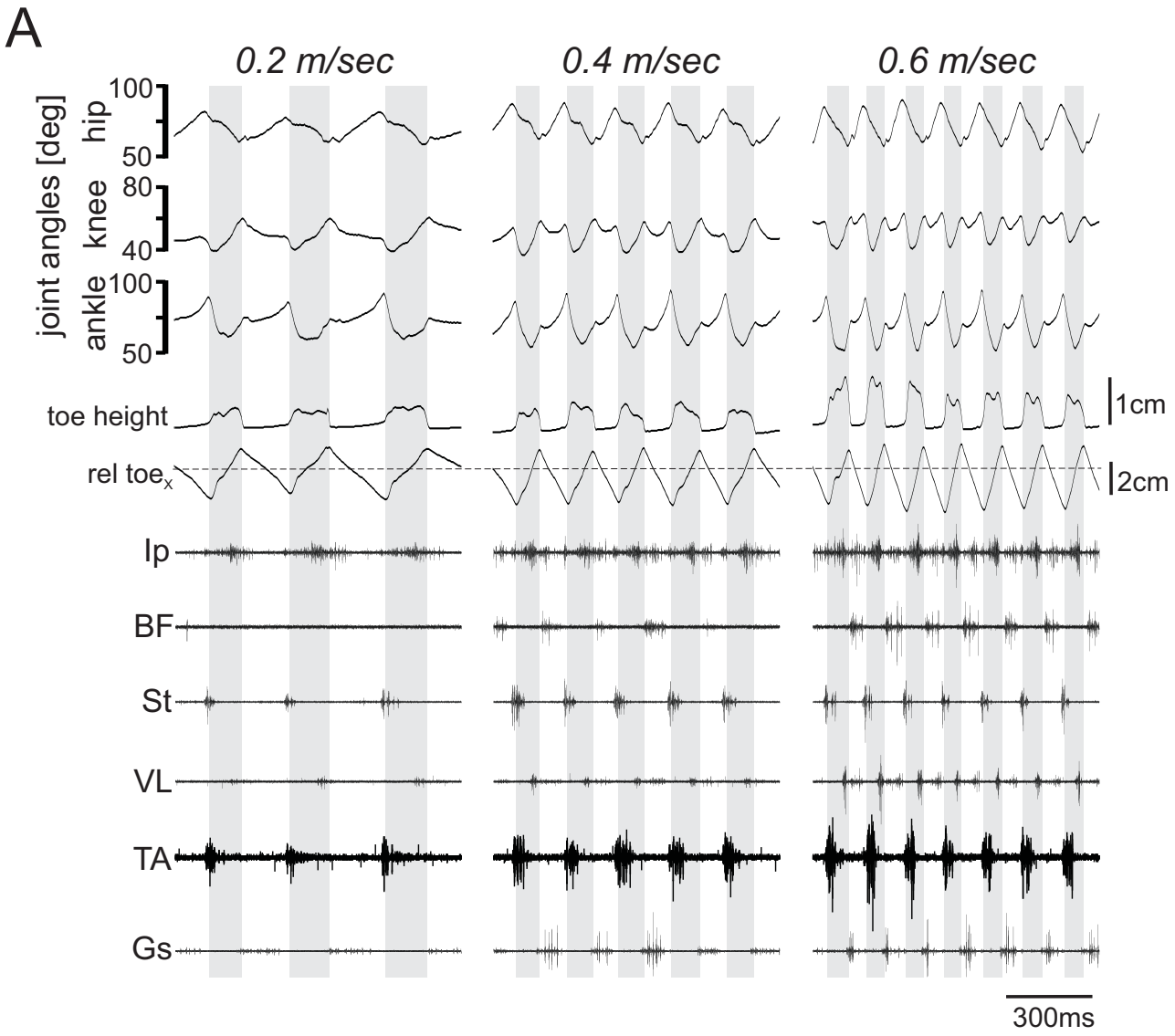


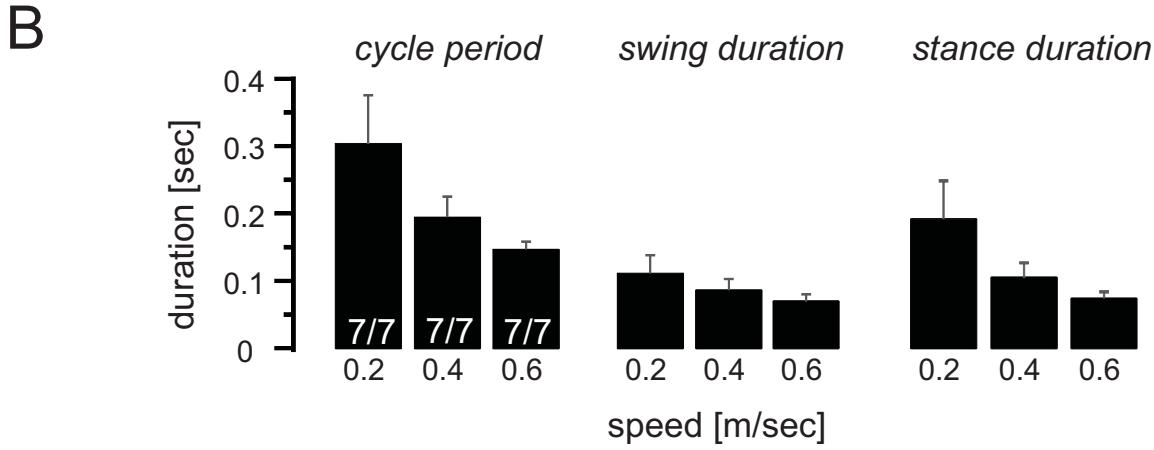


Figure 3

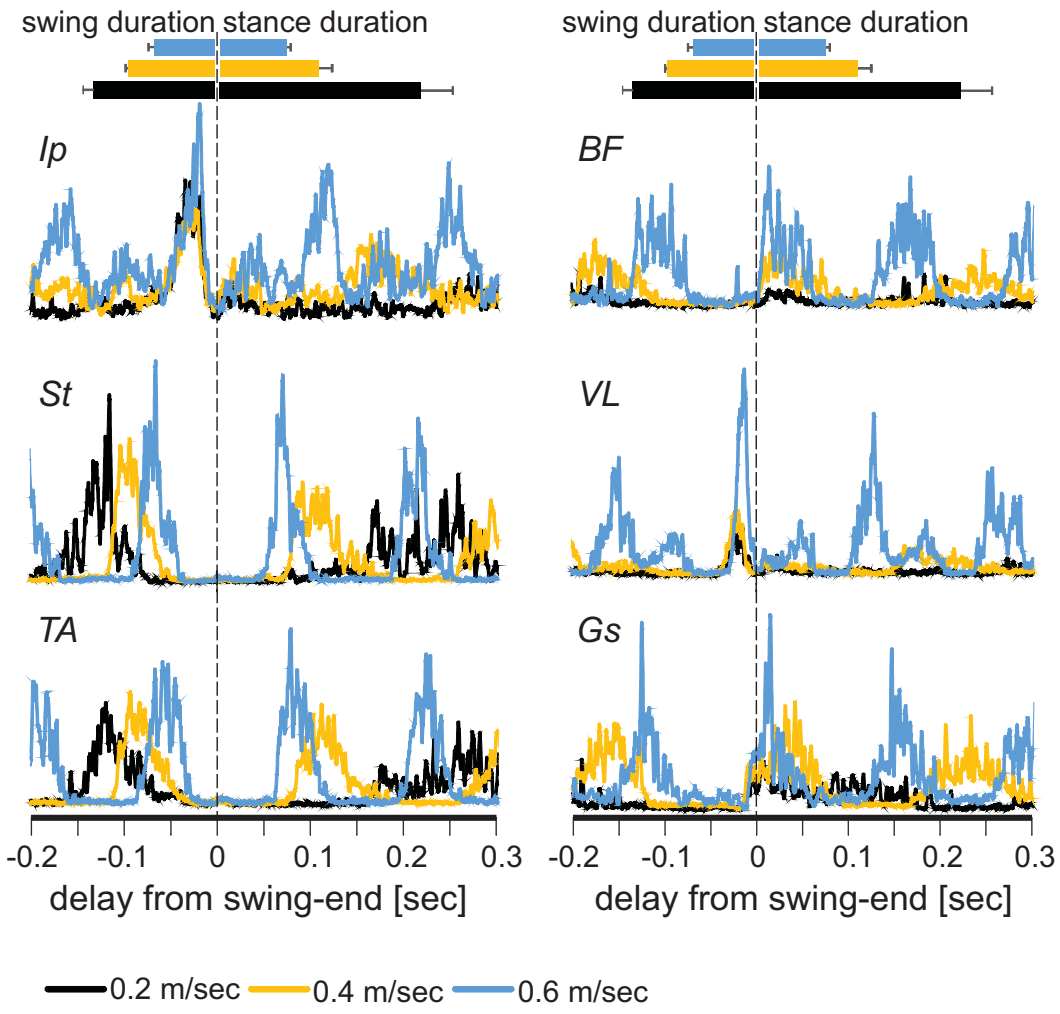


Figure 4

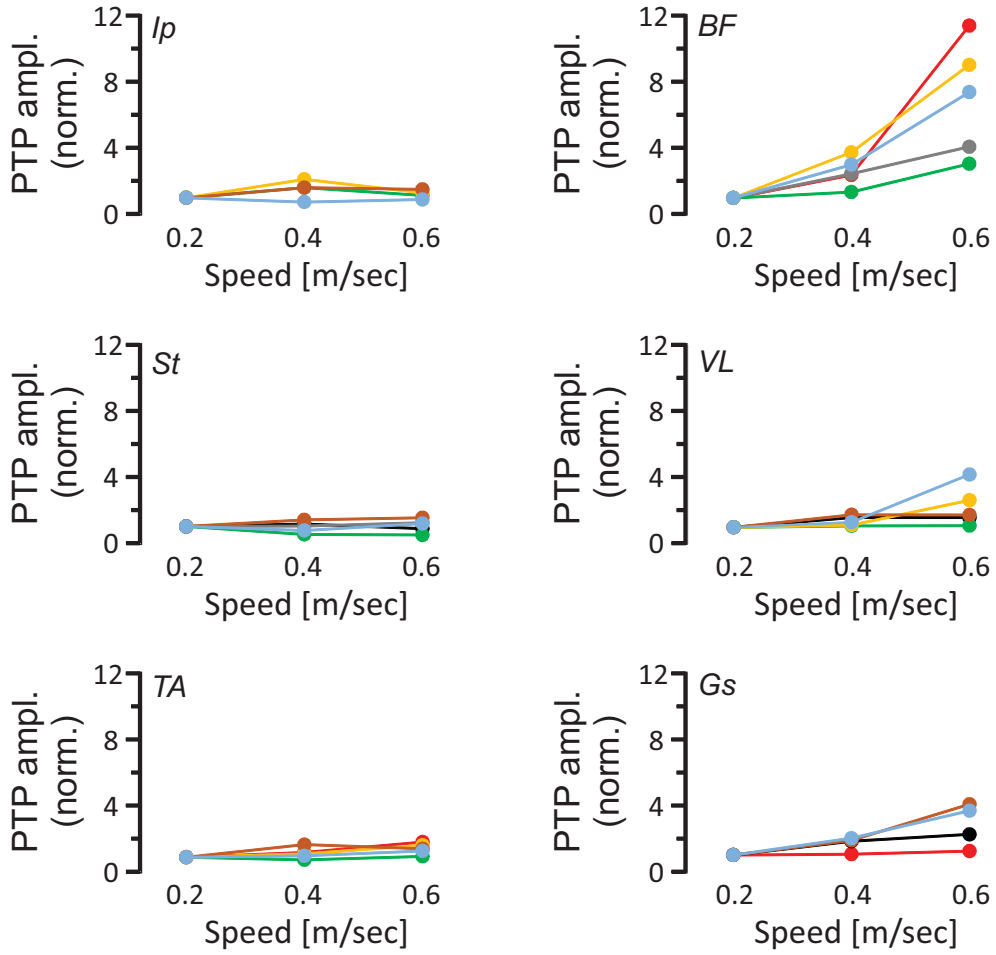
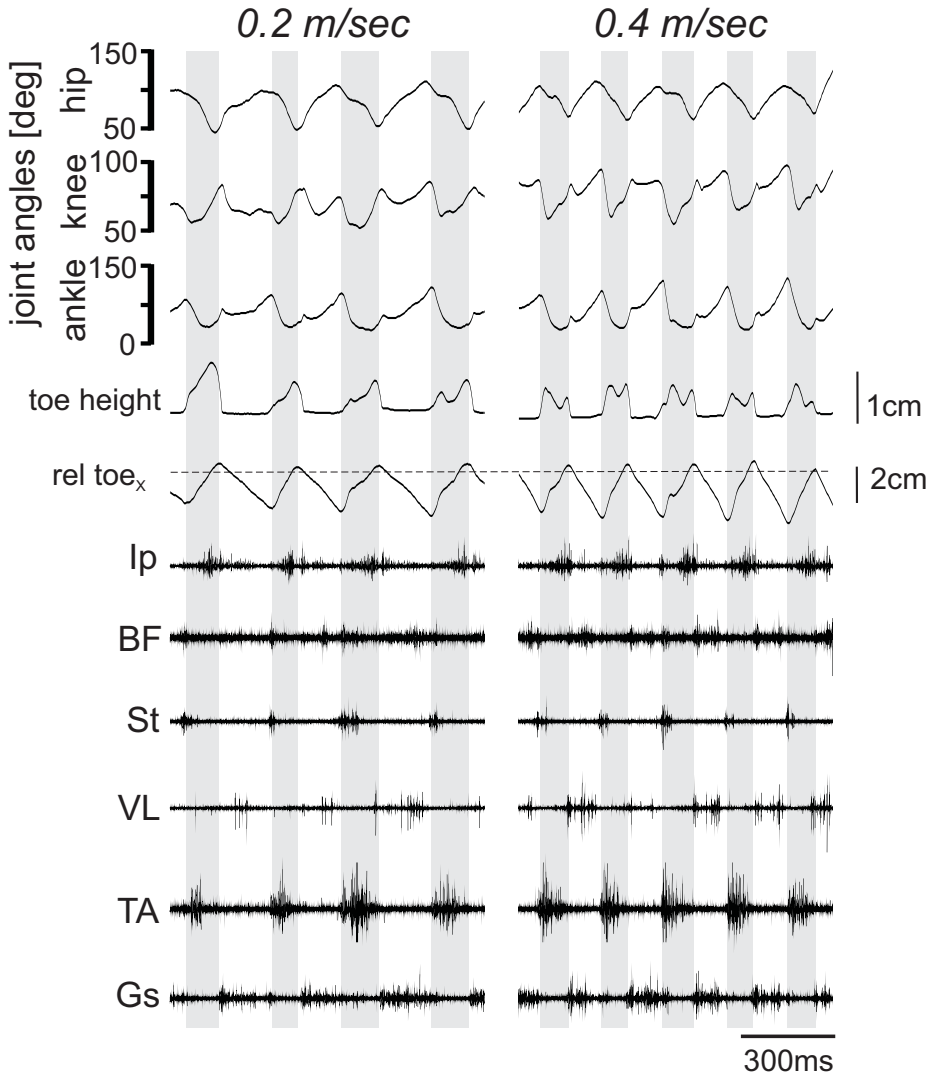


Figure 5

A



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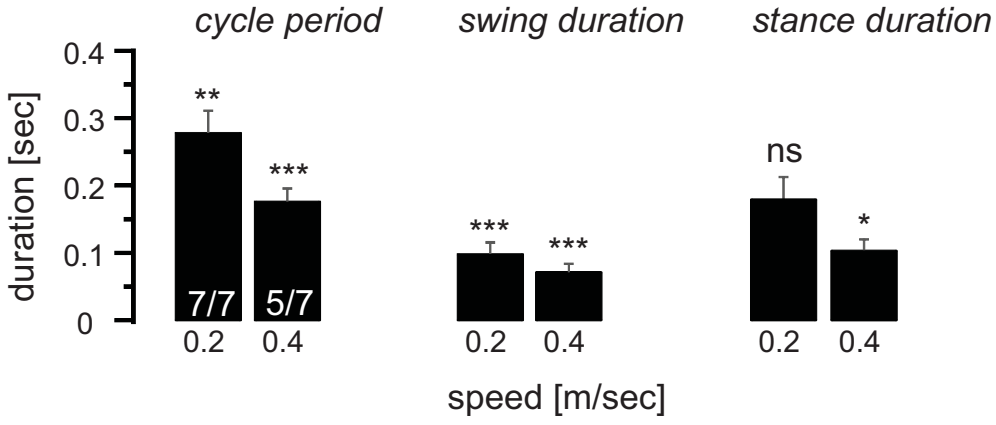

Figure 6

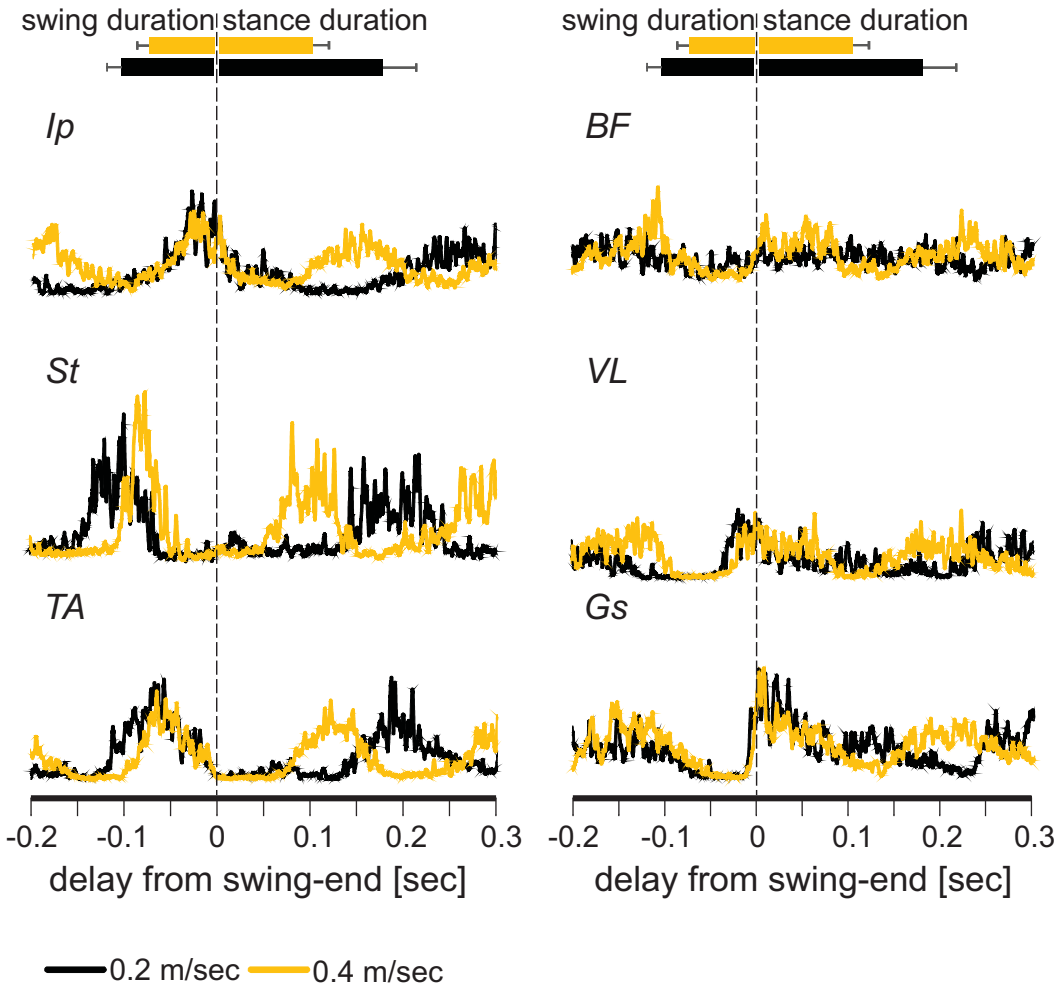

Figure 7

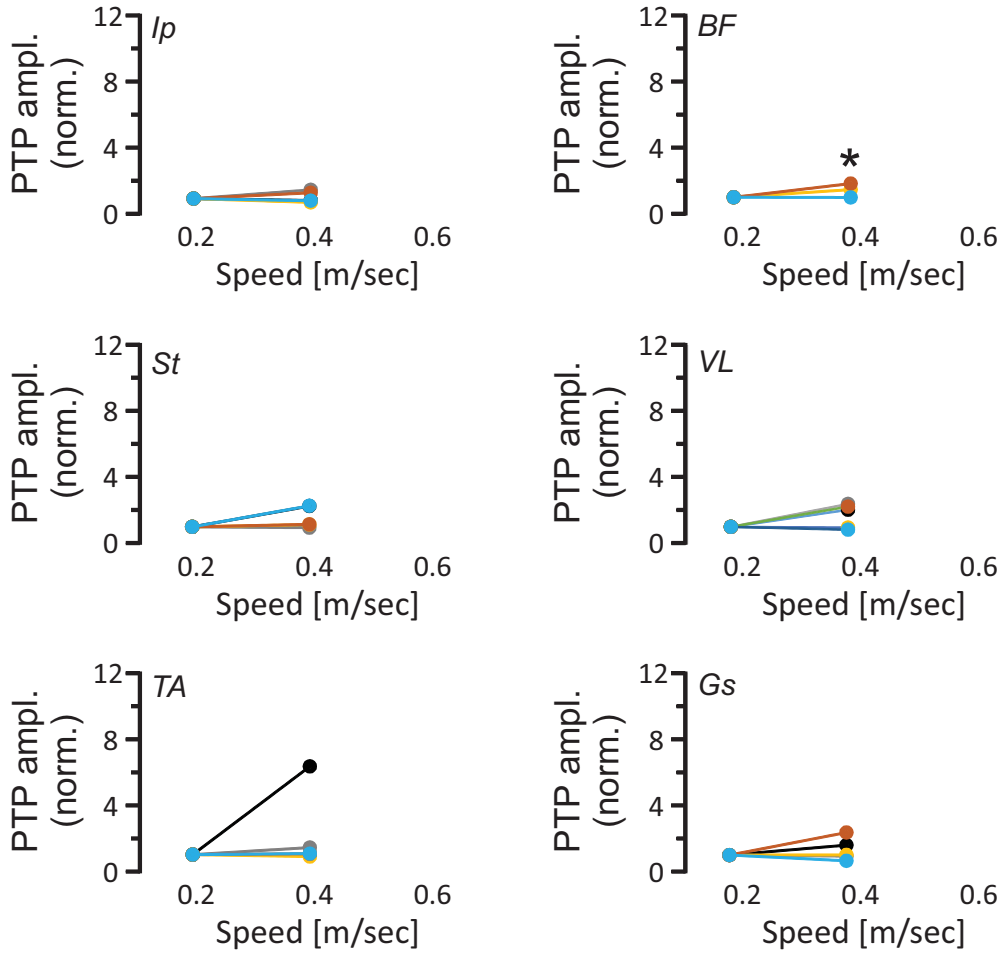

Figure 8

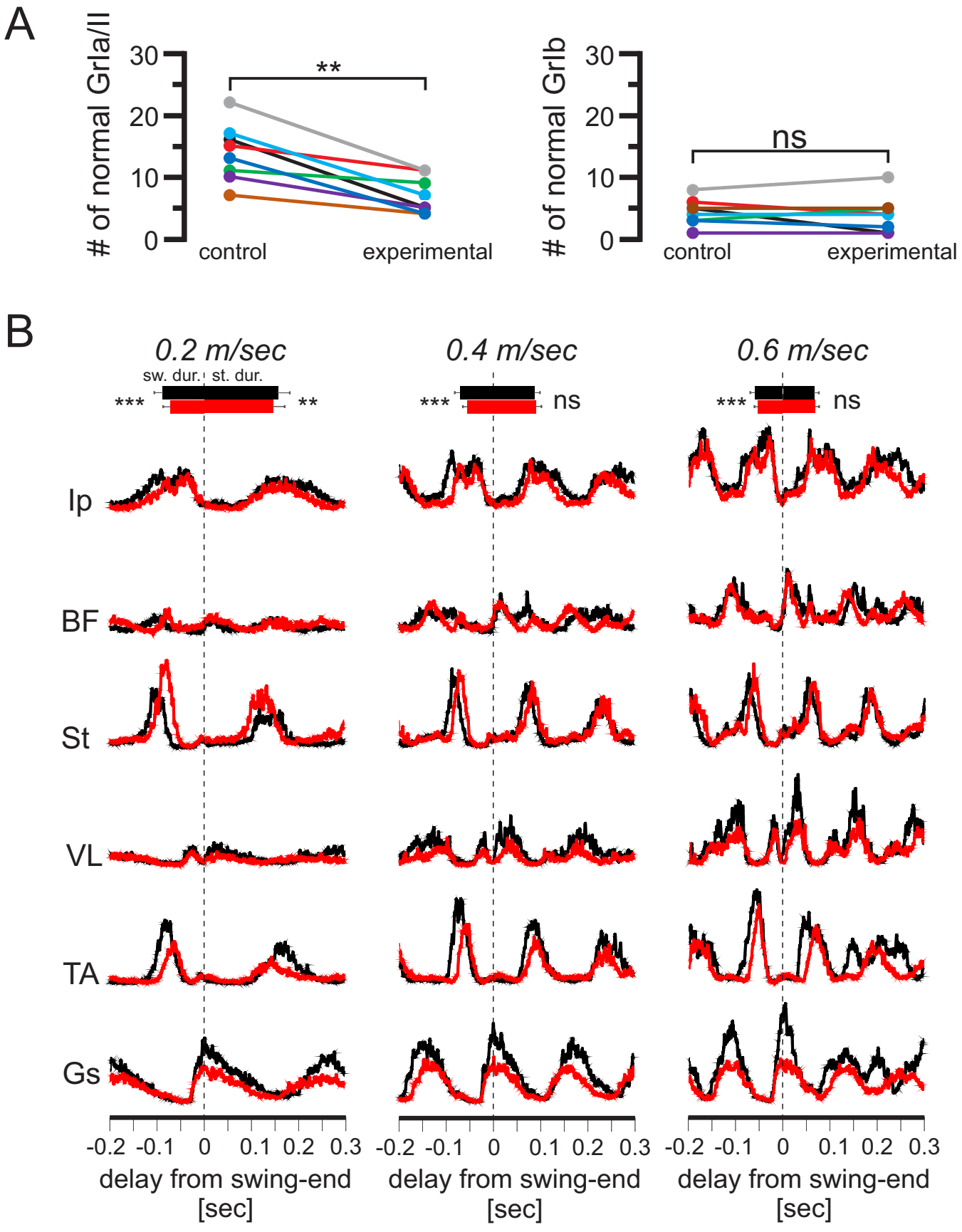

Figure 9

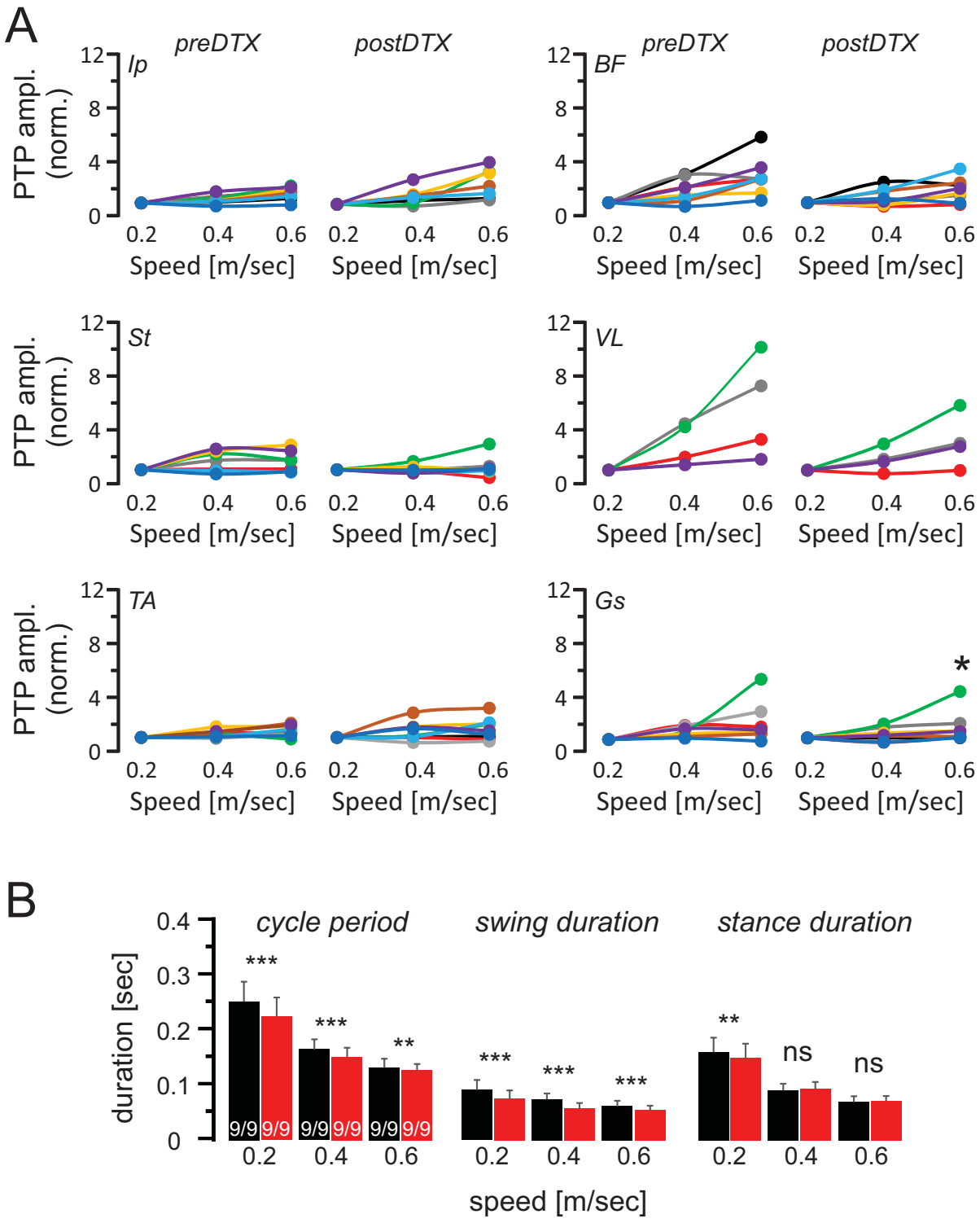

Figure 10

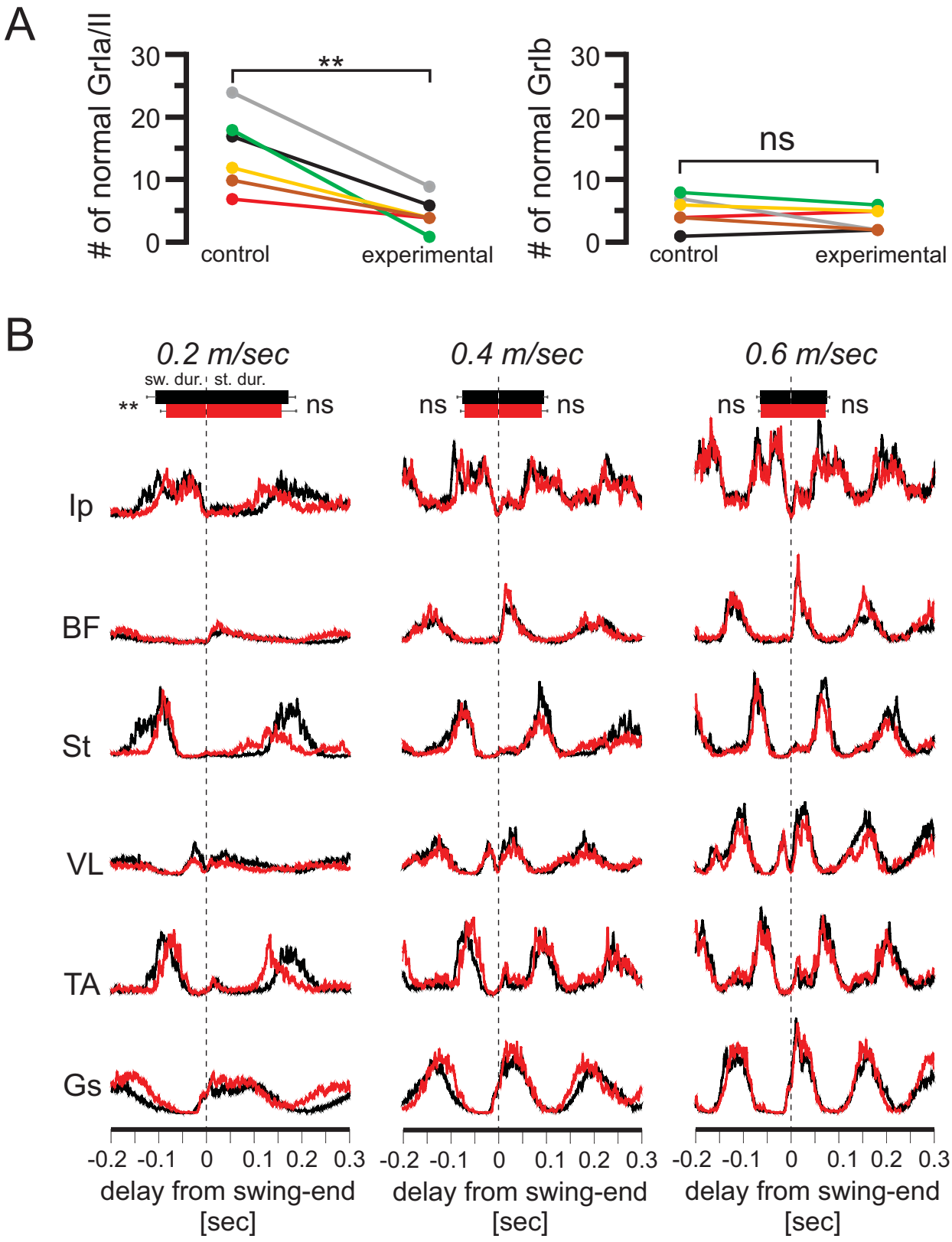

Figure 11

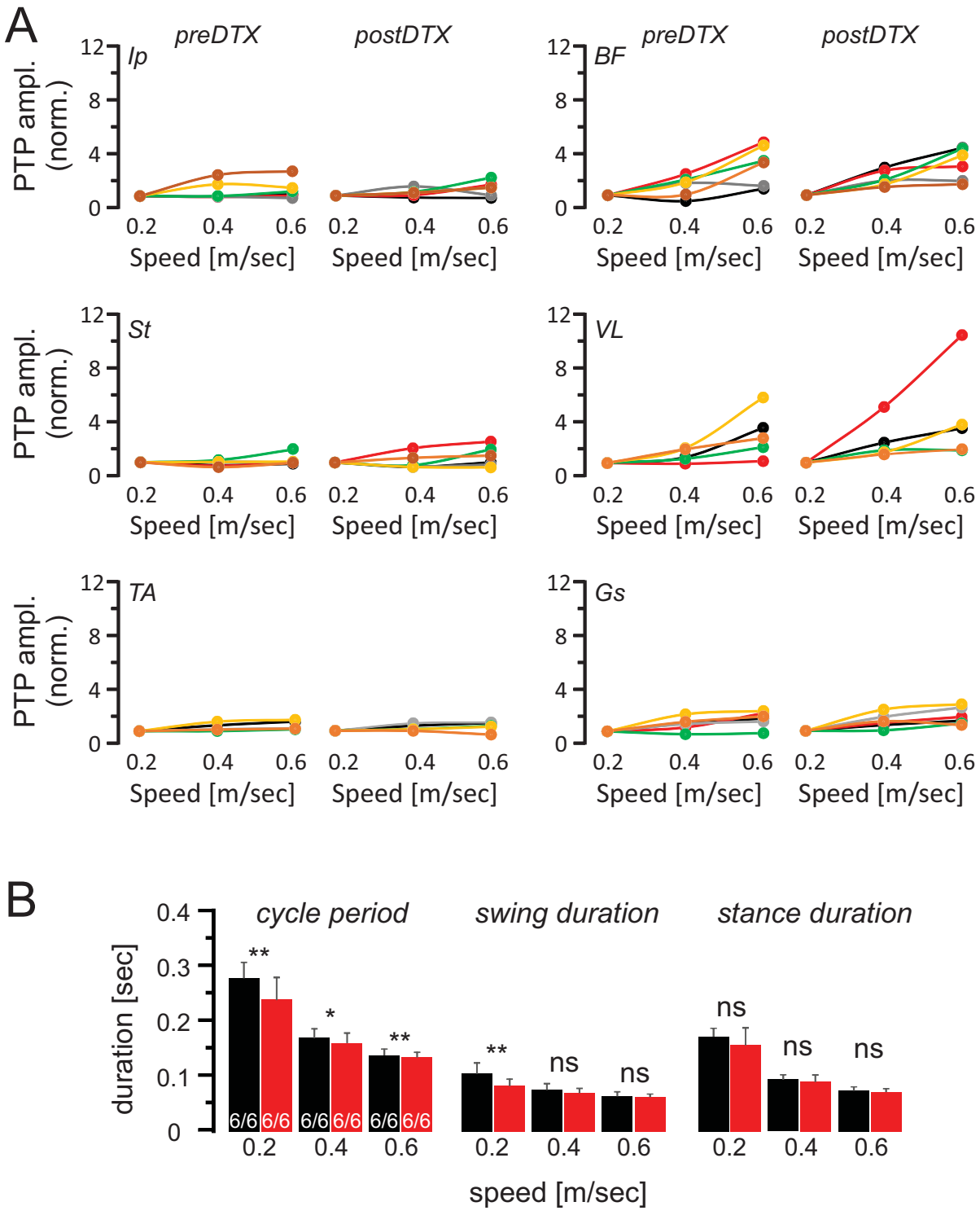

Figure 12