Locomotor Training Alters the Behavior of Flexor Reflexes 
During Walking in Human Spinal Cord Injury 

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34 **ABSTRACT**

35 In humans, a chronic spinal cord injury (SCI) impairs the excitability of pathways mediating early flexor reflexes, and increases the excitability of late, long-lasting flexor reflexes. We hypothesized that in individuals with SCI, locomotor training will alter the behavior of these spinally-mediated reflexes. Nine individuals who had either chronic clinically motor complete or incomplete SCI received an average of 44 locomotor training sessions. Flexor reflexes, elicited via sural nerve stimulation of the right or left leg, were recorded from the ipsilateral tibialis anterior (TA) muscle before and after body weight support (BWS) assisted treadmill training. The modulation pattern of the ipsilateral TA responses following innocuous stimulation of the right foot were also recorded in 10 healthy subjects while they stepped at 25% BWS, in order to investigate whether body unloading during walking affects the behavior of these responses. Healthy subjects did not receive treadmill training. We observed a phase-dependent modulation of early TA flexor reflexes in healthy subjects with reduced body weight during walking. The early TA flexor reflexes were increased at heel contact, progressively decreased during the stance phase, and then increased throughout the swing phase. In individuals with SCI, locomotor training induced the reappearance of early TA flexor reflexes and changed the amplitude of late TA flexor reflexes during walking. Both early and late TA flexor reflexes were modulated in a phase-dependent pattern after training. These new findings support the adaptive capability of the injured nervous system to return to a pre-lesion excitability and integration state.
INTRODUCTION

Two of the reflex changes observed in the human spinal cord following injury are impaired excitability of pathways mediating early flexor reflexes, and increased excitability of pathways mediating late long-lasting flexor reflexes (Roby-Brami and Bussel 1987; Knikou and Conway 2005; Knikou 2007b; Conway and Knikou 2008; Dietz et al. 2009). Specifically, the early flexor reflexes are reduced or can be completely absent at the chronic stage of spinal cord injury (SCI) in humans (Roby-Brami and Bussel 1987; Knikou and Conway 2005; Knikou 2007a), while their increased presence has been linked to improved mobility in individuals with SCI (Hubli et al. 2012). Late flexor reflexes in individuals with SCI have a similar interneuronal reorganization to that reported in acute spinal cats treated with L-DOPA (noradrenaline precursor) or 5-hydroxytryptophan (serotonin precursor) (Anden et al. 1966a, b; Jankowska et al. 1967a, b; Fu et al. 1975). Accordingly, spinal interneurons (termed flexor reflex afferent – FRA – interneurons) mediating the late flexor reflexes are responsible for 3 major roles: 1) they evoke presynaptic inhibition of extensor group Ia afferent transmission to alpha motoneurons of the ipsilateral limb, 2) they inhibit actions of contralateral FRA interneurons, and 3) they facilitate reflex actions of contralateral extensors supporting step progression (Bussel et al. 1989; Roby-Brami and Bussel 1990, 1992). It should be noted that flexor reflex responses evoked at innocuous stimulation intensities in healthy subjects appear at early and not at long latencies, and are modulated in a phase-dependent manner during walking (Duysens et al. 1993; Zehr et al. 1997, 1998; Baken et al. 2005). The phase-dependent modulation of flexor and extensor reflexes during walking in humans is an integral part of locomotion (Knikou 2010b), and its reappearance in neurological disorders can thus be viewed as functional reorganization.
We have recently shown that in individuals with chronic SCI, the phase-dependent modulation pattern of the extensor reflexes (soleus H-reflex) is reestablished, homosynaptic facilitation is reversed to homosynaptic depression, and spinal inhibition exerted at premotoneuronal level is improved after locomotor training (Knikou 2013; Knikou and Mummidisetty 2014). Based on the discussed evidence and our recent findings, we hypothesized that the prevalence ratio between early and late tibialis anterior (TA) flexor reflexes will change after locomotor training, and that these reflexes will be modulated in a phase-dependent manner during walking. Although repetitive step training on a motorized treadmill has shown to improve step progression, step coordination, kinematics of walking, and spasticity in both animals and humans (Lovely et al. 1986; Rossignol et al. 1996; Dietz et al. 1998; Behrman and Harkema 2000; Dobkin et al. 2007; Knikou 2013; Manella and Field-Fote 2013), the specific features of the reorganization of flexor reflexes during walking after locomotor training in individuals with SCI remains poorly understood.

Collectively, in this study, we assessed whether locomotor training alters the behavior of the flexor reflexes during walking in individuals with chronic SCI. The results provide unique evidence that following locomotor training, early flexor reflexes reappear and late flexor reflexes can be altered in amplitude. These new findings support the adaptive capability of the injured nervous system to return to a pre-lesion excitability and integration state.

METHODS

Subjects

Nine individuals with chronic SCI and 10 healthy subjects (6 female, age range 20-45 years) participated in the study. Study participation for SCI individuals varied from 1 month to 3 ½
months depending on the number of locomotor training sessions attended (Table 1). All subjects signed an informed consent form before participation in the study for neurophysiological tests, clinical evaluation, and/or locomotor training, which was approved by the Northwestern University (IL, USA) and the City University of New York (NY, USA) institutional review boards. Subjects' consent was obtained according to the Declaration of Helsinki. Individuals with SCI also participated in studies examining the reorganization of premotoneuronal control and soleus H-reflex excitability after locomotor training (Knikou 2013; Knikou and Mummidisetty 2014), and are identified here with the same code. During the duration of the study, none of the subjects received conventional physical therapy or participated in other research studies.

[Insert Table 1 near here]

Locomotor training

Individuals with SCI received body weight support (BWS) assisted locomotor training with a robotic exoskeleton system (Lokomat Pro®, Hocoma, Switzerland), and were trained 5 days/week, 1 hour/day. The protocol employed to train individuals with motor complete and motor incomplete SCI has been previously published in detail (see Fig. 1 in Knikou 2013). Briefly, training on the first session in AIS A started at 1.58 km/h treadmill speed with 60 % BWS. At each subsequent session the targeted treadmill speed was set to increase by 0.07 km/h and BWS to decrease by 5 %. In AIS C-D subjects, when quadriceps manual muscle test score was ≥3/5, training started at 40 % BWS at 1.98 km/h. The treadmill speed and BWS were targeted to be adjusted by 0.07 km/h and 5 % at each subsequent training session, respectively. When quadriceps and triceps surae strength was increased by a full grade, then
the BWS was decreased by 10%. Based on the TA muscle strength, which was assessed every 5 training sessions, the position of the straps of the ankle braces was adjusted. The ultimate goal in AIS C-D subjects was to reach a treadmill speed of 2.98 km/h at the lowest BWS possible without knee buckling or toe dragging during the stance and swing phases, respectively. On the first day of training, the average treadmill speed was 1.84 ± 0.12 km/h, while on the last day of training it was 2.68 ± 0.16 km/h across subjects. The BWS required before training was 47 ± 0.05 % while after training it was 24 ± 0.07 % across subjects.

**Experimental procedures**

A pulse train of 30 ms (1-ms pulses at 300 Hz) was delivered to the sural nerve (a purely sensory nerve) at the lateral submalleolar region through a stainless-steel bipolar electrode to individuals with SCI while seated with both feet at rest. In healthy subjects, stimulation was delivered to the skin over the medial edge of the right foot innervated by the saphenous nerve and not to the sural nerve, because healthy subjects expressed a great discomfort upon sural nerve stimulation even at low intensities, while noxious stimulation was required to evoke responses in the ipsilateral TA muscle. Stimulation in both cases was delivered by a constant current stimulator (DS7A, Digitimer Ltd., Hertfordshire, UK) triggered with customized LabVIEW scripts. With subjects seated, a bipolar electrode was used to establish the site that elicited a response in the ipsilateral TA muscle, without limb movement, at the lowest stimulus intensity possible. The bipolar electrode was then replaced by two monopolar electrodes (SureTrace, NY) that were secured via a surgical transparent film and maintained in place via pre-wrap and an athletic tape, while the reflex threshold was checked several times during the experiment.
Each subject was then transferred to the treadmill and wore an upper body harness that was connected to an overhead pulley system. While subjects were standing with equal distribution of body weight in both limbs and arms parallel to the trunk, BWS was applied. Thigh and shank segments of the exoskeleton were adjusted based on each subject’s leg length and diameter, and both feet were secured into the foot lifters (SCI individuals only). In all subjects, the reflex threshold during BWS standing was established, and corresponded to the lowest stimulation intensity that evoked an EMG response in the ipsilateral TA muscle without limb or ankle joint movement. In healthy subjects, this intensity ranged from 37 to 110 mA (70.1 ± 8.85; mean ± SEM) and was 3.15 times the sensory threshold (22.2 ± 2.17 mA). In all healthy subjects, 10 reflexes were recorded from the ipsilateral TA muscle at 1.2 times the reflex threshold while standing with 25% BWS at 0.1 Hz. In individuals with SCI, sural nerve stimulation was delivered at 1.1 to 1.5 (1.3 ± 0.04; 140 ± 23 mA) times the reflex threshold before locomotor training, and at 1.05 to 1.46 (1.24 ± 0.03; 137 ± 19 mA) times the reflex threshold after locomotor training, but at equivalent multiples of reflex threshold for each individual with SCI before and after training. Further, after training recordings were conducted at similar BWS levels (47 ± 0.05) and treadmill speeds (1.84 ± 0.12 km/h) to those utilized before training.

During stepping, stimulation was delivered at the same multiples of reflex threshold utilized during standing to evoke ipsilateral TA flexor reflexes. The constant current stimulator was triggered based on the ipsilateral foot switch signals (BIOPAC Systems Inc., Goleta, CA, USA), and was delivered randomly across the different phases (N = 16) of a step cycle every 3 to 5 steps. During stepping, a custom written software program (LabVIEW, National Instruments, Austin, TX, USA) divided the step cycle based on the foot switch signal.
into 16 bins with bin 1 corresponding to heel contact, and bins 8, 9, and 16 corresponding approximately to stance-to-swing transition, swing phase initiation, and swing-to-stance transition, respectively (Knikou et al. 2009; Knikou 2010a). In all subjects, 10 reflexes or more were recorded at each bin (except bin 16). TA EMG and foot switch signals were sampled at 2000 Hz and stored on a personal computer for offline analysis.

Data analysis

Offline data analysis commenced with marking and visual inspection of the foot switch signals for determination of the step cycle phases, followed by marking of the first triggering pulse of the pulse train for determination of stimulation onset within a step. The fullwave rectified band-pass filtered (40-1000 Hz) area of the early (50-ms poststimulus with 50-ms duration) and late (100-ms poststimulus with 400-ms duration) ipsilateral TA flexor reflexes (whichever present) were calculated, grouped for each bin, and averaged (Knikou 2010a). Then, the TA EMG from the nonstimulated steps corresponding to the same time windows and bins was subtracted from the reflex EMGs to remove background EMG activity. The subtracted reflex values were normalized to the maximum TA locomotor EMG observed across all bins of the step cycle, yielding a response activation profile ranging from 0 to 1 (Zehr et al. 1997, 1998, 2012; Knikou et al. 2009; Knikou 2010a). To establish task-dependent changes (standing vs. walking), reflexes were measured as the area of the TA rectified EMG, and were normalized to the mean amplitude of the associated reflex component recorded with the subject standing. The mean amplitude of the normalized TA reflex from each subject was grouped based on the bin number and/or time of testing.
The ipsilateral TA background EMG activity was calculated from the mean value of the
rectified and band-pass filtered EMG for duration of 50-ms, beginning 100 ms before
stimulation for each bin of the step cycle, and was normalized to the maximum TA locomotor
EMG observed across all bins of the step cycle. To quantify changes in muscle and reflex
excitability during walking after training, a modulation index for SCI and healthy subjects for
the overall change in the TA EMG over the step cycle was determined by subtracting the
minimum EMG from the maximum EMG and expressed to the maximum (Zehr and Loadman
2012). This was done separately for the background TA EMG, control EMG, and late TA
reflex EMG of the right and left legs during stepping.

Statistical analysis
The effect of locomotor training on the amplitude of the TA flexor reflexes during walking
was analyzed with a Wilcoxon rank sum test at each bin before and after training for reflexes
normalized to those recorded during standing, and with two-way repeated measures analysis
of variance (ANOVA) (between group factor: time; within-subject factor: bins of the step
cycle) for reflexes represented as normalized subtracted reflex EMGs. The amplitude of the
ipsilateral TA flexor reflexes in healthy subjects during walking was analyzed with one-way
repeated measures ANOVA (within-subject factor: bins of the step cycle), and with two-way
repeated measures ANOVA (between group factor: control/reflex EMG; within-subject factor:
bins of the step cycle). For all statistical tests, when a statistically significant difference was
found, post-hoc Bonferroni tests for multiple comparisons were applied to the data.

The effect of locomotor training on the TA background activity during walking was
analyzed with two-way repeated measures ANOVA (between group factor: time; within-
subject factor: bins of the step cycle). Differences of modulation indices between healthy subjects and individual with SCI on the mean averages of background EMG activity were established with one-way Kruskal-Wallis ANOVA on ranks, while differences between modulation indices of control EMG for each bin of the step cycle were established with two-way Kruskal-Wallis ANOVA on ranks followed by pairwise multiple comparisons when a statistically significant difference was detected.

The mean amplitude of the ipsilateral early and late TA flexor reflexes was plotted on the \( y \)-axis versus the associated TA background activity on the \( x \)-axis, and a linear least-square regression was fitted to the data. This analysis was conducted on data obtained from each subject. Last, to establish the extent to which flexor reflex modulation after training was related to leg motor scores (clinically assessed based on ASIA guidelines), the normalized flexor reflexes before training were subtracted from those obtained after training, grouped for stance and swing phases of gait and a linear relationship to the percentage of change in the motor scores observed after training was completed. Effects were considered significant when \( P < 0.05 \).

\section*{RESULTS}

\textit{Flexor reflexes during walking after locomotor training in SCI}

Locomotor training altered the behavior of TA flexor reflexes during walking in individuals with chronic SCI. Waveform averages of the ipsilateral TA flexor reflexes of a representative subject (R10) while stepping during sural nerve stimulation before and after locomotor training are presented in Fig. 1. In the right leg, the late TA flexor reflexes were modulated in a phase-dependent pattern after locomotor training. Specifically, the late TA flexor reflexes
were profoundly increased at stance-to-swing and swing initiation phases (bins 8, 9) compared
to the other phases of the step cycle. Further, at heel contact (bin 1) and late swing (bins 13,
14) phases, the onset of the TA reflex EMG was decreased by 40 ms after locomotor training
(Fig. 1A). In the left leg, however, the modulation pattern of the ipsilateral late TA flexor
reflexes after training was different from that recorded in the right leg. The late ipsilateral TA
flexor reflexes in the left leg in this subject were profoundly increased after locomotor
training regardless of the step cycle phase (Fig. 1B).

[Insert Figure 1 near here]

The average amplitude of the ipsilateral late TA flexor reflexes from all individuals with
SCI during BWS assisted stepping from the right and left legs are shown in Fig. 2. In Figs. 2A
and 2D, the late TA flexor reflexes recorded from the right and left legs are normalized to the
associated flexor reflexes recorded with subjects standing, while in Figs. 2B and 2E the
reflexes are normalized to the maximum ipsilateral TA locomotor EMG across all bins having
subtracted the control EMGs. Locomotor training significantly reduced the amplitude of the
ipsilateral late TA flexor reflexes of the right leg throughout the step cycle (bins 2-14; \( P <
0.05 \), Fig. 2A). In the left leg, locomotor training re-established a phase-dependent modulation
of the late TA flexor reflexes by increasing their amplitude at late stance phase (bins 6, 7) and
swing phase initiation (bin 9) \( (P < 0.05; \) Fig. 2D). A similar reflex adaptation pattern in both
legs was observed in the normalized subtracted late TA flexion reflexes (Figs. 2B, 2E),
supporting for non-symmetric adaptation of the late TA flexion reflex in the right and left legs
after locomotor training.

Repeated measures ANOVA showed that the difference in mean TA reflex values was
statistically significant different before and after training \( (F_{1,15} = 2.53, P < 0.001 \) for data
shown in Fig. 2A; $F_{1,15} = 4.03$, $P = 0.046$ for data shown in Fig. 2B). The flexor reflex amplitude in the right leg at the swing phase of walking was positively correlated to the percentage of change of the associated motor scores after training ($R^2 = 0.56$, $P = 0.04$), a relationship that was not found for the stance phase ($R^2 = 0.17$, $P = 0.4$) (data not shown graphically). Similarly, the late flexor reflex amplitude in the left leg at the swing phase of walking was positively correlated to the percentage of change of the associated motor scores after training ($R^2 = 0.55$, $P = 0.04$), but not for the stance phase ($R^2 = 0.0024$, $P = 0.96$) (data not shown graphically). Thus, it is likely that asymmetric gains in lower extremity strength (10 and 6.4 % increase in motor scores of right and left legs, respectively) may have led to asymmetric reflex modulation changes.

The mean amplitude of the ipsilateral TA background activity from the right leg before and after training is indicated in Fig. 2C. The level of the right TA background EMG activity did not vary across bins ($F_{15} = 0.72$, $P = 0.76$) or before/after locomotor training ($F_1 = 3.09$, $P = 0.08$) (2-way ANOVA). The right late TA flexor reflexes were not linearly related to the right TA background activity before ($R^2 = 0.33$, $P = 0.04$) or after ($R^2 = 0.34$, $P = 0.35$) locomotor training. The mean amplitude of the ipsilateral TA background activity from the left leg before and after training is indicated in Fig. 2F. The level of the left TA background EMG activity did not vary across bins ($F_{15} = 1.1$, $P = 0.35$) or before/after locomotor training ($F_1 = 0.51$, $P = 0.47$). The left late TA flexor reflexes was not linearly related to the left TA background activity before ($R^2 = 0.007$, $P = 0.87$) or after ($R^2 = 0.04$, $P = 0.55$) locomotor training.
Ipsilateral early TA flexor reflexes were present during walking after locomotor training in 3 out of 9 individuals with SCI (R03, R13, and R14) in the right leg, and in one individual with SCI (R14) in the left leg. Data from these 3 subjects are shown in Fig. 3. Nonrectified waveform averages of early TA flexor reflexes during BWS assisted walking recorded from the right leg before and after locomotor training are indicated in Fig. 3A from subject R14. It is apparent that the shape, latency, and amplitude of these reflexes were altered throughout the step cycle after training.

In Fig. 3B, the subtracted normalized early TA flexor reflexes recorded from the right leg before and after locomotor training are presented. Locomotor training promoted reflex depression in stance phase and reflex facilitation during the early- mid-swing phases of the step cycle ($P < 0.05$ for bins 6 and 10-15 pre/post, Fig. 3B). The early TA flexor reflexes during walking from the right leg were not linearly related to the right TA background activity during stepping before ($R^2 = 0.2, P = 0.22$) or after ($R^2 = 0.12, P = 0.11$) locomotor training. In one subject (R14), early TA flexor reflexes were not present when recorded during seated before or after locomotor training, but reemerged during BWS assisted stepping after locomotor training (Fig. 3C). The increased amplitude of the early TA flexor reflexes at heel contact and early swing phase (bins 1-4, 10, 11; Fig. 3C) cannot be attributed to peripheral sources because the level of the left TA background activity did not vary across the step cycle (Fig. 2F).

Flexor reflexes during walking with partial BWS in healthy subjects
To determine the modulation pattern of the TA flexor reflexes in healthy subjects under similar experimental conditions utilized for SCI subjects, 10 healthy subjects stepped at 25% BWS with a treadmill speed set at 2.89 km/h. The average latency of the TA reflexes with subjects seated or standing was $75 \pm 5$ ms (including the pulse train duration), and only early reflexes in the ipsilateral TA muscle were present during standing or during walking in all tested subjects. Waveform averages of nonrectified early TA reflexes from one representative subject during standing and stepping are indicated in Fig. 4A. It is apparent that the early TA flexor reflexes were modulated in a phase-dependent pattern under conditions of reduced body weight. Specifically, the early TA flexion reflex was completely depressed during the stance phase (bins 3-8), and facilitated throughout the swing phase (Fig. 4A). A linear relationship between the right TA early flexor reflex and the right TA background EMG activity was found ($P < 0.0001$; data not shown graphically).

The average amplitude of the ipsilateral early TA flexor reflexes recorded from the right leg from all healthy subjects is shown in Fig. 4B as percentages of the reflex values recorded during standing, and in Fig. 4C as subtracted reflex EMGs normalized to the maximum TA locomotor EMG. One-way ANOVA showed that the early TA flexor reflexes were statistically significant different across bins ($F_{15} = 10.6$, $P < 0.001$), being statistically significant different from control reflex values at bins 10, 11, 12, 15, and 16 (Fig. 4B). Similar findings were observed for the normalized subtracted TA reflexes ($F_{15} = 59.2$, $P < 0.001$), which were significantly increased at heel contact (bin 1), then progressively decreased reaching control EMG values at late stance phase, followed by a significant facilitation at swing-phase initiation and throughout the swing phase (bins 9-16, $P < 0.05$) (Fig. 4C).
level of the right TA background activity from all subjects is indicated in Fig. 4D. The early TA flexor reflexes recorded from the right leg were linearly related to the right TA background EMG activity (both normalized to the maximum TA locomotor EMG) \((R^2 = 0.42, P < 0.001)\).

Modulation indices of background and control EMG between healthy and SCI subjects

To determine to what extent the modulation pattern of EMG activity in individuals with SCI resembled that observed in healthy subjects we compared the modulation indices of TA background EMG (100 ms before stimulation) and TA control EMG (at similar time windows and bins of nonstimulated steps) in healthy and SCI subjects before and after training. These are indicated in Fig. 5 as the overall mean from all 16 bins of the step cycle. Significant differences between subject groups and within the SCI group before and after training were found. These differences were found for the modulation indices of background TA EMG activity \((P = 0.007, \text{Fig. 5A})\), and control TA EMG \((P < 0.001, \text{Fig. 5B})\) between healthy and SCI subjects for the right leg. Differences were also found for the modulation indices of TA control and reflex EMG within the SCI group before and after training for the right leg (Figs. 5B, C). Last, significant differences for the modulation indices in the left TA were found only for the control EMG before and after training \((P = 0.038, \text{Fig. 5B})\) for the individuals with SCI.

DISCUSSION

We hypothesized that locomotor training alters the behavior of flexor reflexes in individuals with chronic SCI. We demonstrate two novel findings on the reorganization of this spinally-
mediated reflex: 1) locomotor training contributed to the appearance of early TA flexor reflexes during walking in both limbs; 2) locomotor training reduced the amplitude of late TA flexor reflexes in the right leg and increased their amplitude in the left leg, and promoted a phase-dependent modulation of these reflexes.

Behavior of TA flexor reflexes after locomotor training in SCI

In individuals with chronic SCI, the late TA flexor reflexes in the right leg were significantly reduced during walking after training (Fig. 2A), and when depression was replaced by facilitation, the onset of the reflex EMG changed (Fig. 1A). The atypical late TA flexor reflex modulation pattern in the left leg before training was replaced by increased reflex excitability that was modulated in a phase-dependent pattern after training (Fig. 2D). These findings suggest for altered behavior of interneurons mediating the late TA flexor reflexes, and demonstrate that FRA pathways were not reorganized in a similar manner in both limbs. Specifically, two asymmetries were noted between limbs. One, the onset of increased excitability in the late TA flexor reflexes occurred at different phases of the step cycle. And two, the late TA flexor reflexes were modulated differently in the right and left legs before training (compare Figs. 1A and 1E). The reorganization of the late TA flexor reflexes we observed here may be related to the motor capacity of each individual with SCI before training (Table 1), the amplitude and prevalence relationship between early and late TA flexor reflexes, the asymmetry found in the late TA flexor reflex modulation between legs before training (Fig. 1), the type of SCI (cervical vs. thoracic), and to the number, localization, and amount of damaged and spared spinal and supraspinal pathways (Rossignol et al. 2009).
Early TA flexor reflexes deteriorate in amplitude as the SCI becomes more chronic (Dietz et al. 2009), and in some cases they might be completely absent (Roby-Brami and Bussel 1987; Knikou 2007a; Conway and Knikou 2008). Our most important finding was that the early TA flexor reflexes reappeared in one individual with AIS D SCI after 44 locomotor training sessions (Fig. 3C), and regained their phase-dependent modulation excitability pattern during walking (Fig. 3B) in 3 out of 9 individuals with SCI. These findings are consistent with the return of the early TA flexor reflex in one individual with SCI while seated after ~16 locomotor training sessions (Hubli et al. 2012). While early TA flexor reflexes are mostly absent during the seated or supine position in individuals with SCI (Knikou 2007a; Conway and Knikou 2008; Bolliger et al. 2010), they were reemerged during walking in 9 individuals with complete SCI, and the late TA flexor reflexes were concomitantly decreased in amplitude (Bolliger et al. 2010). This behavior was likely driven by appropriate locomotor proprioceptive inputs (Bolliger et al. 2010) that changed the excitability state of FRA pathways, a mechanism also applicable to our findings.

It should be noted that the altered behavior of FRA interneurons coincided with improvements in locomotor ability as demonstrated by decreased BWS, increased treadmill speed, and decreased step cycle duration (Knikou 2013) as well as with improvements in locomotor EMG activity and decreased cocontraction of antagonistic muscle pairs (Knikou and Mummidisetty 2014).

Behavior of TA flexor reflexes during walking in healthy subjects

In healthy human subjects, only early TA flexor reflexes were present and were modulated in a phase-dependent manner (Fig. 4C) despite walking with reduced body weight, consistent
with the modulation pattern reported during walking without BWS (Duysens et al. 1990, 1992, 1993). The phase-dependent modulation pattern of the early TA flexor reflexes can be attributed to amplitude modulation of presynaptic inhibition of cutaneous afferent volleys (Eccles et al. 1962; Menard et al. 2002). These early TA flexor reflexes, elicited by innocuous stimulation of the skin, represent cutaneous reflexes and should not be regarded similar to the early TA flexor reflexes elicited by stimulation of a sensory nerve in individuals with SCI, since FRA pathways have a different neuronal organization in individuals with SCI. However, when the modulation of the early TA flexor reflexes in healthy subjects and individuals with SCI is compared, a distinctive difference observed is that in the SCI group neither the early nor the late flexor reflexes were linearly related to the ipsilateral TA EMG background activity. Nonetheless, the depth of modulation of the background TA EMG activity was similar in both SCI and healthy subjects (Fig. 5A). This is likely related to the fact that inhibitory and facilitatory responses independent of the background activity are also present upon stimulation of sensory nerves during human walking (Duysens et al. 1992; Van Wezel et al. 1997), although spinal reflexes during walking may increase in parallel with the ongoing background activity. Based on the differences in the modulation indices of the control EMG between subject groups and limbs of SCI subjects (Figs. 5B, C), and the non-linear relationship of reflex modulation and background EMG activity in neurologically impaired patients (Knikou 2013; Zehr and Loadman 2012), we propose that the relationship between TA background activity and amplitude modulation of early and late TA flexor reflexes may be utilized to establish or predict recovery of walking with locomotor training in individuals with SCI.
Mechanisms of Reorganization of FRA Pathways after Locomotor Training in SCI

The changes in the behavior of early and late TA flexor reflexes during walking in SCI may be driven by separated or combined modifications in the strength of afferent transmission, physiology and connectivity of spinal interneurons, and supraspinal inputs (Thomas and Gorassini 2005; Knikou 2007b; Barriere et al. 2008; Dietz et al. 2009; Kapitza et al. 2012).

In complete SCI, training likely reinforced proprioceptive feedback of receptors that can directly affect the locomotor rhythm such as the hip afferents responding to stretch and plantar cutaneous and group I afferents responding to load (Knikou 2010a, 2012). Changes of afferent feedback integration may have contributed to the increased amplitude of reflexes at swing phase initiation (Figs. 1A and 3A), which is in agreement to the postulated regulation of FRA interneurons by signals transmitting hip position (Knikou et al. 2006), the normalized function of late TA flexor reflexes during walking upon stimulation of plantar cutaneous afferents (Knikou 2010a), and locomotor Ib facilitation (Knikou 2012) in untrained individuals with SCI. Because proprioceptive feedback regulates but does not give genesis to rhythmic movement (Brown 1911), the altered behavior of early and late TA flexor reflexes after locomotor training must be related to changes in the connectivity and physiological properties of spinal FRA interneurons. Studies in animals have clearly shown that locomotor training induces specific changes in spinal neurons, including but not limited to increasing the density of the glycinergic axonal terminals and decreasing the size of both glycinergic and GABAergic axon terminals (Bras et al. 2013), increasing the size of the soma of neurons (Stigger et al. 2011), reversing the proportion of inhibitory to excitatory boutons to alpha and
gamma motoneurons (Ichiyama et al. 2011), and changing the cellular properties of motoneurons and their synaptic input from the spinal white matter (Petruska et al. 2007).

In individuals with motor complete SCI, FRA stimulation induces contralateral soleus H-reflex facilitation and ipsilateral soleus H-reflex depression, while contralateral FRA stimulation decreases the heteronymous Ia facilitation exerted from the quadriceps onto soleus alpha motoneurons thereby affecting transmission of Ia afferents before they synapse with alpha motoneurons (Roby-Brami and Bussel 1990, 1992). Based on this evidence and our current findings, neuronal changes in the spinal stepping generator in humans after locomotor training may explain the reflex adaptation we observed. These changes likely involved FRA interneurons (based on the altered behavior of early and late TA flexor reflexes), alpha motoneurons (based on the improved locomotor EMG pattern of these patients; see Knikou 2013), and improvements in premotoneuronal control (see Knikou and Mummidisetty 2014).

The behavior of cutaneous reflex pathways during walking is influenced by corticospinal inputs in healthy humans (Pijnappels et al. 1998; Christensen et al. 1999), while FRA interneurons provide ascending information to the cerebellum (Oscarsson 1957). Further, early TA flexor reflexes are abnormally modulated during walking in patients with spastic paraparesis (Duysens et al. 2004), while corticospinal inhibition of the late TA flexor reflex was potentiated after locomotor training in one person with motor incomplete SCI (Hajela et al. 2013). Thus, reappearance of the early TA flexor reflexes in motor incomplete SCI subjects could have been mediated, at least in part, by reorganization of corticospinal control (descending monoaminergic neuronal pathways), and by changes in the mutual inhibitory interactions between short and long latency FRA pathways at a spinal level (Anden et al. 1966b; Engberg and Ryall 1966). Fig. 6 summarizes the changes in behavior of FRA
pathways before and after locomotor training in individuals with SCI. It is apparent that further research is needed to outline the neuronal pathways affecting FRA actions on motoneurons that may undergo adaptive changes with locomotor training after SCI in humans.

[Insert Figure 6 near here]

Conclusion

We described here, for the first time reported in the literature, the behavior of flexor reflexes during walking after locomotor training in individuals with chronic SCI. Locomotor training contributed to the reappearance of early TA flexor reflexes during treadmill walking with BWS and changed the amplitude of late TA flexor reflexes. Both early and late TA flexor reflexes were modulated in a phase-dependent pattern after locomotor training. Further research is needed to comprehensively outline the source of this neuronal reorganization so treatments can be optimized. However, regardless of the source, it is evident that locomotor training changes the excitability and integration state of the injured human spinal cord.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.
AUTHOR CONTRIBUTIONS

M.K. conception and design of research; A.C.S., C.K.M., M.K. performed experiments;
M.K. approved final version of manuscript.

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Treadmill training alters FRA pathways


Treadmill training alters FRA pathways


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**FIGURE LEGENDS**

Fig. 1. Single subject late TA flexor reflexes from the right and left leg before and after locomotor training during treadmill walking. A, B: Non-rectified waveform averages of ipsilateral TA reflexes from one representative subject (R10) recorded during walking before (green lines) and after (blue lines) locomotor training from the right (A) and left legs (B). Grey shaded area in both graphs denotes the late TA flexor reflexes. Each step was divided into 16 equal bins based on the signal from the ipsilateral foot switch. Bin 1 corresponds to heel contact. Bins 8, 9, and 16 correspond approximately to stance-to-swing, swing initiation, and swing-to-stance, respectively. In all figures, the step cycle is identified in a similar manner. TA = tibialis anterior muscle.

Fig. 2. Late TA flexor reflexes during walking before and after locomotor training in SCI. A: Overall amplitude of the ipsilateral late TA flexor reflexes from all individuals with SCI recorded from the right leg for each bin of the step normalized to control response values recorded during standing. B: Overall amplitude of the ipsilateral late TA flexor reflexes from all individuals with SCI recorded from the right leg for each bin of the step normalized to the
maximum TA locomotor EMG having subtracted the control EMG at identical time windows and bins. C: Overall amplitude of the right TA background activity normalized to the maximum TA EMG during stepping. D: Overall amplitude of the ipsilateral late TA flexor reflexes from all subjects recorded from the left leg for each bin of the step normalized to control response values recorded during standing. E: Overall amplitude of ipsilateral late TA flexor reflexes from all subjects recorded from the left leg for each bin of the step normalized to the maximal TA locomotor EMG having subtracted the control EMG at identical time windows and bins. F: Overall amplitude of the left TA background activity normalized to the maximum TA EMG during stepping. Error bars denote the SEM. HC = heel contact, SI = swing initiation, S-T-S = stance-to-swing, TA = tibialis anterior muscle.

Fig. 3. Early TA flexor reflexes during walking before and after locomotor training in SCI. A: Nonrectified waveform averages of TA flexor reflexes from one representative subject (R014) recorded during stepping before (green lines) and after (blue lines) locomotor training. Grey shaded area denotes the early TA flexor reflex. B: Amplitude of ipsilateral early TA flexor reflexes from all subjects recorded from the right leg for each bin of the step normalized to the maximal TA locomotor EMG having subtracted the control EMG at identical time windows and bins. C: Amplitude of ipsilateral early TA flexor reflexes recorded from the left leg of subject R014 normalized to the maximum control EMG having subtracted the control EMG at identical time windows and bins.

Fig. 4. Ipsilateral early TA flexor reflexes during walking in healthy subjects. A: Nonrectified waveform averages of the early TA flexor reflexes from one healthy subject (CT25A) during...
standing and walking at 25 % BWS. In this subject, the TA reflexes were completely depressed during the stance phase (bins 3-8), and facilitated throughout the swing phase. 

B: Amplitude of ipsilateral early TA flexor reflexes from all healthy subjects for each bin of the step normalized to the reflexes recorded during standing. 

C: Amplitude of ipsilateral early TA flexor reflexes from all subjects for each bin of the step normalized to the maximum TA locomotor EMG having subtracted the control EMG at identical time windows and bins of the step cycle. 

D: Mean TA background activity normalized to the maximum TA EMG during stepping. Error bars denote the SEM. HC = heel contact, SI = swing initiation, S-T-S = stance-to-swing, TA = tibialis anterior muscle.

Fig. 5. Modulation indices of background, control, and reflex EMG in SCI and healthy subjects. 

A: Modulation indices for background EMG. 

B: Modulation indices for control EMG. 

C: Modulation indices for reflex EMG corresponding to the late TA flexor reflexes for individuals with SCI only. Modulation indices are indicated as the overall mean from all bins of the step cycle for healthy (black bars) and SCI subjects before (green bars) and after (blue bars) training for the right and/or left tibialis anterior (TA) muscle. *Differences between healthy and SCI subjects or within the SCI group before and after training at $P < 0.05$. Error bars denote the SEM.

Fig. 6. Schematic illustration of FRA pathways before and after locomotor training in SCI. 

Before locomotor training, polysynaptic actions of late FRA INs on TA MNs are strong, while polysynaptic actions of early FRA INs on TA MNs are weak. After locomotor training, polysynaptic actions of early and late FRA INs on TA MNs reverse and inhibitory actions

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between early and late FRA INs are potentiated. Solid and dotted lines represent strong and weak connections, respectively. FRA = flexor reflex afferents, INs = interneurons, MNs = motoneurons, TA = tibialis anterior.
Ipsilateral late TA flexor reflexes in SCI

A

Bins

Heel contact

1

2

3

4

5

6

7

8

Stance-to-swing

9

10

11

12

13

14

15

Swing initiation

16

Swing-to-stance transition

Could not be evoked

Right leg

B

Left leg

Before locomotor training

After locomotor training
Ipsilateral late TA flexor reflexes in SCI

A. Right leg

B. Ipsilateral late TA flexor reflexes (% reflex values during standing)

C. Subtracted normalized TA late flexor reflexes

D. Left leg

E. Ipsilateral late TA flexor reflexes in SCI

F. TA background EMG activity

Control EMG

Reflex EMG or BGA

Before training

After training

* Before training

* P < 0.05, before vs. after
Ipsilateral early TA flexor reflexes in SCI

A

B

Right leg

Control EMG
Reflex EMG
Before training
After training

P < 0.05, before vs. after

C

Left leg

Control EMG
After training

* P < 0.05, before vs. after

100 mV

400 ms
Ipsilateral early TA flexor reflexes in healthy subjects

A

Standing
Stepping

Bins

Heel contact

Stance-to-swing

Swing initiation

Swing-to-stance transition

1

5

6

7

8

9

10

11

12

13

14

15

16

150 mV

400 ms

B

* P < 0.05, early TA reflex vs. control EMG

Control TA EMG

Early TA reflex EMG

HC

Stance phase

SI

Swing phase

P < 0.05

C

Subtracted normalized early TA

Control TA background EMG

Early TA reflex EMG

HC

Stance phase

SI

Swing phase

P < 0.05

D

Subtracted normalized early TA

Control TA background EMG

Early TA reflex EMG

HC

Stance phase

SI

Swing phase

P < 0.05
Before Locomotor Training

After Locomotor Training
Table 1. Characteristics of SCI participants

<table>
<thead>
<tr>
<th>ID</th>
<th>Gender</th>
<th>Age (yrs)</th>
<th>Post Injury (yrs)</th>
<th>Level</th>
<th>Cause of SCI</th>
<th>AIS scale</th>
<th>Clonus</th>
<th>ASIA (light touch)</th>
<th>ASIA (pin prick)</th>
<th>ASIA (motor)</th>
<th>Medication</th>
<th># of training sessions</th>
</tr>
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<tbody>
<tr>
<td>R03</td>
<td>M</td>
<td>24</td>
<td>3.0</td>
<td>T10</td>
<td>GSW</td>
<td>A</td>
<td>1 1</td>
<td>72</td>
<td>72</td>
<td>0</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>R10</td>
<td>F</td>
<td>52</td>
<td>11</td>
<td>T7</td>
<td>Fall</td>
<td>D</td>
<td>1 0</td>
<td>78</td>
<td>78</td>
<td>16</td>
<td>24</td>
<td>Neurontin: 27mg Baclofen: 60mg</td>
</tr>
<tr>
<td>R13</td>
<td>F</td>
<td>39</td>
<td>7.0</td>
<td>T4</td>
<td>Transverse Myelitis</td>
<td>C</td>
<td>3 3</td>
<td>112</td>
<td>74</td>
<td>9</td>
<td>2</td>
<td>Gabapentin: 0.3g Baclofen: 20mg</td>
</tr>
<tr>
<td>R14</td>
<td>M</td>
<td>25</td>
<td>0.5</td>
<td>C5-C6</td>
<td>Diving</td>
<td>D</td>
<td>1 1</td>
<td>112</td>
<td>110</td>
<td>25</td>
<td>13</td>
<td>None during the study</td>
</tr>
<tr>
<td>R15</td>
<td>M</td>
<td>37</td>
<td>1.0</td>
<td>C1</td>
<td>Spinal Tumor</td>
<td>C</td>
<td>3 2</td>
<td>64</td>
<td>64</td>
<td>12</td>
<td>5</td>
<td>Not known</td>
</tr>
<tr>
<td>R16</td>
<td>M</td>
<td>49</td>
<td>2.5</td>
<td>C5</td>
<td>MVA</td>
<td>C</td>
<td>0 0</td>
<td>64</td>
<td>34</td>
<td>17</td>
<td>12</td>
<td>Baclofen: 15mg</td>
</tr>
<tr>
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<td>M</td>
<td>21</td>
<td>3.0</td>
<td>T10</td>
<td>GSW</td>
<td>D</td>
<td>3 3</td>
<td>105</td>
<td>105</td>
<td>13</td>
<td>15</td>
<td>Baclofen: 60mg Gabapentin: 50.9g</td>
</tr>
<tr>
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<td>M</td>
<td>29</td>
<td>2.0</td>
<td>C7</td>
<td>MVA</td>
<td>D</td>
<td>1 1</td>
<td>86</td>
<td>86</td>
<td>25</td>
<td>21</td>
<td>None</td>
</tr>
<tr>
<td>R20</td>
<td>M</td>
<td>55</td>
<td>3.0</td>
<td>T6-T7</td>
<td>Blood clot during spinal surgery</td>
<td>C</td>
<td>2 3</td>
<td>82</td>
<td>82</td>
<td>8</td>
<td>9</td>
<td>None</td>
</tr>
</tbody>
</table>

i Level of SCI corresponds to neurological injury level. For each subject, the American Spinal Injury Association (ASIA) standard neurological classification of SCI for sensation (sensory light touch and pin prick; out of 112 maximal points) is shown and evaluated as 0=absent, 1=impaired, and 2=normal. ASIA motor score (out of 50 maximal points for each leg) is indicated for the left leg (LL) and right leg (RL) based on the manual muscle test of key muscles and evaluated as 0=No contraction, 1=Flicker or trace of contraction, 2=Active movement, with gravity eliminated, 3=Active movement against gravity, 4=Active movement against gravity and resistance, 5=Normal
power. Values for ASIA sensory and motor scores are indicated from clinical evaluation tests conducted before training. Ankle clonus was assessed as follows: 0 = reaction, 1 = mild, clonus was maintained less than 3 s, 2 = moderate, clonus persisted between 3 and 10 s, 3 = severe, clonus persisted for more than 10 s. Medication for each subject is indicated as total mg taken per day. C=cervical. T=thoracic. MVA=motor vehicle accident. GSW: gunshot wound. M=male. F=female. LL=left leg. RL=right leg.