ONLINE METHODS

Animals and animal care. All procedures followed the US National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* and were approved by the Animal Use Committee at the University of California, Los Angeles and the Veterinary Office of the Canton of Zurich. These experiments were conducted on adult female Sprague-Dawley rats (~300 g), housed individually on a 12-h light/dark cycle with access to food and water *ad libitum*. **Supplementary Figure 2** describes the time line of the surgical and experimental procedures, as well as the different groups of rats that were included in the present study.

Surgical procedures and post-surgical care. All procedures have been described in detail previously[2,12,19]. Briefly, a combination of ketamine (100 mg per kg) and xylazine (10 mg per kg) was used to induce anesthesia, which subsequently was maintained with isoflurane (1–2.5%) via facemask. Under aseptic conditions, a partial laminectomy was performed over spinal segments L2 and S1. Teflon-coated stainless steel wires (AS632, Cooner Wire) were passed under the spinous processes and above the dura matter of the remaining vertebrae between the partial laminectomy sites. After removing a small portion (~1 mm notch) of the Teflon coating to expose the stainless steel wire on the surface facing the spinal cord, we secured the electrodes at the midline of the spinal cord at spinal level L2 and S1 by suturing the wire to the dura mater above and below the electrode (**Supplementary Fig. 1**). A common ground wire (~1 cm of the Teflon removed at the distal end) was inserted subcutaneously in the mid-back region.

Bipolar intramuscular EMG electrodes using the same wire type as above were inserted bilaterally in the mid-belly of the soleus and deep (close to the bone) mid-belly of the tibialis anterior muscles, or unilaterally into the deep mid-belly of the vastus lateralis, distal deep compartment of the semitendinosus and medial deep region of the medial gastrocnemius. All electrode wires were connected to a percutaneous amphenol connector cemented to the skull of the rat. The proper location of the epidural and EMG electrodes was verified post-mortem. After 2–3 weeks of recovery from surgery, baseline recordings were obtained (see below). The rats then underwent a second surgical procedure to receive the SCI. A partial laminectomy was made at a mid-thoracic level (~T7) and the spinal cord was completely transected. Gel foam was inserted into the gap created by the transection as a coagulant and to separate the cut ends of the spinal cord. The completeness of spinal cord transections was verified by two surgeons by lifting the cut ends of the cord during the surgery as well as histologically post-mortem.

Pharmacological and EES interventions. To encourage locomotion via EES, we delivered rectangular pulses (0.2 ms duration) at 40–50 Hz[19] using a dual-output Grass S88 stimulator (Grass Instruments) through two constant-voltage isolation units (Grass SIU5, Grass Instruments) connected to the L2 and S1 epidural electrodes. The stimulation intensity (1–4 V) was adjusted to obtain optimal facilitation of stepping visually. Pharmacological modulation was induced by administrating quipazine (0.2–0.3 mg per kg) intraperitoneally and 8-OHDPAT (0.05–0.3 mg per kg) subcutaneously[9]. Serotonin agonists were injected 10 and 15 min before locomotor training and behavioral testing, respectively.

Locomotor training. An upper-body harness was used to position the rats over a treadmill belt and to partially support their body weight during bipedal locomotion (**Supplementary Fig. 1**). An automated, servo-controlled body-weight support system measured and provided the optimal amount of body-weight support that each rat needed to step. Rats were trained every other day, 20 min per session for 8 weeks, starting 8 d after spinal cord transection. Locomotor training was enabled by EES at S1 and L2, by quipazine and 8-OHDAPT, and by the combination of both. Treadmill belt speed was set at 9 cm s^{-1}. Nontrained rats were housed similarly to the trained rats, but did not receive serotonin agonists or EES and were not placed in the treadmill apparatus, except for the final testing session when all rats were tested under the same conditions.

Spinal cord reflexes. Monosynaptic motor potentials were evoked during bipedal standing with constant weight bearing (20% of body weight) by delivering rectangular pulses (0.5-ms duration) through the S1 electrode at 0.2 Hz[49]. Before the injury, we identified the stimulus intensity that elicited the largest monosynaptic responses in the absence of direct muscle responses (direct stimulation of the motor nerve, equivalent to M waves), typically 1.5–2-fold greater than motor threshold[49], and used this intensity to test reflexes at 1 and 9 weeks post-injury. The efficacy of monosynaptic inputs to flexor and extensor motoneuron pools was measured as the peak-to-peak amplitude of averaged ($n = 10$) motor-evoked potentials recorded from the tibialis anterior and soleus muscles bilaterally (10 kHz).

Kinematics and EMG analyses of locomotion. Three-dimensional video recordings (100 Hz) were made using four cameras (Basler Vision Technologies) oriented at 45° and 135° bilaterally with respect to the direction of locomotion or the motion capture system VICON by means of 8 infrared television cameras (200 Hz). Reflective markers were attached bilaterally at the iliac crest, greater trochanter, lateral condyle, lateral malleolus, MTP and the tip of the toe (**Supplementary Fig. 1**). SIMI motion capture software (SIMI Reality Motion Systems) and Nexus (Vicon) were used to obtain three-dimensional coordinates of the markers. The body was modeled as an interconnected chain of rigid segments and joint angles were generated accordingly.

EMG signals (2 kHz) were amplified, filtered (10–1,000-Hz bandpass), stored and analyzed off-line to compute the amplitude, duration and timing of individual bursts. To evaluate coactivation between muscles, we generated probability density distributions of normalized EMG amplitudes of specific pairs of muscles during continuous treadmill stepping sequences, as described previously[3]. In some recordings (**Fig. 4**), vertical reaction forces were monitored using a biomechanical force plate (2 kHz, HE6X6, AMTI) located below the treadmill belt (**Supplementary Fig. 1**). During the long bouts of recording under different load and speed conditions, a rigid circular (diameter, 0.5 mm) stick was inserted between the rat's limbs to prevent them from crossing.

Ten successive step cycles were extracted for both the left and right hindlimbs from a continuous sequence of treadmill stepping for each rat under each condition. A 10-s interval was used when no stepping movements were observed. A total of 135 parameters quantifying gait timing, joint kinematics, limb endpoint trajectory and EMG activity were computed for each gait cycle according to methods described previously (**Supplementary Table 1**)[2,3,50].

Gait parameters. Gait cycles were defined as the time interval between two successive paw contacts of one limb. Successive paw contacts were visually defined by the investigators with an accuracy of ±1 video frame (100 Hz). The onsets of the swing phases were set at the zero crossings of the rate of change of the elevation angle of the limb axis (virtual segment connecting the crest to the MTP), that is, at the onset of forward oscillation. Cycle duration and stance and swing durations were determined from the kinematic recordings. Footfall patterns were used to compute the coupling between the hindlimbs. In particular, the time at which the contralateral limb contacted the treadmill belt was expressed as a percentage of the duration of the ipsilateral gait cycle and represented in polar coordinates (**Fig. 1**). On these polar representations, 50% indicates that the limbs move out of phase, whereas 0% typically corresponds to jumps after which both feet contact the treadmill belt simultaneously or to the absence of movements. Interlimb coordination was statistically computed as the r value at $t = 0$ of the cross-correlation function between the oscillation of the left and right limb axis. An r value of −0.5 indicates that both hindlimbs oscillate perfectly out of phase. The variability of all parameters was measured as the coefficient of variation computed separately for left and right hindlimbs.

Limb endpoint trajectory. Limb endpoint trajectories were analyzed on the basis of the *xyz* motion of the MTP marker. The computed spatial parameters included stride length, three-dimensional linear path, step height, and distance at swing and stance onset between the limb endpoint and the hip in the direction of walking[50]. The extent of paw dragging during swing was computed as the time during which the paw was in contact with the treadmill belt after swing onset. Intensity and direction of foot velocity at swing onset were computed as the module and pitch angle of the MTP velocity vector in the sagittal plane (direction of walking), respectively. Limb endpoint acceleration at swing onset and maximum limb endpoint velocity during swing were measured. We used principal component analysis to quantify spatial consistency of hindlimb endpoint trajectory. Spatial coordinates of hindlimb endpoint trajectory were extracted from the selected sequence of stepping and separated into their x, y and z components. For each gait cycle, each step component was resampled to 100 data points, thus removing the temporal information. The data were then arranged into three $m \times n$ matrices, with each column containing the data for a single step and each row containing

the interpolated position values at each time step. Matlab (Mathworks) scripts were written to extract principal components from each dataset. The consistency of limb endpoint trajectory during swing was measured as the amount of variance explained by the first principal component.

Stability. Stance width was measured for each gait cycle as the perpendicular distance (medio-lateral plane) between left and right MTP markers at the time of paw contact. The degree of pelvis stability was evaluated as the s.d. of pelvis oscillations in the sagittal and medio-lateral planes during the analyzed sequence of stepping.

Hindlimb kinematics. Maximum joint angle positions in flexion and extension were computed as the minimum and maximum values of each angle over the time course of each gait cycle, respectively. The amplitude of joint angular excursions was measured as the difference between maximum positions in flexion and extension. We used cross-correlation functions to assess the coordination among joints. Cross-correlations among all unique pairs of degrees of freedom (that is, hip, knee, ankle and MTP) were calculated and maximum *r* values extracted to quantify the degree of correlation among a given pair of joints[1]. The timing in the coupling between oscillations of adjacent segments (thigh, leg, foot and toe) was quantified for each gait cycle as the relative lag at the maximum *r* value of the cross-correlation function between both angles. To quantify the degree of similitude in the profile of joint angles and joint angle velocities between pre- and post-injury stepping patterns, we computed cross-correlation functions between mean waveforms. The procedure was repeated for each rat, joint angle, joint angle velocity and side. The highest positive *r* value determined the degree of similitude between pre- and post-injury waveforms.

Variability of joint motion. To assess the variability of joint angles across consecutive gait cycles, we computed the s.d. of time-normalized joint angles every 10% of the normalized time base. Variability of each joint angle from both left and right sides was measured as the mean of s.d.

PCA. Performance of gait implies the rhythmic repetition of stereotypical patterns of leg motion. Several parameters need to be used to thoroughly characterize a given gait pattern; here, we computed 135 variables that provided detailed quantification of kinematic and EMG features underlying specific patterns of locomotion. Such high dimensionality substantially complicates the extraction of relevant parameters to account for differences between experimental conditions. However, reduction of such multidimensional datasets can be achieved via multivariate statistical analysis such as PCA. PCA is mathematically defined as an orthogonal linear transformation that transforms the original dataset to a new coordinate system, such that the variance is maximized on each new coordinate axis. Here, data were analyzed using the correlation method, which adjusts the mean of the data to 0 and the s.d. to 1. This is a conservative method and is appropriate for variables that differ in their variance. For each rat and condition, we computed the averaged values of the 135 original variables (**Supplementary Table 1**) and ordered them in a matrix, with the variables along the columns and the observations (data from each rat under a given condition) along the rows. Principal components were then extracted on different sets of conditions (for example, combinations of interventions or different locomotor training conditions). A few principal components, typically three, were sufficient to account for more than 50% of the total variance of the studied samples, demonstrating the high correlation between the variables describing gait. The degree of similarities and differences between the rats and conditions was evaluated as the difference in the factor coordinates of each observation (scores) on each principal component axis. To visualize the differences between rats and conditions, we plotted coordinates from each rat under a specific condition (observation) in the new space created by the first three principal components. In this representation, the distance between the data points increases (nonlinearly) with the difference between the underlying locomotor characteristics, allowing us to readily visualize differences and similarities between rats and conditions. Next, we identified the variables that contributed to account for the differences between the experimental conditions. This classification is based on the analysis of factors loadings, that is, correlations between each variable and each principal component. To display the results of this analysis, we created a color-coded representation (**Figs. 1n** and **4f**) of correlation values for all the computed variables with principal components. In this representation, variables that correlate positively or negatively with a given principal component identified clusters of parameters that account for a specific difference between conditions. Accordingly, each principal component tends to explain distinct differences between rats and experimental conditions.

FOS immunohistochemistry. FOS immunoreactivity of spinal neurons was determined as previously described[2]. Briefly, after a 45-min bout of continuous hindlimb bipedal stepping under pharmacological (quipazine and 8-OHDPAT) and EES (S1 and L2) interventions, the rats were returned to their cages and were anesthetized and killed by intracardial perfusion of 4% paraformaldehyde (wt/vol) in phosphate buffer about 60 min later. After perfusion, the spinal cords were grossly dissected with the vertebral column and post-fixed overnight (4 °C). Spinal cords then were dissected carefully and cryoprotected in 30% sucrose (wt/vol) in phosphate buffered saline (PBS) for at least 3 d. Segments L1 through S2 were mounted and frozen and 30-µm coronal sections were obtained using a cryostat. We processed one section of each spinal segment for locomotor-trained, nontrained and noninjured rats simultaneously for FOS immunoreactivity. Free-floating sections were submerged in 0.3% H_2O_2 (wt/vol, 10 min) and subsequently washed in PBS (three times for 10 min). Sections were incubated in the primary antibody to the c-fos protein product FOS (rabbit, polyclonal; Santa Cruz Biotechnology) at 1:1,000 for 48 h (4 °C). Sections then were washed in PBS (30 min) and incubated in the secondary antibody (biotinylated goat antibody to rabbit; Jackson ImmunoResearch) at 1:100 for 1 h. After PBS washes (30 min), sections were incubated for 1 h in a 1:100 Vectastain Elite ABC (avidin–biotin peroxidase complex) solution (Vector Laboratories). Sections were washed again in PBS and reacted with diaminobenzidine solution (Sigma-Aldrich) for about 2–3 min. Finally, sections were rinsed in PBS (30 min), mounted on microscope slides, air dried, counterstained with Methyl Green and coverslipped with Permount. FOS-positive nuclei were counted manually using camera lucida techniques and superimposed onto Molander's cytoarchitectonic maps of the rat lumbosacral cord. Counts were performed blindly for each spinal segment and for each rat.

Muscle weights. After perfusion, the left hindlimb was dissected blindly and the following muscles and muscle groups extracted: triceps surae (soleus, medial gastrocnemius, lateral gastrocnemius), quadriceps (rectus femoris, vastus lateralis, vastus medialis, vastus intermedius), tibialis anterior, plantaris and extensor digitorum longus. After removal of excess fat and connective tissues, the muscles or muscle groups were weighed and normalized to the body weight of the rat at the time of perfusion.

Statistical analyses . All data are reported as mean values ± s.e.m. One way ANOVAs, repeated-measures ANOVAs or one way repeated-measures ANOVAs were used, depending on the conditions, to test differences between groups and/or conditions on the experimental parameters. The factors examined were the presence of EES (L2 and/or S1) and serotonin agonists (quipazine and/or 8-OHDPAT), body sides (left, right), time points (pre-injury and 1 and 9 weeks post-injury), training status (nontrained, trained), and task conditions (standing, stepping, load, speed, direction). *Post hoc* differences were assessed using the Newman-Keuls test. The software package Statistica was used for all analyses.

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