J Comp Physiol A (2013) 199:1105–1116
DOI 10.1007/s00359-013-0853-3

ORIGINAL PAPER

Removing sensory input disrupts spinal locomotor activity in the early postnatal period

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Received: 7 June 2013 / Revised: 30 August 2013 / Accepted: 1 September 2013 / Published online: 17 September 2013

Abstract Motor patterns driving rhythmic movements of our lower limbs during walking are generated by groups of neurons within the spinal cord, called central pattern generators (CPGs). After suffering a spinal cord injury (SCI), many descending fibers from our brain are severed or become nonfunctional, leaving the spinal CPG network without its initiating drive. Recent studies have focused on the importance of maintaining sensory stimulation to the limbs of SCI patients as a way to initiate and control the CPG locomotor network. We began assessing the role of sensory feedback to the locomotor CPG network using a neonatal mouse spinal cord preparation where the hindlimbs are still attached. Removing sensory feedback coming from the hindlimbs by way of a lower lumbar transection or by ventral root denervation revealed a positive correlation in the ability of sensory input deprivation to disrupt ongoing locomotor activity on older versus younger animals. The differences in the motor responses as a function of age could be correlated with the loss of excitatory activity from sensory afferents. Continued studies on this field could eventually provide key information that translates into the design of novel therapeutic strategies to treat patients who have suffered a SCI.

Keywords Central pattern generator · Locomotion · Mouse · Spinal cord · Sensory input

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Introduction

For decades, it has been known that networks of interneurons located within the spinal cord are responsible for generating locomotion. These networks interact with specific sensory information to allow the reinforcement as well as modifications in the organization of locomotor behaviors (Grillner 1981). The connected ensembles of spinal neurons are defined as central pattern generators (CPGs) and are capable of generating locomotor-like neural activity independently of supraspinal (descending) and afferent (peripheral) input (Grillner and Zangger 1975). The basic principles of CPG function have been extensively studied in invertebrate model systems and locomotion has been detailed in a primitive fish—the lamprey (see Grillner 1981; Grillner et al. 1995; Marder 1998). In terrestrial mammalian systems of overground locomotion such as the cat, it has been shown that a spinal cord transection does not hinder the capability of relearning or re-developing hindlimb stepping in the absence of input from the brain (Lovely et al. 1986; Barbeau and Rossignol 1987; De Leon et al. 1998).

While sensory afferents are not essential for producing the patterned locomotor output in animals deprived of descending (brain) inputs, afferent input is an important source of feedback from the ongoing movements. Afferent inputs are involved in the modulation and correction of parameters such as speed, step cycle, burst duration and interlimb coupling in an ongoing locomotor pattern (see Rossignol et al. 2008). Moreover, in the absence of descending input, locomotion can be recruited and controlled by an ongoing sensory afferent input from the lower limbs (see Edgerton et al. 2008). Studies conducted by these groups and others have demonstrated that combinational mechanical (treadmill and robotic training),

pharmacological (agonists of excitatory pathways and/or antagonists of inhibitory pathways; neurotrophic factor application) and electrical (muscle and/or epidural) strategies re-instate near normal locomotion in animals, and have provided much optimism on the improvement of locomotor function in humans with a spinal cord injury (SCI) (Rossignol 2006; Harkema 2007; Rossignol et al. 2008; Edgerton et al. 2008; Gerasimenko et al. 2008; Courtine et al. 2009; Harkema et al. 2011).

Most previously and presently published scientific work regarding the understanding of the organization of the mammalian spinal CPG network for locomotion at the cellular level have involved studies using the rodent isolated spinal cord preparation (for recent reviews, see Dougherty and Kiehn 2010; Guertin 2009). Physiological and molecular genetics experiments in this preparation have started to shed light on the basic circuit organization responsible for walking in mammals. Even though these experiments have been essential in the development of our present understanding of the organization of the mammalian neuronal network controlling locomotion, the effects of sensory information to spinal locomotor network function have been largely unexplored. We began assessing the role of sensory feedback to the locomotor CPG network using a novel neonatal mouse spinal cord preparation, where the hindlimbs are still attached (Hayes et al. 2009); the loss of descending brain and brainstem inputs was mimicked by a cervical decapitation, and the loss of sensory input was induced by either an upper sacral transection or by ventral root denervation. We show that removing sensory feedback coming from the hindlimbs by way of a lower lumbar transection or by ventral root denervation revealed subtle and, in some cases, drastic changes in motor neuron burst amplitude and even cessation of the locomotor rhythm itself. There was a positive correlation of age with the ability of sensory input deprivation to disrupt ongoing locomotor activity, with the majority younger animals (P0–early P1) not showing significant effects after sensory input deprivation as compared to older animals (late P1–P2), where over 80 % showed significant reduction in motor activity including complete cessation. The disruption of locomotor activity in older animals was correlated to the loss of excitatory sensory drive as recorded from dorsal nerve roots. These results suggest a developmental relationship between spinal motor function and its dependence on sensory input in the absence of supraspinal inputs.

Materials and methods

Experiments were performed using spinal cords of 0- to 2-day-old (P0–P2) ICR mice (Charles River, Wilmington, MA). Animals were killed by rapid decapitation. The spinal cord was exposed by ventral laminectomy under ice-cold (4 °C) oxygenated (95 % O_2–5 % CO_2) low-calcium Ringer solution composed of (in mM): 128 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 0.25 $CaCl_2$, 1.3 $MgCl_2$, 3.25 $MgSO_4$, 25 $NaHCO_3$, and 22 D-glucose. The spinal cord from segments C5 to S3 was pinned ventral-side up using part of the dorsal part of the rib cage and superfused with oxygenated normal Ringer solution composed of (in mM): 111 NaCl, 3.08 KCl, 25 $NaHCO_3$, 1.18 KH_2PO_4, 1.25 $MgSO_4$, 2.52 $CaCl_2$, and 11 D-glucose. In order to assess the effects of sensory input on locomotor activity, a whole spinal cord preparation still connected to the hindlimbs was used (Fig. 1). The preparation used in this proposed work was adapted from an isolated in vitro neonatal rat spinal cord oriented dorsal-up with intact hindlimbs developed recently by Hayes et al. (2009).

Locomotor-like activity was evoked by perfusion with mouse Ringer solution containing a combination of 5-HT (9–12 μM), NMDA (6–9 μM) and dopamine (18 μM). Small-diameter suction electrodes were placed: (1) on both L2 ventral roots to monitor alternating flexor activity in the motor pattern from the right and left sides, (2) on both L5 ventral roots to monitor alternating extensor activity or (3) on the L2 and L5 ventral roots to record alternating motor activity between flexor and extensor motor neuron pools as previously done (Kiehn and Kjaerulff 1996). Sensory input deprivation was induced by performing a lower lumbar–upper sacral transection with an ultra-sharp scissor; this cut will include the whole spinal cord and partial removal of surrounding ribcage. We additionally deprived sensory input by cutting all lumbar and upper sacral ventral roots with ultra-sharp micro-scissors; this will automatically disrupt any noticeable lower limb muscle contractility. All experiments include at least five preparations ($n = 5$ or greater) at each time point (15, 30 and 60 min) after performing either the sacral transecion or the ventral nerve root denervation sensory deprivation methods. Ventral and dorsal root recordings were band-pass-filtered (100 Hz–1 kHz) and recorded using an AC amplifier (Model 1600 from A-M systems). The locomotor rhythm was allowed to stabilize over 20 min, after which the sensory deprivation cutting methods were used. Serotonin (5-HT), *N*-methyl-D-aspartate (NMDA) and dopamine were purchased from Sigma and diluted in regular mouse Ringer.

Data analysis

Locomotor-like activity was recorded in the intact spinal cord preparation during bath application of 5-HT, NMDA and dopamine. Clampfit 9.0 (Molecular Devices), Excel (Microsoft, Seattle, WA), and Spike 2 (Cambridge Electronic Design, Cambridge, UK) were used for data

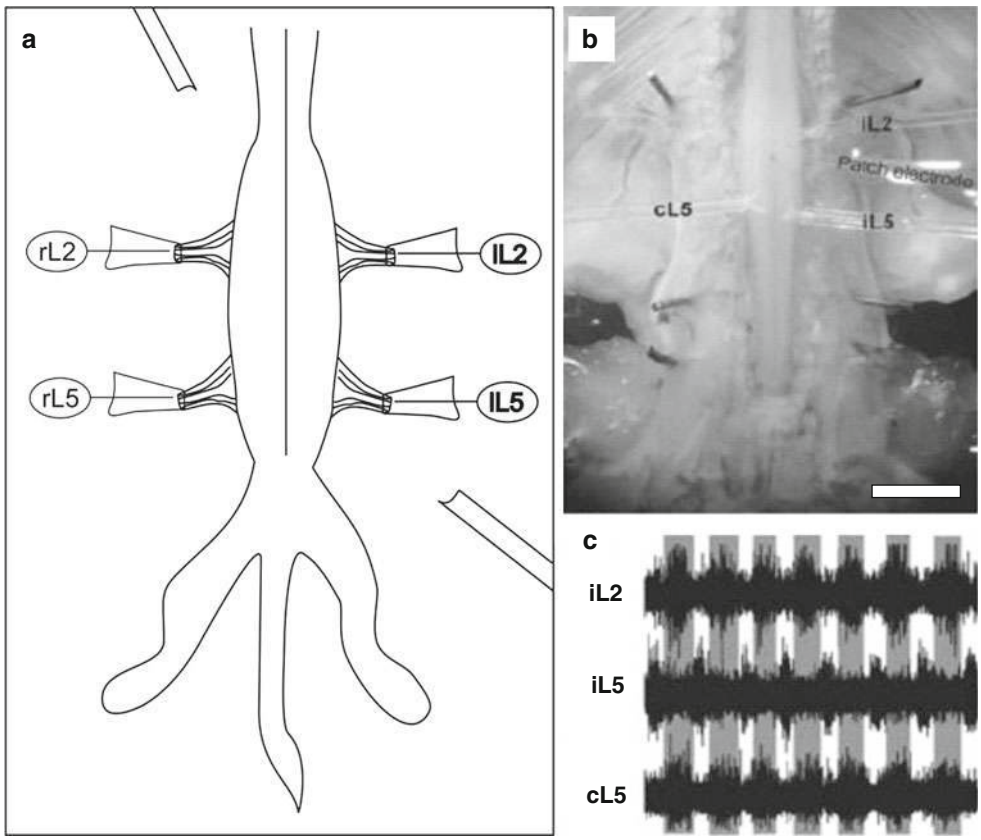

Fig. 1 Spinal cord hindlimbs-attached preparation. **a** Suction recording electrodes were placed to monitor motor activity from ventral root nerves before, during and after perfusion of drugs. **b** Actual preparation showing simultaneous extracellular recordings. **c** Extracellular recordings showing rhythmic locomotor-like activity from lumbar nerve 2 and the left and right 5th lumbar nerves (*scale bar* in **b** *panel* 3 mm)

analysis. A cycle of motor nerve activity started at the onset of either an L2 or L5 ventral root burst and ended at the onset of the next L2 or L5 ventral root burst recorded from the same nerve; these onsets were determined by a custom-made program in Spike 2 (courtesy of Dr. Thomas Cleland, Cornell University) to detect when the rectified signal exceeded the average noise level between bursts by a preset amount. Measurements of burst amplitude (measured from trough to crest) were determined by analysis of rectified and normalized L2 or L5 activity using Spike 2 software. Averages of burst amplitude were determined from all locomotor bursts that occurred once a stable pattern of locomotor-like activity had been established (after a minimum of 15–20 min of stable locomotor activity was recorded). Circular statistics (Zar 1974) were used to determine the coupling strength between opposing L2 and L5 ventral roots. Left or right L2 bursts occurring over a continuous 5 min interval were selected, and their phase values were calculated in reference to either the onsets of each left or right L5 burst, respectively (they were always located in the same side in order to have alternation). Phase values were determined by dividing the latency between the onset of the first L2 burst and the following burst in L5 by the step cycle period (time between the reference L2 burst and the next L2 burst). Locomotor steps in which the ipsilateral L2 and L5 roots were completely out of phase (i.e., appropriate flexor–extensor alternation) had phase values of approximately 0.5. Those completely in phase (co-bursting) had phase values of around 0. The r values are a measure of the concentration of phase values around the mean value for alternation (0.5). An r value of 1 indicates that all the phase values are 0.5, whereas an r value of 0 indicates that the phase values are distributed randomly.

Statistical comparisons between experimental conditions were made using one-way repeated measures ANOVA followed by a Fisher's LSD post hoc test if the data were normally distributed and had equal variance. Otherwise, the data were compared using a Mann–Whitney rank sum test. Results were considered statistically significant at $p < 0.05$. Data are expressed as mean ± SD. Figures were compiled using Sigma Plot 10, Photoshop and Corel Draw.

Results

We performed our experiments using mice that were between 0 and 2 days postnatal (P0–P2). A motor pattern was obtained neurochemically using serotonin (5-HT; 9–12 μM), the glutamate analog NMDA (6–9 μM) and dopamine (18 μM) as previously published (Kiehn et al.

1996). Alternating locomotor-like activity was assessed with extracellular nerve recordings and visual confirmation of alternating hindlimb movements. We performed sensory deprivation experiments in only those spinal cord hindlimbs-attached preparations which showed stable rhythmic hindlimb activity (over 20 min) after adding the drug cocktail.

Limb deafferentation was achieved via a complete transection at the upper sacral level (around S1–S2) just above the hip bones of P0 animals. The rationale for using a sacral transection stemmed from previous studies in which an SCI was produced by either a compression or a cut of the spinal cord at a specific spinal level in cats and rodents (see Edgerton et al. 2008; Rossignol and Frigon 2011). We expected that by severing the spinal cord, as well as all ventral and dorsal roots, we will induce an almost immediate stoppage of the flow of afferent (sensory) and efferent (motor) information, thus perturbing the ongoing (drug-induced) locomotor rhythm. We checked for any significant changes in the either burst amplitude or the phase of the locomotor pattern (alternating versus non-alternating) at 15, 30 and 60 min after performing the sacral transection. After 1 h of deafferentation, no significant changes were seen in burst amplitude (Fig. 2a1; $n = 5$) or locomotor coordination between the left and right L2 or between the right L2 and the right L5 ventral roots (Fig. 2a2; $n = 5$). The average burst amplitude in control P0 preparations was 4.1 ± 2.8 mV. After 60 min of sensory deprivation, the average burst amplitude in these P0 preparations changed to 3.8 ± 1.5, which was not statistically significant. However, the same deafferentation protocol on P1 mice induced a progressive decrease in burst amplitude (Fig. 2b1; $n = 5$) coupled with a loss in the alternating phase of the pattern (Fig. 2b2; $n = 5$). A locomotor-like rhythm could not be seen in three of the five preparations within an hour after the cut (Fig. 2b; $n = 5$). The average time that it took to initiate the progressive loss of the locomotor pattern was 37 ± 8.5 min. The average burst amplitude in P1 control preparations was 3.3 ± 1.5 mV. After 60 min of sensory deprivation, the average burst amplitude in these P1 preparations changed to 0.5 ± 1.3, which was statistically significant. It is important to mention that even though two preparations did not completely lose rhythmic motor activity after sensory deprivation, the denervation still had significant effects on burst amplitude and in the alternating phase of the pattern (data not shown) on these preparations. In P2 preparations, deafferentation led to an even more rapid dissolution of rhythmic motor activity (within 20 ± 9.5 min) with one preparation losing all rhythmic activity within 15 min following deafferentation (Fig. 2c1, 2; $n = 5$), including a complete cessation of rhythmic motor activity in four of five preparations with one of five preparations not losing alternating activity, but still showing a significant reduction in burst amplitude (data not shown). The average burst amplitude in control P2 preparations was 5.5 ± 3.3 mV, which was significantly reduced to 1.5 ± 1.7 mV after 60 min of sensory deprivation.

All preparations were left with ongoing perfusion of drugs (5-HT, NMDA and DA) for at least an hour with no recovery of rhythmic activity. In order to eliminate the possibility that the loss of the locomotor rhythm could be due to over-excitation caused by prolonged exposure to the drugs, control experiments were performed after obtaining a stable rhythm. No sacral transection was made and the preparations were left untouched for at least 90 min to 2 h with continuous drug perfusion. None of the preparations ($n = 3$) displayed a loss of the locomotor rhythm, although a small but significant decrease in burst amplitude and the speed of alternating motor activity (the hindlimbs never stopped alternating in all preparations tested) was seen after 1 h of continuous perfusion in two of the preparations (data not shown) which could be attributed to preparation fatigue (lack of oxygenation to more internal areas of the cord).

Since the sacral transection method included cutting the spinal cord and ventral/dorsal nerve roots (including some muscle and bone from the rib cage), we repeated these experiments using a less invasive sensory deprivation method to eliminate the possibility that the observed effects were due to an injury-related effect rather than from sensory input deprivation. Instead of a sacral cut (transection), we repeated sensory deprivation experiments on P0–P2 spinal cord hindlimbs-attached preparations by cutting all lumbar and sacral ventral nerve roots at the point where they exit the spinal cord. This manipulation will interrupt the flow of efferent (motor) drive from the spinal CPG network to the hindlimbs which stops muscle contraction (including cessation of rhythmic alternating hindlimb movements) and lead to a cessation of afferent (sensory) input through the still intact dorsal nerve roots. The rationale for cutting just the ventral roots was to interrupt the flow of efferent (motor) control to the hindlimb muscles, thus reducing and eventually stopping the flow of afferent (sensory) information "feeding" back in the spinal CPG network through the dorsal nerve roots due to the loss of muscle contraction. Even though we did not interrupt the flow of cutaneous (skin)-related information since the afferent fibers (dorsal nerve roots) were left intact, we expected to obtain similar results as with the sacral transection method. This prediction is based on our theory that the disruption of locomotor activity is due to the deprivation of muscle spindle and Golgi tendon-related sensory information (not cutaneous-related sensory input) through the cessation of motor output by cutting the ventral nerve roots, thus interrupting the motor–sensory information loop.

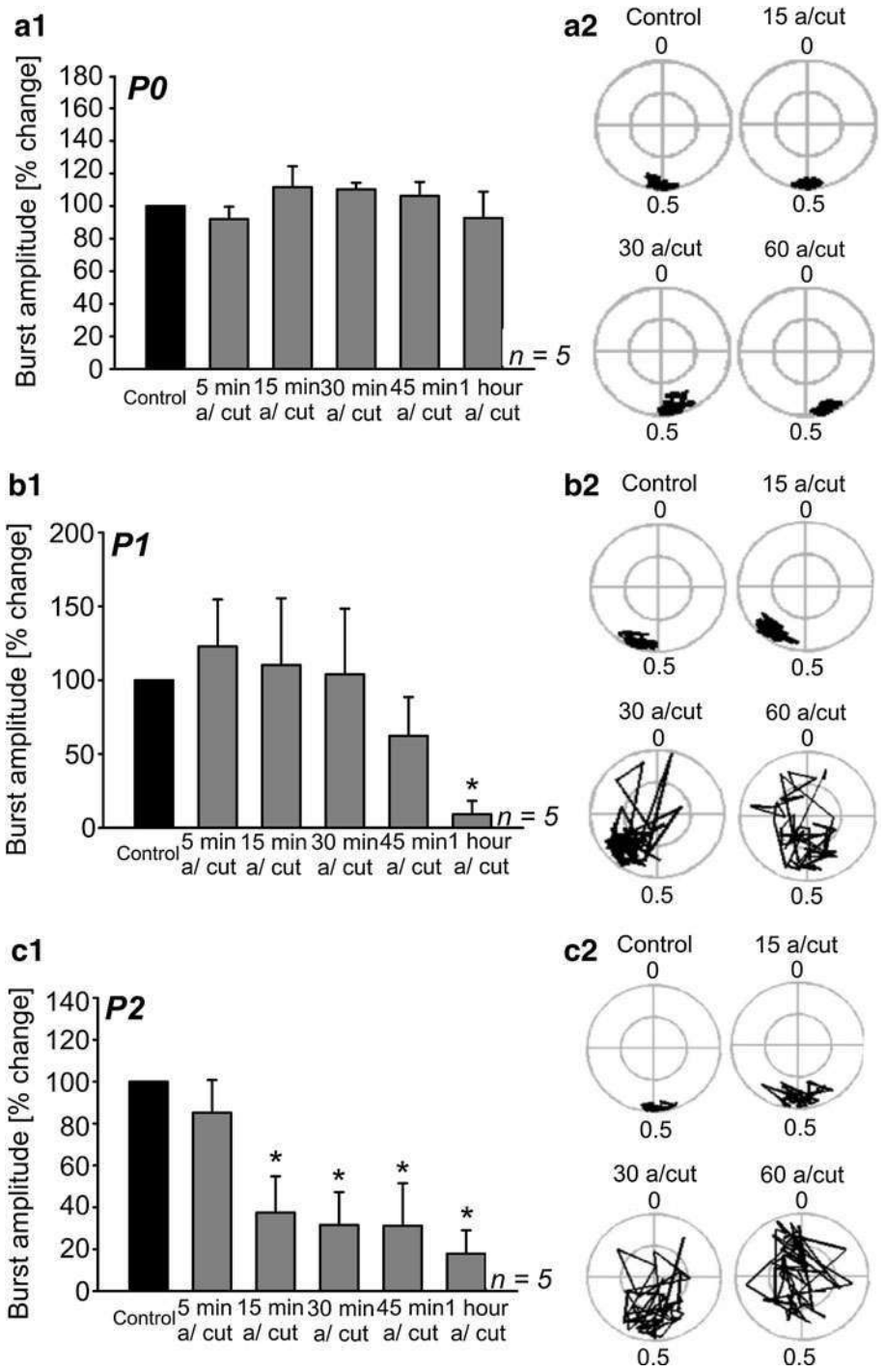

Fig. 2 Effects of sensory deprivation on P0, P1 and P2 mouse spinal cord hindlimbs-attached preparations. All measurements were performed 15, 30 and 60 min after sensory deprivation via sacral transection. **a1** Mean data on the effects of the loss of sensory input on the burst amplitude of the ventral L2 and L5 nerve recordings from P0 mouse spinal cord preparations ($n = 5$). **a2** Circular plots showing the effects of sensory feedback to the phasing between the rL2 and rL5 ventral roots. (Note: cycles of motor burst activity located at 0.5 are considered in alternation, while 0 is considered in synchronous activity.) **b** Similar recording as in **a**, but from a P1 mouse spinal cord lower limb-attached preparation. **c** Similar recording as in **a**, but from a P2 mouse spinal cord lower limb-attached preparation ($n = 5$ for each age group)

Following 20 min of stable drug-induced (5-HT, NMDA and DA) alternating locomotor activity, all lumbar and sacral ventral roots were severed, eliminating the alternating locomotor activity in the hindlimbs of the animals due to the loss of muscle contractions. Sensory deprivation via ventral nerve root cuts had no significant effects on four of the five P0 preparations with just one of the five preparations losing rhythmic motor activity within 30 min after cutting (Fig. 3a1, 2). The average burst amplitude in control P0 preparations was 2.0 ± 1.7 mV which was reduced to 1.9 ± 1.3 mV after 60 min of sensory deprivation, but this result was not statistically significant. Sensory deprivation via ventral nerve root cutting had significant effects on three of the five preparations in P1 mice with two of the five preparations losing locomotor-like activity within 60 min after sensory deprivation (Fig. 3b1, 2). The average burst amplitude in P1 preparations was 3.1 ± 1.4 mV which was significantly reduced to

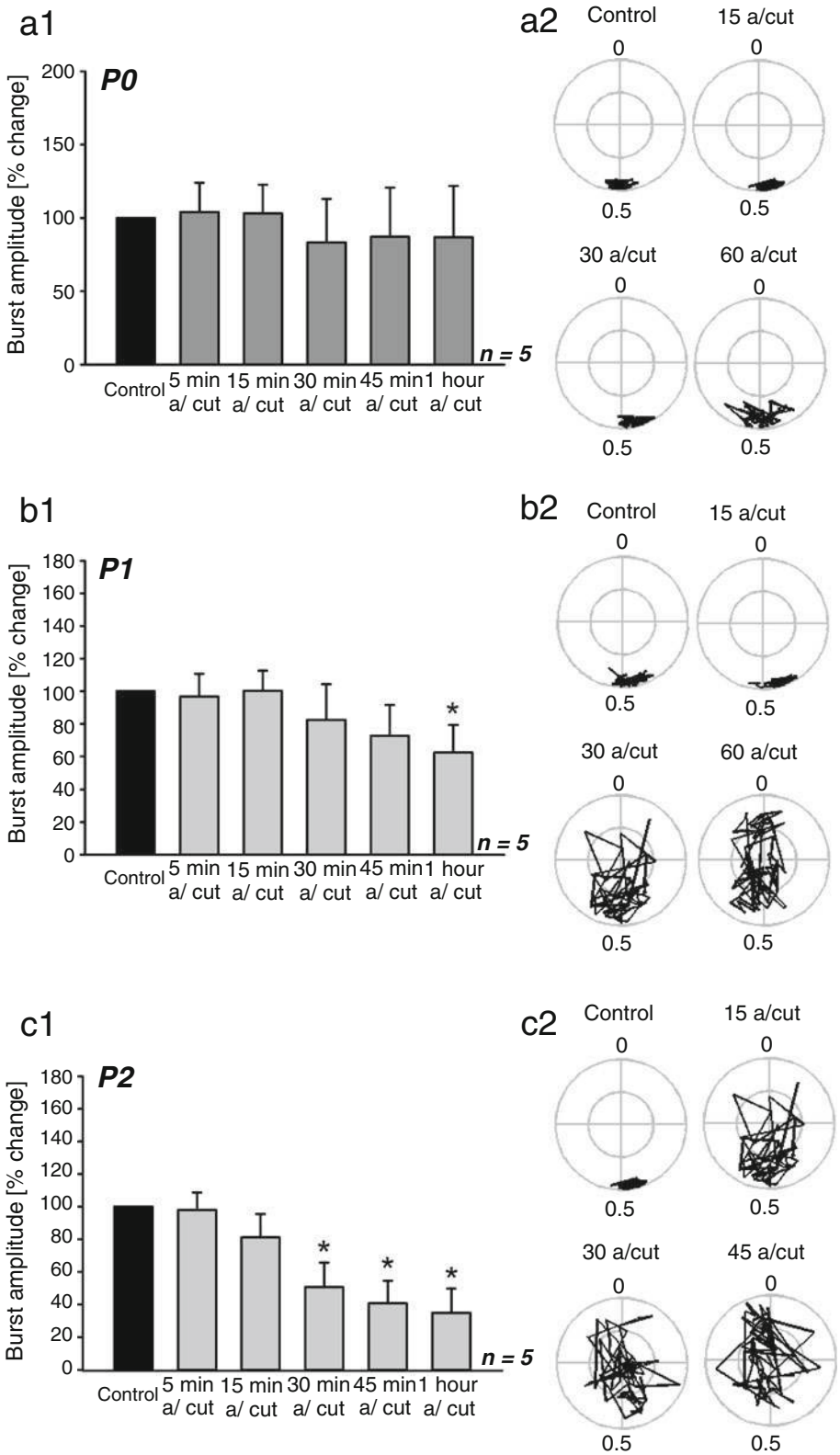

Fig. 3 Effects of sensory deprivation on P0, P1 and P2 mouse spinal cord preparations. All measurements were performed 15, 30 and 60 min after sensory deprivation after cutting all lumbar ventral nerve roots. **a1** Mean data on the effects of the loss of sensory input on the burst amplitude of the ventral L2 and L5 nerve recordings from P0 mouse spinal cord preparations ($n = 5$). **a2** Circular plots showing the effects of sensory feedback to the phasing between the rL2 and rL5 ventral roots (Note: cycles of motor burst activity located at 0.5 are considered in alternation, while 0 is considered in synchronous activity). **b** Similar recording as in **a**, but from a P1 mouse spinal cord lower limb-attached preparation. **c** Similar recording as in **a**, but from a P2 mouse spinal cord hindlimbs-attached preparation ($n = 5$ for each age group)

1.7 ± 1.2 mV after 60 min of sensory deprivation. Finally, we tested the effects of ventral nerve root cutting and subsequent sensory input loss on the drug-induced rhythmic motor patterns of P2 spinal cord hindlimbs-attached preparations. Sensory deprivation induced a cessation of locomotor activity in four of the five preparations. Loss of activity was seen as early as 15 min after sensory input deprivation with an average loss of discernible rhythmic

motor activity at 30.2 ± 8.1 min after ventral nerve root cutting (Fig. 3c1, 2). The average burst amplitude in P2 preparations was 2.8 ± 1.3 mV which was significantly reduced to 0.6 ± 0.9 mV, after 60 min of sensory deprivation. A comparison of the magnitude of the effect of depriving sensory input via sacral transection and ventral nerve root cuts reveals that, after 60 min, a sacral transection decreased burst amplitude by about 85 and 73 % in P1 and P2 animals, respectively. Although the sacral transection method decreased burst amplitude by a higher percentage in P1 than in P2 animals, the effect produced by this sensory input deprivation method was always faster in P2 than in P1 mice (37 ± 8.5 min in P1 mice versus 20 ± 9.5 min in P2 mice; Fig. 2). Depriving sensory input in P1 and P2 animals via ventral nerve root cuts revealed that this method reduced burst amplitude by 45 and 79 % in P1 and P2 animals, respectively. As in the case of the sacral transection method, cutting all lumbar and sacral ventral nerve roots decreased burst amplitude and eventually disrupted locomotor activity faster in P2 than in P1 mice (47.8 ± 7.9 in P1 mice versus 30.2 ± 8.1 min in P2 mice; Fig. 3). Although there were qualitative differences regarding the effects of sensory deprivation on P0–P2 spinal cord hindlimbs-attached preparations using a sacral transection versus ventral nerve root cutting, there was still a positive correlation between animal age and robustness of the effects on sensory input loss on an ongoing rhythmic locomotor pattern of activity. These results suggest that the spinal locomotion CPG neuronal network is developing toward a more sensory-dependent state with age.

In order to elucidate potential mechanisms that could explain this age-dependent loss of spinal locomotor activity after losing sensory inputs in P2 animals, we explored the possibility that the disruption of motor activity could be attributed to a loss of excitatory drive from sensory afferents. To assess this possibility, we recorded motor activity from ventral roots of P2 animals as previously done, but additionally recorded activity from both (left and right) dorsal nerve roots at the L4 level and measured any changes in the frequency of the activity measured from both types (ventral/dorsal) of nerve roots. Previous studies have shown that electrical stimulation of dorsal nerve roots from the mid to lower lumbar levels can elicit bouts of spontaneous locomotor-like activity lasting up to several minutes (Gabbay et al. 2002; Lev-Tov et al. 2010) which prompted us to use the L4 dorsal nerve root to monitor afferent (sensory) activity. We measured the frequency of the firing activity recorded from the dorsal root and simultaneously recorded drug-induced (5-HT/NMDA/DA) locomotor-like activity from the ventral nerve roots before, during, and after cutting all lumbar and sacral ventral roots. We found that the loss of afferent activity always preceded the disruption of locomotor-like activity as seen by a significant reduction in burst amplitude as early as 5 min after the cut (Fig 4; $n = 5$). We additionally attempted to regain locomotor-like activity in P2 animals after ventral nerve root denervation by increasing the concentration of the drug cocktail (5-HT/NDMA/DA). The rationale behind augmenting the concentration of locomotor-inducing drugs was that if the loss of alternating locomotor activity was due to the interruption of afferent (sensory) input, the raised concentration of the drug cocktail should compensate for this apparent void in excitatory drive left after stopping the flow of motor and sensory information through ventral root denervation. We sequentially changed the concentration of each of the drugs by 1 μM increments up to 5 μM (from 9 μM 5-HT/6 μM NMDA/18 μM DA up to 14 μM 5-HT/11 μM NMDA/23 μM DA) in 20-min time period (each new concentration of drugs was maintained in the bath for at least 20 min). Although there was an increase in overall nerve root activity, increasing the concentration of drugs failed to recover alternating locomotor-like activity in all P2 animals tested (Fig. 5, $n = 5$). This result suggests that this apparent loss of excitatory drive to the CPG network after denervation could be due to the progressive loss of a sensory-related neuromodulatory transmitter and not just from less flow of locomotor-related excitatory neurotransmitters, as those present in our drug cocktail, through the motor–sensory information loop.

Overall our results support the idea that when the spinal locomotion CPG network has to interact only with sensory feedback due to the loss of supraspinal inputs (through injury for example), the network develops a more sensory-dependent state of spinal motor activity which could be correlated to the developmental maturity of the sensory and motor spinal networks (Fig. 6). Potential mechanisms explaining this apparent loss of sensory drive to the spinal locomotor network are now discussed.

Discussion

It has been well studied and documented that networks of interneurons located within the spinal cord are responsible for generating locomotion and that these networks interact with specific sensory information to allow the reinforcement as well as modifications in the organization of locomotor behaviors (Grillner 1981). After suffering an SCI, many (and in some cases all) descending fibers (axons) from the supraspinal centers are severed or become non-functional, leaving the CPG network controlling locomotion without its initiating and controlling drive (input). Recent studies have focused on the importance of maintaining sensory stimulation to the lower limbs, via physical therapy for example, on SCI patients as a way to initiate and control the CPG locomotor network (Harkema et al.

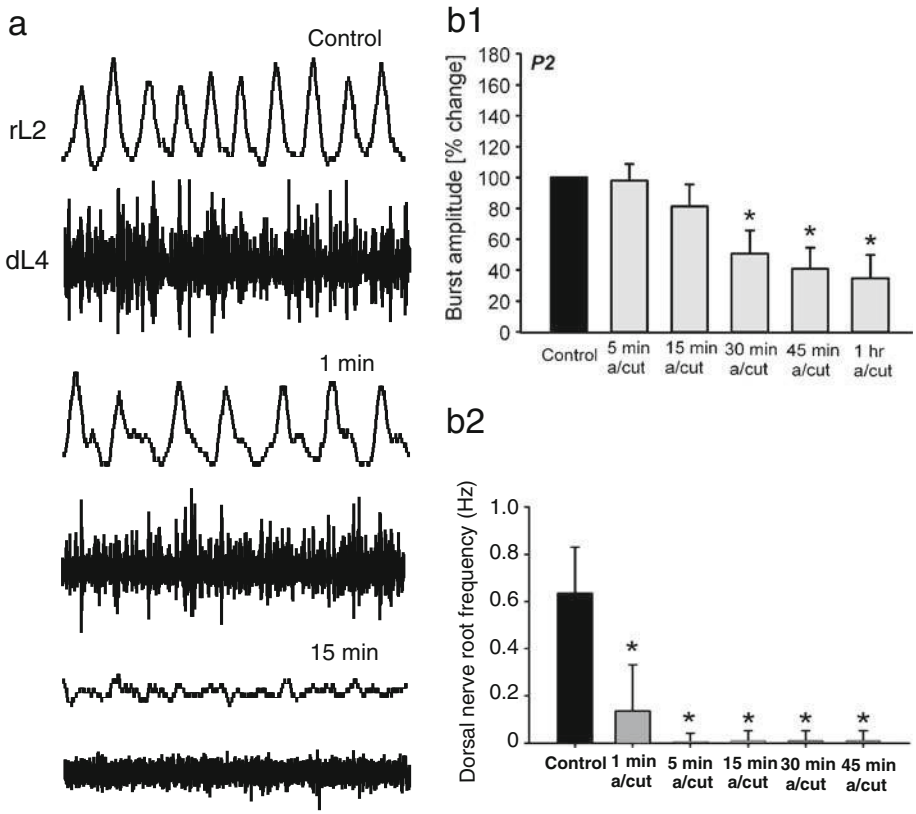

Fig. 4 Comparing ventral nerve activity to dorsal root activity before and after sensory deprivation via ventral nerve root cuts on P2 mice. **a** Rectified ventral root activity from the right second lumbar nerve root and raw dorsal root activity from the right 4th lumbar dorsal nerve root before and, 1 and 15 min after cutting all ventral nerve roots ($n = 5$). **b** Mean data on the effects of loss of sensory input on burst amplitude of P2 mouse spinal cord lower limb-attached preparations (**b1**) and the firing activity as recorded from both L4 dorsal roots (**b2**) ($n = 5$)

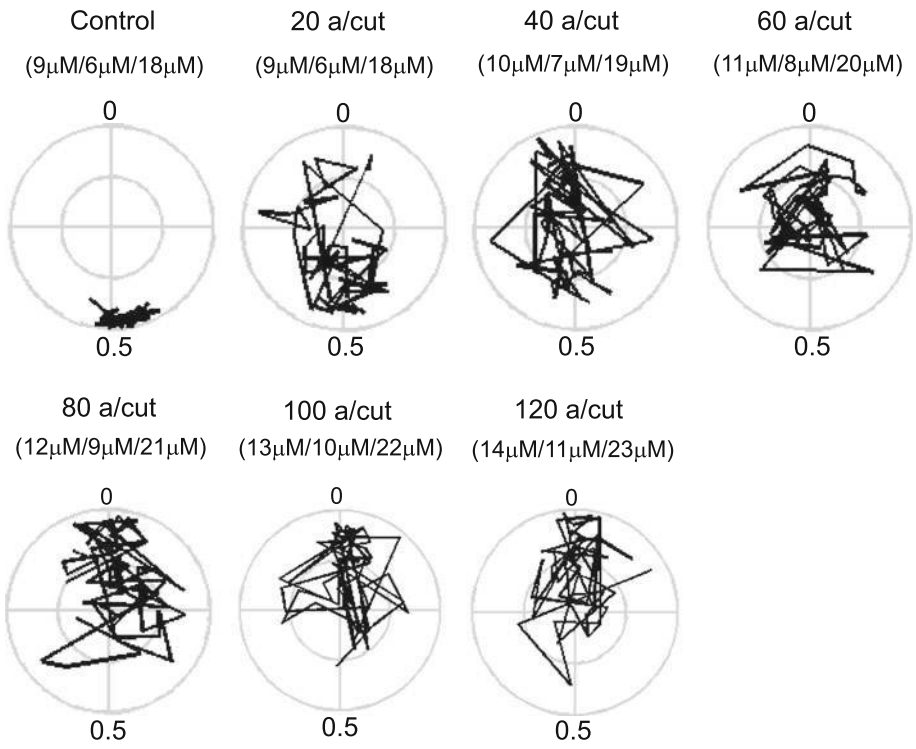

Fig. 5 Attempting to regain alternating locomotor-like activity after sensory deprivation by increasing the concentration of 5-HT, NMDA and dopamine (locomotion-inducing drug cocktail) in spinal cord hindlimbs-attached preparations of P2 mice. After the disruption of the recorded locomotor-like rhythm was achieved via sensory deprivation (ventral root denervation), we proceeded to raise the initial drug cocktail concentrations (5-HT: 9 μM/NMDA: 6 μM/dopamine: 18 μM) by 1 μM increments up to 5 μM (from 9 μM 5-HT/6 μM NMDA/18 μM DA up to 14 μM 5-HT/11 μM NMDA/23 μM DA) in 20-min time period and check (via extracellular ventral root recordings) if the alternating motor pattern was recovered with a higher concentration of the drugs ($n = 5$)

2011; Courtine et al. 2009; Lavrov et al. 2008; Edgerton et al. 2008), but studies on the importance of sensory input to locomotor activity in the absence of supraspinal inputs are lacking. We studied the effects of sensory deprivation on spinal locomotor CPG network function using a recently developed spinal cord hindlimbs-attached preparation

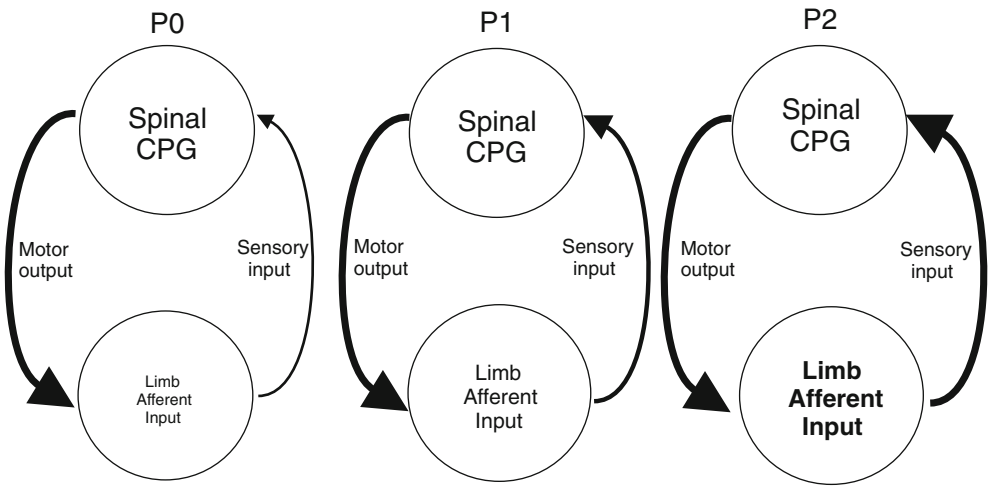

Fig. 6 A diagram of the potential mechanism in which stopping the flow of sensory input disrupts an ongoing pharmacologically-induced locomotor pattern. The included diagram is intended to suggest a potential mechanism in which the depriving sensory input to a spinal CPG network was activated pharmacologically in the presence of limb afferent feedback and no supraspinal inputs. We suggest that the developmental maturity of the sensory network could explain why depriving sensory input in a P0–early P1 animal did not produce a significant perturbation of motor output, since apparently this motor pattern was mostly produced by the intrinsic circuitry of the spinal CPG network (*arrows*). In contrast, in the late P1–P2 animals, the spinal CPG network could have produced a motor pattern which depended in part on the afferent feedback from the limbs (*arrows*). We suggest that this potential mechanism is enhanced in the absence of supraspinal inputs and the source of this afferent signal which is lost after sensory input deprivation is yet to be elucidated

(Hayes et al. 2009). We sought to understand the role of sensory input to spinal locomotor function as the animal begins to actively interact with its environment on its first day after birth by disrupting sensory input via a sacral cord transection or through ventral nerve root denervation.

Our previous work with the isolated spinal cord (Díaz-Ríos et al. 2007; Zhong et al. 2006a, b) has shown that fictive locomotion can be elicited with a combination of 5-HT, NMDA and dopamine, and that this rhythm can last for many hours as long as the concentration of drugs in the bath is kept constant with perfusion. So then why would a drug-elicited locomotor rhythm stop its alternating activity after depriving it of sensory feedback from the hindlimbs? We would like to discuss two possible explanations: (1) locomotor activity induced pharmacologically in a spinal cord preparation with the hindlimbs attached could result in a motor pattern produced by a spinal CPG network that is more dependent on sensory feedback than a CPG network which relies on the synaptic properties of its component neurons to produce a "fictive" locomotor pattern (isolated spinal cord); (2) the deprivation of sensory information via a cut of all afferent (dorsal) roots "feeding" back to the CPG network could lead to a "spinal shock"-like state (seen previously after an SCI; see Dietz 2010, 2011 for recent reviews) which could explain the "shutdown" of network activity after a lower sacral transection.

Dorsal root injuries, which can result in the partial or complete loss of connections between the sensory system and the spinal cord, can lead to sensory impairments, including both the loss of sensation and the development of neuropathic pain in the affected limb (Cragg et al. 2010). Our data do not support that the observed effects on locomotor activity were due to an injury-related effect of cutting the spinal cord due to the following facts: (1) we never saw any immediate effects on motor activity after cutting (data not included), these include any hyper-excitability on the motor output immediately after cutting or any other noticeable effect; (2) preparations were deprived of supraspinal input via cervical decapitation in ice-cold low-calcium Ringer and the preparation was dissected and prepared in this Ringer before being moved to our recording chamber and perfused with regular Ringer solution (see "Materials and methods") in the presence of the drug cocktail. This procedure will take around 45 min to 1 h and then the preparation is continuously perfused with these drugs, even during and after the sacral transection, which should counteract any "spinal shock-like" state induced by the decapitation procedure or spinal cord cutting. Still, to assess the possibility of an injury-mediated effect due to the cutting of the spinal cord we began using a ventral nerve root cutting procedure, thus maintaining the spinal cord and all dorsal nerve roots intact. By not severing the spinal cord or any dorsal roots, we avoided any type of peripherally injury-mediated effects. Effects from ventral nerve cutting on locomotor activity were similar to our sacral transection experiments, thus supporting our observations.

We believe that by removing sensory feedback to an ongoing locomotor pattern, the deprivation of sensory information (via sacral transection or ventral root denervation)

could result in the disruption of a locomotor CPG network which had "learned" to produce locomotion in a sensory feedback-dependent manner. This concept will support the idea that in situations where there is no descending input to the spinal cord, the control of locomotion could be directed from specialized spinal networks directed by sensory afferent input from the lower limbs (Edgerton et al. 2008; Lavrov et al. 2008; Courtine et al. 2009; Rossignol and Frigon 2011; Rossignol et al. 2011). In addition, our results confirmed a positive correlation between loss of afferent activity, as recorded from dorsal nerve roots using suction electrodes, and the subsequent disruption of locomotor activity in all P2 animals tested (Fig. 4). This further suggests that by severing the ventral roots, which caused a subsequent loss of muscle contraction, we disrupted the afferent (sensory) and efferent (motor) activity loop which led to an apparent loss of sensory drive to the CPG network resulting in a disruption of locomotor-like activity. One possible source of this sensory drive could be via a yet-to-be identified neuromodulator such as a neuropeptide.

The tachykinin neuropeptide Substance P (SP) is abundant both in the periphery and in the central nervous system (CNS), where it is usually co-localized with one of the classical neurotransmitters, most commonly serotonin (5-HT; Marson 1989; Thor et al. 1988; Arvidsson et al. 1994). Earlier and more recent work have linked SP to a centrally and peripherally located modulator of locomotor behavior in model systems such as the lamprey and rat (Piercey et al. 1981; Barthe and Clarac 1997; Ullström et al. 1999; Svensson et al. 2001; Kozlov et al. 2001; Parker and Bevan 2007; Thörn Pérez et al. 2009). Application of SP to the isolated lumbosacral spinal cord of newborn rats induced slow rhythmic activity in quiescent preparations (no other drugs present in the perfusate), and during drug-induced fictive locomotion, SP application increased locomotor cycle frequency and burst duration including a stabilizing effect by the neuropeptide on the locomotor rhythm (Barthe and Clarac 1997). Other neuropeptides which have been linked to the dorsal horn and sensory neurons include Neuropeptide Y and GABA (Parker and Grillner 1996; Ullström et al. 1999). NPY has the opposite effect of tachykinins in that it reduces the excitability of spinobulbar interneurons and presynaptically reduces dorsal cell-mediated glutamatergic synaptic transmission (Parker et al. 1998). GABA is colocalized with NPY in bipolar neurons in the dorsal column (Parker et al. 1998). The GABAb receptor agonist baclofen has complementary effects to NPY in depressing sensory synaptic transmission (Christenson et al. 1988; Parker et al. 1998). Based on the observed inhibitory effects of Neuropeptide Y and GABA on sensory synaptic transmission, these are unlikely candidates for explaining the loss of rhythmic motor activity due to sensory input deprivation.

In summary, these experiments suggest that the effects of sensory deprivation on an ongoing drug-induced locomotor pattern could be related to a disruption of what seems to be a "hybrid" network consisting of the locomotor CPG network whose component neurons are located within the lumbar spinal cord and sensory feedback coming from the hindlimbs. The disruption of motor output in older animals could be attributed to a spinal CPG network which is more dependent in sensory input (Fig. 6) due to a maturing motor and sensory circuitry. We suggest that these effects are enhanced in the spinal cord hindlimbs-attached preparation used in this study since the spinal CPG circuitry can only access sensory input and not supraspinal inputs as could be the case in severe cases of cervical or thoracic SCI. These results are supported by recent studies that have shown that after inducing an SCI via a complete transection of the nerve cord in cats and rodents, combinations of serotonergic agonists and epidural electrical stimulation were able to acutely transform spinal networks from nonfunctional to highly functional and adaptive states as early as 1 week after injury (Dorofeev et al. 2008; Courtine et al. 2009). The variability in the motor responses as a function of age recorded after the loss of sensory feedback could be correlated with the developmental maturity of the locomotor CPG network and a use-dependent relationship with sensory information. We are currently gathering data on slightly older animals (P3–P5) than those included in this present study (P0–P2) to contrast and compare any potential differences regarding the effects of sensory deprivation on locomotor behavior in the absence of supraspinal inputs. Other studies are also ongoing, including the use of electromyographic recordings (EMGs) to correlate muscle activity to CPG motor output (Tysseling et al. 2013), to elucidate the potential mechanism mediating the effects apparently induced by the disruption of sensory input to an ongoing centrally initiated motor output.

Acknowledgments We thank Thomas Cleland for Spike 2 scripts used in data analysis and to Alex Kwan for Spike 2 scripts used in the generation of the circular plots. We would also like to thank Ernesto Cabezas, Jeidiel De Leon, Marla Rivera Oliver and Nikol Matos Vergara for their helpful comments on the manuscript. We would additionally like the thank the University of Puerto Rico Puerto Rico Institutional Animal Care and Use Committee for the approval of the animal protocols used for the experiments included in this work in accordance with National Institutes of Health guidelines. The present study was supported by the Craig Neilsen Foundation Grant 124554, the Research Centers in Minority Institution at the University of Puerto Rico—Medical Sciences Campus (RCMI-UPR-MSC) Grant G12RR03051 and a Minority Biomedical Research Support-Research Initiative for Scientific Enhancement (MBRS-RISE) pre-doctoral Grant R25GM061838 to JeanMarie Acevedo-Rosario.

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