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·Review·

Anatomical and electrophysiological plasticity of locomotor networks following spinal transection in the salamander

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Recovery of locomotor behavior following spinal cord injury can occur spontaneously in some vertebrates, such as fish, urodele amphibians, and certain reptiles. This review provides an overview of the current status of our knowledge on the anatomical and electrophysiological changes occurring within the spinal cord that lead to, or are associated with the re-expression of locomotion in spinally-transected salamanders. A better understanding of these processes will help to devise strategies for restoring locomotor function in mammals, including humans.

Keywords: salamander; spinal cord; locomotor recovery; regeneration

Introduction

Many studies on the post-lesional plasticity of the central nervous system (CNS) of adult salamanders have dealt with the molecular and cellular events underlying spinal cord regeneration following tail amputation[1-6]. Regeneration in a context more relevant to human spinal cord injury (SCI) (i.e. after spinalization at the trunk level) has recently been investigated using labeling and imaging techniques[7], and the main finding is that meningeal and glial cells interact to form a permissive environment for axon regeneration after SCI in salamanders. This is in contrast to adult mammals, in which meningeal and glia cells form barriers (glial scars) that contribute to the prevention of axon regeneration[8-10]. Several non-permissive or inhibitory factors that prevent axonal regeneration in adult mammals have been found[11]. Differences between salamanders and mammals with regard to some of these factors, especially those controlling regeneration of the descending pathways known to activate the spinal locomotor networks, remain to be investigated. However, the molecular and cellular aspects of post-lesional plasticity of the spinal tracts are beyond the scope of the present review. Here, we summarize the anatomical and electrophysiological changes occurring within the spinal cord that lead to, or are associated with the re-expression of locomotion in salamanders after full transection of the spinal cord. A better understanding of these changes may provide insights regarding some of the conditions necessary for restoring locomotor function after SCI in higher vertebrates, including humans.

Model Organisms Used for the Study of Locomotor Recovery after SCI and Regeneration

Spinal neuronal circuits continuously process information received from the brain and peripheral sensory inputs. For instance, the central pattern generator circuitry for locomotion (locomotor CPG) can be activated and/or modulated by supraspinal or afferent inputs[12, 13]. This specialized network can be activated even in the isolated spinal cord, thus underlining the crucial role of the cord in gait generation. Following severe SCI, descending brain commands are disconnected from the locomotor CPG. Networks below the lesion can no longer be activated by the brain, and as a result, paralysis occurs in the region controlled by the spinal cord below the lesion. The subsequent degree of axonal regeneration and recovery of locomotor function below the lesion depends both on

ontogeny and phylogeny.

Anuran amphibians, reptiles, birds, and mammals can spontaneously regenerate the spinal cord only as larvae or embryos, and this capability decreases with the progression of development[10, 14]. By contrast, in some adult vertebrates such as lampreys[15], teleost fish[16], and urodele amphibians[17-20], severed axons from descending neurons can spontaneously re-grow across the lesion site, and progressively extend within the sub-lesional spinal cord. The extent of regenerated axons exceeds 10 mm after a sufficiently long recovery time (>6 months post-transection)[19-22]. The number of re-growing axons projecting to the sub-lesional spinal cord also increases with time[20]. Interestingly, neither new brainstem neurons (neurogenesis) nor axon collaterals from unlesioned neurons (sprouting) contribute to the restoration of descending projections after spinal cord transection in salamanders[21], fish and larval lampreys[23, 24].

Behavioral, kinematic and electrophysiological studies have provided evidence that the post-lesional locomotor recovery observed in species with a high regenerative ability is imperfect, even after a long period of recovery[17, 19, 25, 26]. For example, abnormal body undulations and sub-lesional electromyographic (EMG) patterns occur during recovered swimming in spinally-transected lampreys and salamanders[19, 20, 25]. This is likely because the number of regenerated axons innervating the sub-lesional spinal cord is lower than that in intact animals, even after a recovery period as long as several months[27-29]. Moreover, inappropriate incorporation of the regenerated axons into the sub-lesional spinal networks could occur. This might be related to short- and long-term alterations of the cellular and network properties that occur in the spinal cord caudal to the injury site. Indeed, this region should be considered as a “new spinal cord”, with neurons endowed with new electrical membrane properties, new expression patterns of neurotransmitter receptors, and new connectivity[30].

In the freshwater turtle the completely transected spinal cord is reconnected, and it has been suggested that this constitutes a suitable vertebrate model system for studying the plasticity of the locomotor CPG after complete SCI[31]. However, locomotor recovery in this species is incomplete and restricted to stepping. Moreover, further investigation is required to demonstrate a direct causal relationship between axonal regeneration and the recovery of stepping. Indeed, one should stress that visual inspection and/or kinematic analysis of locomotor behavior cannot be used to accurately infer the participation of regenerated axons in the locomotor recovery after SCI (see below).

Following a lesion, not only the descending pathways, but also the ascending axons from spinal neurons that convey signals from the locomotor CPG (internal feedback)[32-34] and/or sensory information from the body region caudal to the transection site are axotomized. In several lower vertebrates, only a fraction of the ascending axons regenerate after spinalization (salamander[35], goldfish[36], larval anuran[37], lamprey[27]). Therefore, it is likely that a significant reorganization of the neuronal networks also occurs above the lesion site because of the loss of some of these ascending fibers[38].

In the present review, we focus on the neuronal mechanisms underlying the post-injury plasticity of the spinal locomotor CPG in adult salamanders.

The salamander is an excellent experimental model system to address this issue since: (1) it is the only tetrapod that can recover locomotor function in adulthood after full transection at all rostro-caudal levels of the spinal cord[20]; (2) it displays terrestrial and aquatic locomotor modes, and this gives the opportunity to compare the post-lesional recovery of the two modes; (3) a significant amount of data on locomotion in intact animals is available to evaluate the quality of the recovered locomotion[39]; (4) its CNS can survive several hours *in vitro*, and this allows the use of electrophysiological methods to investigate at the cellular level the alterations of the locomotor circuits after SCI[40-42]; and (5) the global architecture of the locomotor CPG for swimming and stepping has been deciphered and modeled[43, 44].

Recovery of Locomotor Patterns in Spinally-transected Salamanders

Earlier behavioral[17, 18] and kinematic studies[20] have revealed that in adult salamanders the ability to walk and swim can recover ~3 months after a complete spinal cord transection. Re-transection of the cord after recovery from the first transection results in paralysis, once again, of the body part controlled by the cord below the lesion. This suggests that the recovery of locomotion results from the re-establishment of descending inputs across the

transection[18, 20].

However, since muscle activation was not recorded in these studies, the relative contributions of muscle contractions and passive factors to locomotor recovery were unknown. In a previous study, we made EMG recordings during swimming and terrestrial stepping in the same adult salamander before and at various times (up to 500 days) after a complete transection of the spinal cord at the trunk level (between the two girdles)[19]. We used this approach to assess the time-course and completeness of locomotor recovery as reflected in the various locomotor parameters (cycle duration, intersegmental coordination, inter-limb coupling) that had been previously documented in intact animals[45, 46].

Our results evidenced that 2–3 weeks after spinalization, locomotor movements and EMG activity were limited to the forelimbs and the body rostral to the transection. Thereafter, the locomotor movements and EMG activity returned at progressively more caudal levels, and the animals reached stable locomotor patterns 3–4 months post-transection. Several locomotor parameters (cycle duration, intersegmental phase lag, inter-limb coupling) measured at various recovery times after spinalization were compared to those in intact animals. These comparisons revealed transient and long-term alterations in the locomotor parameters both above and below the transection site. These alterations were much more pronounced for swimming than for stepping, revealing differences in adaptive plasticity between the two locomotor modes.

Recovered locomotor EMG activity was immediately abolished by a re-transection at the site of original spinalization (Fig. 1). This reveals that the re-innervation of the spinal cord below the transection site by descending brain and/or propriospinal axons primarily accounts for the locomotor recovery in spinally-transected salamanders, and the regenerated axons made sufficient appropriate synaptic contacts with neurons of the locomotor network caudal to the transection. Interestingly, behavioral, electrophysiological, and morphological experiments conducted in larval lampreys and fish have shown that the descending re-growing axons form functional synapses associated with swimming recovery[22, 47-49].

However, during the very early stages of recovery, mechano-sensory coupling across the transection could also be involved in the re-activation of the locomotor CPG[50, 51]. At more advanced stages, when the caudal portion of the body begins to recover some movements, the proprioceptive signals from the hindlimbs might enhance the excitability of the CPG[52]. The role of afferent signals in the re-activation of the CPG might be addressed in a comparative study of the fictive locomotor patterns expressed *in vitro* by spinal cords isolated from intact and chronic spinal salamanders (see[53] in the cat).

Interestingly, in fish[54] and cats[55] locomotor recovery following a complete cord transection can be significantly facilitated by daily training sessions. This emphasizes the crucial role of proprioceptive afferent inputs in the re-activation of the locomotor CPG. Salamanders would be a suitable model system to identify the neuronal events induced by training during swimming and stepping. Interestingly, in fish, swim training improves axonal regeneration across the SCI[54].

Regeneration of Descending Pathways in Spinally-transected Salamanders

In salamanders, many descending neurons, including reticulospinal neurons that transmit the locomotor drive from the mesencephalic and diencephalic locomotor regions to the spinal locomotor CPG, regenerate axons[19-21, 28]. Chevallier *et al.*[19] further demonstrated that some of the regenerated axons come from glutamatergic and serotoninergic (5-HT) immunoreactive cells within the reticular formation. We have also provided evidence that regeneration of cholinergic descending propriospinal neurons occurs after complete spinal transection in salamanders (Fig. 2 and see[19, 21]).

Glutamatergic Neurons

Glutamatergic reticulospinal neurons are critical for initiating locomotion (command neurons) in all vertebrates[56]. From this, it is clear that the primary focus in different animal models that seek to establish the functional regeneration of descending locomotor drives from the brainstem across an SCI should be on the reticulospinal pathways[57].

The spontaneous regeneration of reticulospinal glutamatergic axons in salamanders could contribute to the activation of the locomotor networks below the lesion. It is possible that only non-specific connectivity to the neuronal circuitry caudal to the lesion is sufficient to turn this part of

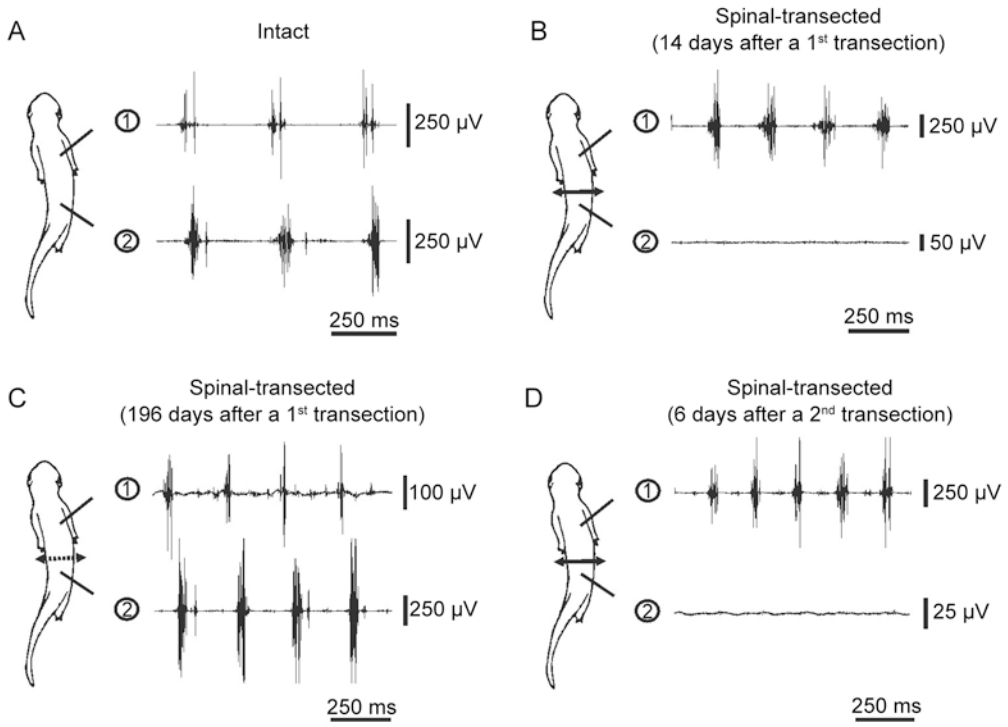

Fig. 1. Recovery of swimming in salamander with spinal transection. In each panel, a drawing of the body shape of the salamander and position of the recording EMG electrodes (left) accompanies the EMG recordings (right). EMG recordings were from two myomeres on the same side of the animal. One myomere was located above, and the other below the transection site (indicated in the drawings with arrows). A: Pattern of EMG activity characteristic of swimming in an intact animal. B: Fourteen days after spinalization, the rhythmic EMG activity was restricted to above the transection. C: At 196 days after spinalization the pattern of muscle activity appeared similar to that recorded before spinalization (compare with A). D: After complete swimming recovery, a second spinalization was performed at the same level as the first one. Within six days after the second spinalization, the rhythmic EMG activity was restricted to above the spinal transection. Recordings are from the same animal (adapted from[19]).

the locomotor CPG on and off. One reason is that in the isolated spinal cord of salamanders (as well mammals), the locomotor CPG can be turned on by bath-application of glutamate agonists, a non-specific mode of activation[40]. The use of patch clamp and calcium imaging techniques most likely will help to address this issue.

Serotoninergic Neurons

Salamanders have a well-developed intraspinal 5-HT system, as well as a supraspinal raphé 5-HT system[58]. Although 5-HT by itself fails to initiate locomotion, it shapes both the swimming and stepping patterns expressed by the salamander spinal cord *in vitro*[58, 59]. In particular, 5-HT agonists increase both the duration and intensity of the locomotor bursts in intact animals. Therefore, the axons of reticulospinal 5-HT neurons that regenerate across the lesion could contribute to the restoration of the sub-lesional EMG activity in transected animals by modulating the non-linear membrane properties of 5-HT-dependent neurons[60]. In contrast, there is no experimental evidence that some of the intraspinal 5-HT neurons regenerate axons after transection. Interestingly, there is compelling evidence that these neurons are involved in the spinal control of locomotion in mammals[60].

Associated with SCI is a marked alteration in the 5-HT control of the swimming rhythm in salamanders. Indeed, application of 5-HT to 1–2 lumbo-sacral segments of the sub-lesional cord induces a dose-dependent increase in the swimming rhythm in recovered spinal salamanders, while a similar application to intact animals induces a decrease in the swimming rhythm (Figs. 3 and 4). But a similar local application of 5-HT in intact or spinal-transected salamanders fails to significantly alter the stepping rhythm

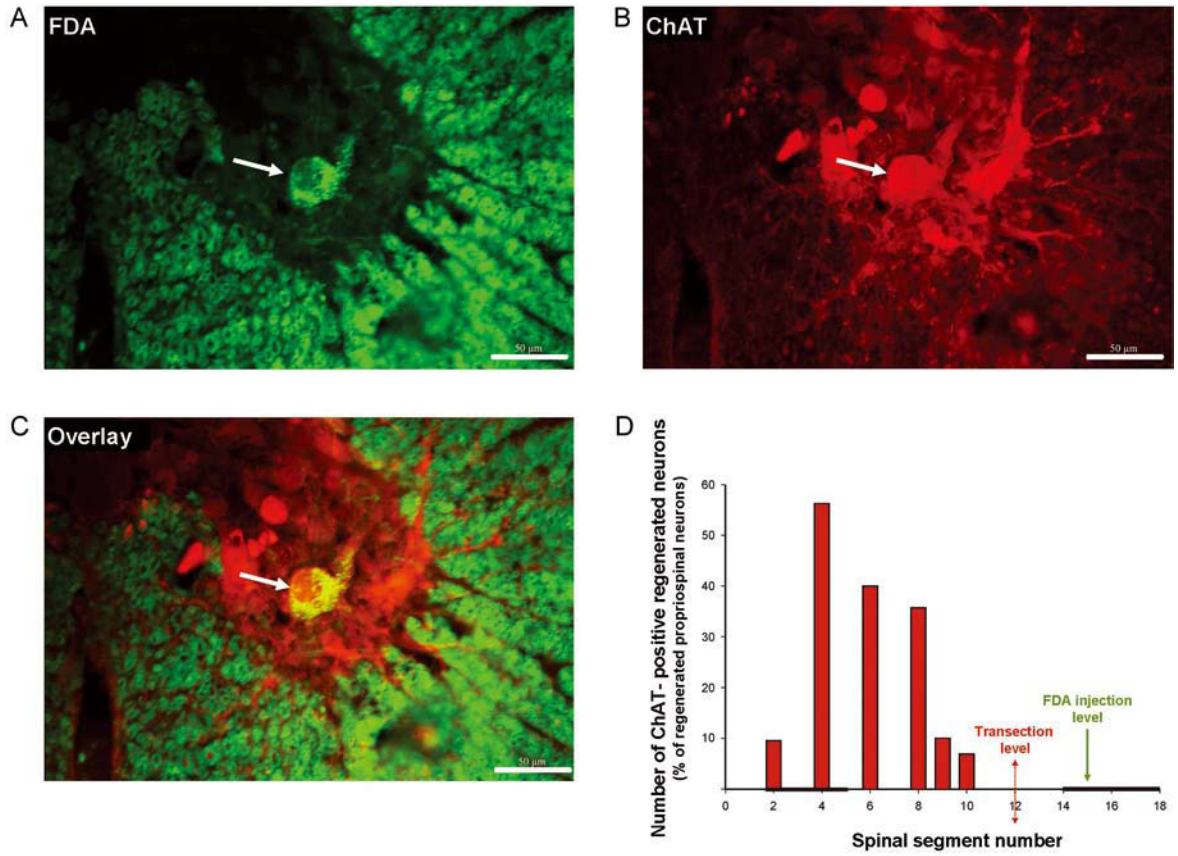

Fig. 2. Axonal regeneration of descending cholinergic propriospinal neurons in salamander with spinal transection. A: A single propriospinal neuron (arrow) was retrogradely labeled with FDA (fluorescein dextran-amines) applied to the cut surface of the 15th spinal segment 180 days after transection at the level of the 12th segment. B and C: This neuron exhibited ChAT immunoreactivity. Scale bars, 50 µm. D: Distribution histogram along the spinal cord of the ChAT-immunoreactive cells that project to the FDA application site. Red and green arrows refer to the levels of transection and FDA application, respectively. The thicker lines indicate the brachial and crural plexuses. Same experiment as in A–C. (unpublished data by Céline Philippe).

(not illustrated). These results are also consistent with the differential degree of post-lesional plasticity of swimming and stepping networks in salamanders (see above). Finally, 5-HT can play a critical trophic role in regeneration in addition to its function in neuromodulation[61, 62], but this remains to be investigated in the present context.

Cholinergic Neurons

The role of regenerated cholinergic propriospinal neurons in the re-establishment of locomotor function in spinally-transected salamanders remains an open question. One possibility is that they contribute to recovery by relaying the descending locomotor drive in the most caudal levels of the cord. Therefore, cholinergic propriospinal neurons would be an important target for strategies designed to produce locomotor recovery[63, 64].

Recovery of Motoneuron Excitability in Spinally-transected Salamanders

Motoneurons (MNs) are the output stage of the locomotor networks and consequently transduce neuronal activity to movement. As MNs can be identified by simple retrograde tracer injection into muscles or ventral roots, these cells are easier to study (anatomically and electrophysiologically) than the interneurons of the locomotor networks. The intrinsic firing properties of MNs depend on the ionic conductances that they express. These conductances are under neuromodulatory control that can reshape the MN

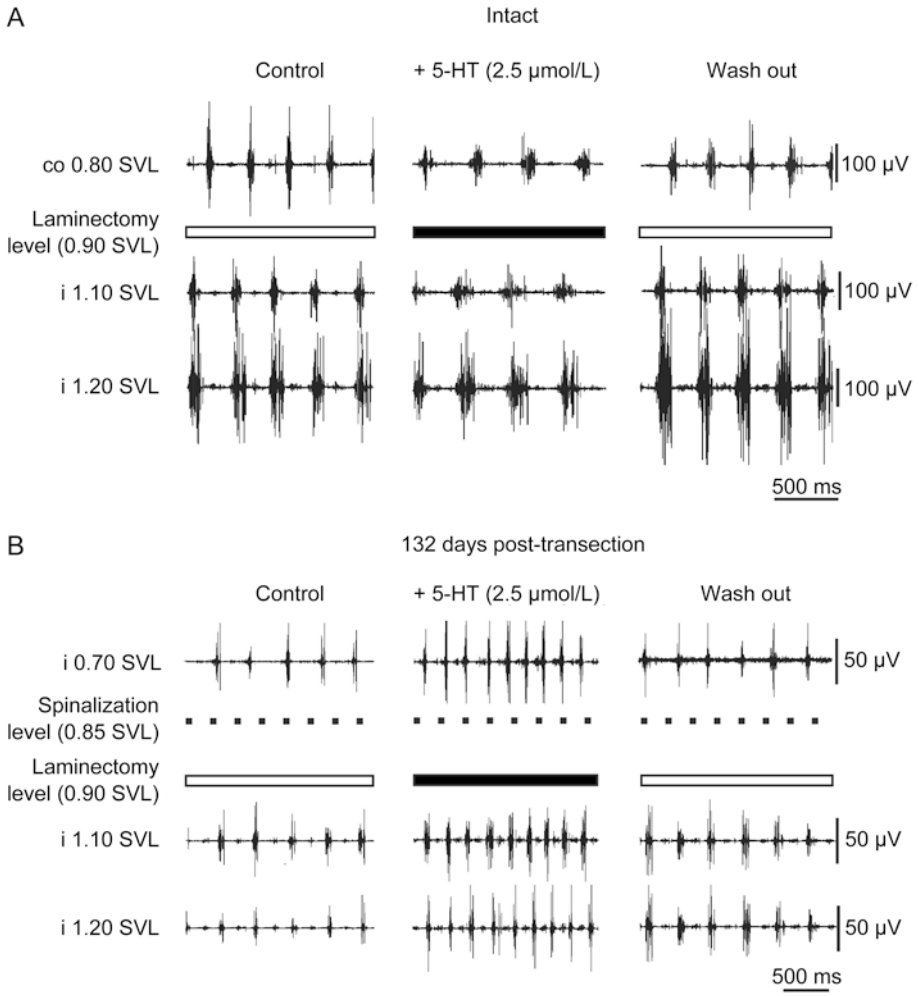

Fig. 3. ***In vivo*** **effects of serotonin on the swimming EMG pattern before and after a spinal lesion in the same salamander. A: In each panel, the EMG recordings are from three myomeres in the intact state. The rostro-caudal positions of the myomeres are expressed as fractions of the snout-vent length (SVL). i, ipsilateral; co, contralateral. The bar indicates the position of the segmental laminectomy allowing a local application of serotonin directly to one spinal segment. B: Same representation as in A for EMG recordings. Data are from the same individual, 132 days after spinalization. The dotted line refers to the position of the transection (unpublished data by Ianina Amontieva-Potapova).**

firing pattern, and hence the output of the locomotor CPG[65, 66], for tuning and adapting the locomotor drive to changing conditions[67]. Since the neuromodulatory descending systems can be disrupted after SCI, strategies aiming at restoring locomotor movements need to address not only the premotor circuits that provide the locomotor drive but also the modulatory system that ensures that MNs are sufficiently excitable to respond to that drive.

Among the neuromodulators, acetylcholine (ACh) provides ongoing modulation of locomotor activity in many vertebrates[64, 68], including salamanders[69]. Since cholinergic propriospinal cells can be used as a relay of the locomotor drive, we have performed a comparative analysis of the cholinergic modulation of the excitability of MNs in intact and spinally-transected salamanders[70, 71]. We found that ACh increases the gain (input-output relationship) of hindlimb MNs *via* activation of muscarinic receptors in intact and transected salamanders. Our data further revealed that the gain of MNs is larger in early spinal (<2 weeks post-lesion) than in late spinal (3–4 weeks post-lesion) animals, while the value of the gain in late spinal animals is close to that in intact animals (Fig. 5). The muscarinic-

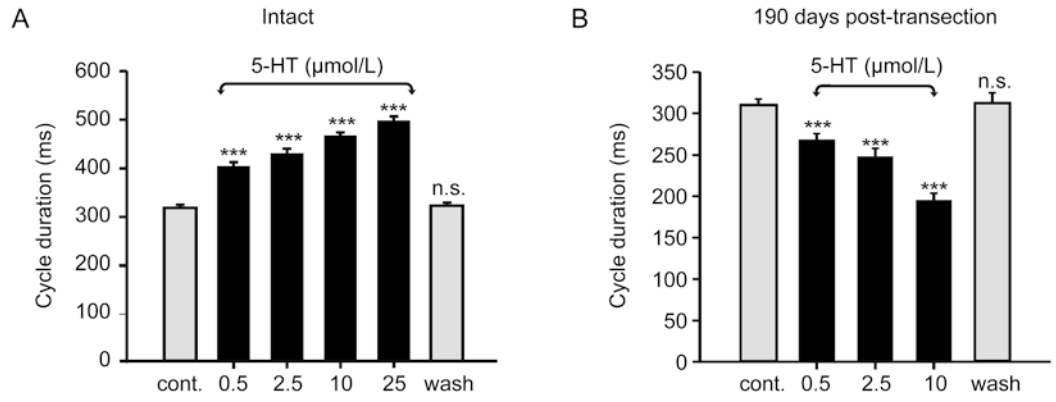

Fig. 4. Quantification of the *in vivo* effects of serotonin on the swimming rhythm in the intact (A) and spinal (B) states in the same salamander. Data are presented as mean ± SD. ***P <0.001; n.s., not significant (unpublished data by Ianina Amontieva-Potapova).

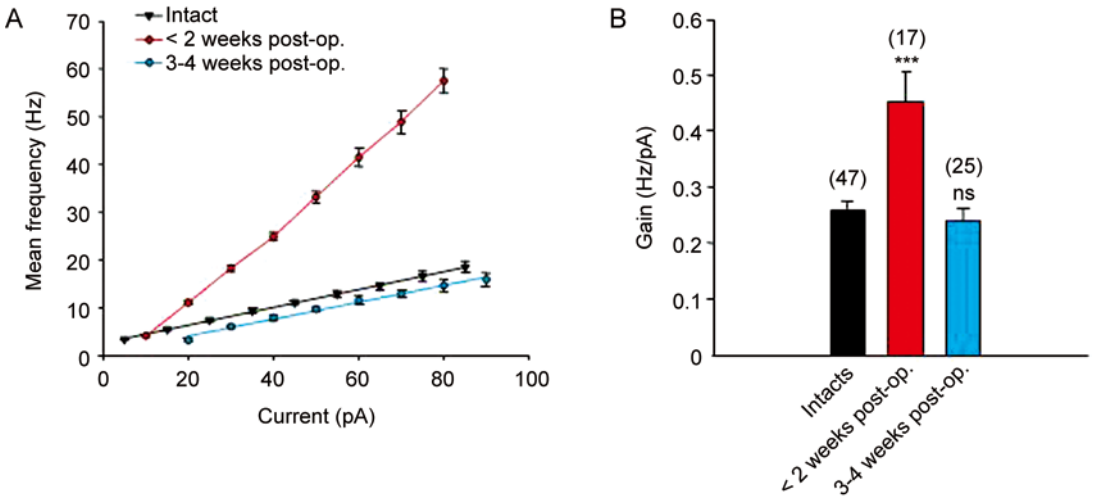

Fig. 5. The excitability of hindlimb motoneurons (MNs) changes with time after spinal transection at the trunk level. A: Frequency–current (*f–I*) relationship for one MN recorded in an intact animal (black line) and two MNs recorded at different post-lesional times in spinally-transected animals (red and blue lines). Note that the transection was performed between the two girdles (0.70 SVL level). The slope of the *f–I* relationship (gain) was steeper for the MN of the animal spinalized less than 2 weeks before recording, indicating a transient higher responsiveness to the same injected current. B: Mean values of the slope of the *f–I* plot for 17 MNs recorded <2 weeks after spinalization (red bar), 25 MNs recorded between 3 and 4 weeks after spinalization (blue bar), and 47 MNs recorded in intact animals (black bar). Data are presented as mean ± SEM. ***P <0.001; ns, non-significant (adapted from[70, 71]).

induced increase in the excitability of hindlimb MNs primarily results, both in intact and spinal animals, from a reduction in the medium-duration afterhyperpolarization (mAHP) amplitude[70, 71]. This result is consistent with previous studies in cat and mouse[72, 73] that demonstrated that the mAHP of spinal MNs is a target for modulation during locomotor behavior. Our results also provided evidence that the proportion of hindlimb MNs that express plateau potentials and after-discharges (bistability[66]) during muscarinic activation is strongly increased following spinalization. The increased MN excitability in transected animals could explain the hindlimb and tail hyper-reflexia observed after spinalization and before locomotor recovery.

It could be argued that the regeneration of descending cholinergic propriospinal neurons across the transection (see above) explains the MN gain reverting to normal four weeks after spinalization. However, previous anatomical and electrophysiological studies did not find re-

innervation of the cord below the lesion 3–4 weeks post-transection[19, 20, 58]. Further investigations are therefore needed to identify the underlying mechanisms.

In addition, since several neuromodulators affect the excitability of spinal MNs during locomotion (see recent review[74]), it is important to determine how they contribute, individually and in conjunction, to locomotor recovery after SCI.

Conclusion

The adult salamander is an excellent experimental model to study the post-lesional plasticity of motor systems in vertebrates. Indeed, the neurobiological study of locomotor recovery in spinally-transected salamanders provides valuable information on the mechanisms required to regain locomotor function after SCI. Furthermore, the spectacular capacity of salamanders to regenerate neurons also applies to midbrain dopaminergic neurons following a chemical lesion, and this has been used to create a new parkinsonian animal model in which the recovery of motor behavior is associated with dopaminergic neuron regeneration/neurogenesis[75]. In the context of SCI, much more work is needed to understand the reorganization of the salamander's locomotor networks in the spinal state. Rewiring of the networks, modifications in the balance between various transmitter systems, alterations in the intrinsic properties of axotomized descending neurons, and the contribution of sensory information are some important avenues to explore. It is hoped that the knowledge gained from these future studies will serve to target therapeutic approaches for rehabilitation in people with spinal injuries.

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