

Motor deficits and recovery in rats with unilateral spinal cord hemisection mimic the Brown-Séquard syndrome

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Cervical incomplete spinal cord injuries often lead to severe and persistent impairments of sensorimotor functions and are clinically the most frequent type of spinal cord injury. Understanding the motor impairments and the possible functional recovery of upper and lower extremities is of great importance. Animal models investigating motor dysfunction following cervical spinal cord injury are rare. We analysed the differential spontaneous recovery of fore- and hindlimb locomotion by detailed kinematic analysis in adult rats with unilateral C4/C5 hemisection, a lesion that leads to the Brown-Séquard syndrome in humans. The results showed disproportionately better performance of hindlimb compared with forelimb locomotion; hindlimb locomotion showed substantial recovery, whereas the ipsilesional forelimb remained in a very poor functional state. Such a differential motor recovery pattern is also known to occur in monkeys and in humans after similar spinal cord lesions. On the lesioned side, cortico-, rubro-, vestibulo- and reticulospinal tracts and the important modulatory serotonergic, dopaminergic and noradrenergic fibre systems were interrupted by the lesion. In an attempt to facilitate locomotion, different monoaminergic agonists were injected intrathecally. Injections of specific serotonergic and noradrenergic agonists in the chronic phase after the spinal cord lesion revealed remarkable, although mostly functionally negative, modulations of particular parameters of hindlimb locomotion. In contrast, forelimb locomotion was mostly unresponsive to these agonists. These results, therefore, show fundamental differences between fore- and hindlimb spinal motor circuitries and their functional dependence on remaining descending inputs and exogenous spinal excitation. Understanding these differences may help to develop future therapeutic strategies to improve upper and lower limb function in patients with incomplete cervical spinal cord injuries.

Keywords: spinal cord injury; Brown-Séquard syndrome; locomotion; spontaneous recovery; monoamines

Introduction

Cervical incomplete spinal cord injury leading to sensorimotor deficits in the upper and lower extremities has a high incidence among patients with spinal cord injury (McKinley *et al.*, 2007).

Despite this, animal studies focusing on functional recovery after spinal cord injury mostly use experimental models of thoracic spinal cord lesions, thereby neglecting the problem of intractable motor dysfunction of the forelimbs. Rats use their forelimbs extensively for many tasks including locomotion, as well as fine

motor skills such as reaching, grasping, seed manipulation and sensory perception of the environment (Webb and Muir, 2005). Despite obvious differences in locomotion between bipedal humans and quadrupedal vertebrates like rats, several remarkable similarities in gait physiology and the neuronal programme controlling locomotion can be found (Vilensky, 1987; Duysens and Van de Crommert, 1998; Dietz and Harkema, 2004; Courtine *et al.*, 2005). Recent functional MRI studies revealed a surprising resemblance of the supraspinal locomotor control between humans and quadrupedal mammals, indicating a conservation of locomotor control in humans despite their transition to bipedalism (Jahn *et al.*, 2008). Sensory integration during locomotion and activity of locomotor-related brain regions (subthalamic, cerebellar and mesencephalic locomotor region, etc) in humans strongly resembled the locomotor network found in the feline system. Primates and humans also have a spinal central pattern generator, as extensively shown for cats, rodents and other vertebrates (Grillner and Wallen, 1985; Dietz and Harkema, 2004). The central pattern generator network is a self-sustained spinal system producing locomotor-like neural activity, which is controlled and modulated by sensory afferents and supraspinal commands (Grillner and Wallen, 1985). Moreover, quadrupedal interlimb coordination between upper and lower extremities has been shown to also occur in humans during bipedal locomotion (Dietz *et al.*, 2001; Balter and Zehr, 2007). Lastly, electrophysiological assessment of reflexes suggested a basic similarity in spinal locomotor circuitry between humans and quadrupeds (Duysens and Van de Crommert, 1998). These conserved characteristics of neuronal control of locomotion qualify quadrupedal animals as well suited models for many aspects of human spinal cord injury.

Unilateral spinal cord lesions produce a Brown-Séquard syndrome characterized by ipsilesional motor weakness or paralysis and loss of proprioception with contralesional deficits in pain and temperature sensation (Little and Halar, 1985; Roth *et al.*, 1991). Interestingly, patients with Brown-Séquard syndrome often recover good leg control and locomotion (Taylor and Gleave, 1957; Little and Halar, 1985; Roth *et al.*, 1991). In contrast, arm and hand functions recover only marginally and remain severely impaired or fully paralysed in these patients (Levi *et al.*, 1996). Only a few studies have investigated motor functions after lateral spinal hemisection in rats and monkeys. They showed a similar discrepancy in fore- and hindlimb recovery (Webb and Muir, 2002, 2005; Martinez *et al.*, 2009, 2010; Rosenzweig *et al.*, 2010). The underlying mechanisms for such dissimilar functional recovery of upper and lower motor functions are not understood.

Local spinal circuits, including central pattern generator networks, remain mostly intact below a spinal cord lesion, but lose their descending inputs, both the specific commands running mostly over the cortico-, rubro-, vestibulo- and reticulospinal tracts, and the unspecific modulatory bulbospinal inputs carried by the serotonin, dopamine and noradrenaline fibres. Local pharmacological application of dopamine, noradrenaline, serotonin or their agonists has been shown to partially substitute for these missing global excitatory inputs. Completely spinalized animals of different species (including mice, rats, cats and monkeys) showed remarkable transient activation of hindlimb central pattern

generators and locomotion under these conditions (Forsberg and Grillner, 1973; Barbeau and Rossignol, 1991; Fedirchuk *et al.*, 1998; Feraboli-Lohnherr *et al.*, 1999; Antri *et al.*, 2003, 2005; Guertin, 2004; Landry and Guertin, 2004). Monoaminergic bulbospinal projections are important for initiation and modulation of locomotion from fish to men (Grillner, 2003). After severe spinal cord injury, upregulation of various monoaminergic receptors occurs, which then can be pharmacologically targeted by locally applied monoaminergic drugs (Giroux *et al.*, 1999; Lee *et al.*, 2007; Hayashi *et al.*, 2010). Although application of monoaminergic agonists has powerful physiological effects in the hindlimb of animals with complete spinal cord injury, only very little is known about their potential in animals with partial spinal cord injury. After severe thoracic contusion injury in rats, voluntary hindlimb locomotion was not improved by injection of direct serotonergic agonists, which highly facilitate locomotion in spinalized rats (Feraboli-Lohnherr *et al.*, 1999; Antri *et al.*, 2003, 2005), but locomotor improvement was achieved by application of the serotonin precursor 5-hydroxytryptophan (Hayashi *et al.*, 2010). In cats, responses to monoaminergic agonists seem to depend on the type and extent of the lesion (Brustein and Rossignol, 1999). Effects of monoaminergic drugs on impaired forelimb functions have not been studied so far.

The purpose of the present study was to investigate locomotor recovery of fore- and hindlimbs in adult rats with precise C4/C5 unilateral spinal cord hemisections and to study the effects of intrathecally applied monoaminergic agonists on locomotor performance. Detailed kinematic assessment of locomotion revealed large differences between the hindlimbs, which rapidly developed robust and regular locomotion, and the ipsilesional forelimb, which showed massive and persisting functional deficits. Specific monoaminergic agonists applied in the chronic phase after spinal cord injury induced considerable modulation of distinct parameters of hindlimb locomotion. In contrast, forelimb locomotor networks showed almost no responsiveness to the same agonists. This study highlights remarkable differences between fore- and hindlimb motor circuitries in response to the strong reduction of supraspinal input and to monoaminergic drug treatment.

Materials and methods

Experimental setup

Adult female Lewis rats (180–220 g) received a precise and complete unilateral C4/C5 hemisection. Five animals were used for locomotor assessment. Spontaneous locomotor recovery was analysed during the first 28 days after spinal cord injury, whereas pharmacologically induced locomotor modulations were studied in the same animals in the chronic phase (starting on postoperative day 31). Eleven animals were used to investigate the monoaminergic innervation of the spinal cord in the intact situation, and 4 and 28 days after the lesion. Animals were housed in groups of three to four per cage and kept at a 12:12 h light:dark cycle with food and water *ad libitum*. All experimental procedures were approved by the veterinary office of the canton of Zurich, Switzerland.

Surgical procedures

Spinal cord injury and postoperative care

Animals were anaesthetized with a mixture of Hypnorm (0.125 mg/200 g body weight, Janssen Pharmaceuticals) and dornicum (0.75 mg/200 g body weight, Roche Pharmaceuticals). After laminectomy at C4 vertebral level, the right cervical spinal hemisection was transected with a surgical sapphire knife. The dorsal roots were spared from the transection. The animals received postoperative care including analgesics (Rymadil, 2.5 mg/kg body weight, Pfizer AG) for 3 days following surgery and antibiotics (Baytril, 5 mg/kg body weight, Bayer AG) for 7 days.

Intrathecal catheter implantation

A subdural catheter enabling intrathecal application of monoaminergic agonists was implanted 28 days after spinal cord injury. After partial laminectomy at T3 vertebral level, a thin catheter (32 gauge, Recathco) was inserted into the subarachnoid space and pushed rostrally to the spinal segment C7. The catheter was sutured to the paravertebral musculature to avoid dislocation of the tube. The distal end of the catheter was attached to a subcutaneous connector/backmount (Plastics One Inc.), which was sutured to the lower back musculature of the rat. The connector system was accessible from outside and allowed intrathecal bolus injections of defined volumes in awake animals. Catheter implantation did not lead to any obvious impairment of locomotion.

Pharmacological treatment

All drugs were dissolved in sterile water. Monoaminergic agonists were applied in the period from 31 to 49 days after spinal cord injury. The injected volume for each drug solution was 20 μ l, followed by 20 μ l of NaCl 0.9% (B. Braun Medical AG) to flush the drug into the CSF. The dead space of the catheter/backmount system was 12 μ l. Each drug was injected only once (except for drugs used in combined injections) to avoid pharmacological tolerance. Drug injections were separated by at least 48 h to avoid possible interactions with previously applied drugs. Depending on the particular drug, actions (if observed) peaked within the first 10 min and generally declined gradually over a period of minutes to few hours. The cannula system was flushed daily with 20 μ l saline solution to prevent clogging. Dosages of individual drugs were derived from earlier publications and were first tested in intact animals to avoid undesirable side-effects. For each drug, two different dosages were tested, of which the higher was behaviourally evaluated. The different drugs were injected at the following dosage: apomorphine (67 μ g/animal), clonidine (25 μ g/animal), methoxamine (90 μ g/animal), quipazine (40 μ g/animal), 8-OHDPAT (52 μ g/animal), combined quipazine and 8-OHDPAT (20 μ g quipazine and 26 μ g 8-OHDPAT/animal), combined apomorphine and quipazine and 8-OHDPAT (22 μ g apomorphine, 13 μ g quipazine and 17 μ g 8-OHDPAT/animal). Due to similar body weights of the animals (222–238 g), the dosages were not adapted to the body weight.

Locomotor quantification

Kinematic analysis

Kinematic assessment of locomotor performance was analysed as described in detail previously (Zörner *et al.*, 2010). In brief, the animals had to walk through shallow water (3 cm water height), while their performance was recorded with a frame rate of 200 Hz by a mobile

high-speed camera (Basler A504kc Color Camera, Basler AG). As shown earlier, the additional weight support provided by the water massively facilitated locomotion of animals with severe motor deficits (Kuerzi *et al.*, 2010; Zörner *et al.*, 2010). This enabled locomotor quantification of acutely injured animals that otherwise would not have been able to bear their full body weight. It should be mentioned that, due to differing biomechanics, locomotor parameters during wading should not be directly compared with walking, although several basic locomotor features seem to be conserved. A mirror system allowed simultaneous locomotor quantification of different body sites by providing the ventral and both lateral views of the animal. Four to six runs were recorded per animal and condition. Only sequences with continuous locomotor velocity between 0.2 and 0.3 m/s and sufficient lateral stability were selected for quantitative locomotor analysis. Pre-drug conditions were recorded immediately before drug application. Post-drug recordings were performed 10 min after drug application. The skin overlying prominent anatomical landmarks (hindlimb: iliac crest, hip, ankle, metatarsophalangeal joint; forelimb: shoulder blade, shoulder, wrist) was tattooed with a commercially available tattoo device (Hugo Sachs Elektronik, Harvard Apparatus GmbH) to allow permanent identification of the joints. Kinematic analysis of locomotion was performed by a colour based, semi-automated tracking software (Clickjoint V5.0; ALEA Solutions GmbH, www.aleasol.ch).

Locomotor parameters

Paw dragging was defined as contact of the paw with the runway during the swing phase of the step cycle and was expressed as ratio of the number of steps without dragging per total steps (Figs 3A and 4A). Body weight support of the respective limbs was determined by measuring the height of the shoulder or the iliac crest relative to the runway. The height of the iliac crest was measured during its mid-stance phase (temporal middle of the stance phase). The ipsilesional shoulder height was quantified at the time point just before the contralateral forelimb touched the floor and started its stance phase, since there was often no defined swing phase in the ipsilesional forelimb (persistent paw dragging). At this time, the ipsilesional shoulder height is maximally dependent on the weight support of the ipsilesional forelimb. Left–right coordination was defined by calculating the phase dispersion between the particular limbs (Kloos *et al.*, 2005). In a perfectly locomoting animal, one limb starts the step cycle exactly in the middle of the step cycle of its contralateral limb, thus resulting in a phase dispersion of 0.5. In Figs 3C and 4C, interlimb coordination was quantified by measuring the deviation (%) from a perfect ‘out of phase’ rhythm. For evaluation of diagonal forelimb/hindlimb coordination, the deviation from a perfect ‘in phase’ rhythm was calculated (Supplementary Fig. 1). Since there was not always an obvious off-ground swing phase (mainly in the ipsilesional forelimb), step cycles were defined by the onset of limb protraction (initiation of swing phase) and the onset of limb retraction (initiation of the stance phase). Protraction of the ipsilesional limbs was expressed as maximal positive distance of the wrist or toe tip relative to the shoulder or iliac crest, respectively (Figs 3D and 4D). Maximal protraction is found at the transition from swing to stance phase. Analogous to protraction, retraction of the ipsilesional fore- or hindlimb was defined as maximal negative distance of the wrist or toe tip relative to the shoulder or iliac crest, respectively (Figs 3E and 4E). Maximal retraction is found at the transition from stance to swing phase. Total limb excursion was derived from the sum of the absolute values of protraction and retraction of a particular step cycle (Figs 3F and 4F).

Tissue processing

Animals were transcardially perfused with 50 ml Ringer's solution containing heparin (B. Braun Medical AG) followed by 300 ml of a 4% phosphate-buffered formalin solution containing 5% sucrose. Animals used for immunohistochemical analysis were perfused with a formalin solution additionally containing 0.1% glutaraldehyde. The tissue was cryoprotected in a phosphate-buffered 30% sucrose solution for 3 days at 4°C. The spinal cords were embedded in Tissue-Tek® O.C.T.™ compound and frozen at –40°C. Spinal cords were cut in 40-µm thick transverse sections and mounted on slides. Transverse sections used for immunohistochemistry were collected free-floating in 24-well plates filled with cold 0.1M phosphate buffer and were stored in an anti-freeze medium (15% sucrose and 30% ethylene glycol in 50mM phosphate buffer, pH 7.4) at –20°C.

Analysis of lesion extent and quantification of motoneurons

Lesion extent and number of motoneurons were analysed in Nissl-stained transverse spinal cord sections. For the lesion size, the maximal lesion extent was determined for each animal (Fig. 1B). Motoneurons in the cervical spinal cord were counted in 35 transverse sections symmetrically distributed over the cervical spinal segments C1–C8 in three animals. The criteria for motoneuron identification were a clearly Nissl-stained cell body with a diameter of at least 30 µm located in Rexed's lamina VIII or IX.

Immunostaining

Free-floating sections of different spinal segments were incubated for 3 days at 4°C with the primary rabbit antibody against serotonin (5-HT, 1:12 000, rabbit, Immunostar) or tyrosine hydroxylase (1:250, rabbit, Millipore). Primary antibodies were detected with a biotinylated antibody (1:300, goat, Jackson ImmunoResearch Laboratories) overnight at 4°C. After washing with phosphate buffer, the sections were probed with streptavidin Cy2 (1:500; Jackson ImmunoResearch Laboratories) for 45 min at 25°C. For cytoarchitectonic identification of spinal segments and motoneuron pools, the sections were counterstained with NeuroTrace 640/660, a deep-red fluorescent Nissl stain (Invitrogen). After final washing steps in phosphate buffer, sections were mounted, air-dried overnight and coverslipped with Mowiol (Calbiochem).

Densitometric quantification

Three to four animals were analysed for each time point (intact, 4 and 28 days after unilateral hemisection). Confocal image acquisition was performed using the spectral confocal microscope TCS SP2 AOBs (Leica Microsystems) with a ×40 oil immersion objective (HCX PL APO Oil, 1.25 numerical aperture) at maximal antibody penetration depth. Image size was defined as 1024 × 1024 pixels. Gain voltage-based pixel intensity of the immunoreactive fibres was set using LUT Glow (O) function to the optimal signal-to-noise ratio. Only areas exhibiting two or more fluorescent Nissl-stained motoneurons (in Rexed's lamina VIII or IX) were used for quantification of optical fibre density. The obtained images were background-corrected and their optical densities were quantified using ImageJ software (NIH). Bilateral averaged optical density of all intact animals at spinal segment C2 was set as 1.0 relative optical density for the

respective marker. Representative images were only processed by identical minimal contrast enhancement.

Statistics

Immunohistochemical (Fig. 2A and C) and behavioural data of spontaneous locomotor recovery (Fig. 3A–F) were analysed using one-way ANOVA followed by *post hoc* Bonferroni test (with repeated measures test for Fig. 3A–E). For comparison of pre- and post-drug conditions (Fig. 4A–F), we used Student's *t*-test (two-tailed, paired). Data are presented as animal group mean values for every testing session and error bars represent SEM; **P* < 0.05; ***P* < 0.01, ****P* < 0.001.

Results

Analysis of lesion extent

Lesion size was analysed for all animals by determining the maximal lesion extent in Nissl-stained spinal cord sections. The largest and smallest lesions among the animal groups (behavioural and immunohistochemical group) are shown in Fig. 1A and B. Except for one animal that had additional damage in the dorsal quadrant of the contralateral hemicord, all lesions were unilateral with no or minor damage of contralesional fibre tracts. To assess the damage of cervical motoneurons by the lesion, the number of motoneurons in Rexed's lamina VIII and IX of the cervical spinal cord was quantified in three animals. The lesion was highly localized; motoneurons disappeared over a total rostrocaudal distance of 1 mm, which corresponds to around half a cervical segment (Fig. 1C and D). Adjacent cervical spinal segments and the contralesional hemicord did not show any decrease in the number of motoneurons (Fig. 1D). The majority of the motoneurons demonstrated normal morphology with granular Nissl staining. Only a few motoneurons, located in close proximity of the lesion site, revealed abnormal dense Nissl staining (data not shown).

Depletion of serotonin and tyrosine hydroxylase-positive fibres in the cervical and lumbar spinal cord after injury

To assess the depletion of bulbospinal monoaminergic projections after cervical unilateral hemisection, immunohistochemical stainings for serotonin and tyrosine hydroxylase were performed at different spinal levels of intact animals, and animals 4 and 28 days after spinal cord injury. Serotonin and tyrosine hydroxylase antibodies revealed highly specific staining of axons and boutons with only minimal background noise.

In intact adult rats, the spinal serotonin innervation was approximately twice as dense at the lumbar levels L2–L4 than at the cervical levels C2 and C6–C8. The sublesional serotonin fibre density in the contralesional hemicord was unaffected at all time points after spinal cord injury (Fig. 2A and B). Ipsilesional serotonin fibres caudal to the lesion were strongly reduced by the injury, resulting in reduction of serotonin fibre density by 68% at C6–C8 and by

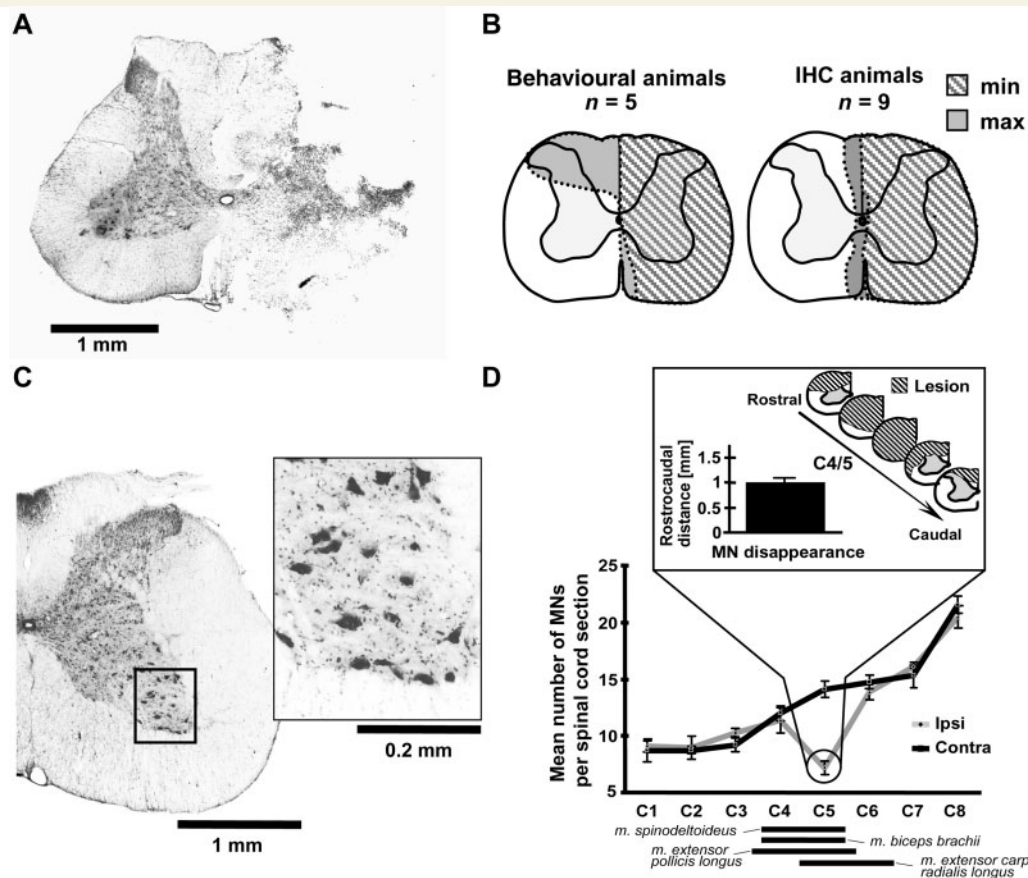


Figure 1 Histological analysis of the spinal cord lesion. (A) Nissl-stained transverse section through the lesion centre at C4/C5. (B) Minimal and maximal lesion extent of animals used for functional evaluation of locomotion (*left*) and animals used for immunohistochemical investigations (*right*). (C) Nissl-stained section illustrating the intact motoneurons (MNs) in the ventral horn of spinal segment C6/C7. (D) Motoneuron counts on ipsilesional (Ipsi, grey line) and contralesional (Contra, black line) hemicord in three animals 7 weeks after unilateral hemisection. The increased number of motoneurons in the caudal cervical segments is due to paw and digit motoneuron pools. The ipsilesional hemicord revealed a dip in motoneurons at level C5, where the lesion was located. Motoneurons disappeared over a total rostrocaudal distance of ~1 mm (*top*). Motoneuron pools of specific forelimb muscles (*m. spinodeltoideus*, *m. biceps brachii*, *m. extensor pollicis longus* and *m. extensor carpi radialis longus*), which span two or more spinal segments (from McKenna *et al.*, 2000), were only partially damaged by the restricted spinal cord injury. Adjacent spinal segments were not affected. Data are presented as mean \pm SEM. IHC = immunohistochemistry.

92% at the spinal levels L2–L4 at 28 days after the injury. The further decline of the serotonin fibre density from postoperative days 4–28 argues against a significant compensatory sprouting of serotonergic fibres in the sublesional spinal cord within 28 days after spinal cord injury.

Similar results were obtained for dopaminergic/noradrenergic fibres visualized by tyrosine hydroxylase immunohistochemistry. In intact animals, a higher density of tyrosine hydroxylase fibres was found at spinal segments C6–C8 than at C2, and the fibre density was again higher in the lumbar enlargement (Fig. 2C). The ipsilesional tyrosine hydroxylase fibres were reduced by 61% in the cervical and by 89% in the lumbar enlargement at 28 days after injury (Fig. 2C and D). In contrast to serotonin, tyrosine hydroxylase-positive fibres demonstrated a partial depletion in the contralesional hemicord caudal to the lesion at 28 days after injury when compared with intact animals (C6–C8: –40%;

L2–L4: –33%). As observed for serotonin, there was no restitution of tyrosine hydroxylase-positive fibre density up to 28 days after spinal cord injury.

Spontaneous locomotor recovery in rats with Brown-Séquard syndrome

The spontaneous locomotor performance in the absence of drug treatment was investigated weekly over a period of 28 days after spinal cord injury. Locomotion was analysed during walking through shallow water (3 cm depth). The specific locomotor parameters analysed were paw dragging, parameters of inter-limb coordination (phase dispersion) and detailed kinematic evaluations of fore- and hindlimb movements, i.e. body height, maximal positive and negative excursion (pro-/retraction), and total limb excursion. During the first few days after spinal cord injury, the

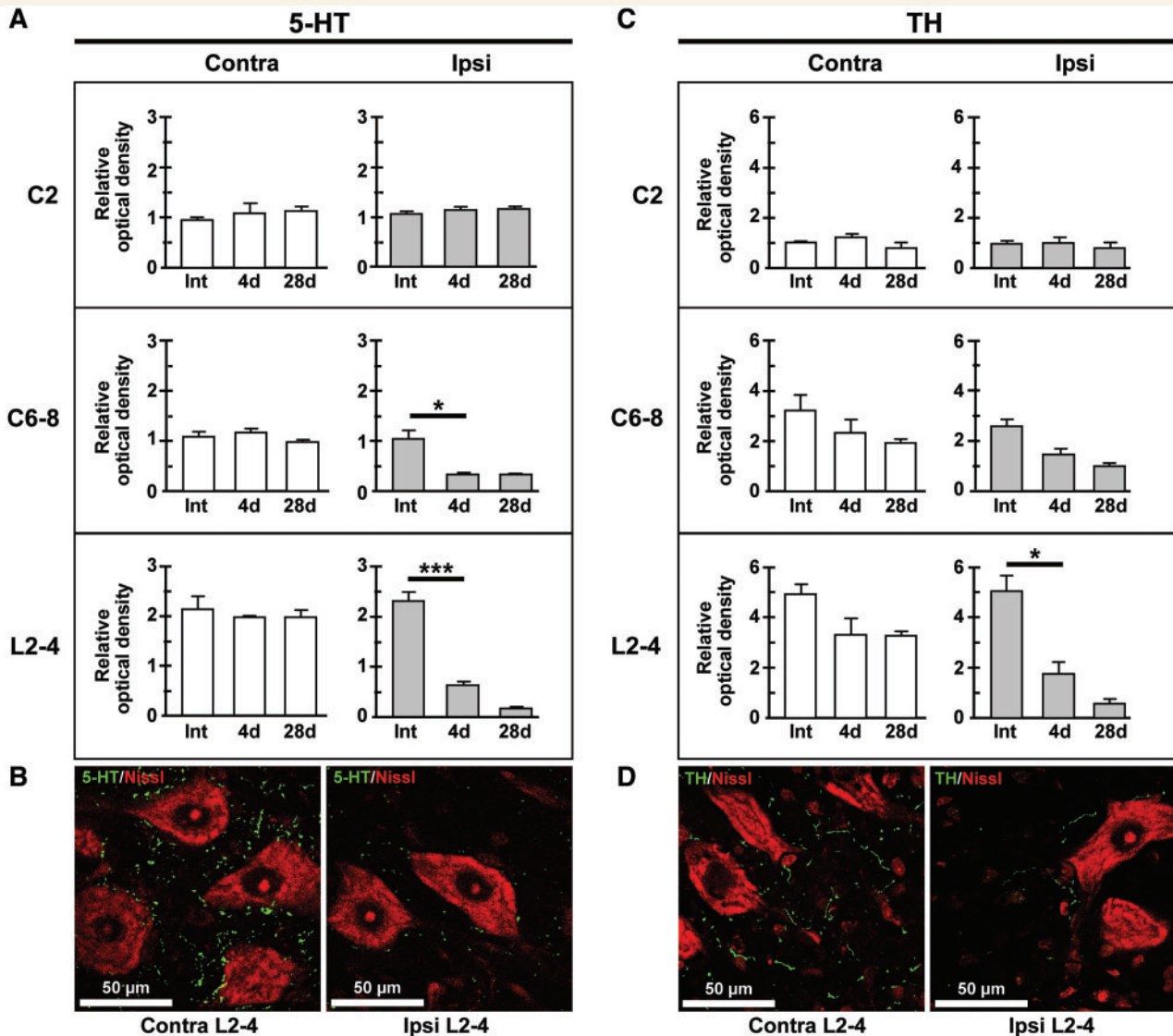


Figure 2 Analysis of serotonin (5-HT) and tyrosine hydroxylase (TH) positive fibres at different levels of the spinal cord. For quantification, densitometric values of all spinal segments were normalized to the average values obtained for intact animals at spinal segment C2. (A) Fibre density of serotonin at spinal level C2, C6–C8 and L2–L4. Serotonin fibre density was higher in the lumbar than the cervical spinal cord in intact animals (Int). Serotonin fibres were progressively lost in the ipsilesional (Ipsi) hemicord 4 and 28 days after injury. The contralesional (Contra) innervation was unaffected below the lesion. (B) Representative images of lumbar serotonin innervation of the motoneuron pool in an animal 28 days after unilateral hemisection. Note the strong reduction of serotonin fibre density in the ipsilesional hemicord (*right*). (C) Tyrosine hydroxylase fibre density at spinal levels C2, C6–C8 and L2–L4 showed increased lumbar versus cervical levels in intact animals. Caudal to the lesion, the tyrosine hydroxylase innervation was mainly reduced in the ipsilesional hemicord, but also partially on the contralesional side. (D) Lumbar ventral horn motoneurons and tyrosine hydroxylase innervation showing dense contralesional tyrosine hydroxylase fibre innervation compared with the strongly reduced fibre plexus in the ipsilesional hemicord (28 days after the lesion). Data in (A) and (C) are presented as mean \pm SEM. Statistical evaluation was carried out with one-way ANOVA with Bonferroni *post hoc* test comparing BL with 4 days (lesion deficit) and 4 days with 28 days (recovery). * $P < 0.05$; *** $P < 0.001$.

animals showed partial to complete paresis of the ipsilesional forelimb and of both hindlimbs with frequent spastic-like periods of high muscle tonus (particularly in the hindlimbs). After postoperative day 7, the hindlimbs recovered at a fast rate and allowed the animal to regain mobility within a few days. In contrast, the ipsilesional forelimb remained in a rigid condition with only minimal weight support function and a small range of motion.

Paw dragging did not occur in fore- and hindlimbs during wading in intact animals. Seven days after injury, proper stepping was abolished and steps without paw dragging were rare or absent for the ipsilesional forelimb. Forelimb dragging persisted over the whole experimental period (Fig. 3A). For the ipsilesional hindlimb, proper steps without paw dragging decreased from 100% to 29% of total steps at 7 days after the lesion. Paw

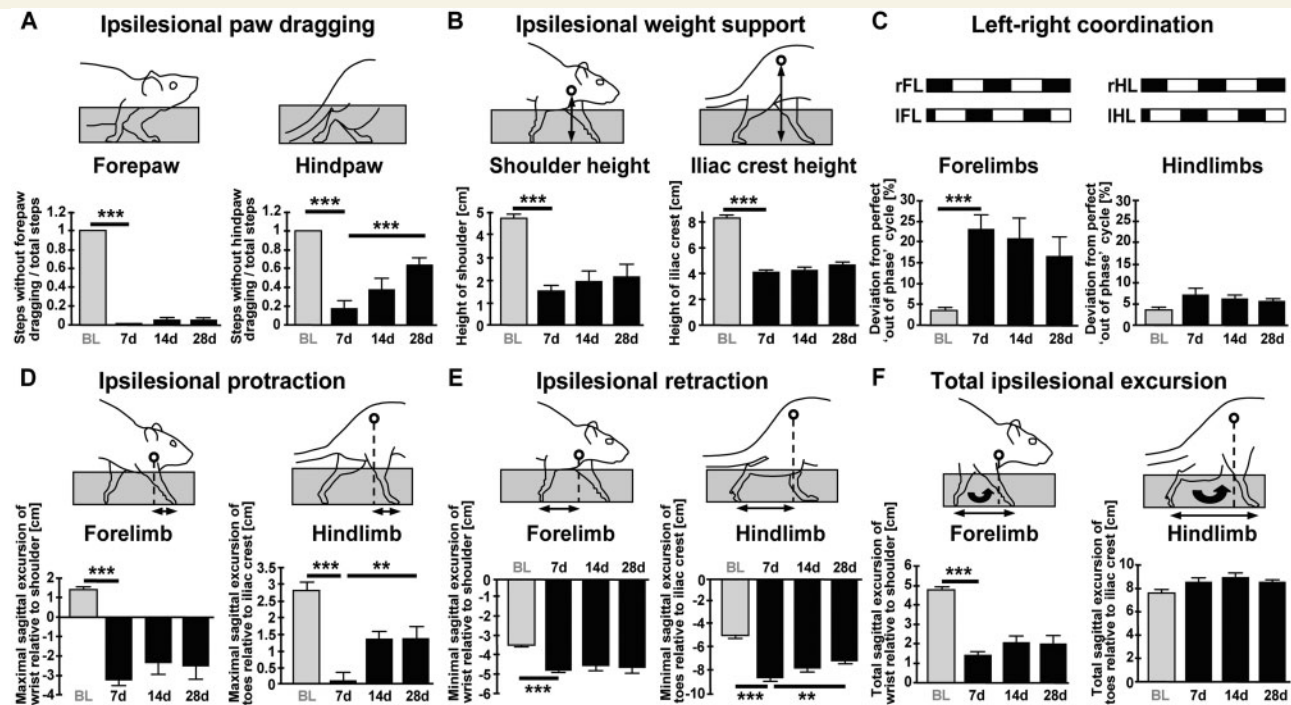


Figure 3 Assessment of spontaneous locomotor performance of fore- and hindlimbs at 7, 14 and 28 days after unilateral cervical hemisection. (A) Ipsilesional limbs showed a strong impairment in stepping resulting in severe paw dragging 7 days after injury. Forepaw dragging constantly persisted over the entire experimental period, whereas ipsilesional hindlimb stepping and dragging recovered significantly after the initial lesion deficit. (B) Body weight support of the ipsilesional fore- and hindlimb was significantly decreased 7 days after injury. There was no significant recovery of weight support in both limbs. (C) Inter-forelimb coordination was distorted acutely after the lesion and showed no significant recovery with time. Inter-hindlimb coordination revealed no significant lesion effect and was close to normal 28 days after spinal cord injury. (D) Protraction of the ipsilesional forelimb was strongly diminished 7 days post lesion and did not recover with time. Hindlimb protraction showed a severe initial decrease 7 days after spinal cord injury, which was followed by significant functional recovery up to 28 days after injury. (E) Retraction of the ipsilesional forelimb was significantly increased and did not recover up to 28 days post lesion. The ipsilesional hindlimb showed a significantly increased retraction acutely after the lesion, which decreased towards baseline by 28 days post injury. (F) Total excursion of the ipsilesional forelimb was significantly decreased by the lesion and did not recover. Total excursion of the ipsilesional hindlimb was not affected by the lesion. Five to 10 representative step cycles were analysed per animal and per condition. Data are presented as mean \pm SEM. Statistical evaluation was carried out with one-way ANOVA repeated measures with Bonferroni *post hoc* test comparing baseline with 7 days (lesion deficit) and 7 days with 28 days (functional recovery). ** $P < 0.01$; *** $P < 0.001$.

dragging of the ipsilesional hindlimb significantly recovered up to 28 days post injury (Fig. 3A).

Body weight support provided by the fore- and hindlimbs was analysed by measuring the height of the shoulder and the iliac crest, respectively. The ipsilesional shoulder height decreased to 33% of the intact weight support 7 days after spinal cord injury (Fig. 3B). There was no significant recovery of weight support in the ipsilesional forelimb during the experimental period. The height of the iliac crest was significantly reduced to 50% of the intact height 7 days after the lesion, and also showed no significant recovery by 28 days post injury (Fig. 3B).

Left–right coordination was analysed by calculating the deviation from a perfect ‘out of phase’ rhythm of the fore- and hindlimbs (Zörner *et al.*, 2010). In intact animals, this ‘out of phase’ deviation was very low (3.5% for the fore- and hindlimbs; Fig. 3C). The alternating stepping pattern in forelimb locomotion was highly distorted 7 days after injury (23.1% deviation). There

was a trend towards functional recovery in inter-forelimb coordination, but this did not reach significance. Very different from the forelimbs, the inter-hindlimb coordination was only slightly impaired after spinal cord injury and reached an almost normal deviation of 5.8% at 28 days post injury (Fig. 3C). To investigate coordination between fore- and hindlimbs, diagonal forelimb–hindlimb coupling was analysed by calculating the deviation from a perfect ‘in phase’ rhythm (synchronous step cycle). Intact animals demonstrated a nearly perfect coupling between the diagonal fore- and hindlimb (Supplementary Fig. 1A). There was a strong impairment of forelimb–hindlimb coupling, which did not recover up to 28 days post injury. This was due to a nearly doubled stepping frequency of the forelimbs as compared with the hindlimbs, which made a proper forelimb–hindlimb coordination impossible (Supplementary Fig. 1B).

Protraction is found during the swing phase of the step cycle when the limb advanced in reference to the body. Protraction of

the ipsilesional forelimb was abolished by the lesion (intact: 1.4 cm; 7 days post lesion: -3.3 cm) without any recovery over time (Fig. 3D). Hindlimb protraction was initially strongly affected (intact: 2.8 cm; 7 days post-lesion: 0.1 cm), but, in contrast, showed a profound functional recovery from postoperative days 7–14 (14 days post-lesion: 1.35 cm). Nonetheless, hindlimb protraction did not recover completely (Fig. 3D).

Retraction is found during the stance phase of the step cycle when the body advances in reference to the limbs. Retraction of the ipsilesional forelimb significantly increased 7 days after injury (intact: -3.4 cm; 7 days after spinal cord injury: -4.7 cm). There was no recovery of forelimb retraction by 28 days after the injury (Fig. 3E). The hindlimb retraction was also significantly increased after injury (intact: -4.8 cm; 7 days post lesion: -8.5 cm), but showed significant recovery up to 28 days after spinal cord injury (-7.0 cm) without, however, reaching baseline values (Fig. 3E).

Total limb excursion is composed of the sum of pro- and retraction. Total ipsilesional forelimb excursion strongly decreased after the injury, which was mainly due to the inability of the lesioned animals to protract their forelimb. The forelimb persisted in a rigid state with a low range of movement (Fig. 3F). The total excursion of the ipsilesional hindlimb was unaffected by the lesion (Fig. 3F). This is due to the fact that reduced protraction was associated with increased retraction leading to a backward shift of the movement with unchanged total values of total limb excursion.

In summary, the spontaneous locomotor performance of rats with unilateral C4/C5 hemisection showed a very unequal recovery for the fore- and hindlimbs. The ipsilesional forelimb showed very severe deficits in all locomotor parameters evaluated, even under conditions where the body weight was optimally supported (wading), and only minor or no recovery occurred with time after the lesion. In contrast, the ipsilesional hindlimb demonstrated a remarkable degree of locomotor performance in most parameters up to 28 days after the lesion in spite of large initial deficits. The locomotor parameters of the fore- and hindlimbs are at least partially influenced by each another. Mainly the poor locomotor function of the ipsilesional forelimb is likely to have an impact on the locomotor pattern of the hindlimbs.

Locomotor responses of fore- and hindlimbs to monoaminergic drugs

The responsiveness of fore- and hindlimb locomotor circuits to dopaminergic, serotonergic and noradrenergic agonists was analysed in the same animals already quantified for spontaneous locomotion, starting at 31 days after spinal cord injury. Agonists acting on the different monoaminergic systems were intrathecally applied via a subdural catheter implanted 4 days prior to injections. Apomorphine was used as a non-selective agonist of the dopaminergic system (agonist of D1 and D2 receptors), clonidine and methoxamine as selective agonists of the α_2 - or α_1 -noradrenergic receptors, respectively, and quipazine (non-specific agonist of 5-HT₂ receptor) and 8-OHDPAT (selective agonist of 5-HT₁ receptor) were used as serotonergic agonists.

Paw dragging of the ipsilesional forelimb was not responsive to any of the applied drugs and constantly persisted throughout the experimental period (Fig. 4A). Conversely, hindlimb dragging became significantly worse with noradrenergic agonists methoxamine and particularly clonidine. The 5-HT₂ receptor agonist quipazine also significantly increased hindpaw dragging (Fig. 4A).

Body weight support was modulated by different monoaminergic agonists (Fig. 4B). The shoulder height of the ipsilesional forelimb was significantly and selectively increased by apomorphine. This effect was not accompanied by a simultaneous increase of weight support in the hindlimbs, indicating a specific modulation of cervical locomotor networks by apomorphine. The height of the ipsilesional iliac crest was negatively affected by clonidine, methoxamine and 8-OHDPAT, all of them significantly decreased the weight support of the ipsilesional hindlimb (Fig. 4B).

Left–right coordination of the forelimbs was largely unaltered by application of any drug (Fig. 4C). Hindlimb left–right coordination was significantly distorted by clonidine. The animals frequently demonstrated stumbling and irregular locomotion. However, combined application of the serotonergic agonists quipazine and 8-OHDPAT tended to stabilize locomotion and improved rhythmic alternation of hindlimb stepping (Fig. 4C). Diagonal forelimb/hindlimb coordination was not altered by drug application (Supplementary Fig. 1C).

Protraction of the ipsilesional forelimb was unaffected by application of any monoaminergic agonist (Fig. 4D). However, protraction of the ipsilesional hindlimb was strongly diminished after application of clonidine (from 1.3 to 0.3 cm). The 5-HT₁ receptor agonist 8-OHDPAT alone and combined injections containing 8-OHDPAT demonstrated a non-significant reduction of the hindlimb protraction (Fig. 4D).

Retraction and total limb excursion did not show significant modulation for the ipsilesional fore- or hindlimb after application of any agonist (Fig. 4E and F).

Taken together, the fore- and hindlimb locomotor networks revealed a very different responsiveness to monoaminergic agonists after unilateral cervical spinal cord injury. The ipsilesional hindlimb showed significant modulation of particular locomotor parameters after application of specific drugs that, in the majority of cases however, negatively interfered with spontaneously recovered hindlimb function. In contrast, the ipsilesional forelimb showed almost no locomotor responsiveness to monoaminergic agonists.

Discussion

High cervical unilateral spinal cord hemisection in adult rats massively impairs fore- and hindlimb functions. Detailed kinematic analysis revealed a highly disproportional recovery of fore- versus hindlimb locomotor performance. The ipsilesional forelimb remained rigid and essentially non-functional, whereas the ipsilesional hindlimb showed substantial functional recovery and performance at 14–28 days post lesion. Dopaminergic, noradrenergic and serotonergic receptor agonists, some of them significantly improving the hindlimb function in complete thoracic spinal cord injury models, were without effect on forelimb locomotion in

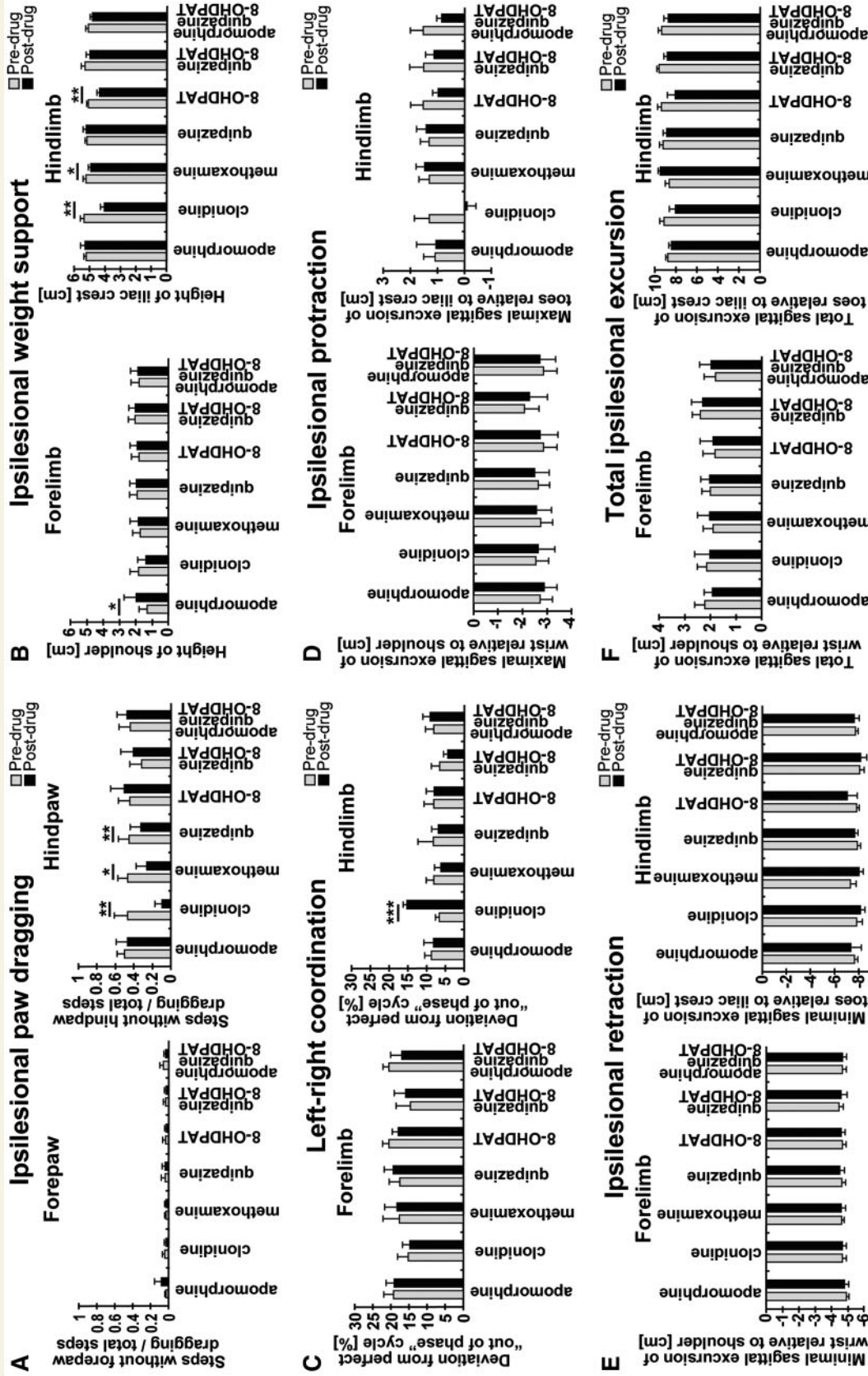


Figure 4 Locomotor responses of fore- and hindlimbs to monoaminergic drugs. (A) Ipsilesional forepaw dragging was unaltered by any monoaminergic agonist. Hindpaw dragging was significantly increased after application of clonidine, methoxamine and quipazine. (B) Injection of apomorphine significantly increased weight support of the ipsilesional forelimb. Clonidine, methoxamine and 8-OHDPAT significantly reduced weight support in the ipsilesional hindlimb. (C) Inter-forelimb coordination was not impaired by monoaminergic drugs. Clonidine significantly perturbed coordination of hindlimbs during locomotion. Combined application of quipazine and 8-OHDPAT revealed non-significant improvement of hindlimb coordination. (D–F) Neither protraction (D), retraction (E), nor total limb excursion (F) of the ipsilesional fore- and hindlimb showed significant modulations by single or combined monoaminergic drug injections. Five to 10 representative step cycles were analysed per animal and per condition. Data are presented as mean \pm SEM. Statistical evaluation was carried out with Student's *t*-test (two-tailed, paired) comparing pre-drug with post-drug condition of a particular drug. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

our hemisection model and had minor, mostly negative effects on hindlimb circuits.

Since acutely after injury, the ipsilesional forelimb and both hindlimbs were severely impaired, wading with partial body weight support was the most appropriate paradigm to study locomotor recovery in these rats. Over 2–4 weeks, the hindlimbs regained a well-controlled locomotor pattern with movement parameters that were close to normal. In contrast, the ipsilesional forelimb remained paretic and rigid with constant dragging often combined with a closed paw. Weight support of the ipsilesional forelimb was permanently reduced and inter-limb coordination remained impaired over the entire experimental period. These findings parallel the clinical observations in humans with incomplete cervical spinal cord injury (including patients with Brown-Séquard syndrome); patients often recover leg function (including walking), but only poor arm and hand functions (Levi *et al.*, 1996). Unilateral cervical hemisection at C7 spinal level in primates revealed an analogous motor recovery between fore- and hindlimbs; hindlimb locomotion recovered extensively, whereas forelimb locomotion and hand function recovered to a lesser extent (Rosenzweig *et al.*, 2010). Thus, the functional outcome after cervical lateral hemisection in rats (especially locomotion) strongly resembles the primate and human situation.

This resemblance in lesion outcome across species with lateral spinal hemisection could rely on two main reasons: first, there is a high functional and anatomical conservation of supra- and intraspinal motor systems among vertebrates. The classical studies by Kuypers and colleagues (Lawrence and Kuypers, 1968; Drew, 1991; Küchler *et al.*, 2002; Lemon, 2008) in macaques showed that the ventromedial (reticulo- and vestibulospinal tract) and the lateral system (rubrospinal tract) reveal similar anatomy and implement analogous motor function as observed in rodents and cats. The corticospinal tract has a more prominent role in motor function in primates/humans as compared with non-primate quadrupeds (Nathan, 1994; Levi *et al.*, 1996; Courtine *et al.*, 2007). Experimental studies agree that interruption of the corticospinal tract in rodents and cats only minimally impairs locomotor function (Alstermark *et al.*, 1989; Muir and Whishaw, 1999). The role of the corticospinal system in human locomotion is less clear, probably due to little restricted damage of this system in clinical cases. Whereas Nathan (1994) postulated that the corticospinal system in humans is the most prominent system for locomotion, other researchers claim that, besides the corticospinal tract, the ventromedial and -lateral reticulospinal fibres are mainly critical for locomotion (Lawrence and Kuypers, 1968; Eidelberg, 1981; Levi *et al.*, 1996). There are also parallels in the propriospinal system between species, especially with regard to the C3–C4 propriospinal pool serving as relay for supraspinal information in cats and monkeys (Alstermark *et al.*, 1981; Pettersson *et al.*, 2007). Evidence for a corresponding C3–C4 propriospinal system in rats is still lacking.

Second, a unilateral hemisection leads to a full unilateral ablation of commands in the spinal cord from each supraspinal system, independent of its specific function. Given the similar anatomy of most descending tracts (Lawrence and Kuypers, 1968; Lemon, 2008), this would result in a similar outcome across species, assuming that supraspinal inputs have a similar role in locomotion.

Motor recovery after unilateral hemisection is thought to result from compensatory sprouting of descending fibres from the intact side of the spinal cord (Weidner *et al.*, 2001; Ballermann and Fouad, 2006; Rosenzweig *et al.*, 2010), from propriospinal relay circuits (Alstermark *et al.*, 1981; Bareyre *et al.*, 2004; Courtine *et al.*, 2008) or from intrinsic re-organization of intraspinal circuits (Barrière *et al.*, 2008). For the latter, Barrière and colleagues (2008) impressively demonstrated that cats with a thoracic lateral hemisection recovered robust hindlimb locomotion. By performing a subsequent complete transection caudal to the hemisection, the authors could show immediate functional hindlimb stepping in the trained cats. This indicates intrinsic re-organization of the lumbar central pattern generator networks after the first hemisection, probably by remaining supraspinal input, as a key element of functional recovery. Given the very different functional outcome observed in the present study after unilateral hemisection, the mechanisms of functional recovery must differ substantially between fore- and hindlimbs, in the quadrupedal rat as the bipedal human.

To investigate direct damage of motor circuits by the cut lesion, histological analysis of cervical motoneuron numbers and morphology were performed. The extent of the lesions was limited affecting motoneurons over a rostrocaudal distance of only 1 mm, which roughly corresponds to half a cervical spinal segment. Since most motoneuron pools innervating particular forelimb muscles span two or more segments (McKenna *et al.*, 2000), direct damage of motoneurons is unlikely to be a major reason for the severe motor deficits of the forelimb after injury. This is further supported by the observation that patients with cervical spinal cord injury at different spinal levels often show very similar motor impairments of arm/hand functions, regardless of the exact location of the lesion (Levi *et al.*, 1996). Moreover, cervical central pattern generator networks operating rhythmic locomotor activity in rats are located predominately in spinal segments C7–T1 (Ballion *et al.*, 2001), well below our C4/C5 lesion.

Interestingly, recent data from retrograde neuroanatomical tracing of descending tracts from the cervical and lumbar spinal cord in intact adult rats revealed that most supraspinal descending systems, such as the corticospinal and most bulbospinal tracts, reveal a considerably higher fibre number ending in the cervical than in the lumbar enlargement (Björn Zörner, manuscript in preparation). Similar observations were reported for reticulospinal fibres in humans (Nathan, 1994). The major exceptions were the monoaminergic nuclei (raphe nuclei, locus coeruleus), which displayed denser innervation of the lumbar spinal cord, as also shown in the present study. These anatomical data strongly suggest that forelimb movements, contrary to the hindlimbs, require more supraspinal commands controlling motor function, which is reflected in the locomotor deficits after unilateral spinal cord injury. Hindlimb circuits, in contrast, function more autonomously based on a prominent central pattern generator controlled by commissural interneurons, some mid-line crossing fibres from the spared tracts, and remaining sensory afferents (Barrière *et al.*, 2008).

Monoamines are key regulators of motoneuron and local circuit excitability in the spinal cord. In intact animals, the density of serotonin and tyrosine hydroxylase-positive fibres was 2–5 times higher in the lumbar than the cervical spinal cord (this study; for

serotonin see also Hadjiconstantinou *et al.*, 1984; Colado *et al.*, 1988). After cervical unilateral hemisection, the serotonin and tyrosine hydroxylase-positive fibres were highly reduced in the cervical and lumbar hemicord. In agreement with Bregman (1987) and Golder *et al.* (2001) (C2 and mid-thoracic unilateral hemisection) but in contrast to Saruhashi and colleagues (1996) (unilateral T8 hemisection), we did not observe a restoration of the ipsilesional serotonin and tyrosine hydroxylase fibre plexus up to 4 weeks after the lesion, but even found a further reduction of the serotonin and tyrosine hydroxylase signals from 4 to 28 days after the lesion. The lack of monoaminergic regrowth after cervical hemisection in this study indicates that monoaminergic fibre sprouting is not a key component of locomotor recovery observed in this lesion model.

After complete transection of the lower spinal cord, monoaminergic agonists have a potent facilitatory effect on hindlimb central pattern generators in mice, rats, cats and monkeys (Forssberg and Grillner, 1973; Barbeau and Rossignol, 1991; Fedirchuk *et al.*, 1998; Feraboli-Lohnherr *et al.*, 1999; Antri *et al.*, 2003, 2005; Guertin, 2004; Landry and Guertin, 2004). Clonidine application in patients with spinal cord injury revealed unclear results, varying from detrimental to beneficial for locomotion (Stewart *et al.*, 1991; Dietz *et al.*, 1995). When we injected agonists of the different monoaminergic systems intrathecally in the chronic phase after the C4/C5 hemisection, particular parameters of hindlimb locomotion were modulated by specific receptor agonists, but mostly in a negative, non-functional direction (increase in dragging, reduction in weight support, deterioration of inter-hindlimb coordination and reduction of hindlimb protraction). The rigid and non-functional forelimb showed almost no reaction to the monoaminergic agonists; an increase in shoulder height and limb stiffness after application of the dopaminergic agonist apomorphine was the only drug-induced modulation observed. The α 2-noradrenergic receptor agonist clonidine showed various detrimental effects on hindlimb locomotion ranging from increased paw dragging, to deteriorated left–right alternation. Similar adverse effects of clonidine on hindlimb locomotion were also found in cats with incomplete thoracic spinal cord injury (Brustein and Rossignol, 1999). The 5-HT₂ receptor agonist quipazine that has been shown to be highly potent in facilitating locomotion of completely spinalized rats (Antri *et al.*, 2003, 2005; Gerasimenko *et al.*, 2007; Ichiyama *et al.*, 2008; Courtine *et al.*, 2009) had only minor effects on hindlimb locomotion in our rats with a unilateral C4/C5 hemisection. Injections of the selective 5-HT₁ receptor agonist 8-OHDPAT induced a significant reduction of weight support, which is well known as 5-HT₁-induced flat body posture in the serotonin syndrome (Darmani and Ahmad, 1999). The dopaminergic agonist apomorphine did not elicit any modulation of hindlimb locomotion.

Our results suggest that the monoaminergic drive may be more important for hindlimb networks, whereas forelimb locomotor networks, which need to flexibly target different muscle groups to execute motor tasks (Harel *et al.*, 2008), are more precisely controlled and driven by supraspinal centres, requiring specific descending input instead of the rather global monoaminergic stimulation. This is further supported by the higher density of monoaminergic fibres in the lumbar compared with the cervical

spinal cord, which is also reflected in the respective receptor densities [5-HT₁ receptors, (Marlier *et al.*, 1991); D1 receptors, (Dubois *et al.*, 1986)]. However, the exact site of drug actions (i.e. segmental level and cell type) cannot be identified by the present study.

A second important conclusion of our results concerns the big differences seen in the drug effects between the incomplete and the complete or ventral spinal cord injury models. In the C4/C5 unilateral hemisection model, the locomotor responses to the monoaminergic agonists were minor and importantly, mostly negative for the hindlimbs. In contrast, rats with complete or large ventral thoracic spinal cord injury reacted to monoaminergic agonists with substantial and mostly positive locomotor responses (Feraboli-Lohnherr *et al.*, 1999; Antri *et al.*, 2003, 2005; Courtine *et al.*, 2009; Filli *et al.*, unpublished results) especially when in combination with epidural spinal cord stimulation (Gerasimenko *et al.*, 2007; Courtine *et al.*, 2009). One reason for these surprising discrepancies might be the differing number, sensitivity and distribution (pre- versus postsynaptic) of monoaminergic receptors in spinal cord injury models of different severity (Rossignol *et al.*, 2001). Hayshi and colleagues (2010) postulated that a certain threshold denervation of monoaminergic fibres must be present in order to induce postsynaptic receptor changes; upregulation of lumbar 5-HT_{2C} receptors was only seen after severe, but not moderate contusion injury in the thoracic spinal cord. Moreover, monoaminergic drugs applied after complete or large ventral spinal cord injury act exclusively on postsynaptic receptors, whereas in our lesion model, the drugs can affect presynaptic (unlesioned terminals) and postsynaptic receptors. Since the function of these receptors could be different depending on their location (i.e. negative feedback role for presynaptic receptors), the differing pre-/postsynaptic receptor balance is likely to affect the pharmacological effects on locomotion. Murray *et al.* (2010) showed that transcriptional changes after complete spinal cord transection led to the production of 5-HT₂ receptors, which are spontaneously active without serotonin. These constitutively active receptors are thought to act as functional compensation for the severe loss of descending serotonergic fibres from the brainstem. Hence, the appearance of these receptors is likely to be dependent again on the severity of the lesion. Lastly, monoaminergic stimulation could negatively interfere with spared locomotor commands, which are present after unilateral hemisection, but absent after complete or large ventral spinal cord injury.

In conclusion, the present study demonstrates a clearly different response of fore- versus hindlimb spinal circuitries upon unilateral ablation of supraspinal commands. As observed in primates and humans, spontaneous motor recovery after cervical unilateral hemisection (leading to the Brown-Séquard syndrome) is considerable for the lower limbs, but often marginal or absent for the ipsilesional upper extremity. Responsiveness to monoaminergic drugs was absent in the forelimb and higher, although mainly functionally negative, in the hindlimbs. Monoaminergic stimulation that exerts positive locomotor effects in animals with complete or ventral spinal cord injury, interfered with the remaining supraspinal command systems in rats with incomplete spinal cord injury. The present study illustrates the urgent need to model cervical incomplete spinal cord injury as a frequent spinal cord injury type in

humans, to better understand the differing control systems of upper and lower extremities as well as potential therapeutic options like the stimulation of spinal monoamine receptors.

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Supplementary material

Supplementary material is available at *Brain* online.

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