Published in final edited form as:

J Neurosci Methods. 2018 October 01; 308: 142–150. doi:10.1016/j.jneumeth.2018.07.014.

Quantification of Motor Neuron Loss and Muscular Atrophy in Ricin-Induced Focal Nerve Injury

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Abstract

Background: Intrasciatic nerve injection of the *Ricinus communis* agglutinin (RCA or ricin) causes degeneration of motor neurons (MNs) with functional deficits, such as those that occur in amyotrophic lateral sclerosis (ALS). The objective of this study was to develop a new comprehensive platform for quantitative evaluation of MN loss, muscular atrophy and behavioral deficits using different ricin injection regimens.

New method: Fluorogold (FG)-guided stereological quantification of MNs, *in vivo* magnetic resonance imaging (MRI) of muscular atrophy, and CatWalk behavioral testing were used to evaluate the outcome of rats treated with different ricin regimens (RCA60 0.5 µg, RCA60 3 µg, and RCA120 6 µg) as animal models of MN degeneration.

Results: FG-guided stereological counting of MNs enabled identification, dissection and robust quantification of ricin-induced MN loss. The RCA60 0.5 µg and RCA120 6 µg regimens were found to be best suited as preclinical MN depletion models, with a low mortality and a reproducible MN loss, accompanied by muscle atrophy and functional deficits evaluated by MRI and the CatWalk method, respectively.

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Author Disclosure Statement

No competing financial interests exist.

Comparison with existing methods: 1) Fluorogold neuronal tracing provides a robust and straightforward means for quantifying MN loss in the spinal cord; 2) MRI is well-suited to non-invasively assess muscle atrophy; and 3) The CatWalk method is more flexible than rotarod test for studying motor deficits.

Conclusion: Intrasciatic injection of RCA60 or RCA120 induces nerve injury and muscle atrophy, which can be properly evaluated by a comprehensive platform using FG-guided quantitative 3D topographic histological analysis, MRI and the CatWalk behavioral test.

Keywords

motor neuron; ricin; sciatic nerve; magnetic resonance imaging; muscle atrophy; neuronal tracing

1. Introduction

Selective degeneration of motor neurons (MNs) followed by muscular atrophy is a pathological hallmark of various severe neurological disorders, including amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA) and spinal bulbar muscular atrophy (SBMA or Kennedy's disease). The underlying disease mechanisms are complex, being an intricate combination of inflammation, glial cell activation, and MN death. In the absence of a cure, stem cell therapy has recently been explored as a new treatment paradigm for MND in experimental animal models (Gowing and Svendsen, 2011; Hedlund et al., 2007; Kerr et al., 2003) and patients with ALS (Glass et al., 2012; Mazzini et al., 2015). For therapeutic development in ALS, nearly all pre-clinical cell transplantation studies have used the transgenic SOD1^{G93A} mouse or rat model (Garbuzova-Davis et al., 2002; Haidet-Phillips et al., 2015; Hemendinger et al., 2005; Klein et al., 2005; Xu et al., 2011). This model manifests itself by an early, global disease onset, rapid progression of MN loss, and near 100% mortality by 120 days of age, complicating the experimental testing of novel therapeutic regimens.

For a pre-clinical model of MND that is more focal and controllable to induce, neurotoxic lectins can be injected to the sciatic nerve (SN) to induce the permanent and selective degeneration of neurons in the central nervous system (Wiley and Kline, 2000; Yamamoto et al., 1985). Ricinus communis agglutinin (RCA, or ricin) is one such toxin which depletes MNs after intrasciatic injection (Wiley and Oeltmann, 1986). It exists in two forms, designated RCA60 (Ricin II) and RCA120 (Ricin I), based on their molecular weight of approximately 60 and 120 kDa, respectively. When injected into nerve fibers, ricin first binds to the cell surface and enters neurons through its B chain. After being retrogradely transported to the MN soma (Spooner et al., 2006), it then irreversibly inhibits protein synthesis through inactivating the 60S subunits of ribosomes by its A chain, causing cells to die (MacConnell et al., 1982; Olsnes et al., 1974). Ricin-induced MN depletion is focal and stable, and thus, is a promising model for the evaluation of the efficacy of stem cell therapy for preclinical MND studies. While this model has been well-characterized by a battery of behavioral tests (Coutts et al., 2010), few detailed studies exist on a precise quantitative evaluation of MN loss and muscular atrophy.

In this study, we injected fluorogold (FG) as a retrograde neuronal tracer to mark sciatic nerve MNs. By establishing a morphological database for coronal sections of the lumbar spinal cord (SPC), we were able to quantify the distribution of lumbar MNs in continuous sections along the SPC in a 3D fashion. We furthermore used MRI to evaluate muscle atrophy and compared the suitability of the CatWalk method side-by-side with the rotarod test for evaluation of behavioral deficits.

2. Materials and Methods

2.1. Animals

Adult female Sprague-Dawley rats (Harlan, Indianapolis, IN), weighing 200 to 250 g were used throughout this study. All experimental procedures were carried out according to guidelines approved by our Institutional Animal Care and Use Committee. Animals were housed under a 12/12 hour light-dark cycle with access to food and water ad libitum. In one cohort, rats (n=5) were subject to a bilateral injection of FG into the sciatic nerve (SN) without RCA injection. In the other 3 cohorts (RCA60 0.5 μ g (n=6), RCA60 3 μ g (n=12) and RCA120 6 μ g (n=6)), RCA was unilaterally (left) injected into the SN with FG injection at the contralateral (right) side. FG was used to indicate the range of MNs contributing to SN and as well as the number of MNs on the contralateral side without ricin injection.

2.2. Sciatic nerve injection and transection

Rats were anesthetized with an intraperitoneal (i.p.) injection of xylazine 10 mg/kg and ketamine 80 mg/kg. The gluteal muscles underwent blunt dissection to expose the SN at the mid-thigh level. The nerve was lifted with the help of a curved scissor. Injections were performed at a shallow angle, using a 33G needle equipped with a Hamilton syringe. Ricin (Vector Laboratories, Burlingame, CA) or 2% FG (Fluorochrome LLC, Denver, CO) in distilled water were mixed with 0.1% fast green (Sigma, St. Louis, MO) to make a final volume of 2 microliters of each mixture (Wiley and Kline, 2000). Solutions were injected over the course of a two-minute period. The needle was repositioned several times to ensure penetration of all major nerve fascicles in the perineural sac. Care was taken to ensure that ricin did not come in contact with neighboring tissues. In another cohort of animals (n=6), the sciatic nerve was transected at the mid-thigh level. After the injection or transection procedure, the nerves and muscles were repositioned and the skin was sutured. The animals were allowed to recover on heating pads and given carprofen (5 mg/kg, subcutaneously) daily for three days.

2.3. Histopathological analysis

At the end time point (1 or 4 weeks), animals were euthanized and perfused with 4% paraformaldehyde (PFA). SPCs were dissected under a surgical microscope (Zeiss Stereo Discovery V8, Germany) and imaged under ultraviolet (UV) light with a Molecular Imager Gel Doc XR System (Bio-Rad, Berkeley, CA). The resulting SPC segments were post-fixed in 4% PFA overnight and dehydrated in 30% sucrose in 10 mM phosphate buffer saline, pH=7.4 (PBS). Tissues were embedded in optimal cutting temperature (OCT) compound, and 40 μ m sections were cut using a cryotome. Sections were blocked with 10% goat serum and incubated with goat anti-choline acetyltransferase (ChAT) primary antibody (Millipore,

Billerica, MA). Sections were rinsed with PBS and incubated with anti-goat Alexa fluor 594 secondary antibody (Invitrogen, Carlsbad, CA). Histochemical and immunofluorescent images were obtained using an Olympus BX51 microscope equipped with an Olympus DP70 camera (Tokyo, Japan). FG was excited by UV light under epifluorescence illumination. Since FG particles diffuse out of the cell body after the immunostaining procedure, each section was photographed for FG visualization before being processed for ChAT staining.

2.4. Image processing and stereological quantification of MNs

A distribution of all FG-positive neurons in the SPC was first reconstructed, followed by the reconstruction of specific MNs using anti-ChAT immunostaining. To this end, every other (for database establishment) or 3rd (for quantification) section of the entire lumbar SPC was consecutively collected for histological processing. MNs were counted for each side of the SPC cross-sections. For ricin-induced animals, every 10th section was collected for histology. To observe the three-dimensional (3D) distribution of FG-positive MNs, SPC cross-sections were imaged with an epi-fluorescent microscope. The full set of SPC images were then aligned along the longitudinal axis with Adobe Photoshop (Adobe, San Jose, CA). Three-dimensional visualization of SPC tissue and FG distribution was achieved by importing the aligned images to Amira 5.4.5 (VSG, Burlington, MA) for iso-surfacing. To visualize the distribution of all lumbar MNs, FG and ChAT fluorescence images for the same cross-section were acquired and overlaid along the longitudinal axis using Adobe Photoshop CS4. FG-positive and non-positive neurons (all positive for ChAT) were annotated by dots of different colors (i.e., red and yellow, respectively). These color dots, along with the outline of the SPC sections, were then used for determining the distribution pattern of MNs in each section, with the images imported in Amira for 3D visualization. The full set of images with annotated colors were then automatically split into two channels when opened in Amira, with each channel showing either FG⁺ or FG⁻ MNs. The whole image stack was first visualized using three orthogonal slices, followed by 3D visualization through iso-surfacing. Image projections were acquired using the Multi Planar Viewer in this software.

2.5. MRI

Four weeks post-ricin injection, *in vivo* MRI was performed on a horizontal 11.7 Tesla MR scanner (Bruker Biospin, Billerica, MA, USA) with a triple-axis gradient (maximum gradient strength = 74 Gauss/cm). A quadrature volume excitation coil of 70 mm diameter was used as radio frequency transmitter, with an 8-channel phased array body receive-only coil for signal acquisition. During imaging, rats were anesthetized with 2% isoflurane mixed with air and oxygen at a 3:1 ratio, and the respiration was monitored using a pressure sensor (SAII, Stony Brook, NY, USA) and maintained at 40–60 breaths per minute. Transverse, sagittal, and coronal T₂-weighted images were acquired using the following parameters: echo time (TE)=40 ms, repetition time (TR)=2000 ms, 4 signal averages, echo train length = 8, in-plane resolution = 0.2 × 0.2 mm, slice thickness = 1 mm, with multiple slices covering the entire legs and lower abdomen. The total imaging time was approximately 30 minutes. After imaging, animals fully recovered within five minutes.

2.6. Behavioral testing

Animals were acclimated to housing and handling and given at least four training sessions for each behavioral assessment. Two examiners participated and were blinded to the treatment groups. The Rota-Rod test (Columbus Instruments, Columbus, OH) was used as a measure of motor performance. After 4 weeks of ricin-treatment, rats were subjected to an accelerating rotarod task in which the speed of the spindle was increased from 2 to 40 rpm over a two-minute period. The time spent on the rod before the first fall or between the first and second fall, was recorded. A CatWalk system (CatWalk 9.1, Noldus Technology, Netherlands) was used to quantify gait parameters when the animal crossed a walkway with an illuminated glass floor. Animals were tested on 12, 24 and 36 days post-ricin injection. For this test, a high-speed video camera is positioned underneath the glass walkway for automatic recording of the paw prints as the animal is walking. During the test, the room was totally darkened. Animals were trained for smooth walkway crossing on the three days prior to the video acquisitions. The following criteria were used for quality control of data collection: (1) a complete walk without a stop across the walkway; and (2) acquisition of a minimum of three such crossings per animal. The used parameters for gait analysis were MaxContactArea (total surface area of the glass floor contacted by the hind paw at the moment of maximum paw-floor contact during the stance phase of the hind paw), MaxContacMax (maximum intensity of the pixels forming the maximum area), swing duration (time that the hind paw is not in contact with the glass floor), stride length (distance between the placement of a hind paw and the subsequent placement of the same paw, swing speed (computed from stride length and swing duration), and base of support (distance between two hind paws, as measured perpendicular to the waking direction)(Hamers et al., 2001). Data from three proper crossings were averaged for statistical analysis.

2.7. Statistical analysis

All data were analyzed using Prism 4.03 software (GraphPad, La Jolla, CA) with the data expressed as mean $\pm$ SEM. A two-tailed standard Student's *t* test was used for comparison of the cell numbers at different levels of SPC cross-sections, the weight of hindlimbs, and the behavioral data. For comparison of more than two groups of data (i.e., number of MNs in rats treated with different ricin regimens), a one-way analysis of variance (ANOVA) test was used with a *post hoc* Neuman-Keuls' test. For all comparisons, $p < 0.05$ was set as being statistically significant.

3. Results

3.1. Distribution of FG-positive lumbar MNs

For labeling of MNs in rat lumbar SPC, FG was injected into the SN, where the particles underwent retrograde transport back to the soma of the lumbar MNs within one week post-injection (Fig. 1A). After dissecting out the spine along with the SN (Fig. 1B), UV illumination was not only able to visualize the SN and its connecting dorsal root ganglion (DRG) (Fig. 1C), but also the ventral roots paralleling the labeled dorsal root connected to the DRG (Fig. 1D), entering the SPC at the level of the L1 vertebrae. The SPC below the L1 vertebrae (corresponding to spinal nerves L3 to L6) was then dissected for histology, and the number of FG⁺ cells was counted for both sides of the SPC. (Fig. 1E). To determine the

overall distribution pattern of FG⁺ MNs, a 3D reconstruction was performed using the central vein as landmark (Fig. 1F, G). From this dataset, the FG⁺ MN distribution pattern was visualized in the XY-plane (transverse plane, Fig. 1H), the XZ plane (sagittal plane, Fig. 1I), and the YZ plane (coronal plane, Fig. 1K). On the XZ and YZ plane, MNs were organized in the form of a helical string, winding along a center line parallel to the longitudinal axis. This distribution was confirmed by sagittal (**Fig. 1J, left**) or coronal (**Fig. 1L, left**) sections. In these two sets of sections, MNs were also discernible under a bright-field phase contrast microscope (**Fig. 1J right, 1L right**).

3.2. Quantitative analysis of normal lumbar MN distribution

To obtain a complete topography of lumbar MNs, every other section was collected and photographed for bright-field imaging, FG⁺ MN distribution, and anti-ChAT immunofluorescent staining (Fig. 2A, with the full dataset available in Supplementary Fig. 1). The majority of MNs could be readily identified under bright-field imaging (Fig. 2B) without the need for further staining, when compared to dark-field FG⁺ MN visualization (Fig. 2C), and dark-field anti-ChAT immunofluorescent staining (Fig. 2D). As FG did not label all lumbar MNs, we further analyzed the spatial relationship between FG⁺CHAT⁺ MNs and MNs that were CHAT⁺ but FG⁻ (Fig. 2E), with image processing for visualization in a 3D coordinate system using Amira (Fig. 2F). The transverse view of the reconstructed SPC revealed the stereotypical spatial relationship of FG⁺CHAT⁺ MNs lying beneath CHAT⁺ MNs that were FG-negative (Fig. 2G). However, the coronal (Fig. 2H) and sagittal (Fig. 2I) views showed an uneven distribution and more complex spatial pattern of lumbar MNs. Quantification of the total number of MNs resulted in a single distribution peak, whereas the number of FG⁺CHAT⁺ MNs exhibited a bimodal distribution (Fig. 2J, K). No statistical difference was found between the distribution patterns from the two sides of the lumbar SPC ($p>0.05$). For each side, there were $1,881\pm32$ FG-positive MNs and $3,237\pm44$ total MNs, respectively (left or right side, $n=10$).

3.3. Ventral horn depletion of MNs following ricin-injection

In order to evaluate ricin-induced MN loss, we first established a distribution map of MNs that contribute to SN (SN MNs) and MNs that do not contribute to SN (non-SN MNs) in control animals (Supplementary Fig. 1). Ricin and FG were injected into the contralateral sides of the SN (Fig. 3A). After four weeks, transverse SPC sections were examined with bright-field, UV-illuminated dark-field, and anti-ChAT immunofluorescent microscopy (Fig. 3B–H). Images were registered with the normal library (see above), and the number of MNs was counted. The normal library alignment ensured that sections from the ricin-injected side could be properly compared to the normal control sections at the same tissue level, reducing the probability of counting errors due to oblique cutting. All three imaging modes demonstrated loss of MNs in the lower part of the ventral horn at the side of ricin injection. For the rats injected with 3 μ g RCA60, there was a significant loss of MNs on the ricin-treated side, from section #4 through section #15 (Fig. 3I, $*p<0.05$, $**p<0.01$, $***p<0.001$, $n=6$). In contrast, 6 μ g RCA120 or 0.5 μ g RCA60 only partially lesioned the MNs at certain range of segments, as evidenced by the significant decrease in the number of MNs from sections #9 through #12 ($*p<0.05$). This differential spatial susceptibility of MNs to ricin-induced injury complicates the quantification of the loss of MNs. A comparison of the total

number of SN and non-SN MNs among the three groups revealed that the 3 μ g RCA60 group had the most significant MN depletion effect compared to the controls ($p < 0.01$, $n = 6$, Fig. 3J). The 6 μ g RCA120 regimen significantly depleted the overall MNs ($p < 0.05$, $n = 6$), although to a less extent compared to the 3 μ g RCA60 regimen. No significant loss of total MNs was found in the 0.5 μ g RCA60 cohort (Fig. 3J). Since ricin is retrogradely transported to SN MNs, a mere evaluation of both SN and non-SN MNs is not a valid comparison, since only SN MNs will be in directly touch with ricins. Differential susceptibility also requires more precise quantification of the loss of MNs at different segments of the spinal cord. As shown in Fig. 3K, limiting the spinal cord analysis to sections that contain SN MNs revealed a more pronounced loss of MNs for the 3 μ g RCA60 group (~75% loss of MNs). This SN MN only analysis also demonstrated a significant loss of MNs for the 6 μ g RCA120 and 0.5 μ g RCA60 groups (Fig. 3L). Taken together, the three RCA regimens all depleted lumbar MNs, but to a different extent. The helical SPC distribution of MNs and the differential spatial susceptibility of MNs to the same ricin regimen makes accurate quantification of MNs a challenging task. However, our stereological quantification strategy uses a normal control library to register spinal cord sections, which reduces errors caused by arbitrary selection of SPC sections.

3.4. MRI and post-mortem examination of SN-innervated muscle atrophy in ricin-injected rats.

MRI was used to visualize the lower trunk and hind limbs of live rats 4 weeks post-ricin injection. Due to the high mortality rate (6/12) and severe local inflammation at the ricin-injection site, we did not perform MRI and behavioral testing for the RCA60 3 μ g group. For the RCA60 0.5 μ g group, there was a dramatic atrophy of the SN-innervated muscles (Fig. 4A, B). The post-mortem anatomical appearance confirmed the MRI observation of gastrocnemius atrophy only on the side of ricin injection (Fig. 4C, D). Both RCA60 0.5 μ g and RCA120 6 μ g induced a significant atrophy of the lower leg muscles ($*p < 0.05$, $n = 6$). No significant difference in effect was found between the two regimens. These findings were further corroborated by leg weight measurements (Fig. 4E).

3.5. Behavioral studies using the CatWalk method and rotarod test

To perform a comprehensive gait analysis, we applied the CatWalk method, which has been shown to detect a multitude of speed-controlled dynamic and static gait deficits after experimental SPC injury and lesioning. We compared three cohorts of rats: ricin-injected rats, SN-transected rats and normal control rats. For normal control rats, the footprints displayed fully spread toes with each individual toe clearly visible (Fig. 5A). In the SN transection group, foot prints only showed the plantar surface without any toes visible (Fig. 5B). The shape of the footprint in the 0.5 μ g RCA60-treated rats showed a pattern similar to that in the SN transection group, except that a vague trace of the toes was visible (Fig. 5C). These changes in footprints indicate gait deficits caused by impairment of muscle function. In normal rats, the gastrocnemius is innervated by the SN and is responsible for plantar flexion of the ankle joint, so that only the toes come in contact with the ground during walking (Fig. 5D). However, when the gastrocnemius loses its innervation from the SN, the ankle joints are no longer supported for the upright position. As a result, the plantar surface comes in contact with the ground during walking (Fig. 5D). Quantitative foot print analysis

further confirmed these changes. Due to the smaller surface area of the plantar surface and the lack of toe contact, the contact area (MaxContactArea) for lesioned groups was significantly decreased compared to control rats (Fig. 5E, $**p<0.01$). The contact force (MaxContactMax) was also reduced for the 0.5 μ g RCA60-injected rats compared to controls ($**p<0.01$), but this was not the case for SN transection. Another prominent deficit was the reduced swing speed in both groups of lesioned rats ($*p<0.05$). While there were no significant changes in stride length, the swing duration in lesioned rats was dramatically increased in both lesioned groups ($*p<0.05$), with as a consequence a reduced swing speed, indicating that it was difficult for rats to move their injured legs due to the wasted gastrocnemius muscle. There was no significant difference among groups for the base of support and stride length as other parameters.

Longitudinal analysis of hind limb footprints revealed dynamic changes after lesion induction (Fig. 5F). At 12 days post-injection (surgery), both the FG- or 0.5 μ g RCA60-injected groups showed gait deficits as a result of the surgery itself: only the back part of the plantar surface was observed. In FG-treated rats, there was a trend toward healing, as at 24 days post-injection, the plantar surface area increased along with the toes. At 36 days post-injection, the footprint from the FG-treated side returned to that of normal control rats. In contrast, the footprints of the ricin-injected animals remained the same throughout the whole temporal observation window. The deficits obtained for 6 μ g RCA120 were similar to that of 0.5 μ g RCA60 injection.

Due to the severe loss of balance, animals were not able to stay on the rotarod equipment until 4 weeks post-ricin injection. In contrast, they could undergo proper behavioral testing with the Catwalk test during the initial (<4 weeks) post-ricin injection period. A significantly reduced performance was observed for both the 0.5 μ g RCA60- and 6 μ g RCA120-injected groups (Fig. 5G), with the CatWalk method showing that both regimens produced behavioral deficits to a similar extent.

3.6. Comparison of the three different ricin regimens

The key features of the three different regimens of ricin injection used in this study are summarized in Table 1. RCA60 3 μ g is the most effective means to obtain the most severe lumbar MN lesions. However, the accompanying inflammation at the injection site and the high mortality make this ricin model impractical for behavioral outcome studies. The other two regimens (RCA60 0.5 μ g and RCA120 6 μ g) caused a less severe MN loss, allowing proper evaluation with the CatWalk or rotarod behavioral tests.

4. Discussion

In this study, we developed a comprehensive analytical platform for evaluating ricin injection as an animal model to study MND. We established a robust procedure for unbiased counting of MNs, which was then applied to compare the effect of different regimens of ricin on the number of lumbar MNs. Two predominant forms of ricin are present in the castor bean plant: Ricin 60 and Ricin 120. RCA 60 (Ricin II, molecular weight of about 65 kDa) consists of a sugar-binding B chain (~34 kDa) mediating cell binding via oligosaccharide residues on the cell surface, and a catalytically active A chain (~32 kDa)

which acts as an RNA N-glycosidase (Bellisola et al., 2004; Lappi et al., 1978). The A chain is linked via a disulfide bond B chain, forming the A-B heterodimer. After internalized by a cell through B chain, this heterodimer is transported to endoplasmic reticulum where the heterodimer is reduced and separated into the two subchains. Then the A chain binds to the ribosome and removes a single adenine from the 28S RNA, preventing further binding of elongation factors, inhibiting protein synthesis and eventually leading to cell death (Chiou et al., 2008). RCA120 (Ricin I) is a 120 kDa heterotetrameric protein consisting of two ricin-like heterodimers linked via a disulfide bond (Sweeney et al., 1997). Although the B chains in RCA 60 and RCA 120 are highly homologous, RCA 120 has a unique A chain, which may explain its weak cell toxicity: 60–2000 times less toxic than RCA 60, depending on the experimental system (Worbs et al., 2015). Both lectins have been shown to act by inhibiting protein synthesis and to cause cell death after retrograde transport in peripheral nerves. For depletion of rat MNs in the SPC through intra-SN injection of ricin, 2 to 8 μ g (Yamamoto et al., 1985) or 1 to 3 μ g (Wiley and Oeltmann, 1986) of RCA60 was initially suggested in the 1980s, which since then became the standard regimen of choice (Griffith et al., 2000; Wiley and Kline, 2000; Wiley and Lappi, 2001). As RCA120 is not as toxic as RCA60, a substantially larger dose of 20–30 μ g is required to achieve the same depletion effect (Yamamoto et al., 1985). Compared to RCA60, RCA120 has been used more frequently to induced degeneration of myelinated axons and neurons in the dorsal root ganglion (Aldskogius et al., 1988; Rivero-Melian and Arvidsson, 1989; Wiesenfeld-Hallin et al., 1987). Our finding that injecting 3 μ g of RCA60 is the most effective among the three regimens is consistent with results from those early studies (Wiley and Oeltmann, 1986; Yamamoto et al., 1985).

There appears to be somewhat of a discrepancy between our findings and a previous study reporting that intra-sciatic injection of 2 μ g or 5 μ g RCA120 can induce substantial loss of MNs (Coutts et al., 2010), since we found 6 μ g RCA120 to be not as efficient as 3 μ g RCA 60 in depleting MNs. It should be note that there are two limitations with the quantification method by Coutts et al. First, their stereological quantification of MNs was incomplete as no information was provided on the spatial distribution (i.e., susceptibility) of the lumbar SPC segments. Second, all MNs from T13 to S2 were included in their quantification. According to our data presented here, this prevents proper quantification as non-SN MNs are included and the spatial location of MNs is important for their susceptibility to injury. This further raises the question of how MN loss in the SPC can be best evaluated. The scattered distribution and helical shape of MN distribution along the longitudinal axis (Fig. 1I–L) results in abrupt changes in cell number in even continuous cross-sections, making it difficult to judge unilateral cell loss without consecutive sampling and stereological quantification. The ratio of the number of FG-labeled MNs to the total number of MNs is also highly variable at different levels of the lumbar SPC, suggesting that the use of the contralateral side as a control needs to be taken with great precaution, due to the variations in sectioning, with oblique cutting causing asymmetry. We believe that a rigid procedure for stereological counting as described here in our study is essential for an accurate evaluation of the loss in number of lumbar MNs. As shown in Figs. 3J–L, the extent of cell loss could vary significantly if different inclusion criteria are being used. Several previous studies on quantitative MN loss were based on only one or two images of SPC cross-sections, as

exemplified for transgenic SOD rodent models as model of ALS (Gurney et al., 1994; Howland et al., 2002). The database (Suppl. Fig. 1) that we have built of consecutive cross-sections of the lumbar SPC may serve as a future reference for the overall distribution of MNs in any given section. The stereological MN counting method that we used in our ricin-induced MND model, along with the FG-labeling method to facilitate the dissection procedure and to identify the affected spinal cord segments, may be further used to standardize the quantification of lumbar MNs in future MND animal models.

To the best of our knowledge, there have been only two studies describing the overall distribution of lumbar MNs in rats dating back from the 1980s. In one study, horse radish peroxidase (HRP) was injected into the hind limb muscle for labeling of MNs in rat SPC (Nicolopoulos-Stournaras and Iles, 1983). In another study, the rat SN was soaked in HRP solution for retrograde labeling of MNs. The number, size, and topographic distribution of the MN populations in the SN were then determined (Swett et al., 1986). Our results are consistent with the major findings in the two above studies in several aspects. For the total number of SN-innervated MNs, we calculated $1,881 \pm 32$ MNs on one side of the SPC, close to the $2,005 \pm 89$ MNs as reported by Swett et al. (Swett et al., 1986). As for the distribution of MNs along the longitudinal axis, the pattern we observed was similar to those that reported in those two early studies. However, the 3D visualization of the MN distribution in our study, which was not possible in the 1980s, makes for a better comprehension of overall topography. One key feature in our study is that we also visualized MNs that are not innervated by the SN in the same cross-section as those who are. This provides a 3D panorama of the topographic distribution of all lumbar MNs.

As for the helical distribution pattern of MNs along the longitudinal axis (Figs. 1I and 1K), a similar pattern was shown previously to exist in rats (Nicolopoulos-Stournaras and Iles, 1983), and another study in cats also reported a similar distribution topography (Burke et al., 1977). Our observation of this helical pattern through sectioning in three orientations (transverse, sagittal, or coronal), along with support from Nicolopoulos-Stournaras et al. and Burke et al., suggests that this might be a general feature of the topography of MNs in the mammalian SPC. The existence of such a helical distribution pattern may be related to the control of muscle movement, given that the labeled MNs belong to one motor neuron pool. The best way to test this hypothesis is to perform optical functional imaging of the MNs in the same motor neuron pool and to examine the coordination of the neuronal activity in such a network. As the field of multiphoton microscopy and optical sensors is rapidly evolving, it is now possible to directly image MNs in live mice (Cartarozzi et al., 2018). Since MNs within a single motor pool also exhibit further subtype distinctions: those innervating fast or slow skeletal muscle fibers (Pun et al., 2006), or α and γ motor neurons (Burke et al., 1977), it would be intriguing to further determine the spatial relationship between different classes of MNs in this helical structure, as α and γ motor neurons can be distinguished using immunospecific markers (Friesse et al., 2009).

There are several traditional behavioral tests that can be used to evaluate the outcome of ricin-induced lesions on the motor pathway, such as the toe spread reflex, the balance beam, the inclined plane, the open field test, and the rotarod test (Coutts et al., 2010). The CatWalk method is an automated gait-analysis system that allows objective quantification of multiple

static and dynamic gait parameters. Due to its objective and quantitative properties, this method has been increasingly used for the assessment of locomotor function in many animal models, including SPC injury (Hamers et al., 2006; Hamers et al., 2001), SN transection (Bozkurt et al., 2008) (Deumens et al., 2007), stroke (Parkkinen et al., 2013), and CNS-based movement disorders (Vandeputte et al., 2010). We performed longitudinal gait analysis using the CatWalk method on rats treated with an intrasciatic injection of ricin or FG. Our gait analysis revealed that the SN on the control side was also damaged (potentially due to the physical tearing force during injection), as evidenced by the change in the shape of footprints. We furthermore found that the CatWalk method was also able to track the healing process (Fig. 5F). This unanticipated damage to the control side could not be detected by traditional behavioral tests (Coutts et al., 2010). Our second behavioral test, the rotarod test, is commonly used for evaluation of locomotor function in rodents (Jones and Roberts, 1968a, b; Pritchett and Mulder, 2003). Although there have been reports that the rotarod test can be applied to rats treated with an intrasciatic injection of ricin as early as one week post-injection (Coutts et al., 2010), in our study the rats could hardly stay on the rotating bar at the starting speed (2 rpm), due to the impaired function in both hind limbs. We found that ricin-treated rats could only perform the rotarod test at one month after injection. Comparing the rotarod test to the CatWalk method, we conclude that the latter is a more flexible and reliable method for continuous monitoring of motor function, with the additional benefit of being quantitative, automated, and sensitive for the detection of locomotion deficits.

However, these behavioral tests only inform us on the overall extent of motor neuron impairment, rather than the extent of the actual MN loss. Considering the different susceptibility of MNs and SN fibers (Rivero-Melian and Arvidsson, 1989; Wiesenfeld-Hallin et al., 1987), it is likely that the behavioral deficits in rats from the 0.5 μ g RCA60 and 6 μ g RCA120 groups were the result of peripheral damage (in nerve or muscle), given the minor loss of MNs in the two groups. Our dose of 3 μ g RCA60 that produces depletion of MNs is associated with a high death-rate along with local inflammation at the injection site, which may be caused by direct toxic effects on the muscles since intramuscular injection can cause necrosis of muscles (Moshiri et al., 2016). Thus, animals treated with 3 μ g RCA60 were not suitable to undergo behavioral testing, and this ricin regimen should be seen as an acute and severe pathological model. In contrast, rats treated with 0.5 μ g RCA60 and 6 μ g RCA120 showed less prominent MN loss, had fewer complications and were able to undergo behavioral testing of the motor pathway. Thus, these two regimens may serve as more realistic models of peripheral nerve damage, in particular to study novel experimental treatment paradigms based on stem cell therapy. Here, our results on the topographic distribution of MNs may provide further guidance for local stem cell injection. Based on our results shown in Fig. 1D, an injection through the space between either vertebral level T13/L1 or L1/L2 will miss the major population of SN MNs, given the fact that the spinal nerves at L3–L6 enter the SPC at vertebral level L1. To choose a level where clustered groups of SN MNs are located, our results shown in Fig. 1 and Fig. 2 suggest that the rostral 1/3 point beneath L1 may be an ideal site for a single injection, as this zone harbors the greatest number of SN MNs within one cluster.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Craig Remenapp and Andres Hurtado from the Kennedy Krieger Institute for their assistance with the CatWalk method, and Mary McAllister for editorial assistance. This work was supported by 2R01 NS045062 and R01 NS091110.

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Highlights

- Intrasciatic injection of RCA60/120 induces nerve injury and muscle atrophy
- Fluorogold neuronal tracing allows 3D analysis of motor neuron loss
- The CatWalk is a more flexible method than rotarod test for studying motor deficits

Figure 1: Distribution pattern of lumbar MNs.

(A) Schematic diagram of experimental layout in for panels A-C. FG particles were injected into the SN for labeling of MNs in the lumbar SPC. (B) Front view of the SPC showing the spinal nerves that join the SN at L4 (yellow arrow), L5 (red arrow), and L6. (C) FG-labeled SN (lower right) and DRGs connecting to spinal nerve L4 and L5 as visualized using UV illumination. (D) FG⁺ ventral roots from L4 (yellow arrow) and L5 (red arrow), as tracked from the illuminated DRGs. In order to include the FG⁺ ventral root, L1 SPC tissue (dashed lines) was further processed for histological examination. Ruler unit (right) = 1 mm. (E) Quantification of all FG⁺ MNs on left and right side of the SPC. Numbers below the x-axis represent sequential numbers of one of three continuous cross sections, starting from the rostral section with the first FG⁺ MN. (F, G) Lateral and top view of 3D reconstruction of the lumbar region along with the distribution of FG⁺ MNs. (H-K) Separate transverse (H), sagittal (I), and coronal (L) sections from the 3D-reconstructed lumbar region. (J, J') Sagittal section of the lumbar region with FG⁺ MNs shown in dark-field (J) or bright-field (J') images. (L, L') Coronal section of the lumbar region with unilateral labeling of MNs by FG, shown in dark field under UV (L) or bright field (L') images. Scale bar in JL' = 1 mm.

Figure 2: Normal topographic distribution and quantification of FG^+ and FG^- MNs in lumbar SPC.

(A) Bright-field (top row), FG (middle row), and anti-ChAT immunofluorescence (bottom row) images, sequentially aligned (every other section) from the rostral to the caudal SPC. (B-D) High magnification images from the sections shown in (A). Arrows and dashed circles represent the same MNs across images. (E) Processed images with FG^+ MNs and $ChAT^+/FG^-$ neurons false-colored in red and yellow, respectively. Scale bar = 200 μm . (F) Images were aligned and imported into Amira for visualization of MNs in the transverse plane (G), the coronal plane (H), and the sagittal plane (I). (J, K) Quantitative distribution and quantification of FG^+ and total number of $ChAT^+/FG^-$ MNs from rostral to caudal for the left (J) and right (K) side of the lumbar region.

Figure 4: Atrophy of SN-innervated muscles.

RCA60 0.5 µg was injected in the left side, and FG in the right side. **(A)** *In vivo* transverse MR images taken from the lower abdomen to the lower limbs (1 mm slice thickness). Numbers indicate slice position. **(B)** *In vivo* MR images for all three anatomical planes, showing muscle atrophy in the left hindlimb. **(C, D)** Post-mortem examination demonstrates degeneration of the gastrocnemius muscle and tibial nerve on the side of ricin injection for both the RCA60–0.5 µg and RCA120–6 µg regimens. **(E)** Quantification of hind limb weight, taken from the hip joint downward (*p<0.05, n=6)

Figure 5: Behavioral testing for ricin-induced locomotion impairment.

(A) Hind paw footprint of a normal rat. (B) Hind paw footprint of a rat with SN transection. (C) Hind paw footprint of a rat 24 days following ricin (RCA60 0.5 μ g) injection. (D) Schematic drawing outlining hind paw positions to explain change in footprints. (E) Catwalk gait analysis with data expressed as the ratio of values for the lesioned hind leg to the contralateral hind leg (* p <0.05, ** p <0.01, n =6). (F) Time course of footprint changes for the ricin-injected or control (FG-injected) side at various time points after injection. (G) Rotarod test of rats treated with different regimens of ricin at 36 days after injection (** p <0.01, n =6).

Table 1:

Comparison of the different ricin toxin regimens used in this study.

	SPC MN loss	SN axonopathy	Inflammation at injection site	Muscle atrophy	Suitability for CatWalk	Suitability for Rotarod	Survival rate
RCA60 3 µg	Robust	+	+	+	–	–	6/12 (50%)
RCA60 0.5 µg	Minor	+	–	+	+	Not until 1 month PI [*]	6/6 (100%)
RCA120 6 µg	Minor	–	–	+	+	Not until 1 month PI	6/6 (100%)

* PI=post-injection