Normal development of spinal axons in early embryo stages and posterior locomotor function is independent of GAL-1

Juana M. Pasquini | Francisco J. Barrantes | Héctor R. Quintá

Departamento de Química Biológica, Instituto de Química y Físico Química Biológica, Universidad de Buenos Aires, Buenos Aires, Argentina

Laboratory of Molecular Neurobiology, BIOMED UCA-CONICET, Buenos Aires, Argentina

Correspondence
Héctor R. Quintá, Departamento de Química Biológica, Instituto de Química y Físico Química Biológica, Universidad de Buenos Aires, Buenos Aires C1113AAD, Argentina.
Email: quintaramiro@hotmail.com

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Abstract
It was recently described that Galectin-1 (Gal-1) promotes axonal growth after spinal cord injury. This effect depends on protein dimerization, since monomeric Gal-1 fails to stimulate axonal re-growth. Gal-1 is expressed in vivo at concentrations that favor the monomeric species. The aim of the present study is to investigate whether endogenous Gal-1 is required for spinal axon development and normal locomotor behavior in mice. In order to characterize axonal development, we used a novel combination of 3-DISCO technique with 1-photon microscopy and epifluorescence microscopy under high power LED illumination, followed by serial image section deconvolution and 3-D reconstruction. Cleared whole Igal-1-/- embryos were used to analyze the 3-D cytoarchitecture of motor, commissural, and sensory axons. This approach allowed us to evaluate axonal development, including the number of fibers, fluorescence density of the fiber tracts, fiber length as well as the morphology of axonal sprouting, deep within the tissue. Gal-1 deficient embryos did not show morphological/anatomical alterations in any of the axonal populations and parameters analyzed. In addition, specific guidance receptor PlexinA4 did not change its axonal localization in the absence of Gal-1. Finally, Gal-1 deficiency did not change normal locomotor activity in post-natal animals. Taken together, our results show that development of spinal axons as well as the locomotor abilities observed in adult mice are independent of Gal-1. Supporting our previous observations, the present study further validates the use of Igal-1-/- mice to develop spinal cord or traumatic brain injury models for the evaluation of the regenerative action of Gal-1.

KEYWORDS
Axonal development, 3-DISCO, Galectin-1, 3-D axonal reconstruction, PlexinA4, RRID: AB_2532998, RRID: AB_10014322, RRID: AB_944890, RRID: AB_2284441, RRID: AB_1841228, RRID:AB_640838

1 INTRODUCTION

Axonal development in the spinal cord is crucial to establish a normal range of motor behaviors such as locomotor coordination involved in reaching and grasping tasks (Zhang et al., 2014). At the molecular level, nerve fibers are guided by repulsive signaling triggered by the Semaphorin protein family secreted at the ventral spinal cord, this step being crucial in the establishment of patterned neuronal connections (Curinga & Smith, 2008; Wright, White, Gerfen, Silos-Santiago, & Snider, 1995; Puschel 1996, Castellani, Chedotal, Schachner, Faivre-Sarrailh, & Rougon, 2000). Therefore, axon guidance results from a direct chemorepellent interaction between Semaphorin 3A (Sema3A) and its specific neuronal receptor complex Neuropilin-1 (NRP-1)/PlexinA4, serving as a cue for guided axonal growth (He & Tessier-Lavigne, 1997; Kolodkin et al., 1997).

Galectin-1 (Gal-1) is an endogenous glycoprotein composed by 14.5 kDa subunits, that binds to glycosylated receptors displaying multiple units of the common disaccharide (Galβ1-4GlcNAc; LacNac) on both N- and O-glycans (Rabinovich & Croci, 2012). Its structure exists in a monomer–homodimer equilibrium which depends on the relative concentration of the two species and the biochemical features of the different host tissues (Cho & Cummings, 1996; Rabinovich & Croci, 2012). While several studies have been published highlighting the...
immunological functions of Gal-1, the link between Gal-1 and axonal growth represents a novel field within neurobiological research.

We have recently reported that the administration of exogenous Gal-1 promotes axonal regeneration and recovery of locomotor activity after a spinal cord injury (SCI; Quinta, Pasquini, Rabinovich, & Pasquini, 2014a, b). Moreover, we dissected the intracellular signaling events which allow Gal-1 to block the inhibitory Sema3A pathway, thus promoting axonal growth (Quinta et al., 2014b). This Gal-1 effect proved to be dependent on dimerization, since the monomeric non-dimerizable form of Gal-1 (M-Gal-1) is unable to promote axonal re-growth (Quinta et al., 2014b, 2016). We have shown that the endogenous fraction of Gal-1 (e-Gal-1) corresponds to the monomeric conformer, which lacks regenerative properties (Quinta et al., 2016). e-Gal-1 fails to reactivate actin cytoskeleton dynamics in the axonal growth cone and is therefore unable to promote axonal re-growth (Quinta et al., 2016).

It has been shown that e-Gal-1 appears early during embryonic development, immediately after the formation of the dorsal root ganglia (DRG), being localized in the cell bodies and terminals of subsets of DRG neurons (Regan, Dodd, Barondes, & Jessell, 1986). However, neither e-Gal dynamics in embryonic tissues nor a possible role of the endogenous lectin in axonal development has been analyzed to date. Within this context, the aim of the present study was to evaluate a potential role of e-Gal-1 in the early development of axons involved in mice locomotion, and the possible consequences of e-Gal-1 depletion on post-natal locomotor function. To address this issue, we evaluated axonal structure in Gal-1 knockout (lgals-1\(-/-\)) and wild type (WT) mouse embryos. The effect of e-Gal deletion was analyzed in different subsets of axons in the spinal cord: (i) motor axons directly involved in locomotion, (ii) commissural axons which are key to coordinated movement, and (iii) sensory axons, known to be responsive to Sema3A. Trigeminal ganglia and its nerves were also analyzed for comparative purposes. Our studies were conducted at embryonic stages 11.5 and 13 (E11.5; E13), an early period in the development of the axonal populations analyzed; in which axons begin to navigate towards their targets.

Using the 3-dimensional solvent-cleared organ (DISCO) technique combined with 1-photon microscopy (Quinta, Pasquini, & Pasquini, 2015; Erturk & Bradke, 2013) we acquired 3-dimensional images of embryos. We showed that axonal development in the spinal cord was unaffected by the absence of e-Gal-1. Axonal length in mutant embryos did not differ from that observed in WT animals. Additionally, no significant differences were observed in the number of axons from DRG or in the shape of growth cones. Moreover, a new method was developed combining 3-DISCO with high magnification epifluorescence microscopy under high power LED illumination followed by serial image section deconvolution. This strategy enabled us to visualize the shape of individual neurofilaments in the axonal sprouting deep in the whole tissue, where no structural differences between lgals-1\(-/-\) and WT mouse embryos were apparent. Finally, in the absence of e-Gal-1, no changes were observed in the axonal localization of PlexinA4, a key receptor of axonal guidance during the embryonic period analyzed. In terms of locomotor ability, lgals-1\(-/-\) mice exhibited a normal behavior, showing no significant alterations in the locomotor coordination.

Collectively our data show that in vivo, spinal axons develop in a normal fashion in lgals-1\(-/-\) mice, suggesting the lack of a role of e-Gal-1 in early axonal development and subsequent locomotor function.

2 | MATERIALS AND METHODS

2.1 Animals

Embryos were collected at E11.5 and E13 from C57BL/6 WT control and C57BL/6 lgals-1\(-/-\) synchronized pregnant mice (provided by animal facility, School of Veterinary at the University of Buenos Aires, Argentina and F Poirier, Jacques Monod Institut, Paris, France; respectively). Animal care and treatment were carried out according to guidelines of the experimental animal care committee of the School of Pharmacy and Biochemistry of the University of Buenos Aires, Buenos Aires, Argentina.

WT and lgals-1\(-/-\) embryos were obtained from WT or lgals-1\(-/-\) homozygous matings, respectively. WT and lgals-1\(-/-\) females were mated on the same date; hence embryos from both genotypes could be collected on the same day and processed simultaneously for the experiments.

Although WT litters are the most commonly used controls for mutant analyses, this alternative breeding strategy was chosen for two reasons: (a) the amount of tissue required for genotyping would seriously compromise the integrity of the extremely small embryos used in our experiments, making it impossible to perform posterior studies in whole embryos; and (b) it is not possible to obtain samples suitable for genotyping after the clearing process. This breeding protocol has already been used for lgals-1\(-/-\) mice (Romaniuk et al., 2012). lgals-1\(-/-\) strain was generated by Dr. F. Poirier (Poirier & Robertson, 1993).

Briefly, by using homologous recombination technique in embryonic stem cells, a null mutation was introduced in the gene encoding the L14 lectin (Gal-1). lgals-1\(-/-\) mice were generated on the MF-1 mouse strain background and then backcrossed to C57BL/6 strain for more than 13 generations. The map of the mouse Lect14 locus is shown in Figure 1a. The Lect14 gene encompasses four exons, encoding 3, 27, and 48 amino acids. A ‘replacement’ vector was constructed containing a neo cassette with a stop codon and a poly(A) addition signal. The recombination process results in the substitution of the region of the gene encompassing exon 2 by the neo cassette, therefore transcription/translation of the mutant allele produces a truncated protein. Homozygous mutant animals were reported to show a complete lack of L14 lectin expression (Poirier & Robertson, 1993; Kobayakawa et al., 2015).

2.2 Genotyping

Genomic DNA from lgals-1\(-/-\) adult mice (male and female) was screened by polymerase chain reaction using a neo primer (3 GGTGCTTGGACAAAAAGAAC 5) and Gal-1 primers (5 CTCAGTGTC-TACATCTGTAAAAATGG 3, and 3 TTCTTTGACATTTGAACCCTATACC 5). The PCR reactions were performed under standard conditions (36
cycles; denaturation at 94°C for 0.5 min, annealing at 55°C for 0.5 min, and polymerization at 72°C for 1 min). Amplification products were visualized following staining of agarose gels with ethidium bromide.

2.3 Surgical procedures

Caesarean section was performed in C57BL/6 WT control and C57BL/6 lgals-1-/- mice. Pregnant females were anesthetized with ketamine (65 mg per kg body weight) and xylazine (15 mg per kg body weight) in a 600 µl solution (Quinta et al., 2014b). Then the embryos were transferred into ice-cold PBS 1X for 10 min.

2.4 Western blot analysis of Gal-1 expression

Gal-1 protein expression was analyzed in the 9-week old male and female mice previously used for mating. Brain and spinal cords from C57BL/6 WT and C57BL/6 lgals-1-/- mice were isolated. Tissues were mechanically homogenized in extraction buffer (60 mM Tris/HCL, pH = 6.8 containing 1% SDS), boiled for 7 min, re-homogenized, boiled for 3 min and centrifuged. Supernatants were electrophoresed on 15% SDS-Polyacrylamide gel. After SDS-PAGE, the proteins were electrophoreted to PVDF membranes (Bio-Rad, Laboratories Inc., Hercules, CA, USA) and blocked with TBS (1X); 0.1% vol/vol Tween-20, 2% BSA for 1 hr. Expression of Gal-1 was evaluated using primary antibody (Table 1) supplemented with 2% bovine serum albumin (BSA) (Sigma-Aldrich, Saint Louis, MO, USA), 0.6% Triton X-100 (Sigma-Aldrich; USA) and horseradish peroxidase-conjugated secondary antibody (Table 1) supplemented with 2% BSA, applied onto the membrane at room temperature. The bands in the membranes were visualized using Image Quant LAS 500 (GE Healthcare Life Sciences, Chicago, IL, USA).

2.5 Whole-mount immunostaining

After incubation in ice-cold PBS 1X, embryos were transferred to glass tubes and incubated for 4 hr in PBSGT solution (PBS 1X, 0.2% gelatin (Sigma-Aldrich; USA), 0.6% Triton X-100 (Sigma-Aldrich; USA) and 0.01% sodium azide (Sigma-Aldrich; USA)) as previously described (Belle et al., 2014). Embryos were then incubated with primary antibodies (Table 1) diluted in PBSGT (300 µl of final dilution per embryo) for 96 hr at 25°C; followed by six washes of 35 min in PBSGT. Next, embryos were incubated in new glass tubes with secondary fluorescent antibodies (Table 1) diluted in PBSGT (300 µl of final dilution per embryo) for 48 hr at 25°C. Finally, a second step of six washes of 35 min in PBSGT was performed. The whole procedure was performed in a roller shaker at 10 rpm. Embryos were stored at 4°C in PBS 1X until the clearing technique was performed.

2.6 Clearing technique

Immunostained whole embryos (E11.5 and E13) were incubated in 2 ml of a mixture of tetrahydrofuran (THF) (Sigma-Aldrich; USA) distilled water (50% (vol/vol)) for 2 hr, then in THF-distilled water (80% (vol/vol)) for 2 hr, in THF (100%) for 1 hr, followed by THF (100%) for 24 hr. Next, embryos were incubated in 3 ml of dichloromethane (100%) (Biopack; Argentina) until they fell to the bottom of the glass vial. Finally, embryos were incubated in 2 ml of BABB solution (mixture of benzyl alcohol (Biopack; Argentina) and benzyl benzoate (Biopack; Argentina) at a ratio of 1:2) until samples became transparent. All the steps were carried out in glass tubes under rotation (10 rpm). The complete process to make embryos transparent was performed in the dark as previously described (Quinta et al., 2015).

2.7 Embryo holder

The holder was fabricated and machined from an aluminum slab as previously described (Quinta et al., 2015) to fit in different microscope stages. The embryos were placed in the holder in sagittal or transverse orientation (depending on the particular specimen) between two glass coverslips and then flattened by mechanical twist and compression.
2.8 | Fluorescent detection of Gal-1 expression

Whole embryos (E13) from WT controls and lgals-1-/− mice immuno-stained (as described above) with Gal-1 and NFM antibodies were placed into the holder in lateral orientation and visualized at low magnification in an Olympus BX51 microscope (Olympus Headquarters Corporate, Philadelphia, PA, USA), equipped with a DP73 Cool Camera and a 4X objective lens with N.A: 0.13 and long working distance.

2.9 | One-photon confocal microscopy

Low magnification 3-dimensional images were obtained using an Olympus Fluoview 1000 confocal microscope (Olympus Headquarters Corporate, USA). Confocal images were collected at two different magnifications (10× objective lens UPLSAPO, N.A: 0.40 and 20× objective lens UPLSAPO, N.A: 0.75). The images were collected taking z-series between 350 and 750 optical slices of Airy unit = 1 airy disk with intervals in accordance with Nyquist theory (optimum overlapping to minimize photobleaching) (Quinta et al., 2015). Images were collected in 16 bits per pixel with scan speeds of 8.0 for 10× magnification and 4.0 for 20× magnification (scanning time per pixel in arbitrary Olympus units). The excitation wavelengths of the lasers used were 480 and 559 nm, respectively, with emission wavelengths of 505 and 567 nm, respectively. The settings of PMT Voltage, Gain and offset were in average: 400, 3125, and 1000, respectively (in all cases arbitrary Olympus units).

2.10 | Epifluorescence microscopy under high power LED illumination and serial image section deconvolution

Three-dimensional images of single axons at high magnification were obtained using a TE-2000-U Nikon Eclipse epifluorescence microscope.
equipped with a piezoelectric (optical step 10 nm) seat in an anti-vibration table. The sample excitation employed was a high power 532 nm LED illumination (Tolket, Argentina). The beam passed through a Cy3 cube (ET545/25x, T565lprx, ET605/70m excitation, dichroic, and emission filters, respectively, Chroma Technology Corp). The stack of z-axis images (average 80 images per axon) was acquired through an apochromatic TIFR objective lens (100×, N.A: 1.49) and a high sensitivity camera (Andor iXon Plus DU-860 EM-CCD) using a 1.0× or a 1.5× projection lens, yielding a pixel size of 160 nm or 106 nm in the image plane, respectively. Images were captured with an acquisition time of 100 ms. SlideBook software (Intelligent Imaging Innovations, Boulder, CO) was used to control the hardware and for initial analysis of the data. Images were subjected to Nearest-neighbor or the more computationally demanding constrained iterative deconvolution procedures and subsequently exported as 16-bit TIF files for further off-line analysis. Prior to performing the 3D reconstruction of single axons, the actual Point Spread Function (PSF) of the composite optical setup was experimentally determined by acquiring a stack of images using 200 nm fluorescent beads (Invitrogen; USA) under identical settings as those employed with the embryo samples.

2.11 | Image processing and analysis

The entire z-axis stacks from embryos collected by confocal microscopy were imported in a calibrated format to IMARIS 3D software v6.3.1 (Bitplane Sci Software; Zurich, Switzerland). 3-dimensional reconstruction of sagittal spinal cord was performed using the Surpass plug-in from IMARIS 3D.

2.11.1 | Axonal length

Quantification of axonal tract length in 3-dimensions was performed using the Measurement point function of IMARIS 3D. Each axonal tract was tracked manually with a measurement point from the beginning of the tract (on each side of the spinal cord) to the end of the tract, creating a template 3D-line with the shape of the axonal tract. Using this strategy, an accurate measure of the axonal tract length was obtained. 20 axonal tracts were measured per embryo and averaged.

2.11.2 | Axonal varicosities

Quantification of axonal varicosities was carried out using a projection of z-stack images from single axons. The count of varicosities was done manually over a trajectory of 90 μm.

2.11.3 | Number of sensory axons/DRG

Quantification of Peripherin positive axonal tracts per DRG neuron was carried out using IMARIS 3D and sequentially applying image volume rotation and ortho slicer visualization plug-ins. Then the images were rotated (real time) in different angles to obtain an accurate quantification of the number of peripherin positive axons per DRG at both sides of each spinal cord segment.

2.11.4 | Fluorescence density

Quantification of fluorescence density in axonal projections and DRG was performed using Fiji software v.1.45 (NIH; Bethesda, MA, USA). Entire 16-bit original images post-confocal acquisitions were opened and z-projected in Max intensity. Then, using polygonal selection the axonal projections or DRGs were delimited and integrated density was measured in the selected areas.

2.11.5 | Floor plate distance

This measurement was obtained using measurement point plug-in from IMARIS 3D. setting the middle of spinal cord as the starting point of the analysis and taking measures at the center and 250 microns up and down in the cranial–caudal axis.

2.11.6 | Image modeling

Representative images from DRG segments, commissural, and trigeminal axons were cropped in the regions of interest using 3D-crop plug-in from IMARIS 3D. The fluorescent signal was transformed into pseudo-color (green or gray) to highlight the axonal tracts and facilitate visualization. Brightness and contrast parameters were almost unchanged compared to the original image.

2.11.7 | Movies

Modeling based on each 3D image from Surpass volume (free form frame by frame) was carried out using Animation plug-in from IMARIS 3D.

2.11.8 | Localization of PlexinA4 and 3D correlation analysis

The comparison between NFM and PlexinA4 localization in axonal projections was performed using the orthogonal view. Z-stack images were imported to IMARIS 3D and split into two individual channels applying Pseudo-color function to each. Finally the Ortho slicer function was applied.

Three-dimensional correlation analysis, scatter plot, and colocalization mask were obtained using a Colocalization threshold plug-in from Fiji software as previously described (Quinta, Maschi, Gomez-Sanchez, Piwien-Pilipuk, & Galigniana, 2010). Confocal z-stack images with optimal overlapping between series were obtained to perform this analysis.

2.11.9 | Axonal diameter

Axonal diameter of white matter tracts was measured on confocal z-stack images imported in IMARIS 3D. Using the Clipping plane function of the software, longitudinal tracts were clipped in z orientation obtaining a coronal view of axons and the axonal tract diameters were measured using the Measurement point function.

2.11.10 | Ultrastructure

To evidence the localization and shape of individual neurofilaments within single axons, high magnification post-deconvolved fluorescence z-images were converted into Thermal spectrum signals (256 color ramp) using Fiji software.
2.12 | Antibody characterization

The details of the primary and secondary antibodies used in this study are summarized in Table 1. All antibodies are commercially available and their characterization has been extensively documented in the literature. In addition, all of them have been used in previous studies from our laboratory. Finally, in our current experiments, these antibodies showed the expected distribution pattern within cells and tissues.

Developing motor and commissural axons were demonstrated using a mouse monoclonal anti-Neurofilament-M (NFM) antibody raised against whole rat NFM protein (Invitrogen, USA; Cat. 13–0700; RRID: AB_2532998), (Lee, Carden, Schlaepfer, & Trojanowski, 1987; Quinta et al., 2014b, 2016). Sensory axons were immunostained with mouse monoclonal anti-peripherin antibody. The antibody was raised against the 4 N-terminal aminoacids of rat peripherin protein (Millipore, USA; Cat MAB1527; RRID: AB_2284441), (Sommer, Shah, Rao, & Anderson, 1995; Quinta et al., 2016). Adult tissue samples were stained for GFAP and βIII-tubulin (neuronal) for histological analysis. Mouse monoclonal anti-βIII-tubulin was generated against a synthetic peptide mimicking a conserved region of the protein comprising aminoacids 436–450 (Sigma, USA; Cat. T8578; RRID: AB_1841228), (Lee, Tuttle, Rebhun, Cleveland, & Frankfurter, 1990; Quinta et al., 2014b). Chicken polyclonal anti-GFAP was raised against isolated bovine GFAP protein – aminoacids 1–428 (Neuromics, USA; Cat. CH22102; RRID:AB_10014322), (Brenner et al., 2001; Quinta et al., 2014b). PlexinA4 receptor was immunolocalized using a rabbit polyclonal antibody raised against a synthetic peptide comprising aminoacids 500–600 of the mouse protein (Abcam, UK; Cat. Ab39350; RRID: AB_944890), (Gutekunst, Stewart, English, & Gross, 2012). The specificity of the anti-PlexinA4 antibody has been further characterized in our laboratory by western blot analysis and immunofluorescence (Quinta et al., 2014b, 2016). Gal-1 was demonstrated by immunohistochemistry using a rabbit polyclonal antibody generated against the N-terminal domain of human Gal-1 (aminoacids 1–45), (Santa Cruz, USA; Cat. sc-28248; RRID: AB_640838), (Zhao, Zhao, Jiang, Zhao, & Chen, 2011). The specificity of the antibody has been confirmed by western blot analysis and immunofluorescence in our laboratory (Quinta et al., 2014b); and was further validated by our current results (immunohistochemistry and western blot analysis) in Gal-1 knock out mice (see results). The specificity of the secondary antibodies was confirmed by omitting the primary antibody. No immunoreactivity was observed in any of these control tissue samples.

2.13 | Behavioral experiments

To assess mice coordinated motor skills we used two tests:

2.13.1 | Rotarod test

Male C57BL/6 WT and C57BL/6 Lgals1−/− mice (6 weeks of age, weighing: 25–28 g) were placed in the Rotarod as previously described (Bergeron, Chagniel, Bureau, Massicotte, & Cyr, 2014; Quinta et al., 2016). The speed used was 12 rpm. Mice were tested for 1 min with a latency period of 10 s. Three sessions were carried out on three consecutive days. Three trials with 4 min of resting time were carried out on each session and values were averaged. Before the evaluation, animals were acclimatized in the sound-attenuated experiment room.

2.13.2 | 90° grid walking test

A straight bridge-shaped metal grid of 25 cm was placed at a 90° angle from the floor, as previously described (Quinta et al., 2016). Mice were placed at the base and the number of foot fall errors—where the hindlimb failed to grasp a bar—was recorded (0–10 scale) per climb. Three repetitions with 2 min of resting time were done for each animal and the values were averaged. Analysis for both tests was performed by two experimenters who were blind to the experimental design.

2.14 | Statistical analysis

Graph-Pad Prism software Version 5.0 (Graph-Pad software, Inc.; La Jolla, CA, USA) was used for data analysis. For each variable/parameter
analyzed (i.e., axonal length, axonal varicosities, number of sensory axons/DRG etc.), values from the same embryo were averaged and considered one independent observation. For histological analyses, eight embryos per condition (i.e., mutant or wt) were analyzed. For behavioral tests, nine adult animals per condition were tested. Data sets were tested for normality using Kolmogorov-Smirnov and D’Agostino & Pearson omnibus tests. Comparisons were then performed using unpaired two-tailed Student’s t-test or one-way analysis of variance (ANOVA) followed by Tukey’s Multiple Comparison Test, where appropriate. Results are presented as mean ± SEM (standard error of the mean).

3 | RESULTS

3.1 | Gal-1 expression

PCR screening of genomic DNA carried out in mutant animals showed a 694 pb band corresponding to the mutated locus (Figure 1b), which was present in every mutant analyzed. Western blot analysis of brain and spinal cord tissues from adult mice showed a 14.5 Kd band corresponding to Gal-1 protein only in WT animals. This band was absent in the tissues from mutant animals (Figure 1c). Immunofluorescent detection of Gal-1 in E13 embryos confirmed the complete absence of Gal-1 signal in mutant embryos. Gal-1 signal was present in WT embryos used as controls (Figure 1d). Figure 2 shows the 3-dimensional whole image acquisition setup used for the analysis of axonal development of these embryos.

3.2 | Axonal development in embryonic day 11.5

First, we evaluated the axonal development in 3-dimensions in Igal-1−/− and WT mouse embryos. Axonal tracts were immunostained with Neurofilament-M, which is expressed in motor axons, sensory ganglia axons and commissural axons. To visualize the 3-dimensional structure of motor and sensory projections as well as DRG, the spinal cords were positioned in a sagittal orientation (Figure 3a).

3.2.1 | Motor axons

Measurement of axonal length of motor projections showed no significant differences between Igal-1−/− and WT mouse embryos (389.4 μm ± 16 vs. 373.4 μm ± 9, respectively), (Figure 3b). In addition,
Figure 4: Evaluation of DRG projections, commissural axons and trigeminal nerves. (a) Representative z-projection images of DRG axons obtained from Igals-1<sup>-/-</sup> and WT mouse embryos immunostained with anti-Peripherin. Scale bar: 100 μm. (b) Bar graph shows the number of Peripherin positive axonal tracts per DRG neuron. (c) Representative images of DRG structure obtained from Igals-1<sup>-/-</sup> and WT mouse embryos immunostained with NFM. Inset below shows the surpass render images for each condition. Scale bar: 100 μm. (d) Bar graph shows the measurement of DRG surface. (e) Bar graph shows the quantification of fluorescence density of DRG z-projections. (f) The images show commissural axons crossing the floor plate at spinal cord level (red arrowhead). Scale bar: 100 μm. Table shows the floor plate distance observed in Igals-1<sup>-/-</sup> vs. WT mouse embryos at three different segments of spinal cord. (g) Representative images of trigeminal structure showing the trigeminal ganglia (TG), proximal projections and branching (yellow arrowheads) as well as its trigeminal nerves (TN). (Voxel size of x, y, and z: 1.59 × 1.59 × 0.59 μm). Insets below show thermal images of the area contained in yellow frames in TN, showing branching patterns in each single axon (yellow arrowheads). Scale bar: 10 μm. Values represent the mean ± SEM (n = 8). ns = not significant using unpaired two-tailed Student’s t-test [Color figure can be viewed at wileyonlinelibrary.com]
fluorescence density of NFM-positive axons was measured. No significant differences between \( \text{lgals-1}^{-/-} \) and WT mice were observed (Figure 3c). In line with these observations, we did not find qualitative differences in the pattern of 3-dimensional axonal projection deep in the tissue in \( \text{lgals-1}^{-/-} \) compared to WT embryos (Movie 1A-B).

Finally we assessed the number of axonal varicosities per axonal projection in \( \text{lgals-1}^{-/-} \) embryos, finding no significant differences with WT embryos (Figure 3d,e). Within the axons, neurofilaments exhibited a normal distribution pattern in both \( \text{lgals-1}^{-/-} \) and WT embryos, with NFM segments flanked by gaps (Figure 2e). This was in agreement

**FIGURE 5** Axonal development on embryonic day 13. (a) Representative surpass images showing a 3-dimensional reconstruction of spinal cord from \( \text{lgals-1}^{-/-} \) and WT mouse embryos immunostained with anti-NFM. (voxel size of \( x \), \( y \), and \( z \): 1.98 \( \times 1.98 \times 0.81 \) μm). (b) Bar graph shows 3-dimensional axonal length measurement of projections. (c) Bar graph shows the quantification of fluorescence density in NFM-positive axons. (d) High magnification (100 \( \times \)) of (a). Yellow frames (1 and 2) show representative surpass render images of single axonal sprouting and NFM pattern from \( \text{lgals-1}^{-/-} \) and WT mouse embryos. (Voxel size of \( x \), \( y \), and \( z \): 0.2 \( \times 0.2 \times 0.5 \) μm). (e) Bar graph shows the number of Peripherin positive axons per DRG neuron. (f) Representative images of DRG structure obtained from \( \text{lgals-1}^{-/-} \) and WT mouse embryos immunostained with NFM. Scale bar: 100 μm. (g) Bar graph shows the measurement of DRG surface. (h) Bar graph shows the quantification of fluorescence density of DRG z-projections. (i) Representative images showing the dynamic growth of commissural axons crossing the floor plate at embryonic day 13. Values represent the mean \( \pm \) SEM (\( n = 8 \)). ns = not significant using unpaired two-tailed Student’s t-test [Color figure can be viewed at wileyonlinelibrary.com]
with the distribution pattern previously described (Trivedi, Jung, & Brown, 2007; Alami, Jung, & Brown, 2009; Quinta & Galigniana, 2012).

3.2.2 | Sensory axons

To analyze sensory projections, transparent embryos were immuno-stained with Peripherin, a specific marker for peripheral axonal tracts (Figure 4a). We found no significant differences in the number of Peripherin positive axons per DRG in \( \text{lgals-1}^{-/-} \) compared to WT embryos (11.5 ± 0.7 vs. 11.0 ± 0.4, respectively), (Figure 4b). DRG from \( \text{lgals-1}^{-/-} \) embryos showed the same rate of 3-dimensional development as that observed in WT embryos, with similar DRG surfaces (22.101 ± 0.2 × 23.352 ± 0.5 \( \mu \text{m}^2 \)). Values represent the mean ± SEM (n = 8). ns = not significant using unpaired two-tailed Student's t-test [Color figure can be viewed at wileyonlinelibrary.com]

3.2.3 | Commissural axons

Commissural axons from \( \text{lgals-1}^{-/-} \) embryos crossed the floor plate at the spinal cord level with an identical pattern to that observed in WT embryos (Figure 4f). Furthermore, the floor plate distance measured in \( \text{lgals-1}^{-/-} \) embryos was the same as that in WT animals from the same embryonic stage (Figure 4f, table).

3.2.4 | Trigeminal ganglia and its nerves

\( \text{lgals-1}^{-/-} \) mouse embryos showed the same 3-dimensional topographical development to that observed in WT embryos, coinciding with the expected degree of development for this stage of differentiation (Shibata et al., 2010; Engelhard et al., 2013). Additionally, individual axons in trigeminal nerves from embryos of both genotypes showed the same pattern of varicosities (Figure 4g).

3.3 | Axonal development on embryonic day 13

Next we followed the axonal development in E13 mouse embryos (\( \text{lgals-1}^{-/-} \) vs. WT). Axonal projections were immunostained with NFM and 3D images were acquired in sagittal orientation.

3.3.1 | Motor axons

When axonal length was assessed no significant differences were observed between \( \text{lgals-1}^{-/-} \) and WT embryos (1089 ± 43 vs. 999.7 ± 50) (Figure 5a,b). Moreover, we did not observe qualitative differences in the 3D pattern of axonal projections (Movie 2A-B). Accordingly, fluorescence density of NFM-positive axons showed no significant differences between \( \text{lgals-1}^{-/-} \) and WT mice (Figure 5c). In addition, both \( \text{lgals-1}^{-/-} \) and WT mouse embryos presented the same degree of axonal sprouting and NFM patterns (Figure 5d).
FIGURE 7 Neuronal localization of PlexinA4 on embryonic day 13. (a) Double immunostaining was performed with anti-NFM (green) and anti-PlexinA4 (red) on Igals-1⁻/⁻ and WT mouse embryos. Representative surpass images showing the 3-dimensional reconstruction of spinal cord. (Voxel size of x, y, and z: 1.98 × 1.98 × 0.5 μm). Magnified orthogonal view shows the localization of PlexinA4 (yellow arrowhead) in the axonal projections. (b) Representative images of forelimb plexus showing the clustered distribution of PlexinA4 (magenta) spread in the axonal shaft (green). Inset below highlights the PlexinA4 localization in the axonal shaft surface (yellow arrowhead). Scale bar: 100 μm for panoramic image and 50 μm for magnification. (c) Images of DRG projections show PlexinA4 localization (magenta) in the DRG, axonal projections and in dorsal root entry zone (DREZ). Scale bar: 150 μm. (d) Magnification images of (c), highlighting the PlexinA4 localization in the DRG and in DREZ- projecting axons (yellow arrowhead). Mask images (white) show the PlexinA4 distribution. Quantification of correlation is shown in the scatter plot inset. Scale bar: 150 μm [Color figure can be viewed at wileyonlinelibrary.com]
3.3.2 | Sensory axons

In agreement with our observations at embryonic day 11.5, no significant differences were found in the number of Peripherin positive axons per DRG in \( \text{lgals-1}^-/- \) when compared with WT mouse embryos (10.4 \( \pm \) 0.70 vs. 11.2 \( \pm \) 0.57, respectively) (Figure 5e). Furthermore, DRG from \( \text{lgals-1}^-/- \) embryos showed the same degree of 3-dimensional development that WT animals, with similar DRG surface (40.600 \( \pm \) 2.500 vs. 40.230 \( \pm \) 2.300) (Figure 5f,g). Moreover, fluorescence density of DRG z-projections showed no significant difference between \( \text{lgals-1}^-/- \) and WT mice (Figure 5h). Of note, an increase in the DRG surface was apparent as neuronal development progressed (E11.5 to E13).

3.3.3 | Commissural axons

Commissural axons of \( \text{lgals-1}^-/- \) embryos at this embryonic stage showed the classical whole array development in the spinal cord, in coincidence with the pattern observed in WT embryos (Figure 5i).

3.3.4 | Trigeminal axons

The length of trigeminal axons measured in \( \text{lgals-1}^-/- \) embryos showed no significant differences when compared to that of WT mouse embryos (344 \( \mu \mathrm{m} \pm 23 \) vs. 294 \( \mu \mathrm{m} \pm 20 \)) (Figure 6a,b). Furthermore, embryos from both genotypes showed a normal distribution pattern of neurofilaments within single trigeminal axons (Figure 6c).

3.3.5 | Neuronal localization of PlexinA4

We evaluated the localization of PlexinA4, a key receptor for semaphorin protein-mediated guidance in E13 embryos. Semaphorin is expressed in these early stages and is key to the correct patterning of the axonal populations studied (Pasterkamp & Kolodkin, 2013). Figure 7a shows a 3-dimensional sagittal view of axonal projections showing equivalent localization patterns for NFM and PlexinA4, in both \( \text{lgals-1}^-/- \) and WT mouse embryos (Figure 5h). Of note, an increase in the DRG surface was apparent as neuronal development progressed (E11.5 to E13).

**FIGURE 8** Quantitative assessment of locomotor coordination. (a) Representative images of locomotor behavior displayed by \( \text{lgals-1}^-/- \) and WT mice, showing a correct positioning of the paw articulation in the Rotarod (yellow arrowheads) in both groups. (b) Graph shows the quantification of coordinated locomotor behavior. Values represent the mean \( \pm \) SEM \((n = 9)\). ns = not significant using one-way ANOVA followed by Tukey’s multiple comparison test. (c) Bar graph shows the quantification of foot fall errors. Arrowheads in the inset show the similar hindlimb position in each condition. Values represent the mean \( \pm \) SEM \((n = 9)\). ns = not significant using two-tailed Student’s t-test. (d) Representative images of spinal cord lumbar segments. Dotted white lines show the limit between white matter and gray matter. Yellow arrowheads point the astrocytes surrounding the axonal tract. Scale bar: 20 \( \mu \mathrm{m} \). Bar graph shows the quantification of axonal diameter. Values represent the mean \( \pm \) SEM \((n = 9)\). ns = not significant using unpaired two-tailed Student’s t-test [Color figure can be viewed at wileyonlinelibrary.com]
that 95% of the PlexinA4 signal observed in \( \text{lgals-1}\) mouse embryos had, in turn, a NFM signal in the vicinity of their focal plane. Similar results were observed in WT embryos (92% PlexinA4 signal was associated with the NFM signal in the vicinity of their focal plane) (Figure 7c,d).

### 3.4 | Postnatal locomotor activity

We next investigated the locomotor behavior in \( \text{lgals-1}\) and WT mice in order to establish a correlation with the previously shown neuroanatomical development. To this aim, we performed the Rotarod test and the 90° grid walking test in 9-week old \( \text{lgals-1}\) and WT male mice. In the Rotarod test, \( \text{lgals-1}\) mice showed correct paw position during the hike (Figure 8a). Additionally, no significant differences in locomotor coordination, assessed as the latency time in the Rotarod, were observed in \( \text{lgals-1}\) compared to WT mice (Figure 8b). These results suggest that locomotor behavior is unaffected in mutant animals. Furthermore, in the 90° grid walking test, \( \text{lgals-1}\) mice showed a minimum number of foot fall errors (1.38 ± 0.56), similar to that observed in WT mice (1.27 ± 0.61) (Figure 8c and insets). In order to establish a histological correlation with the behavioral findings, the axonal projections at the lumbar level of the spinal cord of these animals were analyzed. No significant differences were observed in the axonal diameter nor in the ramification pattern of the astrocytes (Figure 8d), demonstrating that the absence of e-Gal-1 did not modify not only the axonal structure but either the astrocyte architecture.

### 4 | DISCUSSION

The present study was aimed at analyzing the effect of the absence of e-Gal-1 on spinal axonal development and subsequent locomotor activity in mice. We performed an adaptation to the clearing technique described by Chedotal’s laboratory (Belle et al., 2014) with some improvements in the clearing process. In addition, we implemented the 3-DISCO technique to visualize the embryonic development using 1-photon microscopy (Quinta et al., 2015). This technique allowed us to obtain 3-dimensional axonal development with accurate information regarding the number, length, and real orientation of axons. Furthermore, we developed a novel combination of SCO technique with high magnification microscopy, to obtain single intra-axonal structural information of these same embryos, using a combination of epifluorescence microscopy under high power LED illumination and serial image section deconvolution. To improve the 3-dimensional image acquisition deep in the tissue, samples were mechanically compressed to achieve a flat surface of whole embryos. This allowed us to overcome the problems arising from the thick embryo specimens combined with the need to resort to high magnifications.

We evaluated the axonal growth of motor and sensory projections at the spinal cord level during early embryonic developmental stages. At E11.5 and E13 we only found motor and sensory axonal projections with no glial cell signal, consistent with previous observations for the embryonic stages analyzed (Petracca et al., 2016). The axonal length of motor projections was similar between \( \text{lgals-1}\) and WT mouse embryos both at E11.5 and E13, and no difference in axonal fluorescence density was found in these embryonic stages. Moreover, DRG structure and its sensory axonal projections presented a normal growth rate in \( \text{lgals-1}\) embryos. In particular, this observation suggests that in vivo e-Gal-1 does not interfere with Sema3A signaling through NRP-1/ PlexinA4 receptor complex; since developing DRG neurons are known to be sensitive to the inhibitory effects of Sema3A both in vitro (Luo, Raible, & Raper, 1993) and in vivo (Fu, Sharma, Luo, Raper, & Frank, 2000; Wright et al., 1995). Together, these results suggest the lack of an e-Gal-1 function on early spinal axonal development and growth. Likewise, other structures like commissural and trigeminal axons from \( \text{lgals-1}\) embryos showed a normal degree of development, comparable to that observed in WT embryos and to that previously reported for the same embryonic stages (Belle et al., 2014). Interestingly, the structure of individual neurofilaments within single axons of \( \text{lgals-1}\) embryos presented the classical pattern of growing gaps of neurofilament-M (Alami et al., 2009; Trivedi et al., 2007).

Additionally, in the absence of e-Gal-1, PlexinA4 exhibited a normal distribution pattern in segments of forelimb plexus corresponding to motor neuron projections as well as in the DRG and its axonal projections. Of note, PlexinA4 localization was consistent with the reported localization of its ligand, Sema3A (Huber et al., 2005). Finally, we showed that the absence of e-Gal-1 did not alter the normal coordinated locomotion assessed in two independent behavioral tests. This result is consistent with mutant adult animals displaying a normal histology at the lumbar spinal cord and with the morphological/structural observations made at early developmental stages (motor and sensitive projections with normal growth and correct crossing of commissural axons in the floor plate).

Taken together, our results strongly support the lack of a role of e-Gal-1 on early axonal development in the spinal cord (motor, sensitive, and commissural tracts) which correlates with the normal coordinated locomotion displayed by mutant animals in the behavioral tests. Our results support and extend studies from Poirier (Poirier & Robertson, 1993) showing no major neuronal phenotypic abnormalities in these Gal-1 knockout animals.

These observations are in line with our previous results in regenerating spinal axons (Quinta et al., 2014b, 2016), where we have identified the mechanism underlying exogenous Gal-1 promotion of axonal re-growth after SCI and concomitant recovery of locomotor activity. Interestingly this process only occurs when Gal-1 acquires its dimeric conformation. In this quaternary structure Gal-1 binds to PlexinA4 receptor, thus interfering with Sema3A signaling and favoring the reactivation of the actin cytoskeleton dynamics (Quinta et al., 2016). In vivo, e-Gal-1 is present in the tissues at concentrations that favor the monomeric species (Cho & Cummings, 1996; Rabinovich & Croci, 2012). The monomeric lectin lacks the ability to bind PlexinA4 receptor (Quinta et al., 2014a,b) and is therefore unable promote the reactivation of the actin cytoskeleton and subsequent axonal re-growth.

Regarding early axonal guide, prior studies have demonstrated that normal axonal navigation in the spinal cord during development involves several guidance molecules and corresponding neuronal...
receptors. In particular, Plexin receptors present in the neuronal surface mediate signaling from Sema3A, a key anti-attractant molecule for axonal growth localized in the perineuronal interstice (He & Tessier-Lavigne, 1997; Kolodkin et al., 1997; Pasterkamp, Ruitenberg, & Verhaagen, 1999; Tamagnone et al., 1999; Pasterkamp & Kolodkin, 2003; Yoshida, 2012). This interaction guides in 3-dimensions the axonal growth, trajectory and differentiation to reach specific targets (Huber et al., 2005). This repulsive/inhibitory effect of Semaphorin/Plexin signaling is recapitulated after a SCI, when Semaphorin levels rise in response to primary tissue damage inhibiting axonal regeneration. In this respect, the finding that axonal growth during development is independent of the presence of e-Gal-1 is in agreement with the inability of the endogenous lectin to promote axonal re-growth after a spinal lesions in adult mice.

Finally, it is worth mentioning that Igals-1/- mice are currently used as model organisms to study the regenerative properties of exogenous Gal-1, in the absence of the endogenous lectin. However, no studies focused on axonal development had been carried out on these knock-out animals. The present results constitute strong evidence against a role of endogenous galectin on axonal development in the spinal cord; discarding the presence of alterations in the neuronal architecture, as well as in the locomotor behavior provoked by the lack of endogenous Gal-1. In this context, our results provide additional support to studies using Igals-1/- mice models of SCI or traumatic brain injury (Quinta et al., 2014b, 2016; Sirko et al., 2015). Furthermore, they will allow to extend the investigation on the neuro-regenerative properties of dimeric Gal-1 treatment in these traumatic pathologies and potentially other pathologies in the CNS.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

HRQ conceived and coordinated the study; designed and performed the experiments. JMP, FJB and HRQ analyzed the data. HRQ wrote the article. All authors reviewed the results and approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Movie 1. 3-dimensional movies of axonal projection on embryonic day 11.5. (A) and (B) correspond to gals-1 knockout (-/-) and WT mouse embryos, respectively.

Movie 2. 3-dimensional movies of axonal projection on embryonic day 13. (A) and (B) correspond to gals-1 knockout (-/-) and WT mouse embryos, respectively. The movies were obtained using Imaris 6.3.1 bitplane software.

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