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Expression Profile of Flotillin-2 and its Pathophysiological Role after Spinal Cord Injury

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Abstract

Some receptors that block axonal regeneration or promote cell death after spinal cord injury (SCI) are localized in membrane rafts. Flotillin-2 (Flot-2) is an essential protein associated with the formation of these domains and the clustering of membranal proteins, which may have signaling activities. Our hypothesis is that trauma will change Flot-2 expression and interference of this lipid raft marker will promote functional locomotor recovery after SCI. Analyses were conducted to determine the spatio-temporal profile of Flot-2 expression in adult rats after SCI, using the MASCIS impactor device. Immunoblots showed that SCI produced a significant decrease in the level of Flot-2 at 2 days post-injury (DPI) that increased until 28 DPI. Confocal microscopy revealed Flot-2 expression in neurons, reactive astrocytes and oligodendrocytes specifically associated to myelin structures near or close to the axons of the cord. In the open field test and grid walking assays, to monitor locomotor recovery of injured rats infused intrathecally with Flot-2 antisense oligonucleotides for 28 days showed significant behavioral improvement at 14, 21 and 28 DPI. These findings suggest that Flot-2 has a role in the non-permissive environment that blocks locomotor recovery after SCI by clustering unfavorable proteins in membrane rafts.

Keywords

Membrane raft; trauma; lipid raft; regeneration; neurite outgrowth; locomotor recovery

Introduction

Trauma to the spinal cord produces changes at the molecular and cellular levels, such as necrosis due to direct physical contact with the cord, damaged blood vessels that produce changes in blood flow, infiltration of cells from the immune system and ischemia (Hulsebosh, 2005). In addition, it is known that after an injury, a nonpermissive environment develops at the lesion epicenter that blocks axonal regeneration and/or cell survival (Yui and He, 2006). Most of the proteins, and their receptors, which cause a detrimental effect for neurite outgrowth and/or cell existence are present in membrane rafts (Vihanto et al., 2004; Yu et al., 2004). The formation of these microdomains in the plasma membrane produces the compartmentalization (inclusion or exclusion) of several proteins that may be involved in specific cell processes, including the attraction or repulsion of a cell (DeBruin et al.,

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2006) and/or apoptosis (Gajate and Mollinedo, 2001; Hueber, 2003; Gajate and Mollinedo, 2005).

Membrane rafts are small, (10–200 nm) heterogeneous dynamic membrane microdomains enriched in cholesterol and glycosphingolipids that compartmentalize the cellular processes of a specific protein composition to perform a particular task (Lang et al., 1998; Pike, 2006). Membrane rafts facilitates protein clustering, this process allows for conglomerates of proteins to build up in a specific region of the plasma membrane, and this in turns allows for a more effective cellular signaling between the receptors and their targets. Proteins that may be in these microdomains clustered may be involved in apoptosis, endocytosis, secretion, cell surface proteolysis and cell signaling, among others (Gebreselassie and Bowen, 2004; Rajendran and Simons, 2005; DeBruin et al., 2006; Munderloh et al., 2009). The spatio-temporal regulation and activity of many signaling proteins may be modulated through raft recruitment and raft assembly, thus demonstrating the heterogeneity and dynamics of these domains (Lucero and Robbins, 2004).

Among the proteins associated with the formation of membrane rafts are the caveolins and flotillins, which are commonly used as markers of membrane rafts. Of these two proteins, Flotilin-2 (Flot-2) is primarily associated with the plasma membrane because it is palmitoylated and mirystoylated and plays a significant role in the maintenance of membrane rafts (Langhorst et al., 2005). Flot-2 expression is upregulated after optic nerve transection (Schulte et al., 1997). Similar findings were observed with caveolin-1, another membrane raft marker, after spinal cord injury in adult rats (Shin, 2007), in irradiated rat spinal cords (Ahn et al., 2006) and after mechanical injury of PC12 cells (Galbiati et al., 1998). Moreover, the abnormal upregulation of flotillins has been associated with the experimental autoimmune encephalomyelitis (EAE) disease model (Kim et al., 2006) and neurological disorders such as Parkinson's (Jacobowitz and Kallarakal, 2004) or Alzheimer's (Kokubo et al., 2000) diseases. Well-known neurite inhibitors like the Nogo receptor, p75, MAG and OMgp are also detected in membrane rafts' microdomains (Yu et al., 2004; Boynapalli et al., 2005). When membrane rafts are disrupted in-vitro by β -methylcyclodextrin, the repulsive activity that is mediated by the Nogo receptor is reduced (Yu et al., 2004), confirming the presence of proteins that repulse axonal outgrowth in membrane rafts. However, the role of Flot-2 in an animal model of trauma to the cord of an adult vertebrate has not been assessed to date. Therefore, we hypothesize that Flot-2 expression will be altered after SCI and this alteration may provide the conditions to block functional locomotor recovery after trauma.

Material and Methods

C6 glioma Cell Culture

C6 glioma cells (Cell Systems, Seattle, WA) grown in Nutrient Mixture Ham's F-10 media containing 1% antibiotic/antimycotic and 10% FBS were routinely propagated as a confluent monolayer (Silva et al., 2005). Induction of differentiation into astrocyte-like cells was achieved by the addition of 1 mM dibutyryl cyclic AMP (db-cAMP) and the reduction of growth media to 1% FBS (Silva et al., 2005).

Animals and surgical procedures

Adult (200–220 g) female Sprague-Dawley rats (Hilltop Lab Animals Inc, Scottsdale, PA) were anesthetized with intraperitoneal injections of ketamine (Fort Dodge Animal Health Fort Dodge, IA; 87.7 mg/kg), xylazine (Fort Dodge Animal Health Fort Dodge, IA; 4.2 mL/kg) and acepromazine (Vetus Animal Health, Rockville Center, NY; 0.85 mg/kg). A laminectomy at the thoracic T10 level was performed to expose the spinal cord and the

injury was produced by releasing the MASCIS impactor (10 grams) from a height of 12.5 mm (Miranda et al., 1999). Half of the animals underwent sham surgeries (laminectomized: noninjured) and the other half were injured. Animals were sacrificed at 2, 4, 7, 14, and 28 days post-injury (DPI). The rats were euthanized as described before (Cruz-Orengo et al., 2006; Figueroa et al., 2006). Briefly, the animals were deeply anesthetized with pentobarbital (40–50 mg/kg), followed by transcardial perfusion with 0.01 M phosphate buffered saline (PBS: Dulbecco's phosphate buffered saline, Sigma-Aldrich, St. Louis, MO, USA; pH = 7.4). The spinal cord segment containing the lesion epicenter (5 mm) was dissected, and protein extraction performed as described by Lai and colleagues (Lai et al., 2001). All animal work was conducted with the approval of the University of Puerto Rico's Animal Care and Use Committee and followed NIH guidelines to minimize pain or discomfort.

Protein Extraction

The culture medium was removed from the confluent monolayer (~80%), and 5 ml of ice cold PBS was added twice to wash the cells. The PBS was removed, and ice-cold lysis buffer (50 mM TrisCl, 150 mM NaCl, 60 mM octyl-glucoside, 5 mM EDTA, 0.2 mM PMSF, protease inhibitors cocktail including 100 µg/ml each of leupeptin, antipain, bestatin, chymostatin and pepstatin A, pH = 8.0) was added (1 ml per 10⁷-cells/100 mm dish/150 cm² flasks). The adherent cells were scraped off the dish using a cold plastic scraper, and the cells suspension was gently transferred into a pre-cooled microcentrifuge tube and was incubated at 4° C while rocking for 30 minutes. The solubilized samples were centrifuged for 20 minutes at 13,400 g and the supernatant was transferred to a new tube and placed on ice at –20° C. The protein concentration was determined using the Bradford assay, and the sample was diluted in urea sample buffer (4% SDS, 8 M urea, 62 mM EDTA, 0.2% β-Mercaptoethanol and 0.015% bromophenol blue).

Dissected tissue from the spinal cord was homogenized in ice-cold Tris Lysis buffer I (20 mM Tris-HCl, 150 mM NaCl, 5 mM NaF, 1 mM EDTA, 1 mM EGTA, pH = 8.0) that contained 2 µg/mL antipain, 10 µg/mL aprotinin, 5 mM benzamidine, 1 mM DTT, 10 µg/mL leupeptin, 1 mM sodium orthovanadate, 1 mM PMSF, and 10 µg/mL trypsin inhibitors (Lai et al., 2001; Figueroa et al., 2006). The homogenate was centrifuged at 20,817 g for 90 minutes and the supernatant was collected in a new microtube held at 4°C. The pellet was resuspended in lysis buffer with 1% Nonident P-40 for 45 min at 4°C. Following a brief 10-minute centrifugation at 20,817 g at 4°C, the protein concentration of the supernatant was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) protocol, as suggested by the manufacturer's instructions.

Western blots

The protein extract (10 µg) was analyzed in a 15% polyacrylamide-SDS gel and the proteins were electroblotted to a nitrocellulose membrane. To verify transfer and reveal molecular weight markers, nitrocellulose membrane was stained with 0.1% Ponceaus S (made in 0.1% glacial acetic acid) for 10 minutes and then rinsed with PBS 1X three times for 10 minutes each. The membrane was blocked with Blotto (3% nonfat dry milk, 20 mM Tris-NaCl pH = 7.5, 10% Tween-20) for 1 hour at 25°C. Immunodetection was performed by incubating membranes with the monoclonal mouse anti-rat or polyclonal rabbit anti-rat Flotillin-2 antibody (1:250; Pharmigen, BD Biosciences, San Jose, CA, USA or 1:250, Sigma-Aldrich, St. Louis, MO) in blocking solution overnight at 4°C. Membranes were washed and incubated with HRP-conjugated rabbit anti-mouse IgG (1:5000; Sigma-Aldrich, St. Louis, MO) or donkey anti-rabbit IgG (1:10,000; SIGMA-Aldrich, St. Louis, MO) for 1.5 hours at 25°C. HRP-signal was enhanced with SuperSignal West Dura extended version for 1 minute, as per the manufacturer's instructions (Pierce, Rockford, IL, USA), before exposure

and development. The membrane was also probed for GAPDH, as a housekeeping gene (Calvo et al., 2008), with rabbit anti-rat GAPDH (1:5000; Sigma-Aldrich, St. Louis, MO, USA) and goat anti-rabbit IgG (1:10000; Sigma-Aldrich, St. Louis, MO, USA). The development and densitometry analysis of the nitrocellulose membranes was performed using the Versadoc Imaging System (Bio-Rad Laboratories, Hercules, CA, USA) and Quantity One Software (Bio-Rad Laboratories, Hercules, CA, USA).

Analysis of Triton X-100 soluble and insoluble fractions

The spinal cord segment from the T10 level of an adult rat was homogenized in a modified version of the Lysis buffer I (20 mM HEPES, 150 mM NaCl, 5 mM NaF, 1 mM EDTA, 1 mM EGTA, 2 µg/mL antipain, 10 µg/mL aprotinin, 5 mM benzamidine, 2 mM DTT, 10 µg/mL leupeptin, 1 mM sodium orthovanadate, 1 mM PMSF, and 10 µg/mL trypsin inhibitors, 100 mM MgCl₂, pH = 8.0) with a pellet pestle (Kontes Glass Co., Vineland, NJ, USA). After homogenization of the tissue, the samples were centrifuged for 10 minutes at 950 g at 4°C. The supernatant was transferred to a new microtube and treated with ice-cold 1% Triton X-100 (Sigma-Aldrich St. Louis, MO, USA), followed by a brief vortex (5 sec). The TX-100 treated fraction was then incubated on ice for 5 minutes and the sample was centrifuged at 14,000 g for 15 minutes at 4°C. The supernatant was transferred to an ultra-clear centrifuge tube (Beckman Instruments Inc., Palo Alto, CA, USA) and centrifuged in an 80 Ti rotor at 100,000 g for 30 minutes at 4°C with a deceleration of 1,073 g every 4 minutes. After this centrifugation, the supernatant (soluble in Triton X-100 phase) was transferred to a new microtube and the pellet in the centrifuge tube (insoluble in Triton X-100 phase) was resuspended with the same buffer used for homogenization (see above) (Schroeder et al., 1998; Mairhofer et al., 2002; Asano et al., 2009). Samples (20 µg) from each fraction (soluble/supernatant or insoluble/pellet) were diluted in urea SDS-PAGE sample buffer (4% SDS, 8M Urea, 62 mM Tris HCl pH = 6.8, 20 mM EDTA, 5% β-Mercaptoethanol (βME), 0.015% Bromophenol blue), heated and analyzed by SDS-PAGE and Western blot analysis.

Sucrose Density Gradients

The sucrose density gradient was prepared as described by Silva and colleagues (Silva et al., 1999) with some modifications. Solutions of sucrose at 90%, 35% and 5% were prepared in a buffer containing 25 mM MES and 150 mM NaCl (pH = 2.8). Proteins from the T10 epicenter segment of the spinal cord (1 cm long) were washed briefly with ice-cold MBS buffer (25 mM MES, 150 mM NaCl, pH = 3.0) followed by homogenization on a dounce homogenizer with a tight crystal pestle (Kontes Glass Co., Vineland, NJ, USA) containing 2 mL of homogenizing buffer containing Na₂CO₃, to enhance the maintenance/stability of lipid raft domains (25 mM MES, 150 mM NaCl, 100 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 0.5 M Na₂CO₃, 20 mM HEPES, 5 mM NaF, 2 µg/mL antipain, 10 µg/mL aprotinin, 5 mM benzamidine, 2 mM DTT, 10 µg/mL leupeptin, 1 mM sodium orthovanadate, and 10 µg/mL trypsin inhibitors, pH = 8.0) at 4°C (Song et al., 1996; Shah and Sehgal, 2007). The tissue was homogenized with up-down strokes 10–15 times in ice and sonicated with a Microson ultrasonic cell disruptor XL-Series (SPI Supplies, West Chester, PA, USA) during three separate intervals of 10 seconds at 4°C. The homogenate was transferred to a new microtube for additional homogenization with a pellet pestle (Kontes Glass Co., Vineland, NJ, USA) and was centrifuged for 10 minutes at 950 g (4°C). The resulting supernatant (2 ml) and 2 ml of the solution containing 90% sucrose were added to the bottom of a polyallomer centrifuge tube (Beckman Instruments Inc., Palo Alto, CA, USA) and mixed thoroughly. On top of the previous mixture, 4 mL of the 35% sucrose solution was added, followed by 4 mL of the 5% sucrose solution. Any disruptions of the layers already set on the tube were avoided. The gradient was centrifuged on a refrigerated Beckman Coulter Optima L-100 XP ultra-centrifuge (Beckman Instruments Inc., Palo Alto,

CA, USA) at 260,809 g in a SW 41 Ti rotor (Beckman Instruments Inc., Palo Alto, CA, USA) at 4°C for 24 hours. Twelve fractions (1 mL each) were collected and placed in open top ultra-clear centrifuge tubes (Beckman Instruments Inc., Palo Alto, CA, USA) with 2 mL of MBS buffer. The fractions were mixed thoroughly with the buffer, and each tube loaded onto an 80 Ti rotor (Beckman Instruments Inc., Palo Alto, CA, USA). The tubes were then centrifuged at 134,425 g at 4°C for 30 minutes. The supernatant was removed, the pellet was resuspended with SDS-PAGE sample buffer (4% SDS, 8M Urea, 62 mM Tris HCl pH = 6.8, 20 mM EDTA, 5% β -Mercaptoethanol (β ME), 0.015% Bromophenol blue) and heated at 90°C for 10 minutes. The samples were immediately stored at -20°C prior to being analyzed by SDS-PAGE and Western blot analysis.

Tissue processing and immunohistochemistry

This procedure was performed as described before (Cruz-Orengo et al., 2006; Figueroa et al., 2006; Santiago et al., 2009). Briefly, the animals were anesthetized by intraperitoneal administration of pentobarbital (40–50 mg/kg) and transcardially perfused with ice-cold 0.01 M PBS (PBS: Dulbecco's phosphate buffered saline, Sigma-Aldrich, St. Louis, MO, USA; pH = 7.4), followed by a perfusion with cold 4% paraformaldehyde (PFA, Fluka Chemika, Buchs, Switzerland) in PBS. Spinal cords were removed and postfixed in 4% PFA/PBS at 4°C for 3 hours and were then incubated and submerged in 30% sucrose/PBS at 4°C overnight. Approximately, 1.5 cm of spinal cord containing the lesion epicenter was submerged in Tissue-Tek O.C.T. embedding media (Miles Inc., Elkhart, IN, USA). Transverse sections of 20 μ m were sectioned using a Leica cryostat cryocut 1800 (Nussloch, Germany) and mounted on gel-coated superfrost microscope slides (Fisher Scientific, Pittsburgh, PA, USA).

The sections were dried for 30 minutes at room temperature and then washed in cold 10 mM PBS for 5 minutes. Post-fixation of sections was performed in 4% PFA for 5 minutes at 4°C. Sections were washed with PBS three times for 10 minutes each and in a final wash in cold PBS/Triton X-100 (0.1%) for 10 minutes. Nonspecific background was reduced by treating the sections with 0.01 M PBS/3% BSA serum for 3 hours at 4°C. The sections were incubated with primary antibody rabbit anti-rat Flotillin-2 (1:750, Sigma-Aldrich, St. Louis, MO, USA) overnight at 4°C. The following day, the slides were washed with 0.01 M PBS three times for 10 minutes each at 4°C. Diluted secondary antibody in 3% BSA (Rhodamine red donkey anti-rabbit 1:200, Jackson ImmunoResearch Laboratories Inc., West Grove, PA) was added, and slides were incubated for 1 hour at room temperature in a covered area. Cells expressing the Flotillin-2 protein were identified by double-labeling with antibodies against the following specific markers: NeuN (mouse anti-NeuN 1:100, Chemicon International Inc, Temecula, CA, USA) for neurons, GFAP (mouse anti-GFAP 1:100, BD Biosciences, San Jose, CA, USA) for reactive astrocytes, ED-1 (mouse anti-ED-1 1:500, AbD Serotec, Raleigh, NC, USA) for microglia/macrophages, MAG (mouse anti-MAG 1:250, Millipore Co., Billerica, MA, USA) for myelin structures from oligodendrocytes, and NF-200 (mouse monoclonal 1:1000, Chemicon International Inc.) for axonal identification. These antibodies were recognized using donkey anti-mouse ALEXA 488 (1:250, Carlsbad, CA, USA) as secondary antibody. Three washes in 0.01 M PBS for 10 minutes each at room temperature in a dark place were performed. Finally, the sections were protected with Antifade solution (Molecular Probe, Invitrogen, Carlsbad, CA, USA) and a coverslip. The stained sections were visualized with a LSM5 Pascal Laser Scanning Microscope upright confocal system (Thornwood, NY, USA). To discard the possibility of bleeding signal between channels, sequential excitation and capture were performed with tissue containing multiple fluorophores analyzed by laser microscopy.

Intrathecal infusion antisense oligonucleotides

Intrathecal infusion of Flot-2 antisense oligonucleotides was used to demonstrate the role of this protein in the pathophysiology after SCI. As previously reported animals were submitted to the exact same surgery procedure as described above in the surgery section, with an additional laminectomy at the T12 level (Cruz-Orengo et al., 2006; Figueroa et al., 2006). A small incision was made in the dura matter with a 32G needle, and a catheter (internal diameter 0.18 mm; outside diameter 0.36 mm; Alzet, Cupertino, California, USA) was inserted through the puncture until visible on the dorsal area at the T10 level. The catheter was connected to a mini osmotic pump (model 2004, Alzet, Cupertino, California, USA) that delivered a constant flow (0.25 μ l/hr) of oligonucleotide solution for 28 days to the lesion epicenter area of the cord. Thirty animals were used in this experiment. Five sham and 5 injured animals were treated with 5 nmol of antisense or scramble control oligonucleotides, respectively (Integrated DNA Technologies, Coralville, Iowa, USA). Ten injured animals were infused with 2.5 nmol of Flot-2 antisense (AS) oligonucleotides, and 10 lesioned rats were infused with 5 nmol of Flot-2 AS oligonucleotides. Postoperatively, the animals received the same treatment as described previously.

Oligonucleotides design

Flot-2 antisense (AS) oligonucleotide was designed from the Flotillin-2 sequence (NM_031830.1) in the National Center for Biotechnology Information (NCBI) database. The antisense segment covers the ATG initiation codon located in the position 83–85. In addition, the Flot-2 AS oligonucleotide contains 12 nucleotides upstream and 10 nucleotides downstream from the initiation codon. The 25 nucleotides long AS oligonucleotide (5'-GGCAAUUGCCCAUGGUGCCGCUGGC-3') is complementary to the template from the nucleotide in the position 71 to the position 95 of Flot-2. The scrambled control sequence (5'-CUGAGCGACCCACCGGGCAAUUGCC-3') was designed to have the exact G+C content as the antisense sequence but, as evaluated through a BLAST analysis, it did not show complementarities with other RNA sequences of relevance to the rat spinal cord, like flotillin-1, caveolin-1 or any other protein associated to lipid rafts (data not shown). Similar analysis was performed for the antisense oligonucleotides and the results did not demonstrate any complementarities with sequences from flotillin-1, caveolins or any other protein associated to lipid rafts. Modified nucleotides were used to extend the half-life of the oligonucleotides. At the extreme of the oligos, the first and last 5 nucleotides were 2' O-methylated meanwhile the 15 remnant nucleotides located at the center region were phosphorothioate modified.

Behavioral studies

All animals with infusions of oligonucleotides were assessed weekly using the Basso, Beattie and Bresnahan (BBB) open field and grid walking tests, as previously reported (Basso et al., 1995; Santiago et al., 2009). The open field BBB test was performed to evaluate spontaneous open field behavior, which allowed for assessment of each animal's functional locomotor recovery. Animals were evaluated in the open field for a period of 4 minutes by two blinded investigators and scored the rats' behaviors (movement of the hindlimbs, weight support, coordination, etc.). Values of 21 were given for behaviors that were performed normally, as by sham-control rats, and lower values (i.e., 0–2) were given for behaviors that were characteristic of locomotor dysfunction (Cruz-Orengo et al., 2006; Figueroa et al., 2006; Santiago et al., 2009).

In the grid walking test (Merkler et al., 2001), the animals walked over a horizontal ladder with bars that are randomly distanced. The number of errors (a hindlimb slide between the bars leading to the posterior part of the animal falling) was counted for each animal. Sham

animals produced few or no errors, but injured animals had a high number of hindlimb misplacements (Cruz-Orengo et al., 2006; Figueroa et al., 2006; Santiago et al., 2009).

Statistical methods

Statistical analyses were performed by using the GraphPad InStat 3 and Prism 5 software (La Jolla, CA, USA). The data was expressed as the mean $\pm$ S.E.M., and ANOVAs followed by a post-hoc test were performed to determine any significant differences between the groups. Two-tailed unpaired t-tests were used to demonstrate significance in the level Flot-2 expression of animals treated with or without antisense oligonucleotides. Differences were considered to be significant when $p < 0.05$.

Results

Expression of Flot-2 in the adult spinal cord

Identification of Flot-2 protein expression in the adult spinal cord was assessed by Western blot analysis. Mouse anti-rat Flotillin-2 (BD Biosciences, San Jose, CA, USA) and rabbit anti-rat Flotillin-2 (Sigma-Aldrich, St. Louis, MO, USA) were used to recognize this protein in the rat spinal cord and in total homogenates from C6 glioma cells. Both antibodies demonstrated immunoreactive bands in the expected molecular weight range of 42–47kDa (Fig. 1A), thus establishing that Flot-2 is expressed in the spinal cord of adult rats.

One important property of lipid raft proteins is their reduced solubility in Triton X-100 (TX-100) detergent, as reviewed previously (Pike LJ, 2003 and 2004). To support that the immunoreactive band revealed by the Western blot analysis is a characteristic protein of lipid rafts, spinal cord homogenates from adult rats were solubilized with or without TX-100, as reported for other proteins (Mairhofer et al., 2002; Asano et al., 2009). The fractions were analyzed by Western blotting, and the results obtained showed that Flot-2 immunoreactivity appeared in the TX-100 insoluble phase, as would be expected of a lipid raft protein (Rouvinski et al., 2003). The absence of Flot-2 in the TX-100 soluble fraction provided further confirmation that this protein is a lipid raft marker (Fig. 1B).

A sucrose density gradient was performed to assess whether Flot-2 is a protein associated with lipid rafts. Immunoblots performed on homogenates from the spinal cord, which were fractioned through a sucrose gradient, revealed that Flot-2 was present in the fractions 6–9 (Fig. 1C). The immunoreactivity of caveolin-1, another lipid raft marker, was observed in the same fractions of the gradient when an antibody against this protein was used. This result confirm that Flot-2 is a lipid raft protein in cells of the spinal cord, as previously shown in C6 rat glioma cells (Rouvinski et al., 2003; Salgado et al., 2012).

Flot-2 expression in segments of the normal/adult spinal cord

The levels of Flot-2 were analyzed at the cervical, thoracic, lumbar and sacral levels of the rat spinal cord (Fig. 2A). Densitometry analysis of Flot-2 expression (standardized to GAPDH levels (Calvo et al., 2008)) was higher in the cervical area (0.94 ± 0.054 arbitrary units (AU) relative to the other segments of the spinal cord. The amount of Flot-2 expression in the thoracic area was 0.68 ± 0.15 AU, the lumbar area contained 0.47 ± 0.084 AU, and the sacral area presented the lowest amount of Flot-2 expression with 0.073 ± 0.12 AU (Fig. 2B). The results of an ANOVA demonstrated that the decrease in the pattern of Flot-2 expression down the spinal cord axis is significant ($p < 0.001$). In addition, multiple comparison Bonferroni post-hoc tests revealed significant differences in Flot-2 expression between the cervical and lumbar regions, the thoracic and sacral regions ($p < 0.05$), and the cervical and sacral regions ($p < 0.001$) ($n = 4$ for cervical regions and $n = 3$ for thoracic, lumbar and sacral regions). This intersegmental comparison of Flot-2 expression was

normalized to the total amount of protein in each spinal cord segment and did not use the proportion of gray matter, as reported for some neurotransmitters (Yaksh et al., 1988), since this protein is expressed in both regions (white and gray matter).

Profile of Flot-2 expression after trauma to the spinal cord

The temporal pattern of Flot-2 expression was evaluated in control-sham and injured animals at 2, 4, 7, 14, and 28 DPI (Fig. 3A). Ten-micrograms (μg) of protein extract were used in Western blot analysis after evaluating the linear range of protein detection (data not shown). The change in Flot-2 expression in injured animals was evaluated relative to sham animals by densitometry analysis. After standardization of the Flot-2 values to GAPDH levels, the percent of change after injury relative to sham animals was decreased significantly at 2 DPI ($-40.3\% \pm 4.5$), at 4 DPI ($-35\% \pm 13.1$) and at 7 DPI ($-37.3\% \pm 11$). However, the levels of Flot-2 were slightly lower than sham-control animals (normalized to basal levels) at 14 DPI ($-5.4\% \pm 20$). This was followed by a significant increase in expression of Flot-2 at 28 DPI ($41.6\% \pm 11$) (Fig. 3B). Although the levels of Flot-2 at 14 DPI were not significant different to control levels, at this time point the amount of this protein increased significantly relative to 2 DPI. This results shows an increase in Flot-2 that continues until 28 DPI. Statistical analysis with the Student-Newman-Keuls posthoc test showed significant changes in Flot-2 expression between groups ($F(5,71) = 8.576$, $**p < 0.01$, $n = 6-9$ per group and 42 animals in the control group).

Identification of the cell phenotype expressing Flot-2 protein in sham and injured animals

The spatial distribution of Flot-2 expression was evaluated in the spinal cord of the injured and sham rats (Fig. 4). Immunohistochemical studies performed demonstrated the presence of Flot-2 in different cells types. Flot-2 colocalizes in neurons (NeuN staining) of the gray matter (Fig. 4A–F), myelin structures (MAG staining) of oligodendrocytes that surrounds the axons (Fig. 4G–L), axons (Neurofilament-200 staining) (Fig. 4M–R), and reactive astrocytes (GFAP staining) (Fig. 4S–X ($n = 3$)). Flot-2 was also observed in macrophages (data not shown). Co-localization of Flot-2 with macrophages was evident in the spinal cords of injured animals at 2DPI. However, Flot-2 expression comparisons between macrophages of sham and injured spinal cord tissues were not possible due to the fact that macrophage presence in the cords of sham animals is highly rare (data not shown). Qualitative studies demonstrated that immunoreactivity for Flot-2 diminished at 2DPI on the cell types assessed (neurons, oligodendrocytes and axons) with the exception of the reactive astrocytes which showed an increase in Flot-2 immunoreactivity in the white (Fig. 4S–X) and gray matter (data not shown).

Effect of Flot-2 antisense infusion on functional recovery

The role of Flot-2 in the pathophysiology induced by SCI was demonstrated by intrathecal infusion of antisense oligonucleotides for 28 days after trauma. This approach was used successfully in our laboratory to block gene expression (Cruz-Orengo et al., 2006; Figueroa et al., 2006). Knock-down of Flot-2 expression in the injured spinal cord was assessed by Western blotting (Fig. 5A/upper panel). Flot-2 expression standardized to GAPDH levels increased significantly at 28 DPI in the lesioned animal that was infused with the scrambled-control oligos (0.70 ± 0.11 AU) as compared with the basal level of sham animal that was infused with Flot-2 antisense (0.33 ± 0.04 AU), as compared to the data presented in figure 3. The injured animals infused with 2.5 nmol Flot-2 antisense showed a significant decrease in the expression of Flot-2 (0.34 ± 0.45 AU) when compared with the injured animals infused with scrambled-control oligonucleotides (Fig. 5A). Lesioned animals treated with 5.0 nmol Flot-2 antisense oligonucleotides showed a marked decrease in the expression of Flot-2 (0.23 ± 0.15 AU) as compared with the injured group treated with scrambled oligonucleotides (Two-tailed unpaired t-test, $p < 0.05$, $n = 3$) (Fig. 5A/bottom panel).

Behavioral studies (BBB Open Field Test)

The behavioral locomotor assays were performed with the minimum dose of AS oligonucleotides that blocked Flot-2 gene expression. In the open field BBB test (Fig. 5B), the group of injured rats treated with the 2.5 nmol Flot-2 antisense infusion showed a significant functional locomotor recovery at 14 DPI (11.6 ± 1.3), 21 DPI (12.4 ± 0.8) and 28 DPI (15.1 ± 1.2) as compared with the lesioned animals treated with the scrambled-control oligonucleotides at the same time points (14 d= 6.2 ± 0.8 ; 21 d= 8.2 ± 1.0 ; 28 d= 8.7 ± 1.0). Animals in this range (11–15 BBB score) showed extensive movements in their hindlimbs, frequent to consistent plantar stepping and in some cases, frequent coordination of the hindlimb movement. On the other hand, animals with BBB scores between 6 and 8 showed extensive movement of hip and knee articulation, slight movement of the ankles, and even sweeping of the hindlimb. These significantly different patterns of locomotion were not observed at 7 DPI, as injured animals treated with the Flot-2 antisense infusion showed a mean score of 4.9 ± 0.6 and animals that were treated with the scrambled control oligos showed a mean score of 2.3 ± 0.6 . Statistical analyses with ANOVA followed by Bonferroni post-hoc tests showed a significant difference between the Flot-2 AS oligonucleotide and the scramble-control groups ($F(11, 101) = 87.896$, *** $p < 0.001$, $n = 4-10$, Fig. 5B).

Behavioral studies (Grid Walking Test)

A similar pattern of improvement in the Flot-2 AS treated animals was observed in the grid walking test that scored the number of errors (Fig. 5C). These rats showed a significant improvement at 14 DPI (13.3 ± 1.7), 21 DPI (11.3 ± 0.8), and 28 DPI (10.3 ± 1.3) as compared with the errors made by the lesioned animals infused with the scrambled control oligonucleotides at the same time points (14 d= 22.3 ± 1.3 ; 21 d= 18.6 ± 1.9 ; 28 d= 16.4 ± 1.8), respectively. The only time point at which no significant difference was observed was at 7 DPI as injured animals with the Flot-2 antisense treatment showed a mean number of errors of 24 ± 1 , and the animals with the scrambled control treatment showed a similar value. The results of an ANOVA demonstrated a significant effect of Flot-2 AS oligonucleotide (versus rats treated with scramble-control oligonucleotides) on locomotor performance in this test ($F(11, 102) = 77.994$, *** $p < 0.001$, $n = 4-10$; Fig. 5C).

Discussion

Many of the cellular events after SCI, such as axonal outgrowth or apoptosis, primarily depend on cellular signals triggered by membrane receptors. The clustering of receptors and other intracellular signaling molecules in one specific area of the membrane, the membrane raft, makes cellular signaling possible in most cells (Pike, 2006; Vihanto et al., 2006). Flotillin-2 is a single-copy gene that produces a 42–47 kDa protein (Schroeder et al., 1994; Cho et al., 1995; Bickel et al., 1997; Schulte et al., 1997) that is palmitoylated and mirystoylated, and is needed for the structure of the membrane raft to ensure its stability. On the other hand, flotillin-1 (Flot-1) is only palmitoylated, and so this protein has a more mobile nature that permits mobility between the plasma membrane and several membranes of the intracellular organelles. Moreover, studies demonstrate that the stability of Flot-1 requires the presence of Flot-2, but Flot-2 can exist without the presence of Flot-1 (Langhorst et al., 2005). For these reasons, Flot-2 is primarily a marker of cell membrane rafts. However, the expression profile (temporal and spatial) of Flot-2 and the possible role that this membrane raft protein may have after an injury is still unknown.

Our results demonstrated that Flot-2 expression decreases after SCI acutely at 2 DPI, followed by gradual increases towards baseline levels at 4, 7 and 14 DPI surpassing baseline levels at 28 DPI. Immunohistochemical studies demonstrated the presence of Flot-2 in neurons, oligodendrocytes, axons and reactive astrocytes. Moreover, the open field BBB and

grid walking behavioral tests revealed that blockade of Flot-2 gene expression with intrathecal infusion of antisense oligonucleotides into the injured spinal cords improved significantly their functional locomotor recovery confirming a role of Flot-2 in this pathophysiological condition.

Flot-2 was shown to be expressed in the adult spinal cord using Western blot analysis and two different commercial antibodies (Pharmingen, BD Biosciences and Sigma-Aldrich). The probes recognized a single immunoreactive band in the 42–47 kDa region, thus clearly showing the presence of Flot-2 in the adult rat spinal cord. Proteins localized in lipid rafts are insoluble in Triton X-100 (Mairhofer et al., 2002; Pike, 2004; Asano et al., 2009) and should co-fraction with proteins that are characteristic of lipid rafts, such as Cav-1, in a sucrose density gradient. Our results confirmed that Flot-2 in the spinal cord is a membrane raft protein because it co-partitioned into the TX-100 insoluble fractions and co-fractionated with caveolin-1, a widely used lipid raft marker (Schroeder et al., 1998; Rouvinski et al., 2003; Santamaria et al., 2005; Salgado et al., 2012). Therefore, these results confirmed that Flot-2 is present in the adult spinal cord, and among its other functions, this protein could allow the formation of lipid rafts for clustering of receptors that are associated with cell signaling.

Levels of Flot-2 expression varied among the different regions of the spinal cord, as the cervical area contained the highest levels of this protein and the more sacral segments showing progressively lower levels [cervical > thoracic > lumbar >> sacral]. The differences in Flot-2 expression could be related to the diverse populations of cells (interneurons, motor neurons, astrocytes, oligodendrocytes and microglia) in the gray and white matter, or amount of tracts (sensory and motor) across all of the spinal cord segments and their respective roles in each region. Possibly there are specific cell populations which preferably are found in cervical or thoracic regions of the cord which could be using Flot-2 membrane rafts system to cluster specific proteins and perform basal cellular tasks (in the case of sham animals) or specific tasks known to take place after injury, such as reactive astrogliosis, inflammation or apoptosis.

Functional and morphological changes are known to occur in the lumbar region caudal to the lesion epicenter at the T8 level (Garcia-Alias et al., 2010) and the expression pattern of Flot-2 after SCI may modulate those changes. The expression profile of Flot-2 was evaluated at 2, 4, 7, 14, and 28 DPI. This range of time points covers events that take place soon (acute) after SCI through the later (chronic) stages. Flot-2 levels were reduced between 2 and 7 DPI as compared with sham animals. The acute reduction in Flot-2 could be attributed to cellular process, such as necrosis, that occur immediately after the physical injury at the lesion epicenter and which may be due to a decrease in the rate of protein synthesis (Keane et al., 2006). However, the levels of Flot-2 returned to basal levels by 14 DPI. This return to basal levels may be related to the infiltration and migration of cells, such as macrophages and reactive astrocytes, to the lesion epicenter. It was not until 28 DPI that an increase in Flot-2 levels was observed in injured animals as compared with sham animals. The augmented expression observed at this time point may be related to an upregulation of Flot-2 and/or the accumulation of more cells at the lesion epicenter. At 28 DPI, a massive gliosis was observed that expressed Flot-2, which explains the upregulation detected by Western blot analysis (data not shown). The physiological relevance of Flot-2 elevated expression at 28 DPI may be associated to the formation of specialized membrane rafts that cluster proteins with repulsive activity to avoid the formation of inappropriate connections (Cruz-Orengo et al., 2006). However, this cellular response will also affect axonal regeneration indirectly due to the presence of repellent molecules (receptors and/or ligands) in the Flot-2 membrane rafts. Moreover, we could not discard the possibility that apoptotic receptors may also be clustered in Flot-2 specialized membrane rafts to trigger the

programmed cell death of glia and neurons affected by SCI (Davis et al., 2007; Mollinedo and Gajate, 2001; Hueber, 2003; Mollinedo and Gajate, 2005). The discrepancy between the apparent Flot-2 upregulation in reactive astrocytes observed at 2 DPI in the immunohistochemistry studies and the downregulation observed with Western blots could be related to an increase in the expression of this protein mainly in GFAP-positive cells. Specifically, in the Western blot analysis, an increase may have been masked by the massive cell death of oligodendrocytes and neurons related to necrosis and apoptosis at the lesion epicenter.

Immunohistochemical studies have demonstrated that Flot-2 is present in motor neurons, myelin structures of oligodendrocytes, axons, and reactive astrocytes of the adult spinal cord. This finding suggests that Flot-2 may be a ubiquitous molecular signaling protein that is involved in membrane raft formation and functions to maintain basal activity of cells in the adult spinal cord. Moreover, the spatial distribution of Flot-2 in the cells of the spinal cord (as early as 2 DPI) supports the concept that this protein may be involved in the clustering of repellent molecules or the exclusion of attractive proteins for axonal outgrowth. In addition, we cannot discard the possibility that Flot-2 may be involved in the cluster of apoptotic-mediated receptors that induce cell death after injury (Gebreselassie and Bowen, 2004; Davis et al., 2007). Similar results were observed for Flot-1 using immunoblot and immunohistochemical analyses in the spinal cord of rats with EAE (Kim et al., 2006).

Flotillin-2 may have various roles depending on the type of cell. It has been shown that proteins with repulsive activity for neurite outgrowth, such as OMgp, MAG, Nogo and ephrin-ligands, are present in membrane rafts (Yu et al., 2004; Benson et al., 2005; Boynapalli et al., 2005). Reactive gliosis or astrogliosis, after trauma, has been associated with the generation of a chemical and physical barrier to axonal regeneration due to an increase in repulsive proteins that block neurite outgrowth across the lesion epicenter (Fawcett, 2006; Liu et al., 2006; Yiu and He, 2006; Zhang et al., 2006; Busch and Silver, 2007). It is possible that Flotillin-2, as a membrane raft protein, could cluster proteins which are known to be nonpermissive for axonal regeneration after injury. The possibility that reactive astrocytes express apoptotic proteins, like Fas-ligands, in Flot-2 membrane rafts that may interact with apoptotic receptors in other cells (neurons and oligodendrocytes), triggering a cascade of events after SCI could not be discarded. The mechanism of activation of these astrogliotic cells is not well understood. However, it is possible that Flot-2 membrane rafts present in reactive astrocytes as early as 2 DPI have proteins that recognize and internalize a signal that promotes their activation.

Flot-2 has also been observed in macrophages and microglia of the spinal cord. It is known that macrophages at the injury site could be activated by cytokines. The receptors for these molecules may be located in Flot-2 lipid rafts, which facilitate cellular signaling. We could not discard the possibility that this protein may be involved in the phagocytic process, as reported for Flot-1 in the EAE model (Kim et al., 2006). In motoneurons, the expression of Flot-2 could be associated with the clustering of proteins that may activate repellent receptors in regenerating axons. The interaction of the ligand-receptor proteins could initiate a cascade of events that block axonal outgrowth and synapse formation, as have been shown for Eph-ephrin proteins (Klein, 2004; Pasquale, 2005). However, further studies need to be performed in order to elucidate the array of cellular functions mediated by Flot-2 after injury in different cell types.

Antisense studies confirmed that Flot-2 is an important protein in cells at the lesion epicenter of the adult spinal cord and in the reduced locomotor activity observed after injury. Previous results demonstrated that AS oligonucleotides infused intrathecally reached inside the cells of the injured spinal cord and reduced the levels of the genes of interest (Cruz-

Orengo et al., 2006; Figueroa et al., 2006). Similar strategies have been used to study the role of NT-3 in sprouting after injury (White, 2000), c-fos in hyperalgesia (Sugiyo et al., 2001), NMDA receptor subunits (R1, R2C and R2D) in nociception and metabotropic glutamate receptor in long-lasting spontaneous pain after nerve injury (Fundytus et al., 2001). Our Western blot results confirmed the diffusion and blockade of Flot-2 expression with this approach and the absence of an effect on sham animals with an intact blood brain barrier. As observed in the open field BBB and the grid walking tests, the current results suggest that blockade of the Flot-2 upregulation after SCI promotes a positive functional locomotor recovery characterized by spontaneous exploratory movements or initiation of these movements and weight support along with fine motor movement, such as careful paw placement. Interestingly, the recovery by animals treated with the Flot-2 antisense treatment observed in the grid walk test was very similar to the recovery observed in the BBB test in terms of the time points of improvement. Thus, injury may trigger the expression of Flot-2, which facilitates a cascade of cellular events that cluster proteins to produce a nonpermissive environment for neurite outgrowth or prohibits receptors, like trophic factors, from specific microdomains of the plasma membrane. Therefore, axonal regeneration could be inhibited and synapse formation blocked unless a treatment like Flot-2 AS is administered, that could reduce the repulsive activity of proteins in the membrane rafts and promotes locomotor recovery. Further analysis with axonal tracing will be required to monitor neurite outgrowth to support the idea of repulsive proteins associated to Flot-2 membrane rafts after SCI.

In conclusion, this research provides evidence of a pathophysiological role of Flot-2 after SCI. The spatio-temporal pattern of Flot-2 expression in the adult spinal cord, both, prior to and after injury suggested that this protein participates in the harmful environment generated after trauma. Overall, the demonstration that Flot-2 AS oligonucleotides produced a functional locomotor recovery confirms the role of this protein in the cascade of events that produces a unfavorable environment after SCI. Additional experiments should be performed to determine (1) the role of Flot-2 in each cell type that is present at the injury site, (2) which proteins with repulsive activity (like Eph receptors, ephrin ligands, NOGO, MAG or OMgp) cluster in membrane rafts, (3) if blockade of Flot-2 reduces apoptosis or stimulates regeneration, using tracing studies to monitor axonal regeneration, (4) if Flot-2 expression may be involved in neural reorganization and synaptic plasticity in regions caudal to the lesion epicenter that are involved in locomotor recovery via pattern generators, and (5) the spatio-temporal profile of Flot-1 in normal and injured spinal cords.

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Figure 1.

Flot-2 expression in the spinal cord and enriched in fractions characteristic of lipid raft proteins. Total homogenates of adult rat spinal cords and C6 glioma cells were evaluated by Western blot analysis using a rabbit anti-rat Flotillin-2 antibody and a mouse anti-rat Flotillin-2. The same immunoreactive bands were observed for both types of extracts when treated with both commercial antibodies (A), thus confirming the presence of Flot-2 in this tissue. Extracts from adult spinal cords were prepared with 1% Triton X-100 and the insoluble (Ins) and soluble (Sol) fractions were analyzed by Western blot analysis (B). Flot-2 was exclusively expressed in the insoluble fraction, thus demonstrating its insolubility in Triton X-100, a fundamental characteristic of lipid raft proteins. Flot-2 co-localizes with Cav-1, a lipid raft marker, in a sucrose density gradient (C). Spinal cord homogenates were studied by sucrose density gradient and Western blot analysis. Flot-2 was observed in fractions 6, 7, 8, and 9. Cav-1 also co-localized in the latter fractions, thus confirming that Flot-2 is associated with lipid rafts.

Figure 2.

Flot-2 expression in different areas of the spinal cord of the adult rat. Immunoblot studies demonstrated the relative levels of Flot-2 expression in different areas of the spinal cord. The segments studied were cervical (C), thoracic (T), lumbar (L) and sacral (S). An ANOVA confirmed that the levels of Flot-2 expression significantly and progressively decreased along the distance of the spinal cord, with the highest levels of Flot-2 expressed in cervical region and lower levels expressed in the sacral region ($p < 0.001$). Data were analyzed by densitometry and are presented as mean $\pm$ SEM ($n = 3-4$).

Figure 3.

Flot-2 expression profile after spinal cord injury. Flot-2 expression was altered after SCI (A). An initial decrease in this protein was observed in the acute phase after trauma. Flot-2 expression returned to basal expression at 14 DPI, and was upregulated at 28 DPI relative to GAPDH levels at these time points (B). The results of and Bonferroni post-hoc test established significant difference between the samples tested. All of the values presented are the mean $\pm$ SEM (n= 6 – 9 per group and 42 in the control group) **p < 0.01.

Figure 4.

Flotillin-2 expression decreased in motoneurons, oligodendrocytes and axons but increased in reactive astrocytes of the injured spinal cord at 2 DPI. Immunohistochemical studies from the spinal cords of sham (A–C, G–I, M–O and S–U) and injured animals (D–F, J–L, P–R, and V–X) were performed to assess changes in Flot-2 expression (A, D, G, J, M, P, S & V in red color) in motoneurons (B & E, in green color), oligodendrocyte myelin structures (H & K in green color) axons (N & Q, in green color) and reactive astrocytes (T & W) after injury. A decrease in the number of Flot-2 immunoreactive neurons was observed in the injured rats (F, in yellow) when compared with sham rats (C in yellow). The same reduced pattern of expression was observed in myelin structures of oligodendrocytes (L vs. I) and axonal structures (R vs. O). As for the reactive astrocytes, Flot-2 immunoreactivity was observed to increase in injured animals when compared to sham animals (X vs. U). Arrows point to myelin structures of oligodendrocytes. Scale bar= 10 μ m.

Figure 5.

Blockade of Flotillin-2 facilitates functional locomotor recovery and improves fine motor movement after injury. (A) Flot-2 antisense infusion (2.5 and 5 nmoles) for 28 days after injury reduced the levels of Flot-2 as compared with the rats that were injured and treated with the scrambled control oligonucleotides. All of the values presented are the mean $\pm$ SEM (n = 3)*p < 0.05. (B) Rats treated with Flot-2 antisense showed significant improvements in functional locomotor recovery in the BBB open field test after injury as compared with the control (scramble) group, at 7, 14, 21 and 28 DPI. (C) In the grid walking test, rats treated with the Flot-2 antisense showed significantly fewer errors as compared with the control (scramble) group at 14, 21 and 28 DPI. All of the values presented are the mean $\pm$ SEM (n = 4–10) ***p < 0.001, **p < 0.01, *p < 0.05.