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Acute spinal cord injury persistently reduces R/G RNA editing of AMPA receptors

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Abstract

Spinal cord injury (SCI) triggers a complex ischemic and inflammatory reaction, involving activation of neurotransmitter systems, in particular glutamate, culminating in cell death. We hypothesized that SCI might lead to alteration in the RNA editing of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors that govern critical determinants of neuronal survival. To this end, we examined the molecular changes set in motion by SCI that affect the channel properties of AMPA receptors. SCI strongly reduced the level of AMPA receptor R/G editing, involving not only the site of the lesion but also adjacent areas of the cord spared by the lesion. The effects, with changes for some subunits and loci, were observed as long as 30 days after lesioning and may correlate with a partial decrease in enzymatic activity of adenosine deaminase acting on RNA 2 (ADAR2), as deduced from the analysis of ADAR2 self-editing. The reduced editing at the R/G site of glutamate receptor subunits (GluRs) is likely to reduce post-synaptic excitatory responses to glutamate, thus limiting the progression of cell death; however, prolonged suppression of GluR function in later stages may hinder synaptic plasticity. These observations provide the first direct evidence of the potential contribution of RNA editing to excitatory neural injury and recovery after SCI.

Keywords: glutamate receptor, post-transcriptional mechanism, RNA regulation, spinal cord lesion.

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Acute spinal cord injury (SCI) triggers complex ischemic and inflammatory responses that involve excitatory neurotransmitter systems and intracellular signaling (for a review, see, Frigon and Rossignol, 2006) that in turn create an unfavorable environment for synaptic survival and regeneration. Chief among these responses, glutamate release triggers the influx of Ca^{2+} (Choi 1994; Michaelis 1998), leading to detrimental events including cell death (Shapiro 1997), which can then be attenuated by glutamate receptor antagonists (Faden and Simon 1988; Faden *et al.* 1988, 1990; Gomez-Pinilla *et al.* 1989; von Euler *et al.* 1994; Wrathall *et al.* 1994, 1996).

The expression of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) subtype of glutamate receptors has been extensively studied in SCI (Grossman *et al.* 1999, 2001; Grossman and Wrathall 2000; Brown *et al.* 2004; Alilain and Goshgarian 2008; Ferguson *et al.* 2008); how-

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Abbreviations used: ADAR, adenosine deaminase acting on RNA; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; CTR, control animal; GluRs, glutamate receptor subunits; LAM, laminectomized animal; LES, lesioned animal; PBS, phosphate-buffered saline; SCI, spinal cord injury.

ever, their functional state is controlled by alternative RNA splicing, RNA editing, and by targeting and trafficking of receptor subunits at dendritic spines (Dingledine *et al.* 1999), and the roles of these regulatory events have not yet been elucidated. Each of the four AMPA receptors is alternatively spliced in the extracellular region generating the so-called 'Flip' and 'Flop' variants (Sommer *et al.* 1990), which display totally different kinetic properties (Monyer *et al.* 1991; Hollmann and Heinemann 1994; Lambolez *et al.* 1996). In addition to splice variants, the physiologic properties of AMPA receptors are controlled by post-transcriptional RNA editing (Seeburg *et al.* 1998), which modifies one or more translation codons leading to functionally distinct proteins from a single gene. The predominant editing change in mammals is adenosine-to-inosine, catalyzed by the adenosine deaminase acting on RNA (ADAR) 1 and 2 enzymes (Reenan 2001; Bass 2002). With respect to glutamate receptor subunits (GluRs), the editing positions have been named on the basis of the amino acid substitution, such as the Q/R site in AMPA GluR2 and the R/G site in GluR2, GluR3, and GluR4 (Seeburg 2002). The amino acid changes alter channel properties (Egebjerg and Heinemann 1993; Kamboj *et al.* 1995; Swanson *et al.* 1996): for the GluR2 subunits, Q/R site affect receptor maturation and cellular trafficking (Greger *et al.* 2002, 2003) as well as the kinetics of channel gating (Lomeli *et al.* 1994). Ordinarily, GluR2 is fully edited at the Q/R site and the resultant AMPA-GluR2-containing receptors are Ca^{2+}-impermeable, whereas AMPA receptors lacking GluR2 are permeable to Ca^{2+} (Isaac *et al.* 2007). Whether AMPA receptors are permeable or impermeable to Ca^{2+} may profoundly affect neuronal plasticity and survival following injury (Liu and Zukin 2007). Moreover, GluR2, 3, and 4 receptors are edited at the R/G site; this site is located just before the sequences involved in the splicing events forming the Flip/Flop isoforms, and it seems to affect both the splicing events and the desensitisation properties of the AMPA receptor channels (Lomeli *et al.* 1994; Krampfl *et al.* 2002). All these effects have an impact on cell homeostasis that are of potential importance in SCI.

Accordingly, in this study, we examined the molecular changes set in motion by SCI that affect the channel properties of AMPA receptors. We report that SCI leads to altered editing of AMPA glutamatergic receptors consistent with reduced glutamate responsiveness after SCI; in turn, this may lead to the design of therapies that can minimize excitatory damage.

Methods

Animal treatments

All experimental protocols were approved by the Animal Review Committee of the University of Milan and met the Italian guidelines for laboratory animals, which conform to the European Communities (EEC Council Directive 86/609 1987) and the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by United States National Institutes of Health. Adult Sprague-Dawley rats (Charles River Laboratories Inc., Calco, LC, Italy) weighing 240–260 g were kept under standard housing conditions (22 ± 2°C, 65% humidity, lights on from 06:00 to 20:00 hours), with standard lab chow and water freely available. Traumatic SCI was performed as previously described (Gorio *et al.* 2002, 2005), using the UTS-Impactor, at the core of which is a 2.3-mm diameter stainless steel rod that is precisely driven into the spinal cord with specified force and displacement. The movement and impact are monitored by means of a miniaturized piezoelectric dynamometer present within a section of the impacting rod and linked to a computer that drives the device, records, and manages the data. The impounding piston was positioned 1 mm above the exposed cord at T9 and set for an excursion of 3 mm. A force of 1 N was applied for 1 s, followed by the automatic return of the impaction rod. Animals were maintained under halothane anesthesia and positioned over a mat kept at 38°C and, before awakening, were treated with buprenorphine (0.03 mg/kg) for pain management, and penicillin G (10 000 U/kg) to prevent infection. After SCI, the rats were housed two per cage and underwent manual bladder evacuation three times daily. Comparisons were made among animals that had no surgery (control, CTR), laminectomized animals that underwent surgery without spinal cord impaction (LAM), and lesioned animals (LES). Different groups of animals were used for immunocytochemistry as well as molecular analyses (at least five for each group).

Functional assessment

Basal motor activity was evaluated the day before the lesion (day −1) to ensure that the animals enrolled in the study had no initial motor deficits. Motor function was examined at time points ranging from 1 to 28 days post-injury by four blinded observers, and values for each animal were averaged across observers using the methodology described in Basso *et al.* (1995).

Morphology

Three days after injury, animals were anesthetized by inhalation of halothane and transcardially perfused with 1% paraformaldehyde in 0.1 M phosphate buffer pH 7.2, followed by 4% paraformaldehyde for immunocytochemistry. Spinal cords were dissected and post-fixed for 12 h in the same fixative used for perfusion, cryoprotected with 30% sucrose, quickly frozen, and stored at −80°C. At 0.5 mm caudal to the lesion epicenter, the spinal cord was serially cut by means of a cryostat (Carl Zeiss GmbH, Jena, Germany). Serial transverse sections from five animals in each experimental group were stained with thionin and were used as an index of gray matter sparing and to estimate extent of tissue damage (data not shown).

For immunocytochemical staining, the sections were incubated first in 0.05 M NH_4Cl in phosphate buffer for 30 min to quench free aldehyde groups, and then for 30 min in 0.01 M phosphate-buffered saline pH 7.4 (PBS) containing 1% bovine serum albumin and 0.2% Triton X-100. Sections were sequentially incubated with the primary antiserum overnight at ambient temperature, and then with the appropriate secondary antiserum conjugated to a fluorochrome for 2 h; sections were then washed extensively in PBS, mounted with FluorSave™ (Merck, Darmstadt, Germany), and analyzed by

confocal microscopy (Leica TSC2; Leica Microsystems, Heidelberg, Germany).

The following primary antibodies were used: anti-glial fibrillary acidic protein (1 : 400; Covance, Harrogate, UK); anti-β-tubuline III (1 : 150; Covance); anti-glutamate receptor 2 (GluR2; 1 : 150; Chemicon, Temecula, CA, USA). The secondary antibody was 488 goat-anti-mouse IgG (1 : 200; Alexa, Fluor-Invitrogen, Carlsbad, CA, USA), 546 goat-anti-rabbit IgG (1 : 200; Alexa, Fluor-Invitrogen). Nissl's substance was visualized with NeuroTrace™ 640/660 deep red-fluorescent Nissl stain (Invitrogen, Carlsbad, CA, USA). Following a 30-min permeabilization in 0.2% Triton X-100 in PBS, sections were incubated in NeuroTrace™ (1 : 50 in PBS, 40 min at 20–25°C) and washed in PBS-0.1% Triton X-100 for 10 min and in PBS for at least 2 h. Nuclei were counter-stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma, St Louis, MO, USA). In control determinations, primary antibodies were either omitted or replaced with equivalent concentrations of unrelated IgG of the same subclass.

RNA extraction and RT-PCR reaction

Molecular analyses were made at 3, 7, and 30 days after injury ($n = 5$ for each time point). Homogenates were prepared using approximately a 1.5 mm length sample from the spinal cord taken at the epicenter (T9) of the lesion as well as 4 mm caudal and 4 mm rostral to the lesion; corresponding loci were also sampled from the CTR and LAM rats. The spinal cord portions of interest were dissected out rapidly, frozen on dry ice, and stored at −70°C for further analyses. Total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). RNA was recovered by precipitation with isopropyl alcohol, washed by 75% ethanol solution, and dissolved in RNase-free water. RNA quantitation and quality controls were carried out using spectrophotometric analysis and the AGILENT Bioanalyzer 2100 lab-on-a-chip technologies (AGILENT Technologies, Santa Clara, CA, USA). Retro-transcription was performed using the Moloney murine leukemia virus-reverse transcriptase (Invitrogen, Carlsbad, CA, USA). In all 2.5 μg of total RNA from each spinal cord sample was mixed with 2.2 μL of 0.2 ng/μL random hexamer (Invitrogen), 10 μL of 5× buffer (Invitrogen), 10 μL of 2 mM dNTPs, 1 μL of 1 mM dithiothreitol (Invitrogen), 0.4 μL of 33 U/μL RNasin (Promega, Madison, WI, USA), and 2 μL Moloney murine leukemia virus-reverse transcriptase (200 u/μL), in a final volume of 50 μL. The reaction mix was incubated at 37°C for 2 h and then the enzyme was heat inactivated at 95° for 10 min. To perform the PCR reactions, 20 ng of retro-transcribed RNA was mixed with 2.5 μL 10× buffer (Polymed, Florence, Italy), 0.7 μL of 1.5 mM $MgCl_2$, 2.5 μL of 2 mM dNTP, 0.7 μL of each forward and reverse primer, and 1.25 U of Taq polymerase in a final volume of 25 μL. Standard PCR cycle conditions were: one denaturation step for two min at 95°C, followed by 25–35 cycles with 30 s of denaturation at 95°C, 20 s of primer annealing at 60°C, 30 s–1 min of elongation at 72°C following a final extension of 1 min.

Editing level quantitation

The editing level quantitation for AMPA GluR2, GluR3, and GluR4, 5-hydroxytryptamine receptor 2C (5-HTR2C) transcripts, and ADAR2 pre-mRNA was carried out by sequence analysis of gel purified PCR products (Barbon *et al.* 2003). Briefly, in the electropherogram obtained after RT-PCR and sequencing analysis of a pool of transcripts, the nucleotide that undergoes the editing reaction appears as two overlapping peaks: 'A' from unedited transcripts and 'G' from the edited ones. We previously determined that the editing level can be reliably calculated as a function of the ratio between the G peak area and A plus G peaks areas. The nucleotide areas were quantified by the Discovery Studio Gene 1.5 program (Accelrys Inc., San Diego, CA, USA). The mean values and standard errors from each group of animals were used for statistical analysis. To validate the system, a new calibration curve obtained from the five 5-HTR2C editing sites is reported in Appendix S1.

Western blot analysis

The tissues were homogenized using an Ultra Turrax politron (IKA-Labortechnik, Staufen, Germany) in five volumes of a homogenization buffer (25 mM Tris-HCl, pH 7.4, 0.4 mM sodium azide, 0.4 mM phenylmethylsulfonylfluoride, 1 mM benzamidine-HCl, 0.4 EDTA, 0.4 mM EGTA, and 0.25 M sucrose). The homogenate was centrifuged at 1500 *g* for 10 min at 4°C, and the supernatant was used for western blot analysis. The samples (25 μg) were boiled, electrophoretically run on a SDS–PAGE gel, and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). After being blocked at 20–25°C for 3 h with 5% dry milk (Merck, Darmstadt, Germany), the membranes were incubated overnight at 4°C with antibodies against ADAR1 (L-15, 1 : 100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), ADAR2, (1 : 300, Abcam, Cambridge, MA, USA), and β-actin (1 : 1000, Sigma). The membranes were washed and incubated for 1 h with anti-goat antibody (1 : 10 000; Pierce, Rockford, IL, USA) for ADAR1, anti-rabbit antibody (1 : 10 000; Pierce) for ADAR2 and anti-mouse antibody (1 : 10 000; Pierce) for β-actin. The reaction was revealed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce) for ADAR2 and β-actin, and using the SuperSignal West Femto Chemiluminescent Maximum Sensitivity Substrate (Pierce) for ADAR1. Densitometric analysis was performed by Kodak MJ project program. Data were expressed as optical density.

Real-time quantitative PCR and analysis of ADAR2 alternative splicing

To analyze the RNA expression patterns of GluRs and ADAR enzymes mRNAs, we used the Applied Biosystem 7500 Real-time PCR system (Applied Biosystem, Foster City, CA, USA), with TaqMan probes (Id probes: GluR1: Rn00709588_m1; GluR2: Rn00568514_m1; GluR3 Rn00583547_m1; GluR4: Rn00568544_m1; ADAR1: Rn00508006_m1 ADAR2: Rn00563671_m1; Applied Biosystems). The expression of the different genes was normalized based on two neuron-specific housekeeping genes, so as to calculate the expression values relative to the neurons that survived the damage (Grossman *et al.* 1999): Neuronal Specific Enolase (Rn00595017_m1) and neuron-specific beta-3-tubulin (Rn01431594_m1). Each experimental group was composed of five rats, and every determination was repeated in triplicate. The expression ratio of the target genes was calculated using geometric mean values (Pfaffl *et al.* 2002).

Analysis of rat ADAR2 alternative splicing products was performed after RT-PCR amplification of total RNA from each

spinal cord samples; using a primer pair located in exons 3 and 4 relative to the rat sequence (NM_001111056), both the normal and the alternative spliced [+47 nucleotides (nt)] transcripts could be detected. Relative quantitation was performed using the lab-on-a-chip Agilent 2100 bioanalyzer whose software automatically calculates the size and concentration of each separate band and displays the result in real time. The number of cycles was chosen experimentally to fall into the exponential phases of the amplification reaction.

Statistical analysis

Real-time PCR and editing and protein densitometric data were evaluated from each individual animal, and the mean and standard error from each group ($n = 5$) were analyzed first with a global ANOVA (data log-transformed because of heterogeneous variance) including all variables in a single test, so as to avoid the increased probability of a type-I error that would result from multiple tests of the same data set. As justified by the interaction of treatment (CTR, LAM, LES) with the other variables, data were then subdivided for lower-order ANOVA s, followed wherever appropriate, by Fisher's Protected Least Significant Difference Test to evaluate individual values that differed from the corresponding control group. However, where there were treatment effects without interactions, only the main treatment effects are reported. Significance for all tests was assumed at $p < 0.05$.

Results

Motor function recovery

In accordance with our previous results (Gorio *et al.* 2002, 2005), LAM animals showed only a minor initial effect on motor function, with complete recovery within 4 days, whereas in the lesioned group, locomotion was still markedly compromised 28 days after injury (Fig. 1).

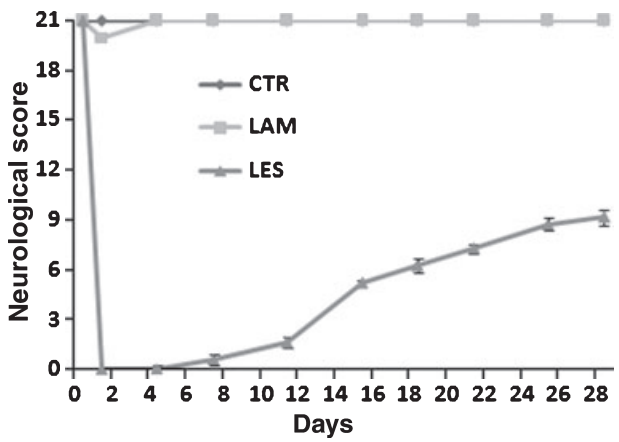

Fig. 1 Locomotor performance using the scale of Basso *et al.* 1995; scores can range from 0 (no observable hindlimb movements) to 21 (normal gait). Values represent mean values and standard errors obtained from five animals in each group.

Morphology

Our paradigm, as already reported previously in several results by our group (Gorio *et al.* 2002, 2005), caused extensive damage to gray and white matter at site of injury with sparing of the ventral and lateral white matter. At 3 mm from the lesion epicenter, there is slight damage of the gray matter around the ependimal canal in the caudal portion, whereas there is no sign of injury rostrally (data not shown).

GluR2s were localized in the deeper layers of the substantia gelatinosa in the dorsal horn and throughout the ventral horn of the spinal cord (Fig. 2, D1 and V1). At higher magnification, the co-localization with Nissl substance confirmed that the GluR2-positively stained cells were indeed neurons (Fig. 2, D2 and V2), and that such expression was maintained after lesioning (Fig. 2, DL and VL sections). Thus, the following evaluations on receptor expression patterns, splice variants, and editing involve receptors located mostly in the neuronal cells.

Global statistical analyses

Subdividing the data into different sub-groupings and performing repeated statistical tests increase the probability of type I statistical errors, and accordingly, we first performed global ANOVA s to ensure that significant treatment differences were detectable and to identify which data subdivisions were justified by treatment interactions with the other variables. For the RNA-editing data, the relevant variables were treatment (CTR, LAM, LES), receptor subunit (GluR2, GluR3, GluR4), time (3, 7, 30 days), locus (epicenter, rostral, caudal), and variant (Flip, Flop). We identified a main effect of treatment ($p < 0.0001$) and interactions of treatment × subunit ($p < 0.0002$), treatment × variant ($p < 0.0001$), treatment × time ($p < 0.0001$), treatment × locus ($p < 0.007$), as well as higher-order interactions among all these variables (not shown). Accordingly, for presentation, we subdivided the data first into the three different subunits and the two variants, and reexamined the treatment effects and interactions with time and locus in lower-order tests.

RNA editing

Figure 3(a) shows that the Q/R site of GluR2, almost fully edited in normal condition, was not affected by SCI in any portions of the cord under the three experimental conditions. On the other hand, for the R/G site, there were significant alterations in receptor editing in the LES animals that was greater at the site of the lesion compared with areas rostral and caudal to the damage. For the Flip variant of GluR2 (Fig. 3b), the editing was reduced overall in the lesioned group ($p < 0.0001$ for the main effect of treatment), but the effect was significantly greater at the epicenter (treatment × locus, $p < 0.0001$); nevertheless, there were significant decrements in both the rostral and caudal segments. The effect was extremely persistent, showing no loss of effect even 30 days after lesioning (no treatment × time

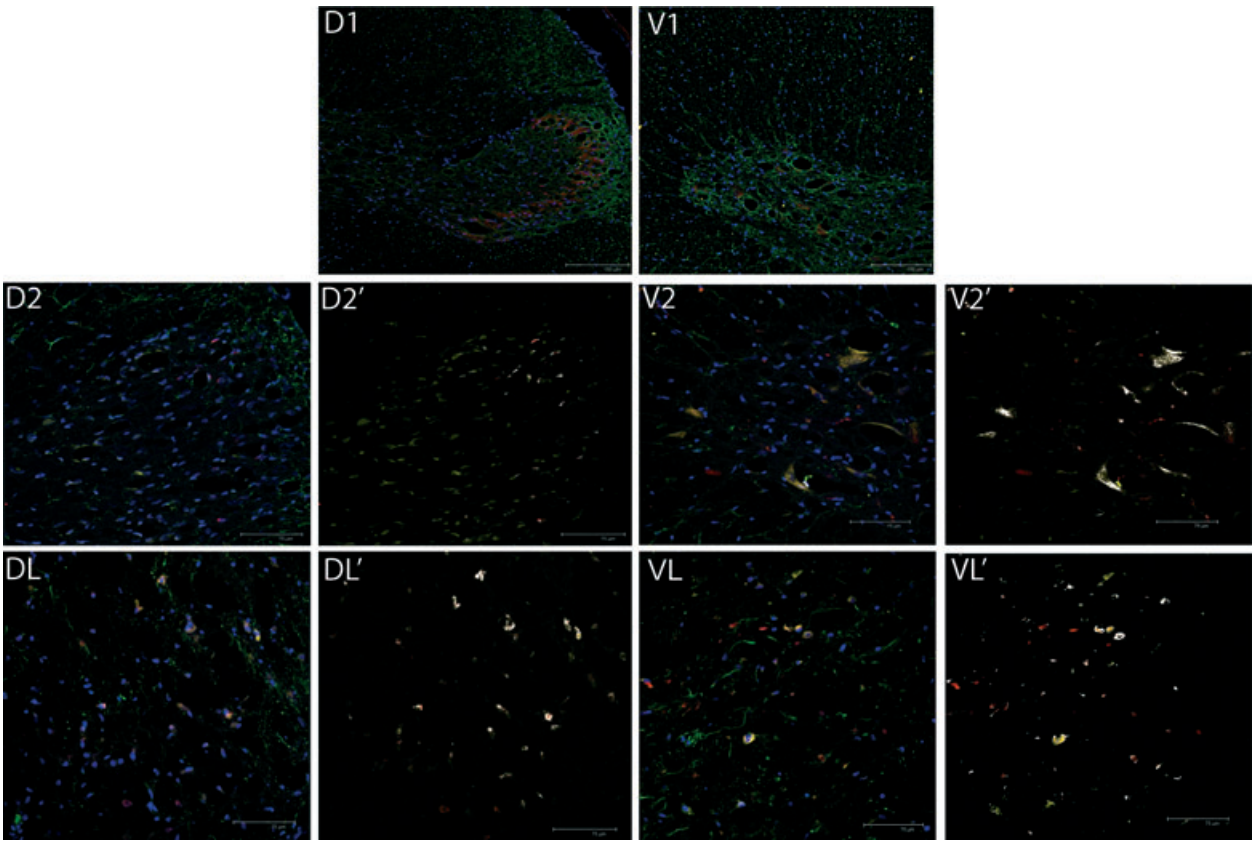

Fig. 2 Confocal images of coronal sections of thoracic spinal cord at close distance (3 mm) rostrally from the lesion epicenter. Pictures in D1 and V1 show glutamate receptor-2 (GluR2; red) and β-tubulin III (green) immunoreactivity in control (CTR) dorsal (D1) and ventral (V1) gray matter of the thoracic cord. Scale bar = 150 μm. Pictures D2, V2 and DL,VL show glial fibrillary acidic protein (green), GluR2 (red), and neurons with Nissl's substance (yellow) of control (D2, V2) and injured (DL, VL) rats, 72 h after SCI, respectively. Nuclei were counter-stained with DAPI (blue). Co-existance of Nissl substance and GluR2 was indicated by the white artificial color (D2′, DL′, V2′, VL′). Scale bar = 75 μm

interaction). Editing was also reduced for the GluR2 Flop variant with basically the same pattern (Fig. 3c): an overall reduction (main effect of treatment, $p < 0.0001$) that was greater at the epicenter than in the rostral or caudal segments (treatment × locus interaction, $p < 0.0001$), but with significant decrements in all three areas individually. The effects of lesioning were equivalent for the Flip and Flop variants, as there were no significant interactions of treatment × variant or of treatment × variant × other variables.

The effects on editing of GluR3 were more complex. There was a significant main effect of treatment ($p < 0.0001$) that again depended on the locus (treatment × locus, $p < 0.0001$), but for this subunit, the effects on the two variants differed (treatment × variant, $p < 0.0001$) and changed over time (treatment × time, $p < 0.0001$), as well as showed more complex interactions: $p < 0.002$ for treatment × variant × time, $p < 0.002$ for treatment × variant × locus, and $p < 0.0001$ for treatment × time × locus. Accordingly, we analyzed the time course of the treatment effects for each variant. For the Flip variant (Fig. 4a), there was a robust decrease in editing at the epicenter of the lesion, with lesser, but significant decrements in the rostral and caudal segments. For the latter two regions, the effects on editing showed signs of recovery by 30 days (treatment × time interaction, $p < 0.0001$), a time-dependence that was distinct from the prolonged deficits at the epicenter (treatment × time × locus, $p < 0.0003$). Effects were far less notable for the Flop variant (Fig. 4b), and the lower magnitude was statistically distinguishable from the greater effect on the Flip variant (treatment × variant, $p < 0.0001$). There was still a significant overall decrement (treatment, $p < 0.0001$) that depended on locus (treatment × locus, $p < 0.0001$), reflecting greater effects at the epicenter. However, in this case, there was no significant effect in the area caudal to the lesion, and effects in the rostral site were limited to the 3 days time point.

Editing of the GluR4s displayed a pattern that was different from that of either GluR2 or GluR3. Again, there was a main treatment effect ($p < 0.0001$) reflecting an overall

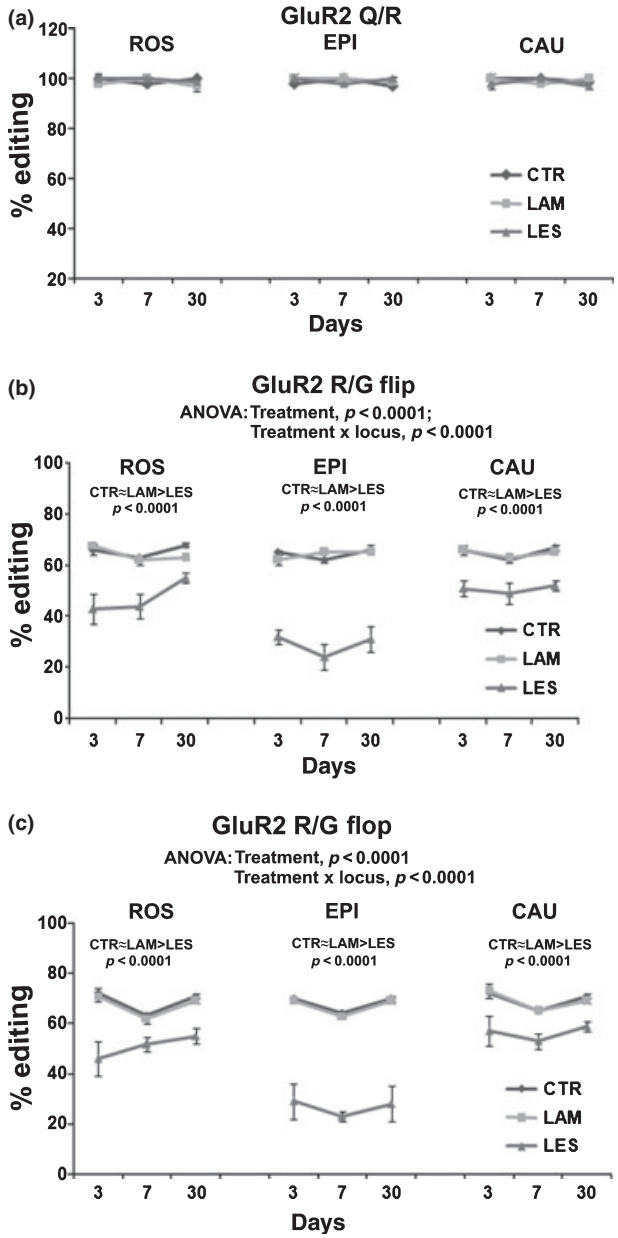

Fig. 3 Evaluation of RNA-editing levels of AMPA glutamate receptor-2 Q/R (a) and the R/G editing site in the Flip (b) and Flop (c) variants for control (CTR), laminectomized (LAM), and lesioned (LES) rats. Different portions of the spinal cord were analyzed: rostral to the lesion (ROS), at the epicenter (EPI), and caudal to the lesion (CAU). Data represent mean values and standard errors obtained from at least five animals in each group at each time point. Multivariate ANOVA appears at the top of each panel and lower-order tests are shown within the panels. We did not assess significant differences for individual time points because of the absence of treatment × time interactions.

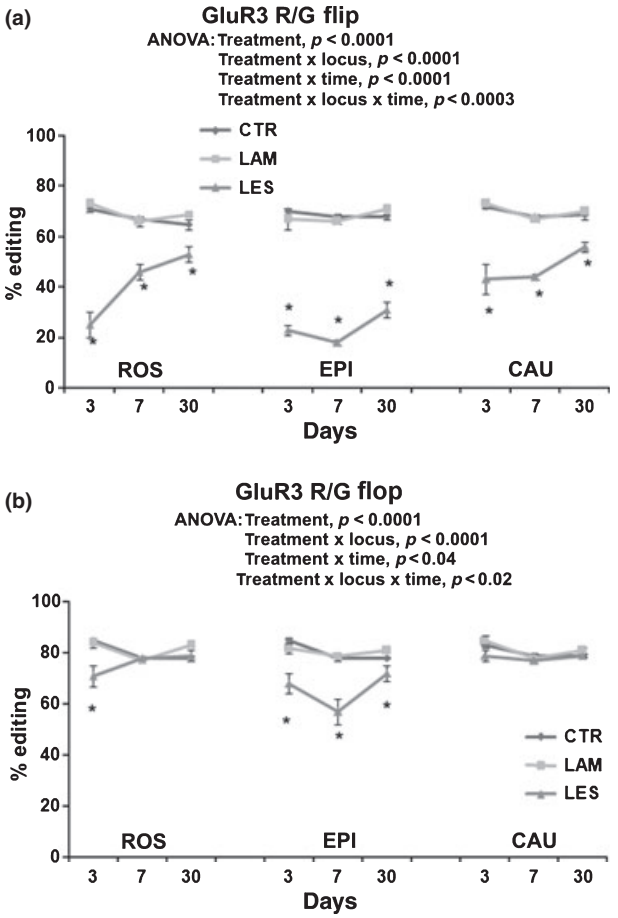

Fig. 4 Evaluation of RNA-editing levels of the AMPA glutamate receptor-3 R/G editing site in the Flip (a) and Flop (b) variants for control (CTR), laminectomized (LAM) and lesioned (LES) rats. Different portions of the spinal cord were analyzed: (ROS) rostral to the lesion (EPI) at the epicenter and (CAU) caudal to the lesion. Data represent mean values and standard errors obtained from at least 5 animals in each group at each time point. Multivariate ANOVA appears at the top of each panel and asterisks denote the individual time points at which the lesioned group differs from the control and laminectomized groups.

decrease in editing as a result of lesioning, with dependence on locus (treatment × locus, $p < 0.0001$), variant (treatment × variant, $p < 0.0001$), and time (treatment × time, $p < 0.03$). For the Flip variant, we again saw a large reduction in editing at the epicenter with lesser effects of rostral and caudal to the lesion (Fig. 5a). Although the effect at the epicenter persisted throughout the post-lesion period, there was a distinct recovery in both of the adjacent sites, so that significant effects were gone by 30 days; the loss of effect was statistically distinguishable from the continued effect at the epicenter (treatment × locus × time, $p < 0.04$). The Flop variant showed an entirely different pattern, with a progressive decline at the epicenter and no effects at either the rostral or caudal sites (Fig. 5b); these differences were reflected in a main treatment effect ($p < 0.0001$) that interacted with time and locus ($p < 0.0001$ for treatment × time, $p < 0.0001$ for treatment × locus, $p < 0.02$ for treatment × time × locus). To distinguish whether the

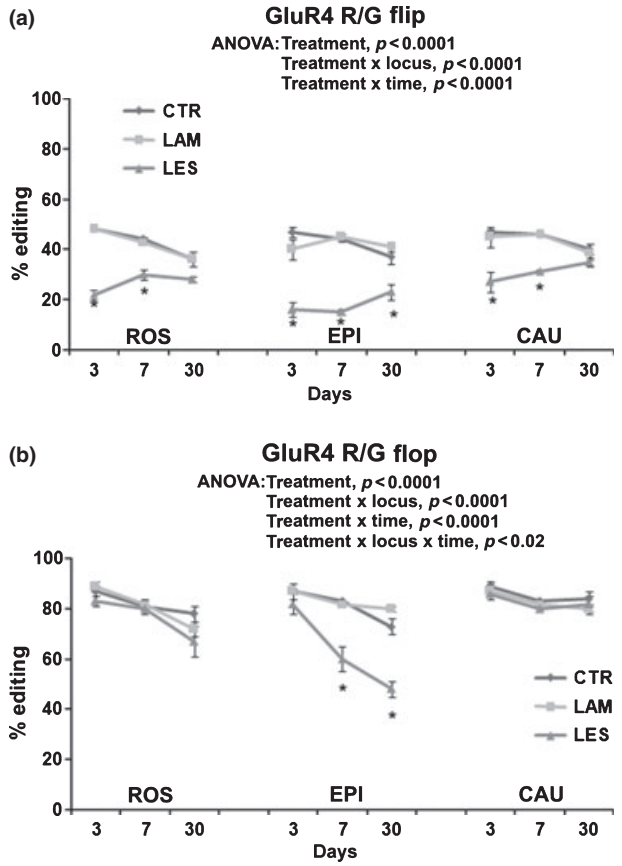

Fig. 5 Evaluation of RNA-editing levels of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate glutamate receptor-4 R/G editing site in the Flip (a) and Flop (b) variants for control (CTR), laminectomized (LAM), and lesioned (LES) rats. Different portions of the spinal cord were analyzed: rostral to the lesion (ROS), at the epicenter (EPI), and caudal to the lesion (CAU). Data represent mean values and standard errors obtained from at least five animals in each group at each time point. Multivariate ANOVA appears at the top of each panel and asterisks denote the individual time points at which the lesioned group differs from the control and laminectomized groups.

treatment effects on editing were selective for the glutamate receptor or instead were due to a general loss of editing capability, we evaluated the mRNA encoding the serotonin 5-HT2C receptor. The five editing sites of 5-HT2C were not affected by SCI (Table S1).

Finally, we assessed the expression levels of the mRNAs encoding the AMPA receptor subunits, GluR1, GluR2, GluR3, and GluR4 (Table S2). In general, the effects, although statistically significant, did not correspond to those seen for RNA editing.

ADAR1 and ADAR2 mRNA and protein expression

It has been reported that the R/G site is efficiently edited by both ADAR1 and ADAR2, whereas the GluR-B Q/R site is only edited by ADAR2 (Maas *et al.* 1996; Melcher *et al.* 1996; Aruscavage and Bass 2000; Wong *et al.*, 2001). To determine if the reductions in AMPA receptor editing reflected alterations in the expression of the enzymes responsible for RNA editing in general, the expression of ADAR1 and ADAR2, both at the mRNA and protein levels, was evaluated.

At the mRNA level, ADAR1 was up-regulated mainly in the epicenter of the lesion, whereas the effects on ADAR2 were far less notable (Figure S1). On the other hand ADAR1 (p110 and p150) and ADAR2 protein expressions were not altered in LES and LAM group compared with control group either at 3, 7, and 30 days post-lesion (Fig. 6a) as shown by the densitometric analysis (Figure S2).

ADAR2 RNA editing and alternative splicing

To test the possibility of a modification of ADAR2 catalytic activity, the self-editing level of ADAR2 at position-1 and of its splicing pattern was analysed. One ADAR2 splicing event results in the inclusion of an additional 47nt cassette at the 5′ end of the coding region, changing the reading frame of the mature ADAR2 transcript. The inclusion of this cassette depends on ADAR2 ability to edit its own pre-mRNA to generate a new intronic 3′ acceptor site.

ADAR2 self-editing (Fig. 6b) was reduced in the lesioned group at the epicenter (effect of treatment, $p < 0.0001$); this effect persisted up to 30 days after lesioning (no treatment × time interaction). Nevertheless, a trend of decrement was observed in both the rostral and caudal segments, becoming significant in the caudal site of the lesion at 30 days ($p < 0.01$).

Following the editing data, the splicing pattern of the canonical and alternative forms was changed. At the epicenter site, the form with the 47nt inclusion showed a relative decrease going from more than 60% of the total rate of transcription to less than 40% (main effect of treatment $p < 0.001$); this effect persisted during the treatment (no treatment × time interaction). A similar pattern, with lower but significant decreases, was found in the rostral segments of the cord at time point 3 days ($p < 0.01$); in the caudal segment, a main effect of treatment was observed ($p < 0.001$).

Discussion

The results found here indicate that SCI produces a decrease in AMPA receptor R/G editing. Further, these changes appear to be a specific regulatory adaptation, rather than simply reflecting the immediate effects of injury itself, as: (i) the effects extend outside the infarcted zone, to include areas of the spinal cord both caudal and rostral to the injury; (ii) the effects were selective for specific GluRs in a pattern distinct from the effects on expression of the GluRs themselves; (iii) many of the effects persisted long after the initial injury; and (iv) the decrease in editing was not shared by a parallel loss of ADAR expression, nor by effects on editing of unrelated

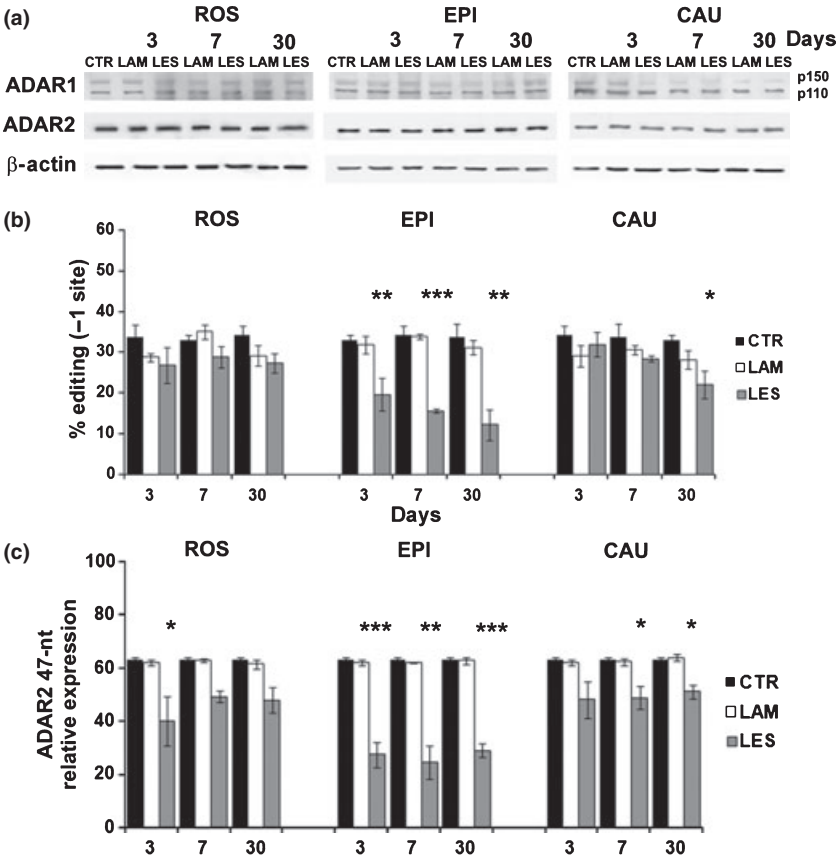

Fig. 6 Adenosine deaminase acting on RNAs (ADARs) enzymes' analysis for control (CTR), laminectomized (LAM), and lesioned (LES) rats. Different portions of the spinal cord were analyzed: rostral to the lesion (ROS), at the epicenter (EPI), and caudal to the lesion (CAU). (a) Representative western blot showing ADAR1 (110 and 150 kDa) and ADAR2 (81 kDa); a β-actin antibody was used as a loading control. (b) ADAR2 self-editing at the intronic position-1. (c) Analysis of ADAR2 alternative splicing using a RT-PCR base strategy. The columns represent the alternative spliced transcripts including the 47nt cassette. Asterisks denote the individual time points at which the lesioned group differs from the control and laminectomized groups.

receptors, such as 5-HT2C, thus showing a selectivity for AMPA receptors.

These observations provide the first evidence of the contribution of RNA editing of AMPA receptors to the dynamic mechanisms set in motion by SCI. In particular, the GluR2 R/G editing site of both Flip and Flop isoforms was dramatically reduced by SCI essentially throughout the entire 30-day period after lesioning, whereas the R/G editing level of GluR3 and GluR4 in their Flip forms showed partial recovery; this specific temporal pattern for RNA editing of AMPA receptors thus points to regulatory adjustments rather than simply representing an acute injury response. The selectivity is further reinforced by the relative lack of effect on editing of the Q/R site of GluR2s suggesting that an increased Ca^{2+} influx through unedited GluR2-containing AMPA channels is not involved in the short- as well as long-term response to SCI. One of the features of GluR2 R/G is to modulate the kinetic properties of AMPA receptor channels in association with alternative splicing at the Flip/Flop cassette (Lomeli *et al.* 1994), thus determining the time course for desensitization and resensitization (Lomeli *et al.* 1994; Krampfl *et al.* 2002). AMPA receptors with the Flip version take longer to desensitize than those with the Flop form; AMPA receptor containing an edited (G) subunit shows a faster recovery rate from desensitization compared with an unedited (R) form. The reduced editing at the R/G site of the Glur2 subunits seen here would slow the kinetic of resensitization of AMPA receptors, thus attenuating the response to the glutamate released in response to SCI. In turn, the reduced activation of post-synaptic receptors would limit calcium-induced activation of post-synaptic neurons, the relevant step mediating excitatory cell death (Choi 1994; Michaelis 1998). In addition, the observation that GluR2 Q/R site is still fully edited after SCI indicates the formation of GluR2 (R)-containing AMPA receptors that, being impermeable to Ca^{2+}, can strongly reduce the excitotoxic effects of glutamate release. The regulatory changes seen here might represent a compensatory response set in motion by neuronal cells to attenuate, at least partially, glutamate excitotoxicity. These modifications would thus achieve the functional equivalent of administration of glutamate receptor antagonists, which are known to reduce the histopathological consequences and functional deficits observed after SCI (Gomez-Pinilla *et al.* 1989; Faden *et al.* 1990; von Euler *et al.* 1994; Wrathall *et al.* 1994); indeed, the administration of AMPA receptor antagonists, such as 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX), attenuates mechanical allodynia-like behavior (central neuropathic pain) and neuronal hyperexcitability following SCI (Gwak *et al.* 2007). Consistent with our results, higher levels

of RNA editing at the GluR2 R/G site in the hippocampus of epileptic patients correlates with a higher frequency of post-synaptic receptor activation through a faster kinetic of resensitization (Vollmar *et al.* 2004).

Although reduced editing at the R/G site of the GluR2 subunits is likely to mitigate neuronal loss evoked by the initial excitatory response to SCI, we observed a prolonged effect that clearly extended well beyond the acute injury phase. Indeed, in most cases, we found persistent reductions of virtually the same magnitude even 30 days after SCI. This effect implies that decreased AMPA receptor responsiveness remains present at the point at which glutamate may actually be needed to initiate and sustain synaptic reorganization, presumably limiting the degree of recovering.

In addition to changes in receptor editing, we found specific alterations in the splicing pattern of the Flip/Flop exon cassette for AMPA receptor mRNAs (see Figure S3). After SCI, we found overall prevalence of the Flip form throughout the cord. As RNA editing is a co-transcriptional event that occurs prior to, and influences splicing (Lomeli *et al.* 1994; Ryman *et al.* 2007), our data showing a reduction in R/G editing coupled to increased splicing of the Flip exon reinforce the hypothesis that SCI has a highly targeted effect on post-transcriptional glutamatergic mechanisms that in turn might influence excitability. However, the mechanisms through which reduced editing takes place (and in turn, the modification in the Flip/Flop splicing pattern) are yet to be determined.

In an attempt to find a molecular explanation for the altered R/G editing herein shown, we explored the possibility of alterations in the function and/or expression of the editing enzymes, ADAR1 and ADAR2, as both enzymes may modify R/G editing levels (Maas *et al.* 1996; Melcher *et al.* 1996; Aruscavage and Bass 2000; Wong *et al.* 2001). ADAR1 was found increased at the mRNA levels, presumably as a consequence of lesion-induced inflammation (Yang *et al.* 2003; Rosenberg *et al.* 2005), but these modifications were not confirmed at the protein level neither for the p110 nor p150 forms. Regarding ADAR2, no statistically significant variations were seen either at the protein or at the mRNA levels. The fact that the expression level of ADAR enzymes is not always consistent with the editing levels of their RNA targets (Maas *et al.* 2001; Jacobs *et al.* 2009; Tan *et al.* 2009; Wahlstedt *et al.* 2009) may depend on several factors: (i) ADAR enzymes could be regulated at post-translational levels such as protein modification or compartmentalization (Desterro *et al.* 2003); (ii) the editing machinery may need a cofactor such as IP6 (inositol hexakisphosphate) or SUMO (small ubiquitin-related modifiers) (Desterro *et al.* 2005; Macbeth *et al.* 2005); (iii) the access of ADAR enzymes to the editing sites may be controlled negatively by a competitive inhibitor, as a propose for the small nucleolar RNA MBII-52 and 5-HT2C editing levels (Vitali *et al.* 2005; Doe *et al.* 2009). This may suggest that regulation of ADAR expression could not be critical for the changes in R/G editing induced by SCI. To this end, we shifted our focus from ADAR expression to ADAR activity and examined the levels of ADAR2 self-editing. ADAR2 has the peculiar ability to edit its own pre-mRNA to generate a new intronic 3′ acceptor site, with the inclusion of an additional 47nt cassette at the 5′ end of the coding region. This 47nt cassette changes the reading frame of the mature ADAR2 transcript (Rueter *et al.* 1999) that codifies for a truncated ADAR2 protein lacking deaminase activity (Rueter *et al.* 1999; Feng *et al.* 2006). Thus, insertion of the 47nt cassette may be a regulatory mechanism by which ADAR2 could prevent its own over-expression to avoid editing at aberrant site. We observed the predominant expression of the +47nt forms (Rueter *et al.* 1999; Slavov and Gardiner 2002; Feng *et al.* 2006) in the spinal cord of control samples and found that SCI induced a relative and persistent down-regulation of the spliced form. These modifications in ADAR2 splicing pattern follow a down-regulation of ADAR2 self-editing at position-1. This decrease in ADAR2 self-editing, however, is not followed by the expected increase in the full length protein, as reported by western blot analyses. These results suggest possible regulation mechanisms at the post-transcriptional and/or translational levels. As this editing position is clearly an ADAR2 specific target, these results suggest a potential deactivation of ADAR2 enzymatic activity. If this holds true, the selectivity of ADAR2 action might be due to a site-specific cellular response to the injury that, through changes in R/G editing and not in other editing sites such as Q/R, leads to a specific modulation of AMPA receptor functions.

In conclusion, our results provide unique insights into the post-transcriptional mechanisms that modify AMPA receptor function after spinal cord lesionings. In turn, the effects on receptor editing and splicing can modulate the consequences of excitatory injury and subsequent synaptic rearrangement, processes that are of critical importance in determining the net outcome after spinal cord damage. Notably, the changes in RNA editing seen here are far larger than those reported previously in epilepsy (Vollmar *et al.* 2004), malignant gliomas (Maas *et al.* 2003), and in sporadic, non-familial forms of amyotrophic lateral sclerosis (Kawahara *et al.* 2004; Kwak and Kawahara 2005). Our finding of a long-term reduction of AMPA receptor RNA editing may prove useful for screening compounds that could reduce initial neuronal loss and/or improve synaptic plasticity during recovery, processes that are of key importance in ameliorating the consequences of spinal injury.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Supplementary Materials and methods.

Figure S1. Real-time PCR analysis of ADAR1 and ADAR2 mRNA expression in different portions of the spinal cord.

Figure S2. ADARs western blot analysis for control (CTR), laminectomized (LAM) and lesioned (LES) rats.

Figure S3. Evaluation of AMPA receptor splicing patterns in different portions of the spinal cord for control (CTR), laminectomized (LAM) and lesioned (LES) rats.

Figure S4. Standard curve to evaluate the editing levels of 5-HTR2C editing sites.

Table S1. editing levels of the 5-HT2C receptor at the epicenter site of control (CTR), laminectomized (LAM) and lesioned (LES) rats 3, 7 and 30 days post-injury.

Table S2. Real-time expression analysis of AMPA receptor mRNAs.

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