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Treatment with albumin-hydroxyoleic acid complex restores sensorimotor function in rats with spinal cord injury: Efficacy and gene expression regulation

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Abstract

Sensorimotor dysfunction following incomplete spinal cord injury (SCI) is often characterized by paralysis, spasticity and pain. Previously, we showed that intrathecal (i.t.) administration of the albumin-oleic acid (A-OA) complex in rats with SCI produced partial improvement of these symptoms and that oral 2-hydroxyoleic acid (HOA, a non-hydrolyzable OA analogue), was efficacious in the modulation and treatment of nociception and pain-related anxiety, respectively. Here we observed that intrathecal treatment with the complex albumin-HOA (A-HOA) every 3 days following T9 spinal contusion injury improved locomotor function assessed with the Rotarod and inhibited TA noxious reflex activity in Wistar rats. To investigate the mechanism of action of A-HOA, microarray analysis was carried out in the spinal cord lesion area. Representative genes involved in pain and neuroregeneration were selected to validate the changes observed in the microarray analysis by quantitative real-time RT-PCR. Comparison of the expression between healthy rats, SCI rats, and SCI treated with A-HOA rats revealed relevant changes in the expression of genes associated with neuronal morphogenesis and growth, neuronal survival, pain and inflammation. Thus, treatment with A-HOA not only induced a significant overexpression of *growth and differentiation factor 10 (GDF10)*, *tenascin C (TNC)*, *aspirin (ASPN) and sushi-repeat-containing X-linked 2 (SRPX2)*, but also a significant reduction in the expression of *prostaglandin E synthase (PTGES) and phospholipases A1 and A2 (PLA1/2)*. Currently, SCI has very important unmet clinical needs. A-HOA downregulated genes involved with inflammation and upregulated genes involved in neuronal growth, and may serve to promote recovery of function after experimental SCI.

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Introduction

Spinal cord injury (SCI) leads to multiple cellular and molecular alterations each following a broad spatiotemporal pattern [1–3]. Although mechanical injury to the spinal cord causes immediate damage to neurons, several pathophysiological changes are induced following the initial acute phase. Mechanical spinal injury also leads to disrupted blood flow associated with bleeding within the immediate vicinity of the injury and ischemia [4], with release of free radicals and toxicity induced by hemoglobin [5]. Acute SCI also involves activation of microglia and astrocytes, and immune cells such as neutrophils (6–24 h), macrophages (24 h to 2 weeks) and T cells [6]. The ensuing neuroimmune response present during the primary and secondary SCI processes, which includes both pro-inflammatory and anti-inflammatory processes, is a relevant component of SCI pathophysiology [7,8] Balanced activity of inflammatory cell types, such as microglia and macrophages, have been shown to improve morphological and functional parameters of SCI [9]. Indeed, microglia and macrophages can change from pro-inflammatory, cytotoxic phenotypes to anti-inflammatory, pro-repair cells types [10], mediated for example by interleukin-4 that facilitates microglia and macrophages to a pro-inflammatory state after SCI [11]. Sometimes, inflamory response improves the regeneration after spinal cord injury. Intraspinal application of diferent proinflammatory drugs, potenciate axonal regeneration [12, 13]. Microglia/macrophages in the injured spinal cord show a M1-like activation state facilitating the proinflammatory state [14].

Comprehensive characterization of the cellular processes activated after SCI and their modification by new therapeutic potential agents, that may ameliorate secondary damage and promote adaptive sensorimotor neuroplasticity, can be achieved using differential gene expression analysis using microarray technology (DNA chips) [15–17]. These studies examine gene expression changes from pooled RNA samples from animals with SCI [18–21] and contribute to our understanding of SCI pathophysiology, including initial upregulation of transcription factors and pro-inflammatory genes, and downregulation of some structural proteins, neurotransmitter receptors and transporters [3].

SCI involves several changes in sensorimotor function below the injury level, including varying degrees of paralysis, and the development of debilitating symptoms and spasticity [22–26]. In addition, spinal injury can cause changes in pain processing, some of which are generated by local pathophysiological mechanisms [27–30]. Taken together these symptoms interfere with successful rehabilitation of residual voluntary motor function following incomplete spinal cord injury [31] and lead to lower quality of life [25, 27–32]. Due to the multiple spinal pathophysiological mechanisms triggered by SCI, novel treatments should be designed to control neuroinflammation and promote growth of residual descending control systems across the lesion [33–38]. In this context, some symptoms of sensorimotor dysfunction following SCI have been related to glial reactivity at the injury site [39, 40], while the restoration of constitutive serotonin and noradrenaline receptors has been reported to be essential for restoring residual motor function [41–43]. Recently, we reported partial recovery of sensorimotor function following T9 contusion SCI in the rat after intrathecal treatment with albumin and ω-9 oleic acid (A-OA) [24]. Immunohistochemical analysis of the lumbar spinal cord revealed that A-OA treatment strongly increased lumbar serotoninergic innervation, and reduced microglia activation and glutamate receptor phosphorylation [24]. Intrathecal injections of A-OA also reduce lesion-induced PPARα immunoreactivity in glia cells [44, 45]. In this context, the modified ω-9 fatty acid molecule, 2-hydroxy OA (HOA), undergoes a slower metabolization compared to OA, due to the fact that hydroxylation of the alpha carbon impairs its degradation through the beta-oxidation pathway [46–47]. Furthermore, oral administration of HOA demonstrated safety and efficacy in the control of cell proliferation and blood pressure in models

of cancer and hypertension, respectively [48,49]. Moreover, oral HOA administration inhibits mechanical and thermal hypersensitivity accompanied by a reduction of microglia reactivity in lumbar spinal dorsal horn following peripheral nerve injury [50].

In the present study, the effect of intrathecal administration of A-HOA on residual lower limb motor function and TA noxious reflex activity up to 28 days following T9 contusion SCI is described. Moreover, injured spinal cord tissue gene expression was analysed using DNA microarray analysis confirmed by RT-PCR analysis in A-HOA and saline-treated treated *Wistar* male rats 1 and 7 days after SCI. This novel treatment induced a marked recovery of the sensorymotor function and pain reduction in rats with SCI. In connection with these effects, we observed downregulation of neuroinflammation-related genes and upregulation of growth factors involved in neurogenesis, among other changes induced by A-HOA treatment. The present study demonstrates that the synthetic lipid HOA is a promising candidate to cover unmet clinical needs of patients with SCI.

Methods

Ten week old male *Wistar* rats (*HsdHan*®:*WIST*, Harlan Laboratories, 250–300 g) with free access to food and water were used. Animals were randomly assigned to different groups following SCI, each of which was administered with an intrathecal bolus. The following 5 experimental groups were planned for microarrays determinations: Control Group without lesion (n = 5), T9 vertebral region (T8 medullar) moderate contusion group treated 1 day or 7 days with saline vehicle (intrathecal, i.t., n = 5), T9 moderate contusion group treated 1 day or 7 days with an A-HOA bolus for 1 day or 7 days (80:0.4 nanomole of HOA and Albumin, respectively, i.t., n = 5). The compounds were administered by local injection in a volume of 10 μl [72] as previously described, immediately following the SCI and every 3 days. For behavioral and electrophysiological reflex analysis, animals were treated during 28 days (10 μl every 3 days, i.t.) as described below [24].

All experimental procedures were approved by the institutional animal experimentation ethical committee [National Hospital for Paraplegic Animal Experimentation Ethical Committee (Register n° V-45-168-296)]. The experiments adhered to the guidelines of the Committee for Research and Ethical Issues of IASP published in PAIN 1983; 16:109–110.

Preparation of the A-HOA complex

The complex was prepared with 20% human albumin (Grifols®), by adding HOA (kindly donated by Lipopharma Therapeutics S.L.). 2-Hidroxyoleic Acid/Albumin solution was diluted to a concentration of 80:0.4 nanomoles in saline (0.9%), as previously described [24].

Experimental animal surgery

Rats were anesthetized with pentobarbital (i.p., 65 mg/kg) and xylazine (i.p., 10 mg/kg). Approximately 90 minutes later, during the experimental surgery process, they received an additional dose containing of 20 mg/kg pentobarbital and 3 mg/kg xylazine. In addition, 0.1 ml of antibiotic was administered (2.5% Baytril, Enrofloxacin, Bayer) after surgery, followed by daily doses during 3 days after SCI.

Commercially available rat intrathecal catheters (ALZT7740Z, Charles River Laboratories, Spain) were implanted (see below) and externalized accordingly [84]. Immediately before surgical implantation, the catheter was re-sterilized with absolute ethanol, and thoroughly washed with sterile 0.9% saline. Following skin incision and blunt dissection of the muscle layers overlying the vertebrae, a small hemi-laminectomy at the vertebral T10 level was performed. The exposed dura-mater was subjected to a small durectomy with iris-type scissors so that the tip

of the i.t. catheter could be inserted rostrally and medially on top of the spinal cord with a final position just below the intended T9 contusion site. The area was cleaned to permit catheter fixture with acrylate cement to the T11 vertebrae. The percutaneous end of the i.t. catheter was finally secured by inserting it through a small cutaneous incision at the base of the cranium, whereupon it was filled with 0.9% sterile saline and tapped with a custom-made nylon filament.

Following intrathecal catheter implantation, a spinal T9 contusion was performed [85]. A bilateral T9 vertebral laminectomy enabled spinal contusion by allowing an 11-gram weight to fall from a height of 12 mm onto a cylindrical flat-tipped impactor with a 2.5 mm diameter placed centrally over the exposed spinal cord above the intact dura. Once the contusion was performed, artificial dura mater was placed onver the injury area (Neuropatch, B. Braun) and the overlying muscle layers were reapposed with a continuous suture stitch and the skin was finally closed with a subdermal suture, both with a 4–0 reabsorbable thread. Rats were carefully observed during recovery, and the bladder was manually expressed daily until recovery of function.

Tissue collection

Tissue was extracted at two specific time points after SCI: at 1 and 7 days after injury. Animals were deeply anesthetized with pentobarbital (Dolethal, 65 mg/kg, i.p., Ref: 737). Dorsal laminectomy was performed to extract thoracic spinal tissue (T7-T9). Spinal tissue was first dissected and placed on a petri-dish on dry ice and median sagittally sectioned with a scalpel blade. The spinal tissue was placed in a 2-ml cryotube (479–0821, VWR International Eurolab SL, Spain) whereupon the sample was homogenized with the aid of a scalpel in 0.5ml of TriZol® Reagent (15596–026, Invitrogen SA, Spain), and then rapidly frozen in liquid nitrogen. The total tissue collection time was no longer than 10 minutes. All the spinal tissue was stored at -80˚C until use.

DNA microarray analyses (Affymetrix, rat genome 230 2.0 arrays)

DNA microarray analyses were performed as described [86]. First, RNA was extracted from each cord sample individually using TriZol® Reagent (Invitrogen, Spain) as described [87]. Spinal cord samples from the contusion area were collected 1 day or 7 days after contusion in animals that had been submitted to treatment with saline vehicle (SCI controls, n = 4) or A-HOA (n = 4) as indicated above. The same type of sample (spinal cord area and amount of tissue) was collected from healthy rats (healthy controls, n = 4). One hundred nanograms of total RNA was used to synthesize double stranded cDNA by reverse transcription and subsequently, biotinylated cRNA was transcribed in vitro and it was fragmented as detailed by the manufacturer (Affymetrix, CA, U.S.A.).

Global RNA analysis profiles were studied using Affymetrix rat genome 230 2.0 arrays (Affymetrix, CA, USA) as previously described [86]. Total RNA was extracted from each cord sample individually using TriZol® Reagent (Invitrogen, Spain), as described [87]. Spinal cord samples from the contusion area were collected 1 day or 7 days after contusion in animals that had been submitted to treatment with saline vehicle (SCI controls, n = 4) or A-HOA (n = 4) as indicated above. The same type of sample (spinal cord area and amount of tissue) was collected from healthy rats (healthy controls, n = 4). Amplification, labeling, hybridization, staining, washing, and scanning of the microarrays followed standardized protocols, with manufacturer-recommended reagents and instruments.

DNA Chip Analysis Software (Cheng Li Laboratory, Department of Biostatistics, Harvard University, Boston, MA, USA) was used to analyze the data. The CEL files were normalized by

the invariant sets method [88, 89], and model-based expression values were obtained using the perfect match/mismatch difference model. Images were inspected for imperfections, and the quality of the data was verified with the outlier detection algorithm as described [88].

Analysis of variance (ANOVA) was used to test for significant differences between experimental groups. The False Discovery Rate tool included in dCHIP was used to detect false positives. Significant changes were identified using the following filtering criteria: statistical significance of $p<0.05$, of which those with $\geq$ and 4-fold change (absolute value) were selected for further analysis; differences of intensities over 100 between baseline and experimental means; detection call of "Present" in the experimental group. Only those genes whose expression met all these criteria were considered regulated with respect to their corresponding group. Non-agglomerative two-dimensional hierarchical clustering was used to analyze the data expression profiles. The Euclidean distance was used to generate clusters, and probe sets were grouped according to similar expression values.

RT-PCR analyses

For the present study, additional real-time RT-PCR was performed to validate additional genes from several major functional classes altered by injury. The same animal samples and RNA extractions used for microarray analyses were used for RT-PCR. RT-PCR was performed for the following genes: *PTGES*, *PLA2GA2*, *PLA1A*, *GDF10*, *TNC*, *ASPN*, *TIMP1*, *FABP4*, *LCN2*, *IL1B*, *EMR1*, *PLTP*, *MOBP*, *COMT*, *CRYAB*, *ARSB*, *NAAA*, *PTPRC*, *AXL and PTAFR*. cDNA synthesis was performed using 100 ng total RNA and the TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Carlsbad, CA, USA). Real-time PCR was carried out in a 7900 HT thermocycler (Applied Biosystems) using 2× Gene Expression Master Mix and Assays on Demand (Applied Biosystems). For comparative analysis, the $2^{-\delta\delta Ct}$ method was used [86].

Motor activity determination

Voluntary hindlimb motor function before and after T9 contusion injury was analyzed in all experimental groups using a Rota-Rod device (4600, Ugo Basile), similarly as described [24]. Briefly, prior to contusion injury, each animal was trained for three days to remain upon a cylindrical surface which rotated at 5 rpm for at least 5 minutes. On the day before SCI control data were obtained by subjecting the rats to the Rota-Rod test, but with the cylinder rotating at a steadily accelerating speed from 5 to 15 rpm during the 5 minutes test duration. Following SCI, rats were tested on day 4 and then weekly thereafter up to 28 days with the Rotarod cylinder rotating at a steadily accelerating speed from 5 to 15 rpm during the 5 minutes test duration, to follow general voluntary motor recovery and the effect of the different treatments strategies.

Tibialis anterior noxious reflex

The methodological protocol for the measurement of TA noxious reflex activity has been described [24]. Briefly, four weeks after spinal cord injury, the rats were anesthetised with isoflurane (2%) in medicinal air (17% oxygen, at 2 l/min, Synthetic medical air, Carburos Metalicos, Spain). The nose was then inserted into a plexiglass adapter (Cibertec S.A., Spain) to administer the isoflurane-air mixture, and atropine was subcutaneously administered. The animal was placed in a supine position on an electric blanket maintained at 37˚C (RTC1 Thermal Regulator, Cibertec S.A.). Hair over the left TA muscle and at the mid-thoracic level was removed and both the trunk and the hindlimbs were extended and fixed into a neutral position with adhesive tape. Bipolar electromyographic responses were recorded using two multi-

stranded Teflon-coated steel electrodes (Cooner Wire, USA) subcutaneaously inserted ca. 0.5 cm into the belly of the Tibialis anterior (TA) muscle of the left limb. In addition, two platinum subdermal electrodes (Astro-Med Inc., Grass Instruments, USA) were inserted into the tip of the fourth toe and secured with adhesive tape. Finally, an earth electrode was inserted subcutaneously between the stimulation electrode and the recording electrode at the level of the left ankle. Prior to beginning reflex EMG measurements, the isoflurane anesthesia level was lowered to 1.2% MAC in medicinal air (1 l/min). Reflex threshold was identified by characterizing the minimal current intensity (mA) required to evoke a clear nociceptive TA reflex EMG response between 0.2 and 1.0 s after stimulation, in over half of ten stimuli. Nociceptive TA reflex activity and temporal summation was evoked during a train of 16 stimuli applied at 1 Hz. Electromyographic data were integrated using the modulus function of the analysis software (Spike 2, CED, UK) between 0.2 and 0.6 s after the stimulus. Integrated reflex EMG data were analyzed after each stimulus and normalised as a percentage of the first reflex response.

Results

A-HOA promotes sensorimotor function recovery in rats with SCI

Four days after T9 contusion SCI in animals treated with saline, motor function (as assessed on the rotarod) was reduced to 1.1±0.1% compared with the pre-lesion control value (100±3%, Fig 1). The experimental SCI group treated with A-HOA also showed similar reduction in the motor activity during the first days after lesion. However, animals treated with A-HOA showed a marked and significant increase in the rate and extent of recovery of voluntary motor function ($p<0.01$, Fig 1). Thus, A-HOA induced a recovery of ca. 70% in motor function after 28 days of treatment. In contrast, rats treated with saline only showed use of the rotarod to below 10% (Fig 1).

Inhibition of noxious TA reflex activity with A-HOA treatment after SCI

TA reflex EMG activity, recorded in response to noxious electrical stimuli, was present in animals with experimental T9 contusion SCI treated with saline vehicle (Fig 2). In animals with SCI treated with saline vehicle alone, the temporal summation of the nociceptive TA flexor reflex was observed up to a maximal value of 1150±200% when compared to the first reflex response (Fig 2). A-HOA had a strong inhibitory effect on temporal summation (Fig 2); thus, post-hoc analysis revealed that temporal summation of the TA nociceptive reflex was inhibited in rats with SCI following treatment with A-HOA. In these A-HOA-treated animals, the maximal TA temporal summation observed was 210±30%.

Gene expression analysis in the spinal contusion area in rats with SCI

Whole-genome expression analysis was performed independently on 4 animal samples (spinal cord T8-T10 contusion area) from each group: control, SCI after 1 day, SCI after 7 days, SCI treated with A-HOA after 1 day, SCI treated with A-HOA after 7 days.

Upon application of the quantification criteria detailed above, DNA microarray analysis revealed marked differences in the gene expression pattern between healthy non-injured rats and those with SCI in the T9 area of the spinal cord both after 1 and 7 days post-lesion (Fig 3). In contrast, rats with SCI and treated with saline showed differences with respect to those that received A-HOA treatment both at 1 and 7 days after lesion (Fig 3). In this context, SCI induced changes in the expression of a very high number of genes (S1 Table). Moreover, ca. 600 genes showed an expression altered over 4-fold with respect to healthy rats (Tables 1 and 2). Interestingly, only 43

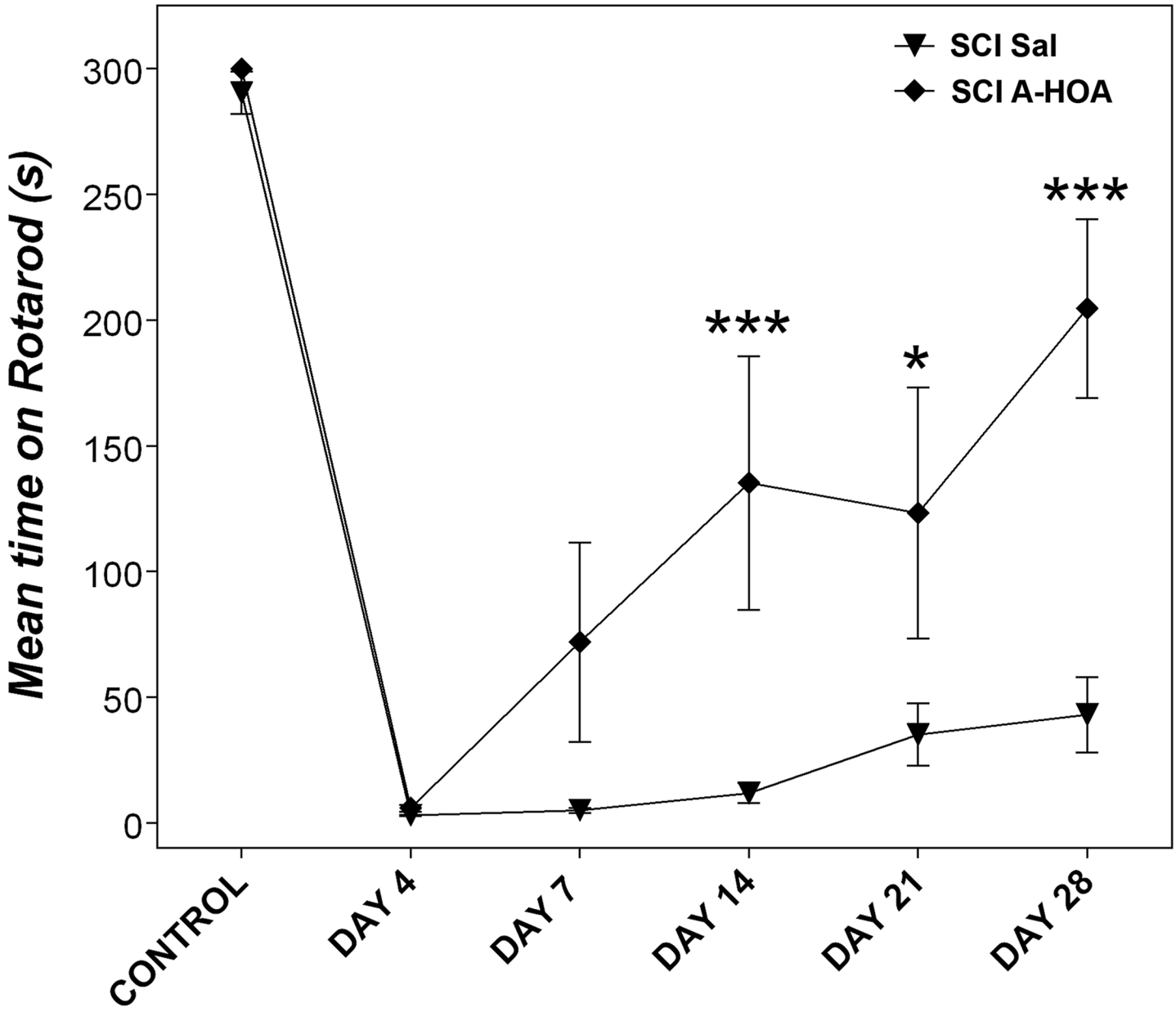

Fig 1. A-HOA promotes early recovery of motor function following T9 spinal cord injury. Longitudinal analysis of the mean (±SEM) time spent on the rotarod following contusion SCI from 4 to 28 days revealed that intrathecal administration of A-HOA (SCI A-HOA, ♦) induced locomotor recovery in contrast to saline vehicle treatment (SCI Sal, ▼). Statistical analysis was performed using a two-way ANOVA. *p<0.05; ***p<0.001. For further details see the materials and methods section.

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genes showed an expression 4-fold lower than healthy controls (Table 2) whereas ca. 550 genes appeared to be overexpressed (Table 1) 1 week after the lesion.

In contrast, treatment with A-HOA only induced changes in the expression of 41 genes, 20 of them overexpressed and 21 underexpressed, in SCI rats (Table 3). Six of these genes were expressed with a difference of more than 4 folds in A-HOA treated rats with respect to saline treated rats (3 genes were overexpressed and 3 underexpressed). Clustering analysis of the data is shown in Fig 3, which graphically represents the differential distribution of samples accrding to the covariance of the expression values obtained for the filtered genes.

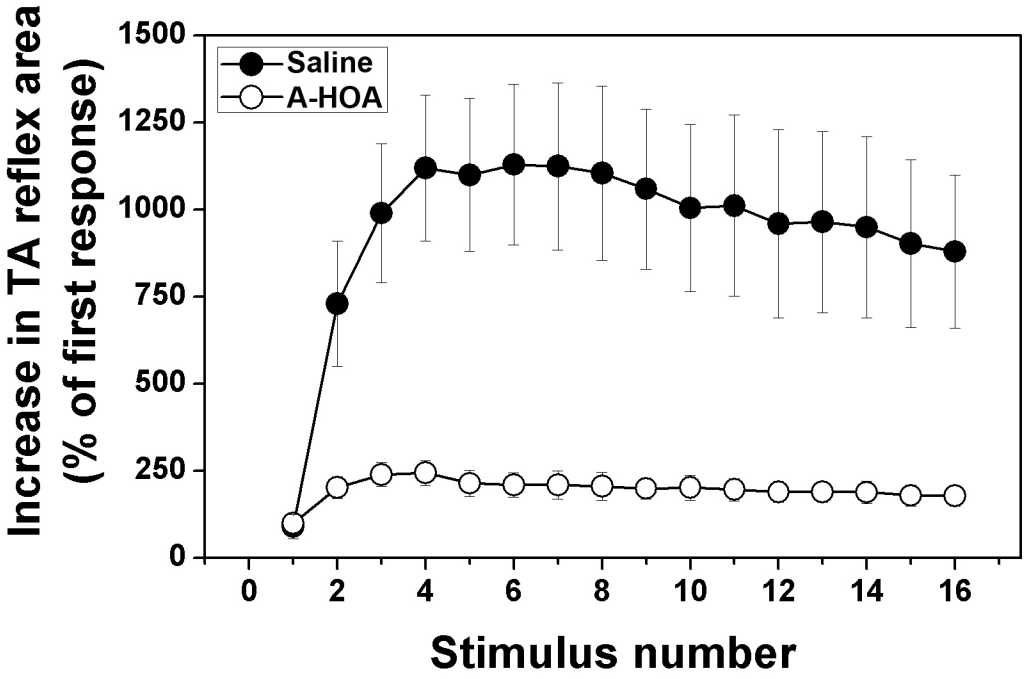

Fig 2. Inhibition of Tibialis Anterior noxious reflex activity in rats with SCI. Quantitative analysis of Tibialis Anterior (TA) noxious reflex temporal summation at 28 days following spinal contusion. Significant ($p<0.001$) inhibition of noxious TA temporal summation in animals with contusion SCI was observed after A-HOA treatment when compared with the group treated with saline vehicle. For further details see the materials and methods section.

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To further validate the changes observed in microarray analyses, we also measured the expression of a number of genes relevant in the context of SCI and the therapeutic effects of A-HOA in nociception control and motor activity (Figs 4–6). In this context, genes such as *TIMP1*, *LCN2 and IL1B* were significantly increased after SCI in the spinal cord lesion area (Fig 4).

In addition to the low number of genes altered in SCI rats treated with A-HOA (Table 3), some of them showed an expression opposite to that of non-treated (Control) SCI rats (Fig 5). An example is the inflammation-related protein, *prostaglandin E (PGE) synthase (PTGES)*, whose expression is markedly and significantly increased in SCI rats (Table 1) but significantly decreased SCI rats after treatement with A-HOA (Table 3, Fig 5). In contrast, *growth differentiation factor 10 (GDF10)* was significantly increased only in SCI rats treated with A-HOA.

Discussion

Spinal injuries have a prevalence ranging from 250–900 patients per million inhabitants in different countries and regional areas [51], and over 90% of them are affected by important losses in voluntary mobility, while spasticity and neuropathic pain affects over 80% of patients with SCI [25, 26, 52]. In this context, there are unmet clinical needs to treat this condition and the symptoms associated with it [51].

In the present study, we showed that intrathecal administration of the A-HOA complex (every third day during 28 days) induced a marked and significant recovery of the voluntary motor function (ca. 70%, Fig 1). Moreover, A-HOA induced a marked and significant reduction with a concomitant inhibition of cutaneous noxious reflex activity and central sensitization to noxious stimuli, which indicates a possible application for spasticity and neuropathic

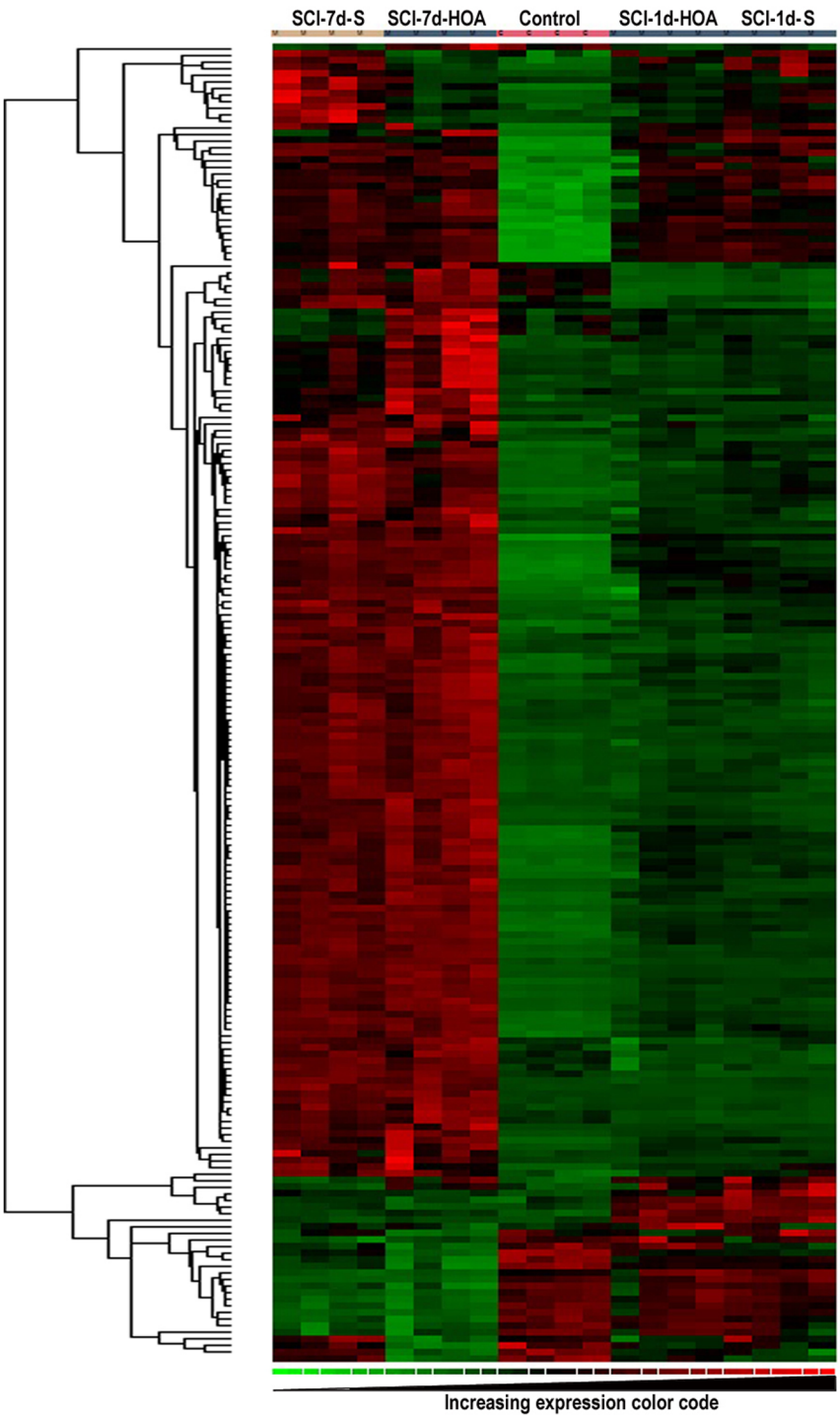

Fig 3. mRNAs differentially expressed in the spinal cord lesion region in rats with SCI. After contusion, total RNA was extracted from the lesion region of rats with SCI or from healthy non-injured controls (Control) 1 day or 7 days (1d and 7d, respectively) and treated with saline vehicle (S) or A-HOA (HOA). mRNA was quantified by microarray analysis. ANOVA following the false discovery rate (FDR) P value correction used to detect significant changes. The figure shows hierarchical clustering in the 5 experimental groups showing the expression levels from green (low) to red (high). Expression levels using the color code indicated at the bottom of the graph is shown for all four animals from each experimental group.

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Table 1. Overexpressed genes in the lesion of rats with SCI (7 days post trauma) compared with non-injured rats.

mRNA species	Fold Change	p
secretory leukocyte peptidase inhibitor	255.25	0.013924
lipocalin 2	177.51	0.005869
CD8a molecule	149.04	0.002310
chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating, alpha)	101.89	0.018744
chemokine (C-X-C motif) ligand 13	73.05	0.005268
Similar to Serum amyloid A-3 protein precursor	67.32	0.033560
Fc receptor-like S, scavenger receptor	57.85	0.000117
Cd68 molecule	54.98	0.000051
chemokine (C-C motif) ligand 2	51.22	0.016908
leukocyte immunoglobulin-like receptor, subfamily B, member 4	49.05	0.006810
apolipoprotein B mRNA editing enzyme, catalytic polypept 1	46.37	0.00006
phospholipase A2, group IIA (platelets, synovial fluid)	42.60	0.024865
interferon induced transmembrane protein 1	42.34	0.000046
chemokine (C-C motif) ligand 20	40.94	0.036242
killer cell lectin-like receptor, subfamily A, member 5 /// Ly49 stimulatory receptor 7	38.31	0.000047
chemokine (C-X-C motif) ligand 11	38.23	0.005826
interleukin 1 beta	36.87	0.003612
C-type lectin domain family 7, member a	33.76	0.000075
killer cell lectin-like receptor, subfamily A, member 5	31.27	0.000007
CD8a molecule	30.33	0.005512
lipopolysaccharide binding protein	28.75	0.001551
Rn.82246.1	28.44	0.001277
cytochrome P450, family 2, subfamily d, polypeptide 1 /// cytochrome P450, family 2, subfamily d, polypeptide 5	28.28	0.019884
folate receptor 2 (fetal)	27.34	0.001246
chemokine (C-C motif) ligand 9	25.80	0.011754
chemokine (C-C motif) ligand 3	25.79	0.000761
immunoglobulin superfamily, member 6	25.78	0.001191
interferon activated gene 204	25.64	0.020116
chemokine (C-C motif) ligand 7	25.41	0.049355
centromere protein F	25.00	0.002765
ribonucleotide reductase M2	24.37	0.013762
CDC28 protein kinase regulatory subunit 2	24.14	0.002132
tumor necrosis factor receptor superfamily, member 1b	23.86	0.000471
leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3	23.34	0.010143
C-type lectin domain family 4, member a3	23.25	0.000736
RT1 class I, locus CE5	23.24	0.023436
epithelial cell transforming sequence 2 oncogene	23.16	0.003377
Rn.17927.1	22.62	0.010525
C-type lectin domain family 12, member A	22.59	0.001627
ribonucleotide reductase M2	22.58	0.001574
Rn.25444.1	22.56	0.005194
ubiquitin-conjugating enzyme E2C	21.97	0.002465
kinesin family member 20A	21.72	0.001638
Rn.11988.1	21.46	0.000510
DEP domain containing 1	21.28	0.004470
CD8b molecule	21.04	0.000303

(*Continued*)

Table 1. (*Continued*)

mRNA species	Fold Change	p
Rn.43019.1	20.53	0.012316
Rn.23777.1	19.72	0.001785
cyclin-dependent kinase 1	19.72	0.001136
topoisomerase (DNA) II alpha	19.48	0.001635
kinesin family member 2C	19.20	0.006305
cathepsin C	19.19	0.003236
matrix metallopeptidase 9	19.11	0.010366
membrane-spanning 4-domains, subfamily A, member 7	19.07	0.003380
phospholipase B domain containing 1	18.73	0.000655
membrane-spanning 4-domains, subfamily A, member 6B	18.62	0.002629
cell division cycle associated 3	18.58	0.003405
complement component 1, q subcomponent, C chain	18.18	0.000637
complement factor properdin	17.90	0.025026
kininogen 1 /// kininogen 1-like 1 /// kininogen 2	17.89	0.003902
killer cell lectin-like receptor, subfamily A, member 17 /// immunoreceptor Ly49si3-like /// hypothetical protein LOC497796 /// similar to immunoreceptor Ly49si1 /// Ly49 inhibitory receptor 5 /// immunoreceptor Ly49si1 /// immunoreceptor Ly49si2 /// immunoreceptor Ly49si3 /// similar to immunoreceptor Ly49si3	17.72	0.006798
Rn.43961.1	17.69	0.003668
chemokine (C-X-C motif) ligand 2	17.60	0.018680
toll-like receptor 2	17.42	0.000143
complement component 2	17.23	0.005947
chemokine (C-X-C motif) ligand 9	17.15	0.010375
regulator of G-protein signaling 1	17.11	0.000034
cystatin F (leukocystatin)	17.02	0.010998
interleukin 6	16.89	0.005156
complement factor D (adipsin)	16.74	0.003805
interleukin 2 receptor, gamma	16.46	0.001355
guanine nucleotide binding protein (G protein), gamma transducing activity polypeptide 2	16.19	0.000590
syndecan 1	15.98	0.003205
nucleolar and spindle associated protein 1	15.87	0.009738
similar to paired immunoglobin-like type 2 receptor beta /// similar to cell surface receptor FDFACT	15.86	0.002416
Rn.46917.1	15.81	0.000073
plasminogen activator, urokinase	15.76	0.002905
SLAM family member 9	15.72	0.000256
ATP-binding cassette, sub-family A (ABC1), member 1	15.50	0.000880
topoisomerase (DNA) II alpha	15.43	0.000894
carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein) /// carcinoembryonic antigen-related cell adhesion molecule 10	15.19	0.016720
Rn.13512.1	14.98	0.001033
EGF-like module containing, mucin-like, hormone receptor-like 1	14.90	0.000140
budding uninhibited by benzimidazoles 1 homolog, beta (S. cerevisiae)	14.62	0.004846
NS5A (hepatitis C virus) transactivated protein 9	14.39	0.004346
Rn.43624.1	14.31	0.029857
stabilin 1	14.25	0.003902
ATP-binding cassette, sub-family A (ABC1), member 1	14.23	0.000984
family with sequence similarity 64, member A	14.17	0.001604
periostin, osteoblast specific factor	13.96	0.023501

(*Continued*)

Table 1. (*Continued*)

mRNA species	Fold Change	p
hypothetical protein LOC689399	13.87	0.004450
membrane-spanning 4-domains, subfamily A, member 11	13.87	0.001427
cyclin A2	13.78	0.006265
myosin IF	13.57	0.001748
Rn.19507.1	13.56	0.000119
activating transcription factor 3	13.34	0.000099
complement component 2	13.33	0.001978
CD14 molecule	13.32	0.011782
protein lyl-1-like // lymphoblastic leukemia derived sequence 1	13.19	0.000007
hypothetical LOC298077	13.14	0.012059
complement component 1, q subcomponent, B chain	13.05	0.007157
family with sequence similarity 111, member A	12.95	0.018706
Rn.41848.1	12.79	0.003937
acid phosphatase 5, tartrate resistant	12.78	0.000007
T-cell receptor beta chain	12.74	0.013690
B-cell leukemia/lymphoma 2 related protein A1d	12.73	0.000147
syndecan 1	12.72	0.001009
CCAAT/enhancer binding protein (C/EBP), delta	12.70	0.008457
CD36 molecule (thrombospondin receptor)	12.62	0.005720
desmocollin 2	12.48	0.007327
Rn.15505.1	12.16	0.005645
cyclin B1	12.07	0.002048
Rn.8244.1	12.02	0.001165
stefin A2-like 3	11.98	0.036969
signal transducing adaptor family member 1	11.96	0.004210
complement component 2	11.91	0.001246
S100 calcium binding protein A11 (calizzarin)	11.88	0.007999
neutrophil cytosolic factor 4	11.86	0.002619
paired immunoglobin-like type 2 receptor alpha	11.81	0.003926
protein regulator of cytokinesis 1	11.80	0.000531
mesothelin	11.76	0.003398
ADP-ribosylation factor-like 5C	11.73	0.002995
metallothionein 1a	11.72	0.000003
DnaJ (Hsp40) homolog, subfamily C, member 22	11.60	0.007681
DEAD (Asp-Glu-Ala-Asp) box polypeptide 60	11.47	0.000193
chemokine (C-C motif) ligand 4	11.20	0.001643
phospholipase A1 member A	11.18	0.004211
prostaglandin E synthase	11.16	0.014772
schlafen 3	11.10	0.012101
protein tyrosine phosphatase, receptor type, C	11.09	0.004743
Rn.8136.1	11.05	0.000548
hematopoietic prostaglandin D synthase	11.00	0.002281
Rho GTPase activating protein 8	10.99	0.029394
cyclin B2	10.90	0.002157
platelet factor 4	10.89	0.003908
Rn.34220.1	10.83	0.003238

(*Continued*)

Table 1. (*Continued*)

mRNA species	Fold Change	p
maternal embryonic leucine zipper kinase	10.82	0.003993
RNA binding motif protein 47	10.82	0.004257
hemopoietic cell kinase	10.74	0.000585
tumor necrosis factor receptor superfamily, member 14 (herpesvirus entry mediator)	10.73	0.005281
chemokine (C-C motif) ligand 6	10.67	0.000429
ADP-ribosylation factor-like 11	10.67	0.000120
family with sequence similarity 105, member A	10.65	0.000859
nucleolar and spindle associated protein 1	10.56	0.012334
bone marrow stromal cell antigen 1	10.55	0.002871
v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian)	10.43	0.015517
Rn.6731.1	10.37	0.000049
Fc fragment of IgG, low affinity IIa, receptor (CD32) /// Fc fragment of IgG, low affinity IIb, receptor (CD32)	10.30	0.002587
kinesin family member 18B /// kinesin-like protein KIF18B-like	10.33	0.004672
cytochrome P450, family 1, subfamily b, polypeptide 1	10.28	0.004820
leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3-like	10.15	0.001043
phospholipid scramblase 1	10.12	0.002010
placenta-specific 8	10.09	0.000391
Rn.17891.1	10.08	0.000220
triggering receptor expressed on myeloid cells 2	10.07	0.000413
chemokine (C-C motif) ligand 5	10.05	0.008970
matrix metallopeptidase 19	10.03	0.000182
tumor necrosis factor, alpha-induced protein 8-like 2	9.96	0.002763
Fc fragment of IgG, low affinity IIa, receptor (CD32) /// Fc gamma receptor II beta	9.90	0.000597
Cd69 molecule	9.85	0.008057
pituitary tumor-transforming 1	9.80	0.001701
cancer susceptibility candidate 5	9.72	0.002957
complement factor B	9.58	0.001416
Granulocyte-macrophage colony stimulating receptor alpha	9.54	0.002564
Fc fragment of IgG, low affinity IIIa, receptor	9.43	0.003885
collagen triple helix repeat containing 1	9.34	0.028215
tumor necrosis factor alpha induced protein 6	9.28	0.016817
neuralized homolog 3 (Drosophila)	9.26	0.00192
Rn.55535.1	9.25	0.000028
unc-93 homolog B1 (C. elegans)	9.23	0.000179
Rn.23529.1	9.22	0.000635
prostaglandin-endoperoxide synthase 2	9.21	0.029610
Rn.3724.1	9.20	0.006151
glucagon receptor	9.20	0.021510
GLI pathogenesis-related 1	9.05	0.000001
C-type (calcium dependent, carbohydrate recognition domain) lectin, superfamily member 6	9.03	0.001373
CCAAT/enhancer binding protein (C/EBP), delta	9.03	0.000440
Rn.21147.1	9.01	0.004375
filamin binding LIM protein 1	8.99	0.000696
plasminogen activator, urokinase receptor	8.98	0.001881
hematopoietic cell signal transducer	8.96	0.000691
Rn.15077.1	8.92	0.000090

(*Continued*)

Table 1. (*Continued*)

mRNA species	Fold Change	p
Rn.24230.1	8.91	0.000671
zinc finger CCCH type containing 12A	8.90	0.000698
solute carrier family 7 (cationic amino acid transporter, y+ system), member 7	8.87	0.000121
schlafen 2	8.84	0.000340
coagulation factor V (proaccelerin, labile factor)	8.83	0.022265
mannose receptor, C type 1	8.77	0.009812
similar to paired immunoglobin-like type 2 receptor beta /// similar to cell surface receptor FDFACT	8.77	0.004090
nuclear antigen Sp100-like	8.64	0.001008
similar to hypothetical protein MGC34760	8.58	0.006961
retinol binding protein 1, cellular	8.51	0.000292
CD86 molecule	8.49	0.000222
stimulated by retinoic acid gene 6	8.49	0.009655
complement component 1, q subcomponent, A chain	8.43	0.000065
phospholipase D family, member 4	8.42	0.005256
TRAF-interacting protein with forkhead-associated domain, family member B	8.39	0.010299
interferon gamma inducible protein 30	8.36	0.000000
hypothetical LOC302884	8.35	0.003418
stimulated by retinoic acid gene 6	8.34	0.011267
pigeon homolog (Drosophila)	8.31	0.002078
Rn.37608.1	8.24	0.001213
strawberry notch homolog 2 (Drosophila)	8.19	0.007075
Rn.34740.1	8.19	0.001824
vav 1 guanine nucleotide exchange factor	8.15	0.000017
kinesin family member 23	8.15	0.002259
phosphorylase, glycogen, liver	8.14	0.000040
crystallin, mu	8.09	0.005020
Rn.63919.1	8.04	0.001672
Rn.16262.1	8.02	0.000590
triggering receptor expressed on myeloid cells 2	8.02	0.000065
RT1 class I, locus CE12	8.01	0.022370
similar to Shc SH2-domain binding protein 1	7.98	0.005740
Rn.79975.1	7.92	0.000304
Rn.14817.1	7.92	0.017410
leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3-like /// similar to paired-Ig-like receptor B /// similar to paired-Ig-like receptor A11	7.92	0.017781
carnosine dipeptidase 1 (metallopeptidase M20 family)	7.90	0.000048
cyclin-dependent kinase inhibitor 3	7.89	0.000257
Rn.22374.1	7.88	0.000658
chemokine (C-X-C motif) ligand 9	7.88	0.024321
toll-like receptor 7	7.86	0.001658
oxidized low density lipoprotein (lectin-like) receptor 1	7.83	0.000996
membrane-spanning 4-domains, subfamily A, member 11	7.81	0.010059
Rn.13339.1	7.81	0.001245
Rn.41691.1	7.77	0.002347
Rn.22530.1	7.75	0.000064
Rn.12095.1	7.69	0.000684

(*Continued*)

Table 1. (*Continued*)

mRNA species	Fold Change	p
similar to RIKEN cDNA 1600029D21	7.69	0.016664
FYVE, RhoGEF and PH domain containing 2	7.61	0.000224
Solute carrier family 37 (glycerol-3-phosphate transporter), member 2	7.61	0.000005
phospholipid transfer protein	7.59	0.000159
Rn.39365.1	7.58	0.004913
TRAF4 associated factor 1	7.57	0.010165
glutathione peroxidase 2	7.57	0.007010
proteasome (prosome, macropain) subunit, beta type 8 (large multifunctional peptidase 7)	7.56	0.015366
dipeptidase 2	7.52	0.002268
fermitin family homolog 3 (Drosophila)	7.50	0.000056
budding uninhibited by benzimidazoles 1 homolog (S. cerevisiae)	7.47	0.002175
stimulated by retinoic acid gene 6	7.45	0.029079
Rn.37608.2	7.43	0.000278
thyrotropin releasing hormone	7.43	0.000139
intercellular adhesion molecule 1	7.40	0.000958
suppression of tumorigenicity 14 (colon carcinoma)	7.33	0.001224
sterol O-acyltransferase 1	7.32	0.002610
mitogen-activated protein kinase kinase kinase 8	7.18	0.001481
guanylate binding protein 4	7.15	0.000187
Fc fragment of IgE, high affinity I, receptor for; α-polypeptide	7.14	0.000300
similar to Myeloid cell surface antigen CD33 precursor (Siglec-3)	7.13	0.001428
UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1	7.13	0.000955
CD36 molecule (thrombospondin receptor)	7.03	0.003678
Rn.12905.1	7.02	0.000052
5-hydroxytryptamine (serotonin) receptor 2B	7.01	0.001990
cytoskeleton associated protein 2	6.97	0.000213
Rn.20457.1	6.95	0.003250
family with sequence similarity 38, member A	6.95	0.004140
NCK associated protein 1 like	6.94	0.000144
solute carrier family 15, member 3	6.93	0.002008
docking protein 3	6.90	0.000116
N-acetylneuraminate pyruvate lyase	6.86	0.000044
coxsackie virus and adenovirus receptor	6.85	0.011197
Bruton agammaglobulinemia tyrosine kinase	6.85	0.000017
H2.0-like homeobox	6.85	0.000012
alanyl (membrane) aminopeptidase	6.85	0.000034
Rn.35760.1	6.83	0.005121
T-cell receptor beta chain	6.76	0.005428
baculoviral IAP repeat-containing 3	6.76	0.002429
thromboxane A synthase 1, platelet	6.74	0.000001
Rn.17796.1	6.73	0.002191
Fc fragment of IgG, high affinity Ia, receptor (CD64)	6.72	0.000680
leukocyte specific transcript 1	6.70	0.000022
cytotoxic T lymphocyte-associated protein 2 alpha	6.68	0.000987
lectin, galactoside-binding, soluble, 3 binding protein	6.66	0.001503
CD8a molecule	6.63	0.001398

(*Continued*)

Table 1. (*Continued*)

mRNA species	Fold Change	p
kinesin family member 11	6.62	0.002488
RAB32, member RAS oncogene family	6.62	0.017143
complement component 5a receptor 1	6.62	0.000004
extra spindle pole bodies homolog 1 (S. cerevisiae)	6.61	0.008779
chemokine (C-C motif) ligand 6	6.60	0.046912
Rn.17556.2	6.57	0.000563
heme oxygenase (decycling) 1	6.57	0.001143
complement factor properdin	6.56	0.000869
glia maturation factor, gamma	6.55	0.000210
Rn.12486.1	6.55	0.006555
Mediterranean fever	6.51	0.001733
Rn.15124.1	6.49	0.000029
tumor necrosis factor alpha induced protein 6	6.49	0.014032
antigen identified by monoclonal antibody Ki-67	6.48	0.000633
Rn.17858.1	6.46	0.005273
membrane bound O-acyltransferase domain containing 1	6.44	0.002281
leucine rich repeat (in FLII) interacting protein 1	6.41	0.000842
cytochrome b-245, alpha polypeptide	6.39	0.000611
cathepsin Z	6.36	0.000395
collagen, type XVIII, alpha 1	6.32	0.003553
transforming, acidic coiled-coil containing protein 3	6.29	0.000330
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	6.26	0.000425
transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	6.25	0.000142
deoxyribonuclease II Alpha	6.25	0.001119
matrix metallopeptidase 9	6.24	0.011841
adipose differentiation related protein	6.19	0.000353
v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	6.18	0.003324
feline sarcoma oncogene	6.15	0.000002
Rn.15220.1	6.14	0.003527
thrombospondin 2	6.14	0.018333
Src-like adaptor	6.14	0.001732
Rn.9477.1	6.14	0.001820
Rn.13529.1	6.13	0.000375
MHC class I RT1.O type 149 processed pseudogene	6.13	0.012003
colony stimulating factor 3 receptor (granulocyte)	6.13	0.002592
bridging integrator 2	6.12	0.012643
adenylate cyclase 4	6.11	0.003223
basic leucine zipper transcription factor, ATF-like	6.08	0.000157
SP140 nuclear body protein	6.07	0.003669
Rn.18190.1	6.05	0.001546
kinesin family member C1	6.05	0.003165
ubiquitin-conjugating enzyme E2T (putative)	6.03	0.000782
transmembrane protein 176[a]	6.03	0.000778
solute carrier family 16, member 3 (monocarboxylic acid transporter 4)	6.03	0.003752
Rho GTPase activating protein 9	6.02	0.000443
Rn.37608.2	5.98	0.000032

(*Continued*)

Table 1. (*Continued*)

mRNA species	Fold Change	p
transmembrane protein 106[a]	5.97	0.000769
Rn.3765.1	5.96	0.000275
Rn.64479.1	5.95	0.000379
Rn.42802.1	5.95	0.007488
translocator protein	5.90	0.000213
Rn.32174.1	5.89	0.000638
Rn.17187.1	5.85	0.000419
protein tyrosine phosphatase, non-receptor type 18	5.82	0.000003
angiopoietin-like 4	5.81	0.000952
FXYD domain-containing ion transport regulator 2	5.79	0.000120
lysosomal protein transmembrane 5	5.78	0.000543
aldo-keto reductase family 1, member B8	5.78	0.002604
Ttk protein kinase	5.77	0.002488
serine (or cysteine) peptidase inhibitor, clade G, member 1	5.75	0.000012
toll-like receptor 1	5.75	0.010590
immunoglobulin superfamily, member 7 /// similar to CLM3 /// similar to dendritic cell-derived immunoglobulin(Ig)-like receptor 1, DIgR1—mouse	5.74	0.000011
caspase 1	5.71	0.000852
Rn.6416.1	5.70	0.003004
chemokine (C-X-C motif) ligand 10	5.69	0.027229
ectonucleoside triphosphate diphosphohydrolase 6	5.67	0.007890
ectonucleotide pyrophosphatase/phosphodiesterase 3	5.66	0.004302
chemokine (C-X-C motif) receptor 4	5.65	0.000004
tissue factor pathway inhibitor 2	5.64	0.000770
myxovirus (influenza virus) resistance 1	5.63	0.000013
schlafen 8	5.62	0.008481
poliovirus receptor	5.61	0.002727
phosphoinositide-3-kinase adaptor protein 1	5.61	0.000218
lymphocyte cytosolic protein 2	5.54	0.000317
Rn.50688.1	5.53	0.000624
tubulin, beta 6	5.53	0.001832
pleckstrin and Sec7 domain containing 4	5.50	0.000073
caspase 8	5.48	0.000226
minichromosome maintenance complex component 5	5.47	0.003464
Rn.24916.2	5.47	0.000026
family with sequence similarity 55, member B	5.46	0.000039
Similar to paired immunoglobin-like type 2 receptor alpha	5.46	0.002178
serine (or cysteine) proteinase inhibitor, clade B, member 1a	5.45	0.009948
peptidylprolyl isomerase C	5.44	0.004347
similar to interferon-inducible GTPase	5.44	0.001651
RT1 class I, locus CE11-like /// RT1 class I, locus A3 /// RT1 class I, locus CE10 /// RT1 class I, locus CE2 /// RT1 class Ib, locus EC2	5.42	0.006253
paraoxonase 1	5.42	0.000231
Rn.7834.1	5.41	0.001241
Rn.2721.1	5.41	0.002768
complement component 4, gene 2 /// complement component 4B (Chido blood group)	5.38	0.000681
Rn.61067.1	5.36	0.009763

(*Continued*)

Table 1. (*Continued*)

mRNA species	Fold Change	p
Rn.13320.1	5.36	0.000665
centromere protein E	5.35	0.006419
Rn.18506.1	5.34	0.002755
Rn.46497.1	5.33	0.000184
metallothionein 2[a]	5.32	0.000001
Cell division cycle 20 homolog (S. cerevisiae)	5.31	0.000164
interleukin 1 receptor, type II	5.29	0.004606
family with sequence similarity 198, member B	5.28	0.000437
antisense RNA overlapping MCH	5.27	0.002693
hypothetical protein LOC308990	5.27	0.001784
Rn.4301.1	5.27	0.000002
Rn.7958.1	5.24	0.003443
interferon regulatory factor 7	5.24	0.000233
Rn.2548.1	5.23	0.000438
dedicator of cytokinesis 8	5.21	0.000069
Rn.35619.1	5.19	0.030130
SH3-domain binding protein 1	5.18	0.000191
Tenascin C	5.18	0.027698
NUF2, NDC80 kinetochore complex component, homolog (S. cerevisiae)	5.17	0.000714
cholesterol 25-hydroxylase	5.16	0.000514
potassium inwardly-rectifying channel, subfamily J, member 4	5.16	0.046190
chemokine (C-C motif) receptor-like 2	5.13	0.000619
RAS protein activator like 3	5.13	0.000006
Rn.37608.1	5.12	0.031019
Rn.13650.1	5.11	0.008676
ninjurin 1	5.11	0.000037
zinc finger, FYVE domain containing 1	5.08	0.003275
glycoprotein, alpha-galactosyltransferase 1	5.07	0.000122
amidohydrolase domain containing 2	5.07	0.001029
Rn.27718.1	5.06	0.000006
Sterol O-acyltransferase 1	5.06	0.000990
ER degradation enhancer, mannosidase alpha-like 1	5.05	0.002228
SPC25, NDC80 kinetochore complex component, homolog (S. cerevisiae)	5.04	0.001127
2'-5'-oligoadenylate synthetase-like	5.03	0.000043
similar to Putative protein C21orf45	5.03	0.004235
STEAP family member 4	5.03	0.000295
TGFB-induced factor homeobox 1	5.03	0.000095
T-cell, immune regulator 1, ATPase, H+ transporting, lysosomal V0 subunit A3	5.00	0.000003
dual specificity phosphatase 2	5.00	0.000215
bone morphogenetic protein 7	4.99	0.004166
integrin, alpha M	4.98	0.016479
asp (abnormal spindle) homolog, microcephaly associated (Drosophila)	4.98	0.005363
CKLF-like MARVEL transmembrane domain containing 3	4.97	0.000391
cathepsin K	4.97	0.000478
capping protein (actin filament), gelsolin-like	4.95	0.000073
Tyro protein tyrosine kinase binding protein	4.95	0.000002

(*Continued*)

Table 1. (*Continued*)

mRNA species	Fold Change	p
v-yes-1 Yamaguchi sarcoma viral related oncogene homolog	4.95	0.012878
DSN1, MIND kinetochore complex component, homolog (S. cerevisiae)	4.94	0.001287
Rn.52525.1	4.94	0.023854
lectin, galactoside-binding, soluble, 3	4.92	0.000033
kelch-like 6 (Drosophila)	4.91	0.000003
RT1 class Ib, locus EC2	4.91	0.002921
receptor-interacting serine-threonine kinase 3	4.90	0.002183
Epstein-Barr virus induced 3	4.89	0.000157
apolipoprotein B	4.89	0.001464
similar to 2310014H01Rik protein	4.88	0.000290
Rac GTPase-activating protein 1	4.87	0.001871
G protein-coupled receptor 84	4.86	0.001056
Fc fragment of IgG, receptor, transporter, alpha	4.86	0.019037
IQ motif containing GTPase activating protein 3	4.84	0.000471
chemokine (C-X-C motif) receptor 4	4.84	0.000035
Rn.40577.1	4.84	0.002763
RT1 class II, locus DMa	4.83	0.003158
interleukin 2 receptor, beta	4.82	0.024770
myosin IG	4.82	0.000030
actin related protein 2/3 complex, subunit 1B	4.82	0.000044
hypothetical protein LOC689399	4.82	0.000397
DnaJ (Hsp40) homolog, subfamily B, member 12	4.81	0.000511
similar to Antxr2 protein	4.81	0.005512
RT1 class Ib, locus EC2	4.81	0.004160
sterol O-acyltransferase 1	4.78	0.000261
ring finger protein 213	4.78	0.006053
Rn.26537.1	4.78	0.027254
TRAF3 interacting protein 3	4.78	0.001619
cell division cycle 20 homolog (S. cerevisiae)	4.78	0.001964
damage-regulated autophagy modulator	4.77	0.004459
Rn.65520.2	4.77	0.022947
Rn.12670.1	4.76	0.000443
DEXH (Asp-Glu-X-His) box polypeptide 58	4.76	0.000643
transcription factor 19	4.75	0.001845
CCAAT/enhancer binding protein (C/EBP), beta	4.74	0.000165
disabled homolog 2 (Drosophila)	4.74	0.013469
Rn.50630.1	4.71	0.001946
Rn.48053.1	4.71	0.004482
serine (or cysteine) proteinase inhibitor, clade B, member 1a	4.70	0.000003
Rn.47647.1	4.69	0.000116
PR domain containing 1, with ZNF domain	4.69	0.000179
Rn.19395.1	4.66	0.000117
leucine rich repeat containing 33	4.65	0.003258
collagen, type IV, alpha 1	4.65	0.003226
poly (ADP-ribose) polymerase family, member 14	4.64	0.009531
procollagen, type VII, alpha 1	4.64	0.000676

(*Continued*)

Table 1. (*Continued*)

mRNA species	Fold Change	p
interleukin 13 receptor, alpha 1	4.63	0.001698
immunoglobulin joining chain	4.63	0.038881
transmembrane protein 37	4.63	0.000398
Rn.18088.1	4.62	0.011206
signal transducer and activator of transcription 1 /// signal transducer and activator of transcription 4	4.62	0.024167
RT1 class II, locus DMb	4.61	0.000056
rCG32064-like	4.61	0.001592
CD40 molecule, TNF receptor superfamily member 5	4.60	0.001388
collagen, type IV, alpha 1	4.59	0.008011
lymphocyte antigen 86	4.56	0.001124
Rn.16900.1	4.56	0.000006
protein tyrosine phosphatase, non-receptor type 18	4.56	0.000296
SHC (Src homology 2 domain containing) transforming protein 1	4.55	0.000236
NDC80 homolog, kinetochore complex component (S. cerevisiae)	4.53	0.026631
Rn.2746.1	4.51	0.009740
interferon induced transmembrane protein 3	4.51	0.000855
epithelial stromal interaction 1 (breast)	4.50	0.000081
Rn.43557.1	4.50	0.012087
septin 6	4.50	0.000066
proteolipid protein 2 (colonic epithelium-enriched)	4.50	0.001055
Rn.12513.1	4.49	0.000389
RT1 class I, locus CE5 /// RT1 class Ib, locus EC2	4.48	0.045842
cysteine-rich intestinal protein	4.48	0.007099
interferon-induced protein with tetratricopeptide repeats 3	4.47	0.000004
transglutaminase 2, C polypeptide	4.47	0.001958
Rn.43420.1	4.47	0.003614
leucine-rich alpha-2-glycoprotein 1	4.46	0.004151
vanin 1	4.45	0.000182
Rn.35620.1	4.44	0.000197
proteasome (prosome, macropain) subunit, beta type 9 (large multifunctional peptidase 2)	4.44	0.002524
syntaxin 11	4.43	0.005689
metallothionein 1[a]	4.42	0.017051
nuclear receptor subfamily 1, group H, member 3	4.42	0.000147
Rn.33681.1	4.41	0.002894
toll-like receptor 4	4.39	0.001768
proline-serine-threonine phosphatase-interacting protein 1	4.39	0.000187
Rn.3724.2	4.36	0.005443
tropomyosin 4	4.36	0.007533
gasdermin D	4.36	0.002804
transmembrane protein 86[a]	4.35	0.000839
chloride intracellular channel 1	4.34	0.000191
Rn.23216.2	4.32	0.004487
T-cell, immune regulator 1, ATPase, H+ transporting, lysosomal V0 subunit A3	4.32	0.000124
fibrillin 1	4.31	0.003306
transmembrane protein 176B	4.29	0.000154
NCK associated protein 1 like	4.27	0.000003

(*Continued*)

Table 1. (*Continued*)

mRNA species	Fold Change	p
tumor necrosis factor (TNF superfamily, member 2)	4.27	0.009351
UDP glucuronosyltransferase 1 family, polypeptide A1, A2, A3, A4, A5, A6, A7, A8, and A9	4.27	0.001679
chemokine (C-X-C motif) receptor 4	4.26	0.000092
purinergic receptor P2X, ligand-gated ion channel 4	4.26	0.000379
Rn.47453.1	4.25	0.008502
Friend leukemia virus integration 1	4.25	0.010038
Rn.41974.1	4.25	0.001107
integrin, beta 2	4.24	0.001368
platelet derived growth factor C	4.23	0.020623
Rn.23216.1	4.23	0.000418
microsomal glutathione S-transferase 2	4.22	0.000185
Phosphoinositide-3-kinase, regulatory subunit 6	4.22	0.000266
G protein-coupled receptor, family C, group 5, member A	4.20	0.010505
collagen, type XV, alpha 1	4.20	0.003684
ADAM metallopeptidase domain 8	4.20	0.001320
six transmembrane epithelial antigen of the prostate 1	4.20	0.004441
B-cell linker	4.19	0.000092
xanthine dehydrogenase	4.19	0.003713
Leupaxin	4.19	0.000488
Rn.24916.1	4.18	0.009254
troponin T type 1 (skeletal, slow)	4.18	0.004580
RGD1565926	4.16	0.003588
Rn.19846.1	4.14	0.002039
cannabinoid receptor 2 (macrophage)	4.14	0.021660
poly (ADP-ribose) polymerase family, member 14	4.14	0.000863
protein tyrosine phosphatase-like A domain containing 2	4.12	0.001265
similar to Protein C8orf4 (Thyroid cancer protein 1) (TC-1)	4.12	0.035565
Pleckstrin	4.12	0.001972
plexin B2	4.12	0.000105
matrix metallopeptidase 7	4.11	0.001393
G-protein signaling modulator 3 (AGS3-like, C. elegans)	4.11	0.000001
tubulin, beta 5	4.11	0.015952
cellular retinoic acid binding protein 2	4.11	0.010182
bridging integrator 2	4.11	0.000080
Transgelin	4.10	0.009167
growth arrest specific 7	4.10	0.007335
B-cell CLL/lymphoma 3	4.09	0.000302
UDP glucuronosyltransferase 1 family, polypeptide A1; UDP glucuronosyl- transferase 1 family, polypeptide A2; UDP glycosyltransferase 1 family, polypeptide A3; UDP glucuronosyltransferase 1 family, polypeptide A5; UDP glucuronosyltransferase 1 family, polypeptide A6; UDP glucuronosyl- transferase 1 family, polypeptide A7C /// UDP glycosyltransferase 1 family, polypeptide A8; UDP glucuronosyltransferase 1 family, polypeptide A9	4.08	0.000178
Rn.19771.1	4.07	0.002784
Rn.3212.1	4.06	0.015501
Granulin	4.06	0.000117
kinesin family member 20B	4.05	0.003643
RT1 class Ib, locus S3	4.05	0.003206
glucosaminyl (N-acetyl) transferase 1, core 2 (beta-1,6-N-acetylglucosaminyltransferase)	4.05	0.005215

(*Continued*)

Table 1. (*Continued*)

mRNA species	Fold Change	p
Rn.20328.1	4.04	0.000013
ferric-chelate reductase 1	4.04	0.007403
chemokine (C-X-C motif) ligand 14	4.04	0.000385
Rn.8685.1	4.03	0.000060
RT1 class Ib, locus S3	4.02	0.000175
PYD and CARD domain containing	4.02	0.000058
serine/threonine kinase 10	4.01	0.000887
similar to CG3880-PA	4.01	0.005699
Glucosamine (N-acetyl)-6-sulfatase	4.01	0.006479
CKLF-like MARVEL transmembrane domain containing 6	4.01	0.000490
purinergic receptor P2Y, G-protein coupled, 14	4.00	0.000299
lymphocyte cytosolic protein 1	4.00	0.000091
peroxisome proliferator-activated receptor gamma, coactivator-related 1	4.00	0.002901

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pain (Fig 2). These results are in agreement with the previous studies showing that A-HOA could reduce pain in rats with SCI [24]. Therefore, A-HOA could constitute a potential treatment for paralysis, spasticity and pain in patients with SCI.

In this context, we observed a dramatic modification of genes within the damaged spinal tissue (Fig 3, Tables 1, 2 and 3 and S1 Table). Thus, the expression of almost 4,000 genes was significantly altered by SCI, in most cases showing upregulation (S1 Table). Considering a 4-fold threshold, ca. 550 gene products were significantly overexpressed (Table 1), whereas only 43 were underexpressed (Table 2). These results indicate that cells in the area of the spinal injury respond by activating several signaling mechanisms. By contrast, treatment with A-HOA induced a limited gene expression regulation. In this context, only 41 genes were significantly up- (20 gene products) or downregulated (21 gene products) in rats with SCI treated with A-HOA with respect to rats treated with saline, with 3 genes being overexpressed more than 4-fold and another 3 gene products downregulated to a similar extent 7 days after injury (Table 3). These results indicate that treatment with A-HOA had targeted only a few regulatory mechanisms over a week after SCI involved in the therapeutic effects mediated by A-HOA.

In the search for the mechanisms involved in SCI pathophysiology and therapy, and also as a means for the validation of the technique, we further evaluated the expression of selected genes using real-time qRT-PCR. We found that all genes whose expression appeared to be higher or lower in DNA microarray experiments also showed the same expression change trend after qRT-PCR quantification, although the absolute values were not identical. These results indicate that the microarray approach used was appropriate to accurately evaluate gene expression alterations. In this context, our results on the pathophysiological alterations induced by SCI agree with previous studies showing relevant expression modulation in genes which regulate diverse functions: stress and apoptosis, inflammation, cytoskeletal proteins, metal response elements, growth factors and receptors, cell cycle and neurotransmission [53]. In this scenario, the relevance of the results obtained in treated rats after SCI also resides in the number of genes regulated by A-HOA associated with motor activity regulation, such as *Aspn (Asporin)*. This gene encodes an extracellular matrix member of the small leucine-rich proteoglycan protein family involved in regulation of cartilage and bones and is altered in patients with vertebral pathologies. Moreover, Aspn has been associated with development of the CNS and therefore it could play a crucial role in the neural damage recovery after SCI and the

Table 2. Downregulated genes in the region of the spinal lesion of rats with SCI (7 days post trauma) compared with non-injured rats.

Gene name	Fold Change	P
NFKB inhibitor interacting Ras-like 1	-4.00	0.003430
Rn.20701.1	-4.01	0.038684
Rn.46464.1	-4.13	0.001306
protein phosphatase 1, regulatory (inhibitor) subunit 14c	-4.15	0.003595
Rn.51610.1	-4.20	0.001321
Rn.60179.1	-4.23	0.004754
peroxisomal biogenesis factor 5-like	-4.26	0.000456
Rn.18590.1	-4.28	0.000839
Rn.55394.1	-4.34	0.004764
Rn.50930.1	-4.37	0.005187
Rn.32812.1	-4.37	0.000528
smooth muscle and non-muscle myosin alkali light chain 6B-like	-4.40	0.002352
ATPase, Ca++ transporting, plasma membrane 2	-4.46	0.010895
ryanodine receptor 2, cardiac	-4.48	0.003419
Rn.62287.1	-4.52	0.000192
Rn.58970.1	-4.53	0.019291
glutamate receptor, ionotropic, N-methyl-D-aspartate 3A	-4.67	0.000241
G protein-coupled receptor 61	-4.70	0.018862
Hedgehog-interacting protein	-4.77	0.010322
Rn.51548.1	-4.78	0.001893
synaptotagmin XII	-4.81	0.002296
Hypothetical protein LOC688535	-4.90	0.008575
Rn.57513.1	-5.04	0.000046
Rn.60594.1	-5.07	0.027516
Rn.50664.2	-5.14	0.004178
Rn.71359.1	-5.23	0.003486
rCG32052-like	-5.48	0.001538
PNMA-like 2	-5.48	0.019266
Rn.46840.1	-5.78	0.000847
serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 3	-5.85	0.000694
Rn.49823.1	-5.93	0.008502
Rn.46754.1	-6.13	0.001703
Rn.32352.1	-6.17	0.000210
glycine receptor, alpha 1	-6.22	0.007860
peroxisomal biogenesis factor 5-like	-6.27	0.015086
outer dense fiber of sperm tails 3	-6.33	0.014749
Rn.59729.1	-6.34	0.004101
Rn.42032.1	-6.71	0.040806
solute carrier family 12 (potassium/chloride transporters), member 7	-6.89	0.001097
Rn.20545.1	-7.13	0.000156
potassium voltage gated channel, Shaw-related subfamily, member 3	-7.18	0.037598
potassium voltage gated channel, Shaw-related subfamily, member 3	-7.60	0.031703
activin A receptor, type IC	-9.13	0.016778

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Table 3. Gene expression modulation in the lesion area of rats with SCI treated with A-HOA compared with saline-treated rats with SCI (7 days post trauma).

Gene Symbol	Gene Name	Baseline mean	Experiment mean	Fold change	P value
Lum	*Lumican*	2648.25	5066.18	1.91	0.010056
Aldh1a2	*aldehyde dehydrogenase 1 family, member A2*	1041.77	556.89	-1.87	0.000260
Ptges	*prostaglandin E synthase*	596.47	205.06	-2.91	0.026784
Pla2g2a	*phospholipase A2, group IIA (platelets, synovial fluid)*	594.04	122.13	-4.86	0.035830
Dusp1	*dual specificity phosphatase 1*	901.48	530.79	-1.70	0.000711
Sfrp4	*secreted frizzled-related protein 4*	415.85	908.59	2.18	0.007671
Gdf10	*growth differentiation factor 10*	38.04	151.32	3.98	0.002760
Apoc1	*apolipoprotein C-I*	631.65	1449.22	2.29	0.026384
Slc6a20	*solute carrier family 6 (proline IMINO transporter), member 20*	404.28	154.00	-2.63	0.016295
Slc6a20	*solute carrier family 6 (proline IMINO transporter), member 20*	2448.44	747.27	-3.28	0.007328
Cyp2d1	*cytochrome P450, family 2, subfamily d, polypeptide 1 /// cytochrome P450, family 2, subfamily d, polypeptide 5*	195.66	32.11	-6.09	0.022354
Pla1a	*phospholipase A1 member A*	826.48	398.31	-2.07	0.014396
Mx1	*myxovirus (influenza virus) resistance 1*	694.35	1388.75	2.00	0.016593
Kng2	*kininogen 2*	17.58	5.79	-3.04	0.007851
Mfap4	*microfibrillar-associated protein 4*	150.67	284.34	1.89	0.002309
Tpm2	*tropomyosin 2, beta*	100.74	281.72	2.80	0.035562
	Rn.3291.1	428.68	206.19	-2.08	0.004151
Aoc3	*amine oxidase, copper containing 3 (vascular adhesion protein 1)*	57.19	172.80	3.02	0.025730
Srpx2	*sushi-repeat-containing protein, X-linked 2*	16.37	82.21	5.02	0.044530
Tnc	*Tenascin C*	530.03	1428.34	2.69	0.019260
	Rn.30828.1	40.14	105.15	2.62	0.017407
	Rn.11906.1	613.35	1579.46	2.58	0.037547
LOC363060	*similar to RIKEN cDNA 1600029D21*	120.51	43.81	-2.75	0.034124
Arhgap8	*Rho GTPase activating protein 8*	28.65	7.32	-3.91	0.048155
Smarcad1	*SWI/SNF-related, matrix-associated actin-dependent regulator of chromatin, subfamily a, containing DEAD/H box 1*	19.38	40.18	2.07	0.008265
Aspn	*Asporin*	102.11	861.26	8.43	0.004732
Kng1	*kininogen 1 /// kininogen 1-like 1 /// kininogen 2*	156.12	46.97	-3.32	0.004672
Cxcl1	*chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)*	653.77	144.62	-4.52	0.030424
	Rn.18275.1	206.25	100.05	-2.06	0.002566
	Rn.12277.1	448.85	177.91	-2.52	0.001260
	Rn.20685.1	30.95	68.69	2.22	0.021731
	Rn.42991.1	835.99	466.74	-1.79	0.000244
	Rn.29413.1	25.16	10.48	-2.40	0.012320
Aspn	*Asporin*	318.16	1833.62	5.76	0.001719
Coch	*coagulation factor C homolog, cochlin (Limulus polyphemus)*	329.19	102.74	-3.20	0.029028
Shisa3	*shisa homolog 3 (Xenopus laevis)*	137.10	390.53	2.85	0.004579
	Rn.49714.1	291.54	125.38	-2.33	0.000343
Gpr182	*G protein-coupled receptor 182*	198.86	91.33	-2.18	0.003590
P4ha3	*Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide III*	73.12	163.65	2.24	0.040062
C1qtnf7	*C1q and tumor necrosis factor related protein 7*	38.47	95.80	2.49	0.003539
	Rn.72710.1	166.69	335.04	2.01	0.012002

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therapeutic effects of A-HOA [54]. More specifically, this gene could be involved in the recovery of the extracellular matrix of the tissue damaged after SCI. In fact, Aspn expression already

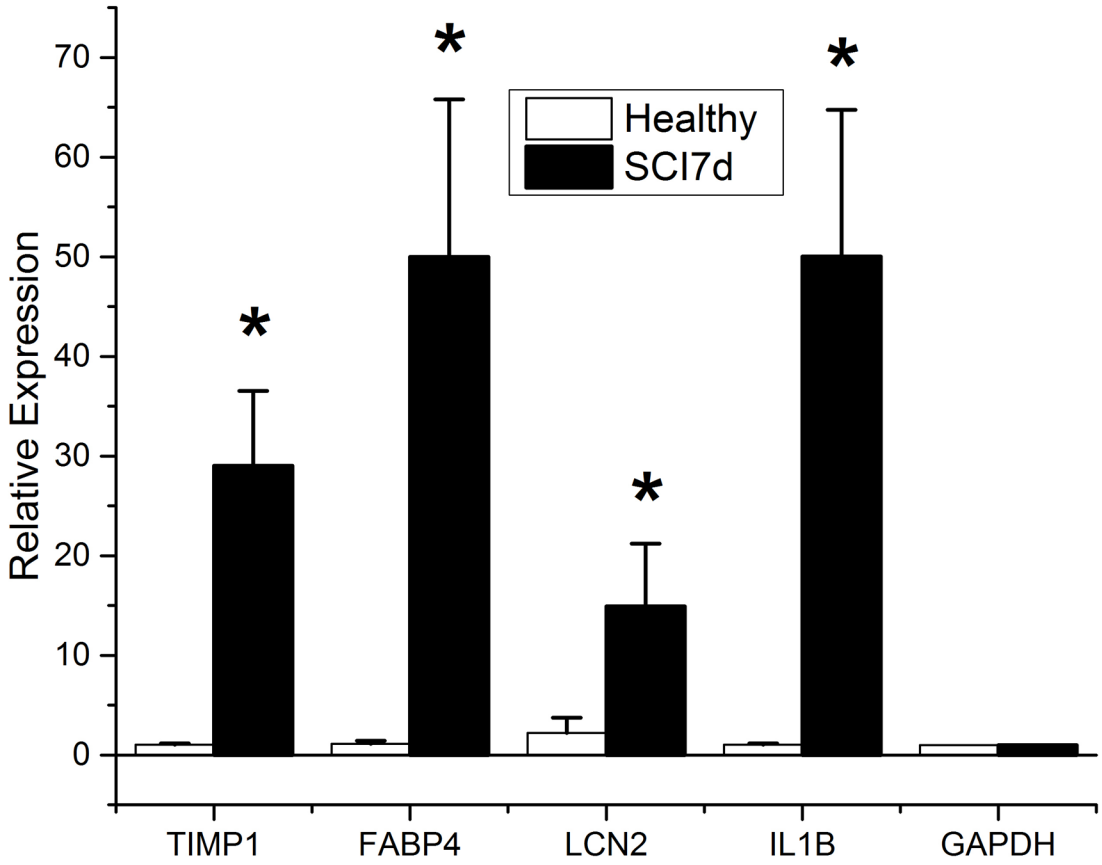

Fig 4. Relative gene expression in SCI rats 7 days after lesion. Levels of the mRNA species indicated were quantified by qRT-PCR in the spinal cord of healthy non-injured rats (open bars) and SCI rats 7 days after contusion (solid bars). The relative expression was calculated from 4 animals using triplicate samples. The samples used were the same as those used for microarray analysis. The relative expression for each gene was calculated with respect to the expression of the housekeeping gene *GAPDH*. *p<0.01.

https://doi.org/10.1371/journal.pone.0189151.g004

is high (ca. 3-fold) in animals treated with saline and treatment with A-HOA causes further increases (ca. 6–8 fold), which indicates the relevance of this gene product in the physiological and pharmacological recovery after spinal injury.

Similarly, *Growth Differentiation Factor 10 (GDF10)* is a member of the bone morphogenetic protein family and the TGF-α superfamily, which is involved in the anti-inflammatory activity of certain cytokines and in alleviating nerve injury-induced neuropathic pain in rats [55]. Moreover, *GDF10* has been recently reported to be a signal for axonal sprouting and neuron functional recovery after stroke [56]. The members of this family are regulators of cell growth and differentiation in both embryonic and adult tissues. Interestingly, this protein is also expressed in adipocytes, where it inhibits adipogenesis [57]. Because the CNS has very high lipid content, it is feasible that this protein could be involved in the lipid metabolism and nerve regeneration. Therefore, the neurotrophic, anti-inflammatory, analgesic and metabolic roles of *GDF10* could play critical roles in the recovery from SCI.

Another protein overexpressed in A-HOA-treated SCI rats was *tenascin C (TNC)*. This protein is involved in regulating the proliferation of both oligodendrocyte precursor cells and astrocytes. *TNC* is present in central nervous system injuries and gliomas [58]. In this context, in *TNC* defficient mice improved axonal sprouting has been observed, suggesting that this protein may interfere with nerve recovery after SCI. However, the fact that A-HOA induces *TNC*

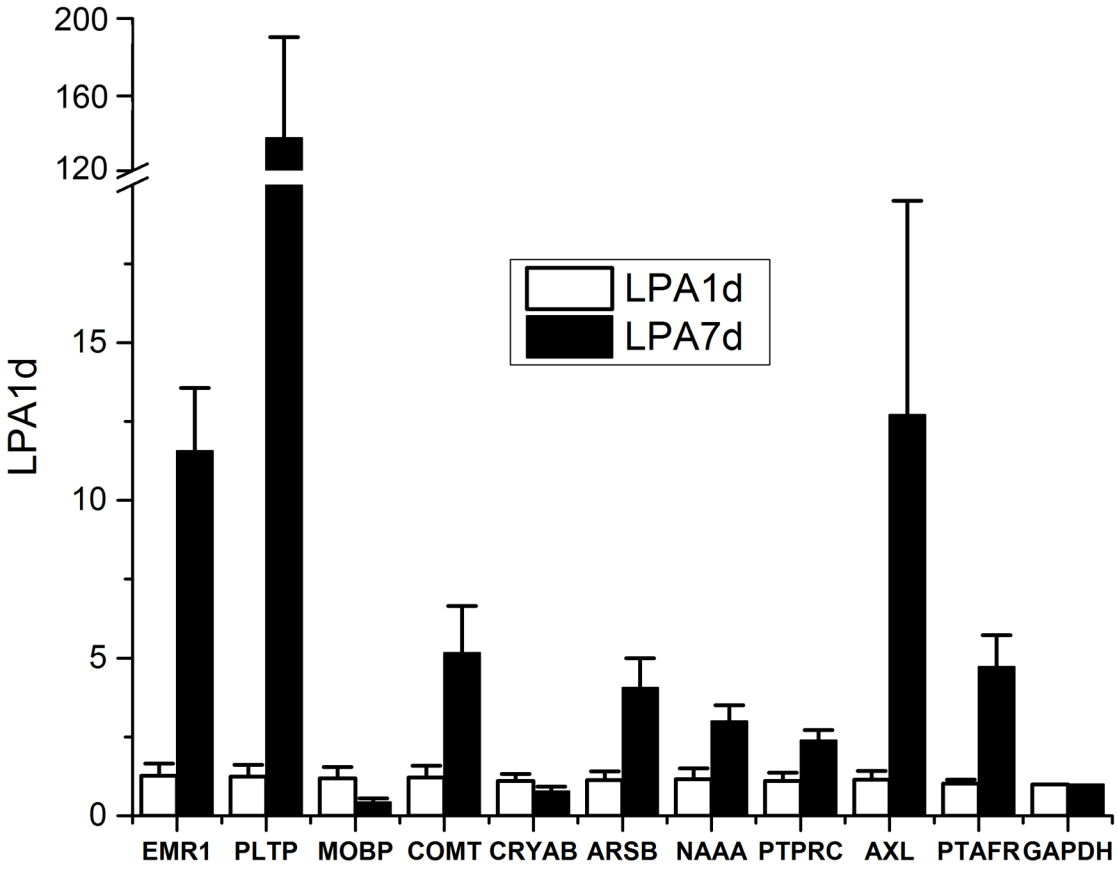

Fig 5. Relative gene expression in SCI rats 1 and 7 days after lesion. Levels of mRNA species quantified by qRT-PCR in the spinal cord of SCI rats 7 days after contusion (solid bars) relative to 1 day expression. The relative expression was calculated from 4 animals using triplicate samples. The samples used were the same as those used for microarray analysis. The relative expression for each gene was calculated with respect to the expression of the housekeeping gene *GAPDH*. *p<0.01.

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overexpression followed by generalized motor recovery could indicate that this is one of the molecular cell events associated with recovery from SCI. *TNC* has also been related to extracellular matrix alterations, accelerated leukocyte infiltration and enhanced axonal sprouting after spinal cord hemisection in *tenascin-C*-deficient mice [58].

Another gene with a markedly and significantly increased expression in A-HOA-treated SCI rats with respect to saline-treated rats was *sushi-repeat-containing protein X-linked 2 (SRPX2)*. This gene encodes a secreted protein with 3 sushi repeats, and has a relevant role in cognitive activities, such as speech and language, as well as in angiogenesis [59,60]. Moreover, alterations in the *SRPX2* gene are associated with bilateral perisylvian polymicrogyria, rolandic epilepsy, speech dyspraxia and mental retardation. In addition, it participates in cell migration and adhesion, activates angiogenesis and promotes synapse formation [61]. These roles suggest that *SRPX2* may play an important role to restablish vascularization and recover synapse loss associated with SCI. In fact, mutations in *SRPX2* have been linked to neurological syndromes with altered neuronal migration [62]. In summary, this evidence suggests that *SRPX2* could play a role in functional recovery in rats with SCI.

Some kind of lipids are able to regulate inflammatory mediators through complex mechanisms to promote or inhibit inflammation [63–68]. In our study, genes related to inflammation, such as *PTGES*, *PLA1 and PLA2* were repressed at least 4-fold. In this context, *PTGES*

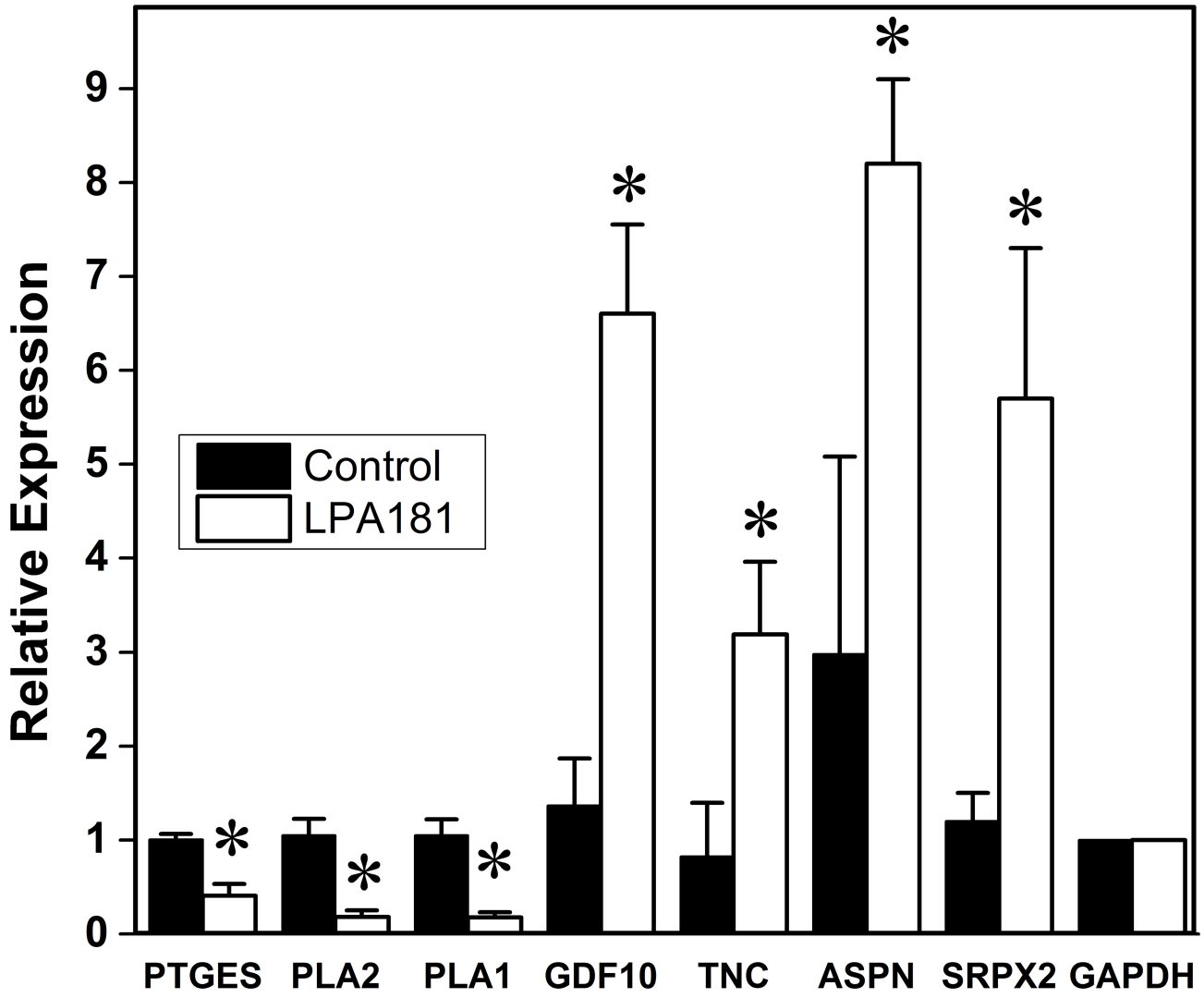

Fig 6. Effect of A-HOA on the relative gene expression of SCI rats 7 days after lesion. Levels of the mRNA species were quantified by qRT-PCR in the spinal cord of SCI rats 7 days after contusion treated with saline vehicle (solid bars) or A-HOA. The relative expression was calculated from 4 animals using triplicate samples. The samples used were the same as those used for microarray analysis. The relative expression for each gene was calculated with respect to the expression of the housekeeping gene *GAPDH*. *p<0.01.

https://doi.org/10.1371/journal.pone.0189151.g006

gene encodes for a glutathione-dependent PGE synthase. The expression of this gene has been shown to be induced by proinflammatory cytokine *interleukin 1 beta (IL-1B) and by tumor suppressor protein TP53*, and may be involved in *TP53*-induced apoptosis. Knockout studies in mice suggest that this gene may contribute to the pathogenesis of collagen-induced arthritis and mediate acute pain during inflammatory responses. In agreement with this, it has been seen that intrathecal *PGE2* administration induces hyperalgesia and allodynia, the latter tactile hypersensitivity effect observed in rats being often associated with neuropathic pain in patients [69, 70]. Moreover, knockout mice lacking the membrane enzyme that produces *PGE2 (mPGES-1$^{-/-}$)* did not exhibit mechanical allodynia, while retained normal nociceptive responses after spinal nerve transection, which demonstrates the involvement of this protein in neuropathic and inflammatory pain [71,72] and in the therapeutic effects mediated by A-HOA. In addition, *PGE2* inhibits microglial migration in the spinal cord, which could

further interfere with SCI therapy [72], so that *PTGES* inhibition by A-HOA would also permit glial cell trafficking [40].

The other two genes, *PLA1 and PLA2*, encode for *phospholipases A1 and A2*, respectively. These two enzymes produce lysophospholipids and fatty acids, such as arachidonic acid, a well-known inflammatory mediator that causes hyperalgesia [73]. On the one hand, it has been reported that *PLA1* plays a relevant role in 1-oleoyl-2-palmitoyl-phosphatidylcholine turnover in neurons, a lipid that regulates localization of signaling proteins to defined synaptic areas [74]. Furthermore, *PLA2* induction after SCI or intrathecal *PLA2* injection itself can cause axon demyelination and focal hemorrhagic pathology, suggesting that inhibition of *PLA2* might be associated with remyelination in the spinal contusion area after treatment with A-HOA [75]. Therefore, the reduction in animals of *PLA2* following treatment with A-HOA may contribute to reduced inflammation, nociception and cell death in the area of SCI.

Inhibition of *PLA2* by Annexins produces a post-traumatic anti-inflammatory effect, suggesting that the therapeutic effect of A-HOA could also be related to the inhibition of progressive tissue damage after SCI, due in part to repression of *PLA1/2* expression [76]. Further studies are required to assess the role played by these target genes in the pro and anti-inflammatory effects related to SCI.

In line with these results, significant changes were found in the spinal lesion area of A-HOA rats treated for 1 and 7 days, respectively (Fig 5). One of the most relevant changes was the alteration in the expression of the *phospholipid transfer protein*, *PLTP*, whose expression was found increased after 7 days of treatment with respect to the first day of treatment both after DNA microarray (13.6-fold; $p<0.001$) or qRT-PCR (over 100-fold change; $p<0.001$) quantification. This result further indicates the relevance of lipids in the pathophysiology and therapy of SCI. However, it should be ruled out the possibility that fatty acids in general could have therapeutic effects against SCI. In this context, it has been clearly shown that cis-monounsaturated fatty acids, such as HOA and its analog, oleic acid, induce changes in the structure and function of membrane lipids and proteins that are not paralleled by other fatty acids with identical (e.g., elaidic acid) or similar (e.g., stearic acid) chemical composition but with different structure [77,78]. Thus, the structure of fatty acids is crucial to modulate the structure of membranes and ensuing signaling events [48, 79, 80]. Membrane-drug interactions play critical roles in the efficacy of certain compounds [81] and the general mechanisms underlying the effects of synthetic fatty acids and related compunds (e.g., A-HOA) on the cell's physiology and gene expression have been summarized elsewhere [82,83].

In summary, in the present study we showed that the complex made between the lipid binding protein, albumin, and the synthetic lipid, 2-hydroxyoleic acid, showed a high efficacy to promote sensorimotor recovery after SCI, having been identified by DNA microarray and RT-PCR analyses a number of genes with a potentially relevant role for therapy. This therapeutic complex (A-HOA) could be of clinical interest for the treatment of motor function, the spasticity syndrome and the control of neuropathic pain in patients with spinal cord injury. Further experimental studies are required using behavioural and histological techniques to identify the role of the new gene targets modulated by A-HOA in this study.

Supporting information

S1 Table. Gene expression modulation in the lesion area of rats with SCI (7 days post trauma) compared with non-injured rats. This table presents approximately 3,900 genes/transcripts that undergo changes over 2 fold when gene expression of SCI (7 days post injury) and non injured animals were compared.
(DOCX)

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Writing – review & editing: Gerardo Avila-Martin, Manuel Mata-Roig, Iriana Galán-Arriero, Julian S. Taylor, Xavier Busquets, Pablo V. Escribá.

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