Matrix metalloproteinases, purinergic signaling, and epigenetics, hubs in the spinal neuroglial network following nerve injury.

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Abstract

The neuroglial network characterizes synaptic transmission and accounts for both cellular elements (neurons and glia) and neural extracellular matrix (nECM) roles. Glial cells, neuron, and nECMnetwork is strongly interconnected, in physiological and pathological conditions as shownin several neurodegenerative diseases. Purinergic activation and matrix metalloproteinases (MMPs) remodeling of the spinal cord is pivotal in maladaptive plastic changes following peripheral nerve injury (PNI). To understand how purinergic and MMPs inhibition may modulate and potentially reverse the neuroglial network failure, we used the spared nerve injury (SNI) model of the sciatic nerve. Molecular and morphological analysis of astrocytic and microglial activation, purinergic and neurotrophic receptors, Histone Deacetylase (HDAC)1, HDAC2 were analyzed to define the pathways in response to the purinergic and MMPs inhibition. The data suggest complex protein interconnections, which are not passively influenced by epigenetics but actively contribute to modify the transcriptomics machinery. The present study contributes to unveiling the spinal network consistency and ultimately encourages new paths for targeted treatments in neurological diseases with benefits of neuroprotection, plasticity, and functional recovery.

Introduction

Glial cells are a well-recognized component of the synaptic complex, together with neurons and the surrounding matrix, constituting a more exhaustive model of synapse than the “classic” neuron-based structure, both in physiology [1] and pathology [2]. The matrix-neuroglial network response to noxious stimuli, early, involves both glial cells and the neural extracellular matrix (nECM), the matrisome [3]. Indeed, the role of astrocytosis and microglial activation in the central nervous system (CNS) pathology has been extensively related to maladaptive changes in neurotransmission, neuron starvation, hyper-oxidation, cytokines release, and enhanced activation of matrix metalloproteinases (MMPs)[4, 5, 6]. Changes inthese components at first affect the synaptic transmission, causing a reversible loss of function, and late involves neuronal viability resulting in maladaptive system re-wiring and permanent damage [7].

Gliopathy and nECM disorders have been shown to play a role essential to understand CNS diseases and target them. As evidence suggests, the purinergic system is entangled with both sensory transmission and neuropathic pain affecting gliopathy [7, 6] and is regulated by MMPs modulation [8], suggesting a multi-level, fine-tuned mechanism.

Adenosine 5' triphosphate (ATP) through the ionotropic class of P2X receptors (P2XRs) is the main putative gliotransmitter involved in the purinergic activation [9]. P2XRs were reported to modulate intracellular Ca2 + currents, neuroglial transmitters release, GFAP upregulation, and inflammatory microglial invasion [9, 6], having a wide range of effects. Spinal cord neurons and glial cells mainly express P2X7 and P2X4, which can increase Ca2 + signaling, induce cellular apoptosis, mediate cytokines release, and, in turn, MMPs activation, among other functions [10, 6]. Spinal cord neurons and reactive

glial cells overexpress purinergic receptors during pathological conditions, which affect the inflammasome and the lesion remodeling in combination with growth factors [11, 12].

Further, MMPs are a pivotal enzyme family for nECM formation, adaptability, and pathological remodeling and recent data suggest a key role of these proteins in CNS diseases [13]. MMPs are secreted as pro-enzymes (pro-MMPs) by various cell types, including CNS residents. Afterward, pro-MMPs are activated by proteolytic cleavage and eventually blocked by tissue inhibitors of MMPs (TIMPs) [14].

Based on the data literature and our previous research, in the present work, we intend to deepen how P2XRs and MMPs are involved in the spinal cord plasticity following spared nerve injury (SNI) of the sciatic nerve, and if they share a modulatory impact. We inject intraperitoneally (i.p.) the oxidized ATP (OxATP), a nonselective P2XRs antagonist, alone or in combination with intrathecal (i.t.) infusion of GM6001 (a broad spectrum MMPs inhibitor). We focused our study on the expression of markers of astrogliosis, as glial fibrillary acidic protein (GFAP), the microglia and macrophages activation, as the ionized calcium-binding adaptor molecule 1 (Iba1), the purinergic (P2X4) and neurotrophic (TrkA and p75) receptors. However, MMPs and P2XRs modulation is not restricted to the extracellular and cytoplasmic level, more, it can influence gene transcription processes, altering chromatin accessibility. Histone deacetylases (HDACs) and DNA methylation could both regulate and in turn be affected by MMPs and P2XRs activation [15, 16].

The present study analyzes molecular hubsin the spinal cord following nerve injury and modulation of MMPs and P2XRs, increasing our level of understanding of neuroglial networking between the nECM, channel receptors, up to the chromatin organization level.

Material And Methods

Animals

Adult (250–300 g; Charles River, Calco, Italy) Sprague Dawley rats (n = 58) were used. Animal care was in compliance with the Italian (D.L. 116/92) and EC (O.J. of E.C. L358/1 18/12/86) regulations on the care of laboratory animals.Each animal was allowed free access to food and water, under a 12/12 h light/dark cycle. Animals were kept in pathogen-free iron-sheet cages with solid floor covered with 4–6 cm of sawdust. We did not use cages with thin-plate floors to avoid exacerbation of the discomfort from the affected hind paw [17].

SNI Model

Sciatic spared nerve injury (SNI) was made according to Decosterd and Woolf[18]. Briefly, each rat (n = 58) was anesthetized with chlorohydratetiletamine (30 mg/kg) during surgery. The sciatic nerve and its three terminal branches (the sural, common peroneal, and tibial nerves) were exposed on the lateral surface of the thigh. The SNI procedure comprised axotomy and tight ligation of the tibial and common peroneal nerves leaving the sural nerve intact. For the sham-operated control (CTR) group (n = 10), nerves

were exposed but not truncated. Muscle and skin were closed in layers. Great care was taken to avoid any contact with or stretching of the intact sural nerve.

Drug Delivery

OxATP treatment was performed according to our protocol[6]: from the day after surgery, animalswere treated with i.p. OxATP (6 mg/kg), dissolved in 100 µl of sterile distilled water (dH2O) or dH20 alone.

The intrathecal lumbar spinal catheter for GM6001 or ACSF administration was positioned during SNI procedure to reduce the discomfort bias, according to Decosterd and Woolf[18]. Briefly, a small opening was made through the laminas of the lumbar spine and a catheter [polyethylene (PE) 10 tubing attached to PE 60 tubing for connection to an osmotic pump] was inserted into the subarachnoid space and directed to the lumbar enlargement of the spinal cord. After anchoring the catheter across the careful apposition of a glass ionomer luting cement triple pack (KetacCem radiopaque; 3 M ESPE, Seefeld, Germany), the wound was irrigated with saline and closed in two layers with 3–0 silk (fascial plane) and surgical skin staples. On recovery from surgery, lower body paralysis was induced by intrathecal lidocaine (2%) injection to confirm proper catheter localization. Each rat was placed on a table, and the gait and posture of the affected hind paw were carefully observed for 2 min. Only animals exhibiting appropriate, transient paralysis to lidocaine, as well as lack of motor deficits, were used for treatments; the free extremity of the catheter was connected to an osmotic minipump and the pump was implanted subcutaneously. Osmotic pumps attached to intrathecal lumbar spinal catheters were filled with GM6001 (Calbiochem, Germany) (180 µg/µl, corresponding to 100 mg/Kg body weight), or vehicle only [artificial cerebrospinal fluid(ACSF)]. The osmotic pumps were model 2001 Alzet (Cupertino, CA) pumps, which pumped at a rate of 1µl/h.

Animals were divided into eight groups:

(I) D3 OxATP (n = 8), SNI rats treated with i.p. OxATP daily from day 1 to 3 and i.t. ACSF;

(II) D8 OxATP (n = 8), SNI animals treated with i.p.OxATP daily from day 1 to 8 and i.t. ACSF;

(III) D3 OxATP + GM6001 (n = 8), SNI rats treated with i.t. GM6001 and i.p. OxATP daily from days 1 to 3;

(IV) D8 OxATP + GM6001 (n = 8), SNI animals treated with i.t. GM6001 and i.p. OxATP daily from days 1 to 8;

(V-VI) D3–D8ACSF groups, SNI animals treated with i.t. ACSF and i.p. dH2O for 3 or 8 days (n = 16);

(VII - VIII) D3– D8control (CTR) groups (n = 10), sham-operated animals.

Antibodies

The following antibodies were used for immunodetection: rabbit antibodies against GFAP (Sigma-Aldrich, Saint Louis, USA); rabbit antibodies Iba1 (Wako Chemicals, VA, USA); rabbit antibodies against histone deacetylase 1 (HDAC1) (Sigma-Aldrich, Saint Louis, USA) and histone deacetylase 2 (HDAC2) (Sigma-

Aldrich, Saint Louis, USA); rabbit antibodies against purinergic receptors type X4 (Immunological Sciences, Rome, Italy); rabbit polyclonal to p75 NGF Receptor (Abcam, Cambridge, England); rabbit polyclonal against TrkA (Chemicon Inc., Temecula, CA, USA); mouse antibodies to ß-Actin (Sigma, Saint Louis, USA).

Tissue Preparation

Rats were deeply anesthetized on day 3 and 8 with an i.p. injection of chloral hydrate (300 mg/kg body weight) and perfused transcardially with saline solution (TrisHCl 0.1 M/ EDTA 6 mM). Spinal cords for western blotting (n = 29) were removed. Spinal cord for immunohistochemistry(n = 29) continued for fixation by 4% paraformaldehyde added to 0.1% glutaraldehyde in 0.01 M phosphate-buffered saline (PBS), pH 7.4 at 4°C. They were removed and postfixed 2 h in the same fixative, then soaked in 30% sucrose phosphate-buffered saline (PBS), and frozen in chilled isopentane on dry ice, as previously described [19]. Serial sections were cut at the slide microtome (25 µm thickness) and collected in cold PBS.

Immunofluorescence (IF)

Immunofluorescence staining was performed according toour protocols[20]. Sections were incubated with the primary antibody: GFAP (1:400),Iba1(1:500),TrkA (1:2000), p75 NGF Receptor (1:500) for 48 h at 4°C. Following washes with PBS, sections were incubated with the appropriate secondary antibody (Alexa Fluor 488 anti-rabbit IgG, Alexa Fluor 546 anti-rabbit IgG; 1:200; Invitrogen, Carlsbad, CA, USA) for 2 h. Sections were mounted and coverslipped with Vectashield (Vector Laboratories).

Western Blotting (WB)

Lumbar spinal cord samples on day 3 and 8 rats were homogenized in 50 mMHepes pH 7.5, 10% glycerol, 10 mM NaCl, 10 mM dithiothreltol, 1% SDS, 5 mMEDTA and protease inhibitors (Sigma Aldrich). Lysates were loadedon a 0.75 mm SDS polyacrylamide minigel (8%-10%-12%), which was electrophoresed at 150 V for 90 min. The proteins were transferred overnight to nitrocellulose membranes at 30 V, 4°C. After blocking of non-specific sites by 5% milk in [20 mMTrisHCl (pH 7.4), 0.2% Tween 20 (TBST)], membranes were incubated overnight with primary antibody anti-GFAP (1:400), anti-Iba1(1:250), anti-HDAC1 (1:500), anti-HDAC2 (1:1000), anti-P2X4 (1:500), anti-TrkA(1:1000), anti p-75 NGF Receptor (1:500),ß-Actin (1:2000). After washing in TBST, membranes were incubated withthe appropriate biotinylated secondary antibody (Vector Labs, Inc.; 1:500) in blocking solution for 60 min at room temperature. Subsequently, they were washed in TBS and processed using the Vectastainavidin-biotin peroxidase kit (Vector Labs, Inc.) for 30 min at room temperature. After washing in 0.05 M Tris-HCl they reacted with 3,3-diaminobenzidine tetrahydrochloride (DAB; Sigma Aldrich, 0.5 mg ml^{-1}Tris-HCl) and 0.01% hydrogen peroxide. Each specific band was acquired and processed for density measurement with the computer-assisted imaging analysis system (MCID 7.1; Imaging Res. Inc.). To compare the differences between control and treatment groups, we first normalized the density of each specific band against the density of the corresponding internal loading band.

Measurements and Statistical Analysis

Measurements of markers in the whole lumbar spinal cord were accomplished using computer-assisted image analysis system (MCID 7.1; Imaging Res. Inc., Canada). All data were collected in a blinded manner; the observer making the measurements was not aware of the group. Data were exported and converted to a density distribution histogram using the Sigma-Plot 10.0 program (SPSS-Erkrath) and presented as the mean ± SEM for all quantitative analyses. Data were checked for normal distribution and homogeneity of variance by the Kolmogorov-Smirnov's and Levene's meantests, respectively. Normal distributions with equal variances were statistically analyzedby using one-way ANOVA for multiple comparisons followed by all pairwise Holm–Sidak post hoc test. The level of significance was always set at $p = 0.05$ (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$).

Individual images were assembled, and the same adjustments were made for brightness, contrast, and sharpness using Adobe Photoshop (Adobe Systems).

Results

Reactive glial populations are selectively modulated by the purinergic inhibitor in combination with the metalloproteinases block.

Iba1 has been used as marker of microglia and macrophages while GFAP showed astrocytes reaction. The Iba1 immunofluorescence analysis showed microglial activation in ACSF animals compared to the control group at D8(ACSF 89.182 ± 4.684, CTR 39.222 ± 3.161, $p \leq 0.001$).D8OxATP treatment decreased microglial activation (64.296 ± 4.421, $p \leq 0.001$), and the combination with GM6001 led it to baseline levels at the same timepoint(46.185 ± 1,685, $p \leq 0.001$)(Fig. 1a-b). The astrocytic reaction following injury was observed at D8(51.815 ± 1.130 ACSF,27.01 ± 2.247 CTR, $p \leq 0.001$), andthe individual treatment withD8 OxATP was effective in quenching it off (30.852 ± 1.382,$p \leq 0.001$). The combined treatment was slightly effective(41.083 ± 3.855, $p = 0.0209$) (Fig. 1a-c).

Western blots for Iba1 showed the increased microglial marker expression in ACSF animals compared to control group (D8 ACSF 0.680 ± 0.069,CTR 0,1872 ± 0.0234, p = 0,003) and its reduction withD8treatmentsversus the ACSF group (D8 OxATP 0.428 ± 0.06,$p = 0.047$; D8 OxATP + GM6001 0.281 ± 0.06,$p = 0.006$)(Fig. 2a-b), confirming the IF results.

GFAP blotting revealed astrocytosis in the ACSF group (D3 1.2921 ± 0.1877; D8 2.5522 ± 0.036) compared to CTR (D3 0.468 ± 0.0213, $p = 0.0052$; D8 0.481 ± 0.0057, $p \leq 0.001$), reduced with D8OxATP administration(1.587 ± 0.376, $p = 0.012$). Surprisingly, the combination of the purinergic and MMPs modulators did not affect significantly the astrocytic reaction(D3 1.554 ± 0.109, D8 2.320 ± 0.015) (Fig. 2a-c).

Taken together these data showed that while damage-related astrocytosis is demodulated by purinergic inhibition without a clear contribution of GM6001 co-administration, the microglial reaction was affected by MMPs and P2XRs simultaneous inhibition.

Class I HDACs are differentially affected by nerve injury and purinergic signaling inhibition, even combined with MMPs modulation.

Cellular activation requires epigenetic modifications and posttranscriptional regulation [21].The deacetylation of the histone amino acids N-ε-acetyl lysine causes the chromatin to enwrap tightly, inhibiting gene expression [22] and allowing cells to react almost immediately to environmental changes both in physiology and pathology. Particularly here we consider HDAC1 and HDAC2.HDAC1 and HDAC2 are class I deacetylases and mainly act as regulators of epigenetic processes.HDAC1 levels were not significantly affected by the surgical procedure after three days (D3 ACSF 1.030 ± 0.141; CTR 0.808 ± 0.171), or after 8 days (D8 ACSF 1.206 ± 0.209), neither following OxATP and GM6001 administration (D8 OxATP 0.909 ± 0.0305; D8 OxATP + GM60010.898 ± 0.0258) (data not shown). HDAC2 was higher in the D8 ACSF group (1.174 ± 0.103) compared to controls (0.320 ± 0.001$p \leq 0.001$). Treatment with D8 OxATP showed a downregulating trend, with statistical significance in the combinatory drug administration (D8OxATP 0.805 ± 0.12$p = 0.066$; D8 OxATP + GM60010.623 ± 0.08, $p = 0.010$) (Fig. 2a-d).These data uncover differences in the role of spinalHDACsbelonging to the same class of enzymes following the nerve damage and treatments inhibiting purinergic receptors and MMPs.

OxATP and GM6001 treatments successfully downregulate the expression of the purinergic receptors.

We considered P2X4R, as one of the most expressed receptors in both CNS and peripheral nerves, especially on microglia, to drive inflammation in pathological situations such as spinal cord damage[12, 23]. We found that P2X4R was increased in D8ACSF group (0.798 ± 0.04, $p \leq 0{,}001$) compared to the sham-operated one (D8 CTR 0.350 ± 0.06), with a significant decrease of the expression in the two groups of D8treatments (OxATP 0.586 ± 0.04, $p = 0.008$;OxATP + GM6001 0.524 ± 0.05,$p = 0.002$) (Fig. 3a-b). These data provide further support to the anti-inflammatory effect of the purinergic inhibition, alsoin combination with MMPs modulation.

Neurotrophin receptors are differentially modulated by nerve injury,and purinergic signaling inhibition, combined with MMPs modulation.

Neurotrophic growth factorsplay a key role in the spinal cord remodeling after lesion, along with purinergic receptors and MMPs [11, 8].

Data concerningthe nerve growth factor (NGF)receptors TrkA and p75correspond with our previous data [24],confirmingthe increase of TrkA expression (the NGF high-affinity receptor),8 days following peripheral nerve injury (0.493 ± 0.064, $p = 0.002$)compared to sham-operated animals(0.178 ± 0.077). Treatments with OxATP alone or combined with GM6001revertedTrkA levels to the baseline (D8 OxATP 0.159 ± 0.01, $p \leq 0.001$; D8 OxATP + GM60010.182 ± 0.037, $p = 0.001$) (Fig. 3a-c).

WB for p75 (NGF low affinity receptor)showed expression levels similar to CTRin D8 ACSF group (D8 CTR 0.180 ± 0.077; D8 ACSF 0.185 ± 0.039)and strongly upregulated by both treatments (D8 OxATP 0.687 ± 0.097, $p = 0.012$; D8 OxATP + GM6001 0.793 ± 0.101, $p = 0.003$) (Fig. 3a-d).

WB data were confirmed by densitometric analysis from IF sections of the dorsal horn of the spinal cord (Fig. 4). The expression of TrkA receptor was increased in D8 ACSF group (D8 ACSF 2.637 ± 0.310; D8 CTR 1.208 ± 0.066, $p \leq 0{,}001$) and reversed to control level with both treatments (D8 OxATP 1.454 ± 0.051, $p \leq 0.001$; D8 OxATP + GM6001 1.4135 ± 0.0705, $p \leq 0.001$) (Fig. 4a-b). Conversely, the expression of the p75 receptor was not affected by nerve injury (D8 ACSF 1.569 ± 0.046; D8 CTR1.486 ± 0.083). However, both the treatments induced a sharp upregulation of the expression level (D8 OxATP 2.001 ± 0.108, $p = 0.024$; D8 OxATP + GM6001 2.891 ± 0.190, $p \leq 0{,}001$), confirmingWB results.These findings suggestan opposite role played by the high and low-affinity NGF receptors during the spinal neuroglial remodeling following nerve injury and the differential modulation of the purinergic and MMPs systems.

Discussion

Substantial modifications occur in the nervous system following nerve injury, depending on the cell features, type of stimulus, and time [25, 8, 26]. However, interactions between glial cells and neurons through the neural extracellular matrix drive the pathological changes after the nerve damage[27].The damage-induced purinergic signaling affects the neuroglial network while the matrisome undergoes a structural remodeling, making both P2XRs and MMPs promising targets for neuropathic pain treatment[28, 29]. Nonetheless, integrated purinergic and nECM modulation could help to better describe the complex synaptic model, adding the matrisome component to the already investigated neuroglial network.In the present study, we used the SNI model to evaluate the spinal cord molecular remodeling at D3 and D8 of treatment with OxATP alone or in combination with GM6001, which inhibit the purinergic activation and MMPs, respectively.However, we detected that the most significant modifications occur at D8, validatingthe relevance of temporal regulation during disease progression [28, 26].

Anti-inflammatory and antigliotic activityproduced by OxATP and GM6001 treatment has beenpreviouslyreportedby our group[30, 6, 31, 8], hence, examining the expression of the pivotal protein in the neuro-inflammatory network, we aim to find one or more keystones that could support the interaction between the matrisome and the channel receptors inhibition. We reported that OxATP reduced GFAP and Iba1 expression, diminishing astrocytosis and microglial activation. However, an interesting outcome is given by the relevant reduction of microglial reaction in the D8by the administration of both OxATP and GM6001. Molecular and morphological analyses have shown that Iba1 is downregulated by inhibiting the purinergic signaling, while GM6001 co-administration seems to further reduce Iba1 expression and partially restore microglial morphology after one week of treatment(Fig. 1). This effect may be due to the contemporary inhibition of intracellular Ca^{2+} influx (mediated by OxATP) and the blockage of nECM maladaptive remodeling (mediated by GM6001), having a selective effect on microglial cells rather than astrocytes.

Activated microglia has been reported to specifically upregulate P2X4R, which drives the inflammation in neuropathic pain models [32, 33].We demonstrated a decrease of P2X4R with OxATP and a possible additional effect given by MMPs inhibition (Fig. 2), supporting a pathway connection between purinergic receptor expression and the nECM modulation, to be in future fully elucidated.

Cells'reaction to insult and inflammatory response involves epigenetic changes. Histones deacetylases or demethylases of lysine residues regulate chromatin access to transcriptional factors with repression or activation of specific genes [34]. While HDAC1 protein expression was not modified by the nerve injury, HDAC2 was upregulated, suggesting that class I HDACs are not functionally equivalent [35]. The spinal upregulation of HDAC2 following SNI was consistently associated with astrocytes and may be involved in the downregulation of glial glutamate transporters GLAST and GLT-1, causing the glutamate spillover in the synaptic cleft and the synaptic dysfunction[6, 36]. Moreover, glutamate metabolism abnormalities have been shown in MECP2 (methyl-CpG-binding protein 2) null astrocytes, a transcriptional co-repressor thatcan bind HDACs[37]. HDAC2 expression was downregulated by purinergic antagonism,as also occurred after the MMPs modulation [8], and theconcomitant administration of the two drugs produceda higher effect, reverting the increase reported in the D8 ACSF group (Fig. 2). Remarkably, the intraspinal infusion of the HDAC inhibitor valproic acid significantly improved rats' recovery following PNI and reduced P2X4 receptor expression on microglia[16]. Moreover, MMP9 downregulationhas been shown in response to subcutaneous administration of valproic acid after spinal cord injury [38].HDAC1 expression, in our previous work, was found upregulated following MMPs inhibition[8],showinga possible positive feedback loop.However, thisexclusive link faded when the animals had combined treatment, suggesting that P2XRs activation could be an intermediate player in selective chromatin processing and matrix remodeling.

Neurotrophic support is determinant for the suitable activity of the neural circuitry, especially after injuries[7, 39], while MMPs inhibition changes neurotrophins availability and their activation or degradation[31], P2XRs modulation could prevent MMPs activation or affect precursor neurotransmitter release and nerve growth factor signaling [40, 41, 42].

The NGF precursor proNGF is proteolyzed by plasmin tomature NGF and then inactivated by MMPs. In previous studies, we demonstrated an NGF enrichment of the ECM by administering GM6001[5, 31].We studied the NGFreceptors, high-affinity TrkA, and low-affinity p75 expression (Fig. 2–3), andwe found an opposite trend concerning the expression of the receptors. The upregulation of TrkA following SNI was foundas previously reported[24, 8].OxATP treatment alone and in combination with GM6001 reduced TrkA expression while determining p75 upregulation. TrkA decrease could be adaptive to the NGF matrisome enrichment, while p75 can generate pro-survival or pro-apoptotic signals depending on co-receptors and different intracellular pathways in the neuronal and glial cell types[43, 7]. Therefore, we previously showed that a p75 upregulation in the dorsal horn was associated with a reduction of neuropathic behavior as well as adaptive plasticity modification to the PNI [24].

This work analyzed the expression of key molecules in the matrix-neuroglial network,contributing to define the impact ofthe purinergic and MMPs inhibition in the spinal cord following PNI. The resulting data suggest complex protein interconnections, which are not passively influenced by epigenetics but actively contribute to modify the transcriptomics machinery. Purinergic and MMPs inhibitory modifications affected cellular/matrix balance at various levels, suggesting a connection between the extracellular matrix and the chromatin rearrangement.The relations between P2XRs, MMPs, and

epigenetics need to be further studiedin future experiments. These may help to identify targeted treatment in neurological diseases with benefits for neuroprotection, spinal plasticity, and functional recovery.

Declarations

Ethics Approval This study was performed in accordance with the guidelines of Italy and Europe for the Care and Use of Laboratory Animals (EU Directive 2010/63). All protocols were approved by the Animal Care and Use Committees of the University of Campania “Luigi Vanvitelli”.

Consent to participate not applicable

Consent for Publication not applicable

Availability of data and materials The datasets and materials generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests The authors declare that they have no relevant financial interests to disclose.

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Authors’ contributions All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Ciro De Luca, Assunta Virtuoso, and Michele Papa. The first draft of the manuscript was written by Ciro De Luca and Assunta Virtuoso. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Disclosure of potential conflict of interest The authors declare that they have no conflicts of interests.

Research involving Human participants and/or Animals This study was performed in accordance with the guidelines of Italy and Europe for the Care and Use of Laboratory Animals (EU Directive 2010/63). All protocols were approved by the Animal Care and Use Committees of the University of Campania “Luigi Vanvitelli”.

Informed consent not applicable

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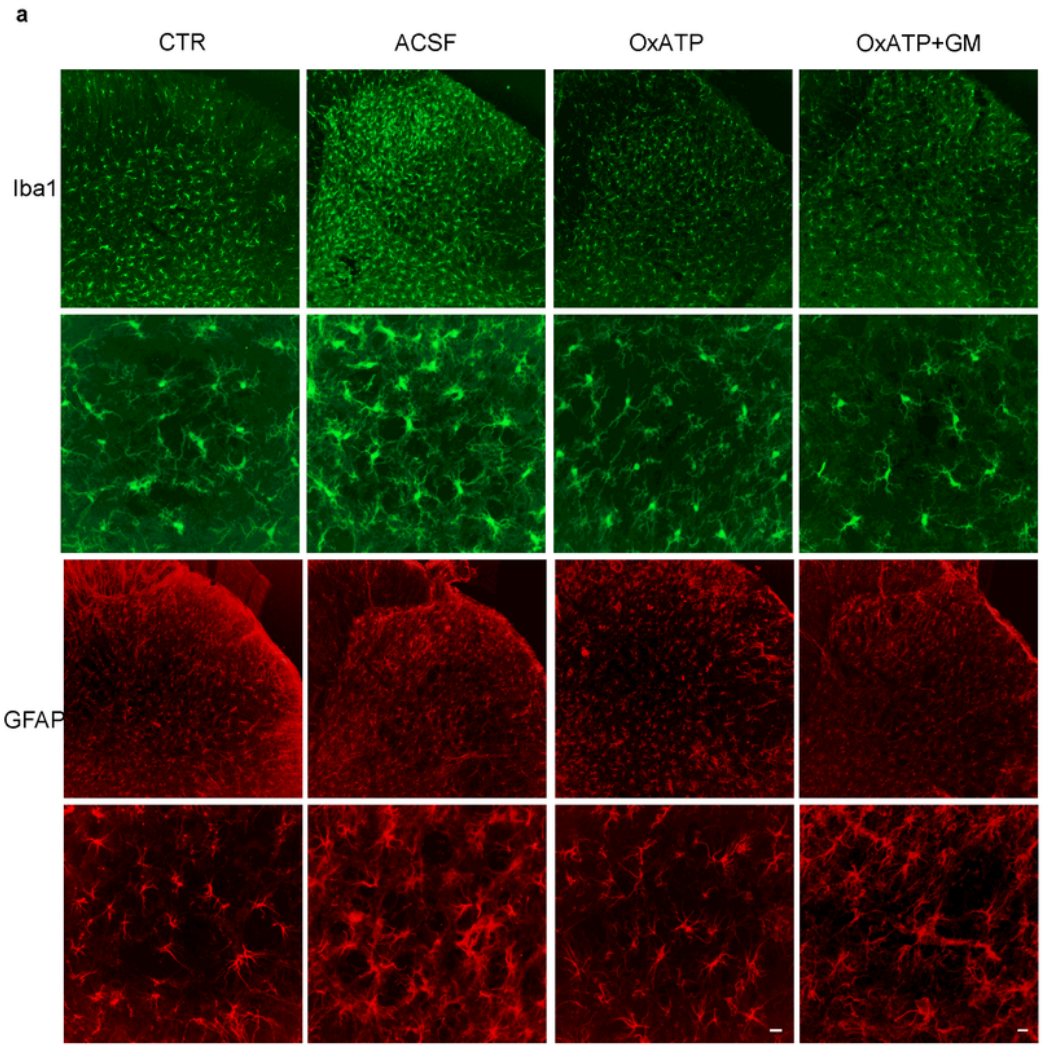
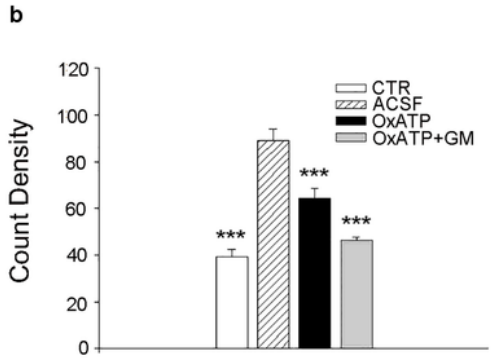
Figures





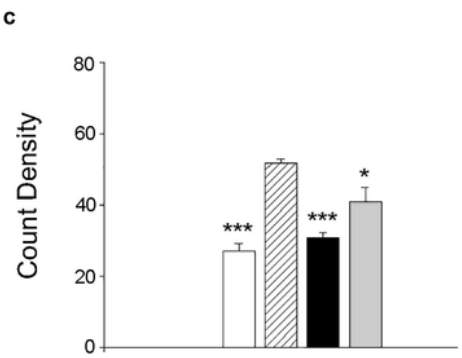


Figure 1

Reactive gliosis is reducedby purinergic inhibition, even in combination with MMPs modulation. a Low and high magnification representative images of immunofluorescence staining for Iba1 and GFAP in the dorsal horn of lumbar spinal cord in sham-operated (CTR) and SNI animals treated with ACSF or OxATP or OxATP+GM6001(GM) for 8 days.(Scale bar: 50 μm). b-c Densitometric quantitation of Iba1 and GFAP fluorescence versus the ACSF group. Data are shown as the mean value of the data distribution (bars) ± SEM, n=4/group (*p≤0.05; **p≤0.01; ***p≤0.001; One way ANOVA followed by post hoc Holm-Sidak correctionfor multiple pairwise comparisons).

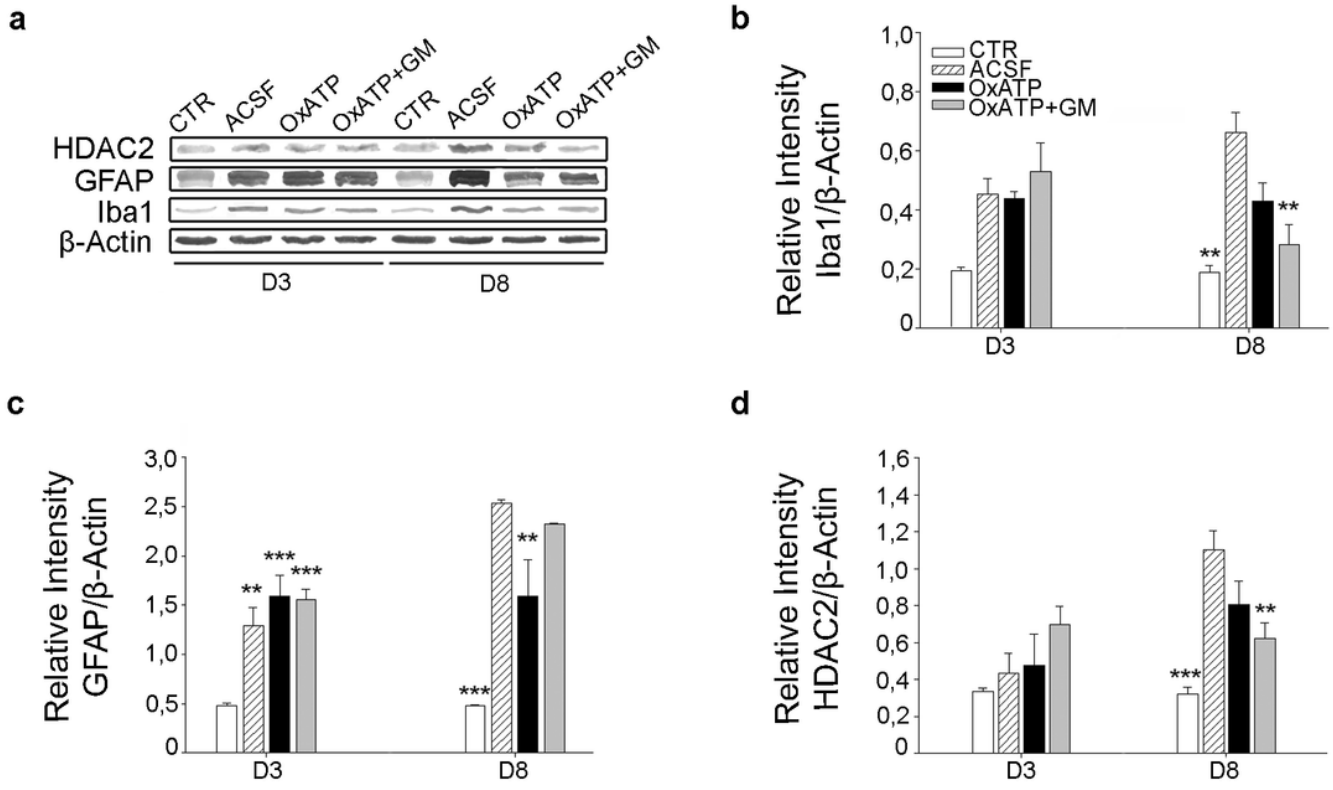


Figure 2

Reactive gliosis and epigenetic targets are affected by OxATP chronic treatment or in combination with GM6001. a Representative immunoblot of Iba 1, GFAP, and HDAC2 in the lumbar spinal cord from sham-operated (CTR) and SNI animals treated with ACSF or OxATP or OxATP+GM6001(GM) at D3 or D8. b-d Corresponding quantitation of Iba1, GFAP, and HDAC2 immunoblotsversus the ACSF group. Normalized data relative to the β-actin content are presented as the mean value of the data distribution (bars) ± SEM, n=4-5/group (*p≤0.05; **p≤0.01; ***p≤0.001; One way ANOVA followed by post hoc Holm-Sidak correction for multiple pairwise comparisons).

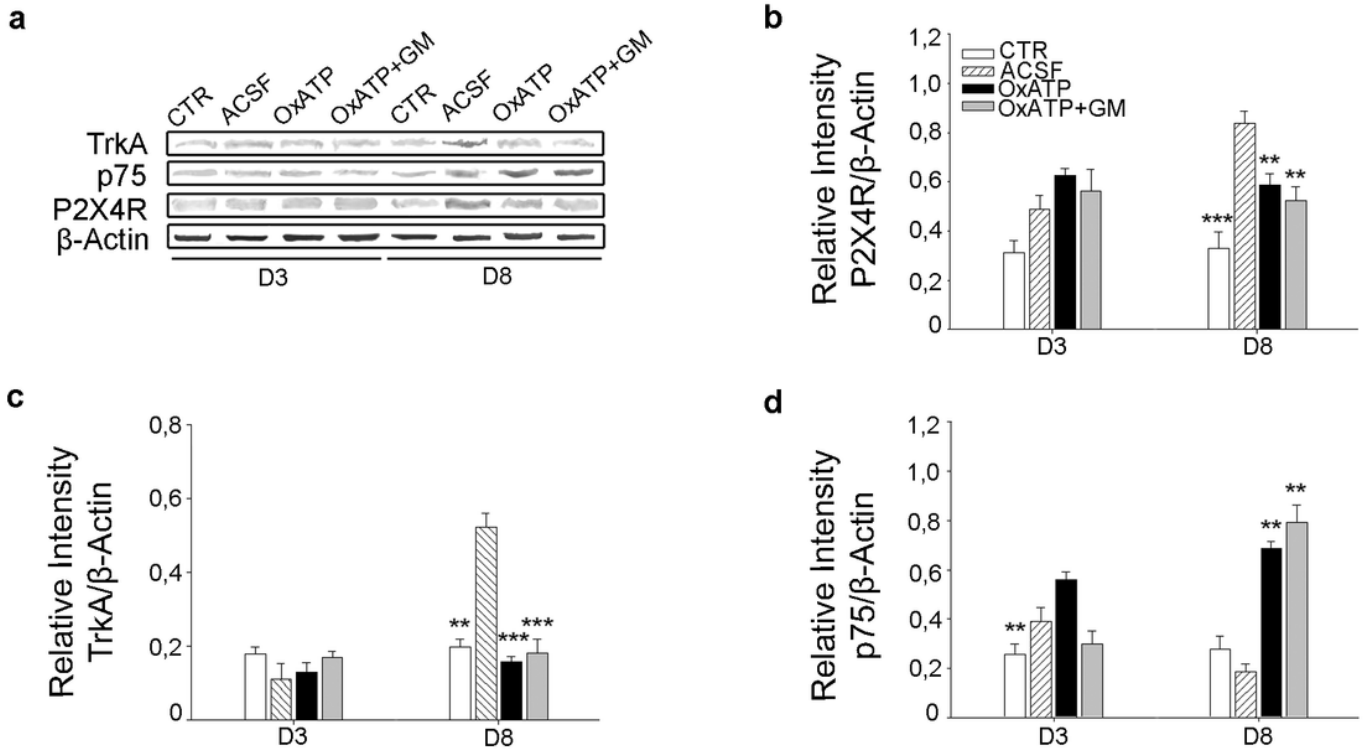


Figure 3

Purinergic and neurotrophic receptor protein expression are modified after SNI and the treatment with OxATP or OxATP+GM6001 (GM). a Representative immunoblot of P2X4R, TrkA, andp75 receptors in the lumbar spinal cord from sham-operated (CTR) and SNIanimals treated with ACSF or OxATP or OxATP+GM6001(GM) at D3 or D8. b-d Corresponding quantitation of P2X4R, TrkA, and p75receptors immunoblots versus the ACSF group. Normalized data relative to theβ-actin content are presented as the mean value of the data distribution (bars)± SEM, n=4-5/group(*p≤0.05; **p≤0.01; ***p≤0.001; One way ANOVA followed by post hoc Holm-Sidak correction for multiple pairwisecomparisons).

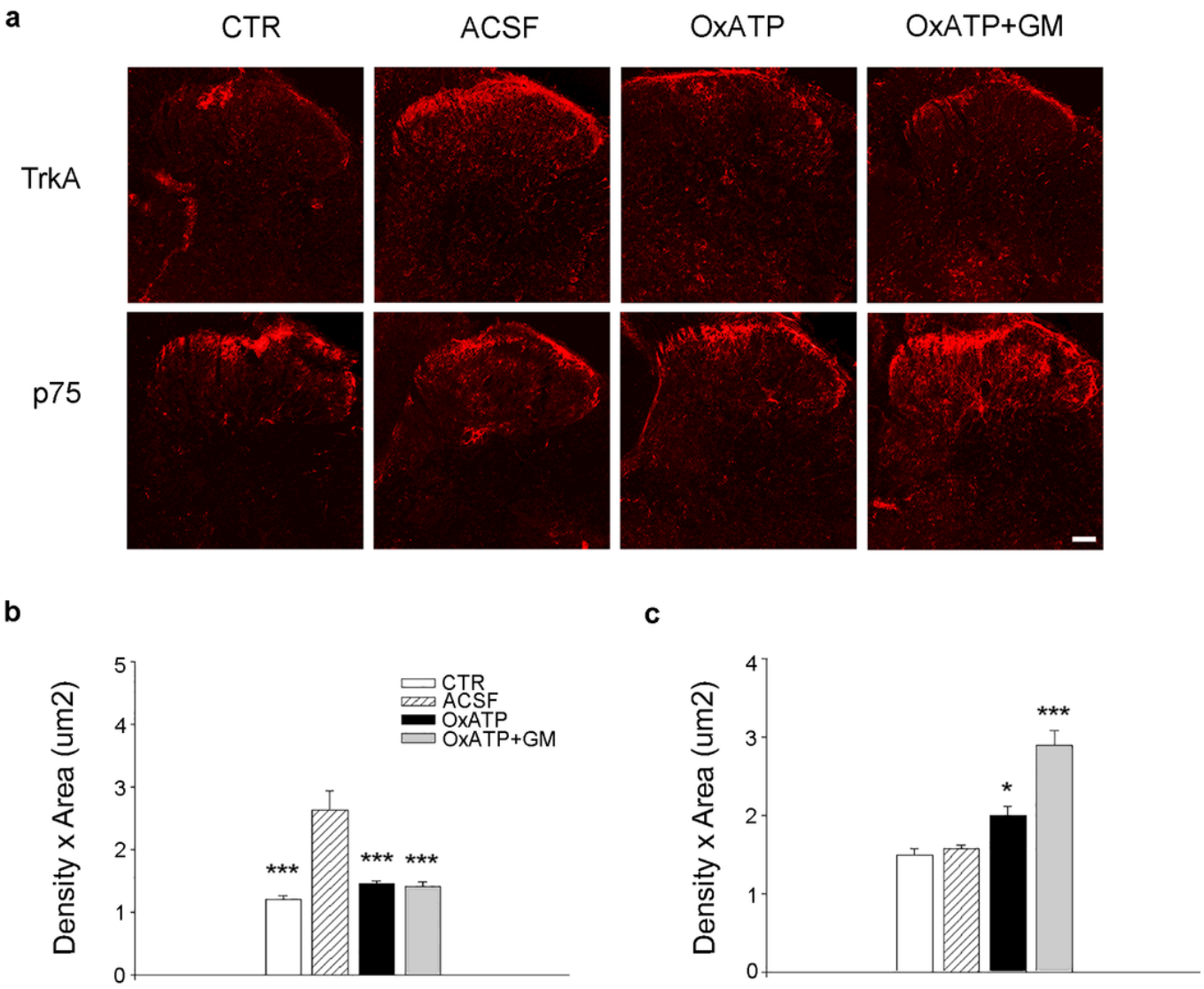


Figure 4

Neurotrophic receptors are differentially modulated by OxATP, also in combination with GM6001. a Representative image of immunofluorescence staining for TrkA and p75 in the dorsal horn of lumbar spinal cord in CTR rats, after SNI and OxATP treatment with or without GM6001 (GM) for 8 days. (Scale bar: 100 μm).b-c Densitometric quantitation of TrkA and p75 fluorescence versus the ACSF group. Data are shown as the mean value of the data distribution (bars) ± SEM, n=4/group (*p≤0.05; **p≤0.01; ***p≤0.001; One way ANOVA followed by post hoc Holm-Sidak correction for multiple pairwise comparisons).