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Valproic acid ameliorates morpho-dysfunctional effects triggered by Ischiatic nerve crush injury-induced by compression model in mice: Nerve regeneration and immune-modulatory pathway

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ABSTRACT

Peripheral nerve injuries are extremely severe and may lead to permanent disability, despite the regenerative capacity of the peripheral nervous system (PNS). To date, there is no established pharmacological therapy capable of predicting functional recovery and alleviation of trauma-related symptoms such as neuropathic pain, inflammation and weakness, which are the main targets for current therapies. In this work we provide new evidence for a therapeutic use of valproic acid (VPA) upon ischiatic nerve injury. Ischiatic nerve-injured mice treated with VPA after lesion, displayed an improvement in pain and motor function associated with an increase in the number of myelinated nerve fibers, and exhibited a more organized microenvironment during regeneration. In addition, VPA treatment also promoted an immunomodulatory capacity, leading to a significant enhancement of neutrophils in the peritoneal cavity, suggesting its role on the sensory and motor recovery after ischiatic nerve injury. This highlights the physiological role of VPA during ischiatic nerve regeneration and contributes to the characterization of innovative pharmacological epigenetic therapy capable of accelerating peripheral nerve regeneration with critical impacts on the clinical practice.

1. Introduction

Peripheral nerve system (PNS) injuries occur frequently, with over 300,000 cases reported in Europe. It is estimated that more than 200,000 peripheral nerve repair procedures are performed annually in the United States (Khalifeh, 2022). Such injuries can be extremely severe, leading to permanent disability, despite the regenerative capacity of the PNS. Approximately 33 % of these patients do not achieve full functional nerve recovery, resulting in partial or total loss of motor and sensory function, chronic pain, muscle atrophy, and weakness. Consequently, peripheral nerve functional deficits can often lead to lifelong morbidities and permanent disability (Wang et al., 2018). In fact, traumatic PNS injuries represent a significant clinical challenge and, therefore, restoring the function of damaged PNS remains a continuous challenge in basic and translational research. Despite considerable efforts to improve existing repair techniques and develop new approaches, the percentage of patients achieving complete functional recovery, and the extent of that recovery, has not significantly increased in almost 70 years (Foy et al., 2022). To date, there is no established pharmacological therapy in clinical practice capable of predicting functional recovery and the quality of PNS regeneration (Bota and Fodor, 2019). Currently available pharmacological treatment options for PNS injuries typically aim to alleviate trauma-related symptoms such as neuropathic pain, inflammation, and weakness, without affecting regeneration kinetics. Thus, many studies aim to characterize innovative pharmacological treatments that can accelerate the rate of regeneration, preserve neuron viability, and enhance axonal precision in target organs.

In this sense, epigenetic therapy emerges as an interesting and promising option for treating PNS injuries. Preclinical studies have

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shown that Histone Deacetylase Inhibitors (iHDAC) such as vorinostat, entinostat, valproic acid, and phenylbutyric acid have modulatory effects on neuroinflammation and make significant contributions to the treatment of neurodegenerative diseases (Gladkova et al., 2023). In the present study, we employed valproic acid (VPA), a classical and efficient iHDAC, at concentrations corresponding to those commonly used in clinical practice (Tremolizzo et al., 2005). Previous data from the literature have demonstrated that VPA stimulates neurite growth, promotes axonal regeneration in specific neurological conditions, and delays neuronal apoptosis in degenerative neurons (Biermann et al., 2010). Additionally, VPA has been shown to protect neurons from glutamate-induced excitotoxicity and injury due to oxygen deprivation and oxidative stress (Göttlicher, 2001; Kong et al., 2017). Remarkably, sciatic nerve transection in rats, followed by oral VPA treatment, resulted in improved regeneration quality and significant enhancement in motor function rehabilitation (Cui et al., 2003). In fact, clinically relevant therapeutic concentrations of VPA produce effects akin to neurotrophic factors, such as the promotion of neuronal growth and survival (Wu et al., 2008).

It has been shown that VPA acts by inhibiting Class I HDACs (HDAC 1, 2, 3, and 8) resulting in gene expression regulation with critical impacts on various biological processes such as cell survival, transcriptional regulation, ionic homeostasis, and cytoskeletal alterations (Nalivaeva et al., 2009). Additionally, it was observed that VPA significantly downregulates pNFκB levels and the release of pro-inflammatory cytokines, suggesting its potential immunomodulatory effects (Nalivaeva et al., 2009). In addition, VPA treatment led to the hyperacetylation of regulatory regions of the Brain-Derived Neurotrophic Factor (BDNF) gene leading to increased BDNF expression, further contributing to the neuroprotective effects of VPA (Shnayder et al., 2023). BDNF is a very well-known protein involved in promoting both adult neuron survival and the differentiation of new neurons, as well as synaptogenesis, in both the central nervous system (CNS) and the PNS, bridging, in this way, the mechanistic effects of VPA and physiological aspects (Brigadski and Lessmann 2020).

In this work we provide new evidence for a therapeutic use of VPA upon ischiatic nerve injury. Ischiatic nerve injured mice, treated with VPA starting 24 hours post injury and during 14 consecutive days, displayed an improvement in pain and motor sensitivity associated with an increase in the number of myelinated nerve fibers, displaying a more organized microenvironment during regeneration. In addition, we showed for the first time that VPA treatment also displayed an immunomodulatory capacity, leading to a significant enhancement of neutrophils in the peritoneal cavity, suggesting its role on the sensory and motor recovery, after ischiatic nerve injury. This study highlights the physiological role of VPA during ischiatic nerve regeneration and contributes to the characterization of innovative pharmacological epigenetic therapy capable of accelerating peripheral nerve regeneration, with critical impacts on the clinical practice.

2. Materials and methods

2.1. Animal proceeds

We have used a total of 68 animals. Home-breed male C57Bl/6 mice ranging from 8 to 10 weeks and weighing between 19 and 25 g were randomly allocated into four experimental groups: sham (group 1; sham), ischiatic injury without treatment (group 2; injury), ischiatic injury treated with saline (group 3; injury+saline) and ischiatic injury treated with VPA (group 4; injury+VPA). Five and eight animals per group were used in all morphological studies and functional experiments, respectively. For cytometry analysis we used 4 animals per group. Mice were deeply anesthetized with ketamine (100 mg/kg) and xylazine (15 mg/kg) intraperitoneally and, using a surgical magnifying glass (Opto FI04, SP, Brazil) an incision on the posterior aspect of the proximal third of the thigh was performed to expose the ischiatic nerve. Nerve crushing was then performed 2 mm below the exit of the ischiatic nerve from the greater ischiatic foramen using a Dumont #5 forceps (Fine Scientific Tools) for 1 minute. Nerve crush injury results in axonotmesis which is characterized by disruption of axons and their myelin sheaths with relative sparing of the connective tissue of the nerve. In the sham group, the same procedure was carried out as described above, but the nerve was only exposed, and no crushing was performed. After the surgical procedures, the muscles were repositioned over the nerve, and the skin was sutured with 6.0 nylon monofilament. Immediately after surgery, animals were kept warm for post-anesthetic recovery and then returned to their cages with free access to water and food, and housed at a 12 h day/night cycle. The experimental protocol complies with ethical procedures and was authorized by the Commission for the Evaluation of the Use of Animals in Research of the Health Sciences Center/UFRJ (Protocol n° 057/19).

2.2. Treatment administration

The treatment was performed intraperitoneally using a syringe and during 14 consecutive days, starting 24 hours post-surgery and at day 21 the mice were euthanized. Sham and injury groups did not receive any injection along the 14 days of protocol; Injury+saline group received 100 µl of PBS (phosphate-buffered saline: NaCl (sodium chloride): 8 g, Na2HPO4 (disodium phosphate): 1.44 g, KH2PO4 (monopotassium phosphate): 0.24 g, KCl (potassium chloride): 0.2 g) and injury+VPA group received 100 µl of VPA in PBS at a concentration of (300 mg/kg (Chen et al., 2018; Depakene, Abbott Laboratories Brazil®, 250 mg).

2.3. Functional assessment

Sensory and motor response assessments were performed during the post-surgical period aiming to grade the sensory/motor disturbance and to evaluate animal recovery. The analyses were conducted one day before surgery and at 7, 14, and 21 days post-surgery. For the gait analysis of the animals, we employed the Ischiatic Function Index (IFI). For this, the animals were placed on a runway covered with white Canson paper (A4 140 mg/m^2). The hind paws were painted with water-based paint, and the animals were allowed to walk leaving imprints of their footsteps. The paws selected for measurements were chosen at a point where the mouse walked with a moderate step. Animals were allowed to walk until we obtained measurable markings (Inserra et al., 1998). For the IFI analysis, we measured toe spread parameters, comprising the distance between the first and fifth toes, and the length of the footprint, encompassing the distance between the third toe and the hind pad. These measurements were obtained from both the experimental and normal legs, and the IFI was calculated using the formula proposed by Inserra et al. (1998).

To analyze the recovery of painful sensitivity following the injury, we employed the pinprick test. For this the mice were placed in acrylic boxes whose floor is a mesh net equal to 5 mm^2 composed of 1 mm thick non-malleable wire, with mirrors positioned 25 cm below the experimentation boxes, facilitating the visualization of the animal's paw. An entomological pin was gently pressed on the plantar surface of the hind feet and five areas on the lateral aspect of the right hind paw, within the innervation area of the ischiatic nerve. Each area was tested twice and the responses were recorded as positive only when reactions such as quick paw withdrawal and vocalization were triggered by the stimulation (Navarro et al., 2007).

2.4. Morphological evaluation

After 14 days, mice were anesthetized, perfused intracardially with 4 % paraformaldehyde in phosphate buffer (0.1 M, pH 7.4) and the ischiatic nerves were dissected. A 0.5 mm segment (0.2 mm distal to the injury site) was obtained from each nerve and immersed in a 2.5 % glutaraldehyde solution in 0.1 M cacodylate buffer at pH 7.4, for at least

24 hours, under refrigeration. Subsequently, the ischiatic nerves were post-fixed for 60 minutes in 1 % osmium tetroxide, containing 0.8 % potassium ferricyanide and 5 nM calcium chloride in 0.1 M cacodylate buffer (pH 7.4), for 60 minutes and then washed for 5 minute in 0.1 M cacodylate buffer at pH 7.4 and placed in a 1 % aqueous solution of uranyl acetate overnight, under agitation. The samples were washed in distilled water for 5 minutes and dehydrated in increasing concentrations of acetone (30 %, 50 %, 70 %, 80 %, 90 %, 100 %). Immediately after dehydration, the material was infiltrated in a 1:1 mixture of 100 % acetone and resin (Poly/Bed® 812, Polysciences), overnight in the stirrer. The material was then infiltrated in 100 % resin for 12 hours, and left at 60ºC for 48 hours for resin polymerization. Semi-thin transverse sections of 500 nm were obtained using an ultramicrotome (MT-6000-XL-RMC, Inc.), placed on glass slides, and stained with toluidine blue. Ultra-thin nerve cross-sections were also processed, with a thickness between 60 and 70 nm and collected on copper grids with 300 squares. For morphological analysis, semi-thin sections were photographed through an optical microscope (Zeiss Axioskop 2 plus) with Axiovision Rel. 4.5 software at 40x magnification. The total number of myelinated nerve fibers and blood vessels were quantified at 100x magnification.

For the quantification of the total number of myelinated fibers and blood vessels, the tibial branch of the ischiatic nerve (n=6) was used. For the quantitative analysis of myelin area, axon area, nerve fiber area, and G-ratio, the same semi-thin sections were photographed in five different fields and analyzed using Image J (1.42q). The G-ratio is a well-known and commonly used parameter to determine the degree of myelination of nerve fibers. This parameter, which has a direct relationship with both the thickness of the myelin sheath and the conduction velocity of the nerve impulse, is calculated as the ratio between the diameter of the axon (d) and the diameter of the nerve fiber (D) (d/D). For the ischiatic nerve, the best value of the G-ratio falls between 0.55 and 0.68 (Chomiak and Hu, 2009).

2.5. Flow cytometry and morphological analysis

On the third day of the protocol, mice from all experimental groups were euthanized, and cells from the peritoneum were collected. To this end, 5 ml of PBS were aspirated using a syringe and injected into the peritoneal cavity of each mouse, followed by a slight massage to allow the cells to detach and recollect them with the same syringe previously used, aspirating them back to the syringe. The goal was to perform a peritoneal wash with PBS to collect the cell populations present there, as the peritoneum was the site of VPA administration. The fluid obtained from the peritoneal cavity, now containing the cells from this body compartment diluted in PBS, was placed into a 1.5 ml eppendorf tube and centrifuged at 1200 g for 5 minutes to pellet the cells obtained by aspiration. After centrifugation, the liquid was discarded, the pellet was resuspended in 1 ml of PBS, and an aliquot of 10 microliters was taken for cell counting in the Newbauer chamber. For phenotypic analysis, cellular suspensions were incubated with Fc-blocking antibody, produced by the 2.4G2 clone obtained from the Rio de Janeiro Cell Bank for 10 minutes at 4ºC. The cell suspension was washed with PBS and incubated with the monoclonal antibodies CD11b FITC (BD Bioscience, 553310) and anti-Ly6G APC (Biolegend, 127613). Samples were analyzed from the positive 7AAD (BD Pharmingen, 559925) cells, which identify only viable cells. Analyses were performed using the FACSAria II cytometer (BD Biosciences) from a total of 100.000 events acquired for each sample through FlowJo software V10.

2.6. Statistical analyses

All results obtained were analyzed using GraphPad Prism 8 (Graph Pad Software, Inc., San Diego, California, USA) through the Two-Way Anova or One-way ANOVA test with Tukey's post-test. Values were presented as mean and standard error. A confidence interval of 95 % was accepted. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

3. Results

3.1. VPA treated mice display an improvement in motor function and pain after ischiatic nerve injury

Fig. 1A shows the time-course of performed experiments. To assess the effectiveness of VPA therapy on pain function, we employed the pinprick test. In the first week, the injury+VPA (3.875±0.125) experimental group demonstrated a statistically significant improvement in painful sensitivity compared to the injury (3.000±0.267) and injury+saline (2.875±0.295) groups. Similarly, in the second week, the injury+VPA group also exhibited a significant improvement in pain function compared to the injury and injury+saline groups, and similar to the sham group (5000±0.000). At the end of the protocol (21 days), all groups reached the maximum score (Fig. 1B).

Aiming to better characterize the motor function in the experimental groups, we analyzed the Ischiatic Function Index (IFI). In the first week after surgery, the IFI showed a deterioration in motor function in the injury (-34.513±8.764), injury+saline (-44.761±9.958) and injury+VPA (-37.251±5.821) groups with a statistically significant difference compared to the sham group. However, in the second week, the injury+VPA group exhibited an improvement in the motor function with a statistically significant difference compared to the injury and injury+saline groups but similar to the sham experimental group without statistical significance. At the end of the protocol (21 days) no significant differences were detected among the experimental groups (Fig. 1C).

Thus, we conclude that VPA leads to an improvement in pain and in motor function upon ischiatic injury.

3.2. VPA treated mice display an increase in the number of myelinated nerve fibers and a more organized microenvironment after ischiatic nerve injury

Aiming to gain more insights into the main morphological changes evoked by VPA treatment after ischiatic nerve injury, we performed histological analysis and morphometric quantifications. For this, the tibial portion of the ischiatic nerves obtained after two weeks (14 days) was used for quantitative and qualitative analyses of the nerve regenerative process. It was possible to observe qualitative differences in the semi-thin sections, highlighting that injury (Fig. 2B,F) and injury+saline (Fig. 2C,G) experimental groups exhibited differences when compared to the sham (Fig. 2A,E) group, which displayed a lower number of myelinated fibers and a more disorganized microenvironment. On the other hand, the injury+VPA experimental group (Fig. 2D,H) displayed a similar morphological pattern in comparison to the sham experimental group, exhibiting an increase in the number of fibers and a more organized microenvironment. In agreement, quantitative analyses revealed a significant increase in the total number of myelinated fibers in the injury+VPA (2344±20.090) group when compared to injury (1541 ±15.70) and injury+saline (1543±7.769) experimental groups (Fig. 2I). The number of blood vessels was increased in the injury+VPA (31.830 ±0.600) when compared to injury (27.000±0.365) and injury+saline (23.830±0.6009) (Fig. 2J). No significant differences were observed among sham and injury+VPA groups.

Semi-thin sections of the regenerating ischiatic nerve did not reveal statistically significant differences in axon area, fiber area, and myelin area among all experimental groups (Fig. 2K, L, M). G-ratio analysis did not show significant differences among experimental groups sham (692.333±84.696), injury (534.500±95.528), injury + saline (438.000 ±55.558) and injury + VPA (524.000±32.082) in the range of 0.55–0.68 (Fig. 2N).

Ultra-thin sections highlighted some relevant ultrastructural differences between the injury+VPA and injury+saline groups. The injury + saline group displayed thinner and more tortuous myelinated fibers, with less proximity to each other, indicating that they were less clustered (Fig. 3A). Additionally, this experimental group exhibited an

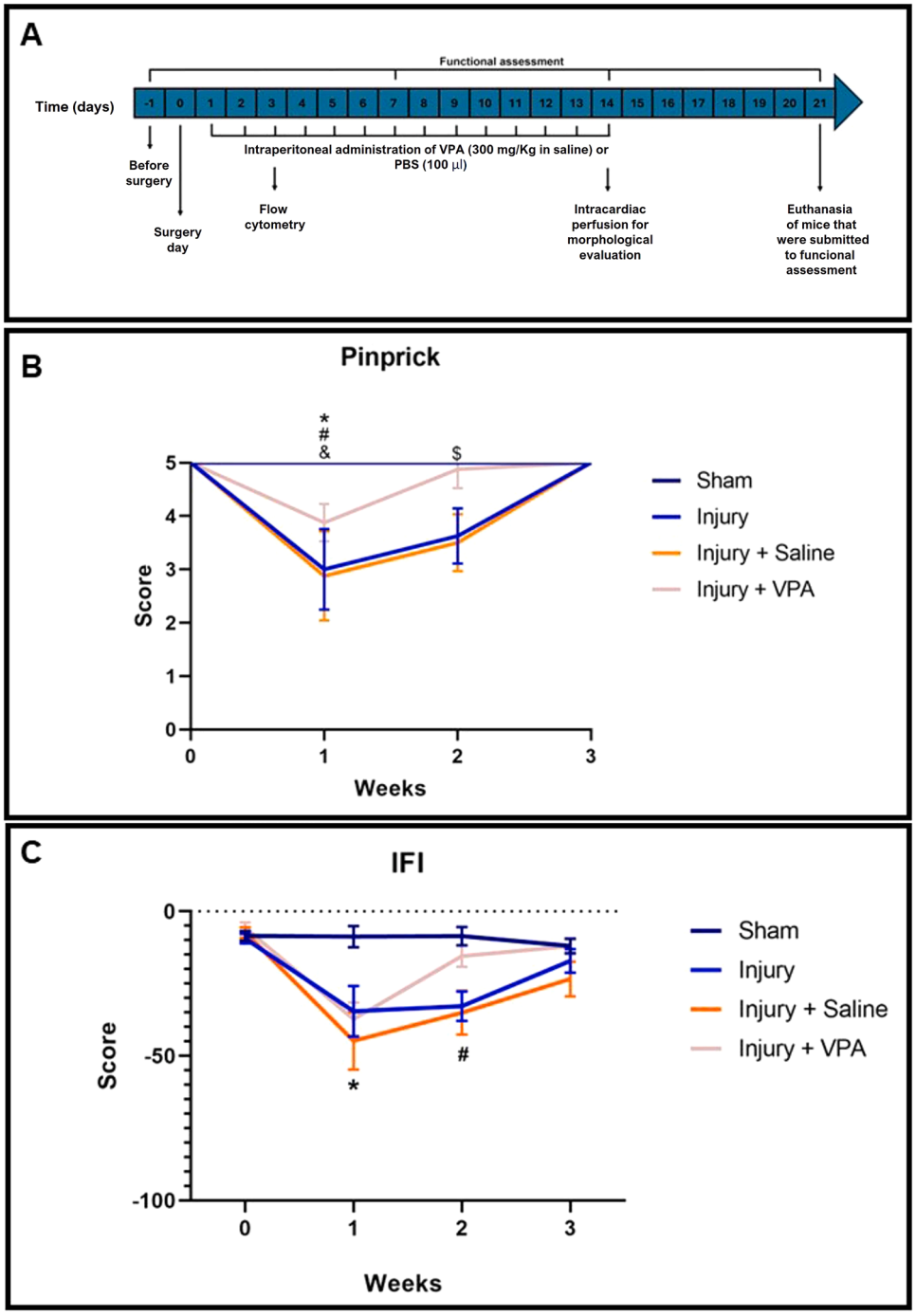

Fig. 1. VPA treated mice promoted an improvement in motor skills and sensitive pain upon ischiatic injury. (A) Schematic diagram of the experimental protocol. (B) Functional analysis using the pinprick test, where * indicates comparison among injury+VPA X injury and injury+saline with sham; # indicates comparison among injury+VPA X sham with injury and injury+saline; & indicates comparison among injury X injury+saline; $ indicates comparison among injury+VPA X sham with injury and injury+saline). (*), (#), (&), ($) $p<0.0001$; (C) Motor function analysis using the Ischiatic Function Index (IFI), where * indicates comparison among injury+VPA, injury and injury+saline and sham, # indicates comparison among injury+VPA and sham X injury and injury+saline), (*), (#) $p<0.0001$. The graph shows the variation in the tests before the injury and in the three weeks following the injury (21 days). (*. Values are represented as mean and standard error of the mean (SEM). N=8. Two-way ANOVA followed by Tukey's post-test.

abundance of myelinated fibers with axoplasm containing disintegrated cytoskeleton (Fig. 3A, arrow) and myelin ovoids, indicating axonal degeneration (Fig. 3A', asterisk). In contrast, ultra-thin sections from injury+VPA group displayed more rounded, thinner and more clustered myelinated fibers, with more compacted myelin sheath (Fig. 3B, arrow head), indicating healthy looking axoplasm (Figure B').

Thus, we conclude that VPA treatment leads to an increase in the number of myelinated fibers and contributes to a more organized microenvironment after ischiatic nerve injury.

3.3. VPA treatment promotes an immunomodulatory capacity on peritoneal neutrophils after ischiatic nerve injury

The literature has shown the key role performed by the cells of the innate immune system in modulating the inflammatory microenvironment after nerve injury focusing on neutrophils (Sas et al., 2020). In this

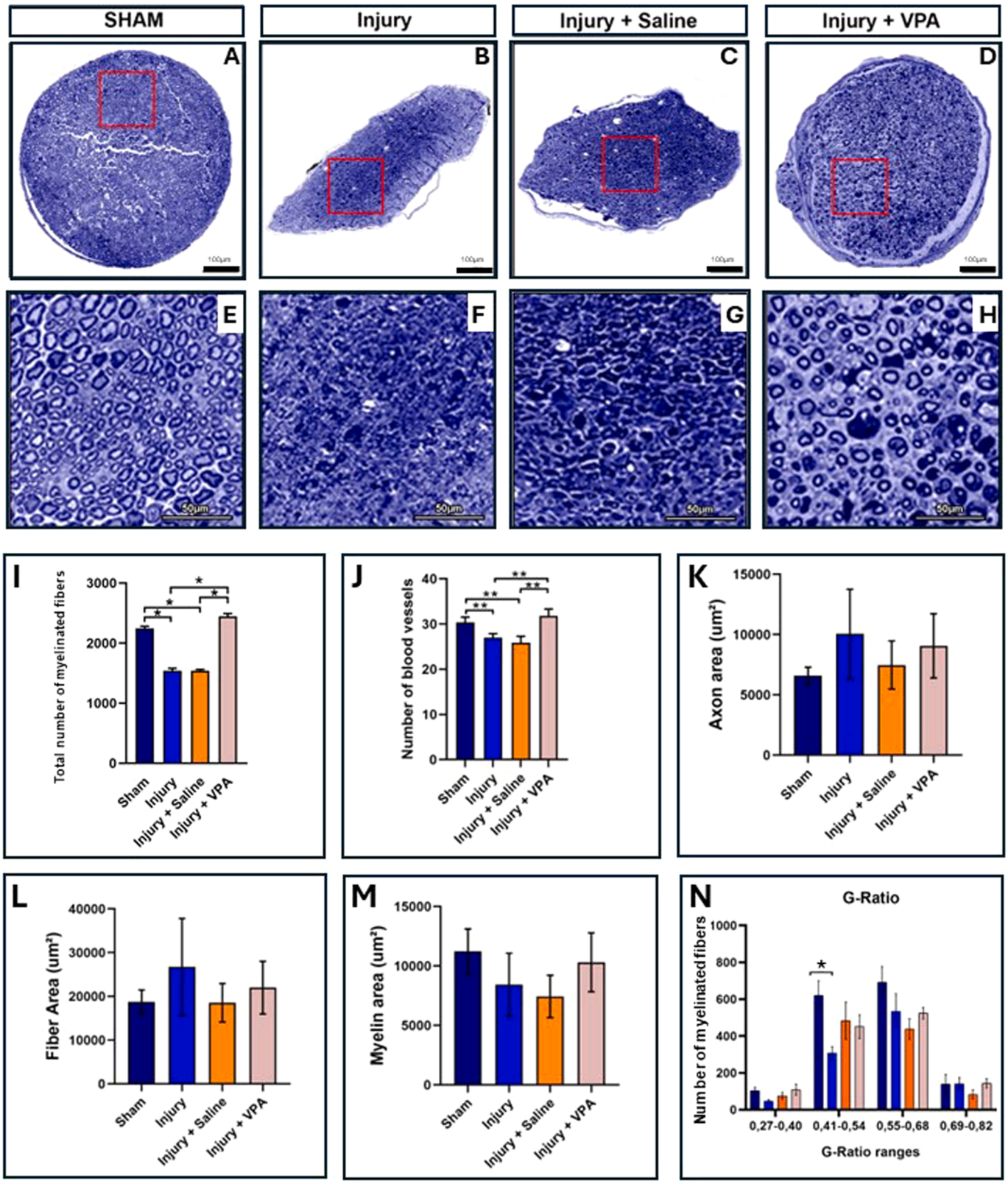

Fig. 2. VPA treated mice promoted an increase in the number of myelinated nerve fibers and blood vessels and a more organized nerve microenvironment after ischiatic nerve injury. Semi-thin sections of the tibial portion of the ischiatic nerve, distal to the injury site, in the Sham (A), Injury (B), Injury+saline (C) and Injury+VPA (D) experimental groups. Higher magnification of the area delimited by the red square in A, B, C, D displays the morphological pattern of the Sham (E), Injury (F), Injury+saline (G) and Injury+VPA (H) groups, respectively. (I) Quantification of the total number of myelinated fibers among experimental groups. (J) Quantification of the number of blood vessels among experimental groups. (K) Quantification of axon area among experimental groups. (L) Quantification of the fiber area among experimental groups. (M) Quantification of the myelin area among experimental groups. (N) Quantification of the number of myelinated fibers given by the G-ratio among experimental groups. N=5. Two-way ANOVA followed by the Tukey's post-test. * $p<0.05$; ** $p<0.01$. Values are represented as mean and standard error of the mean (SEM).

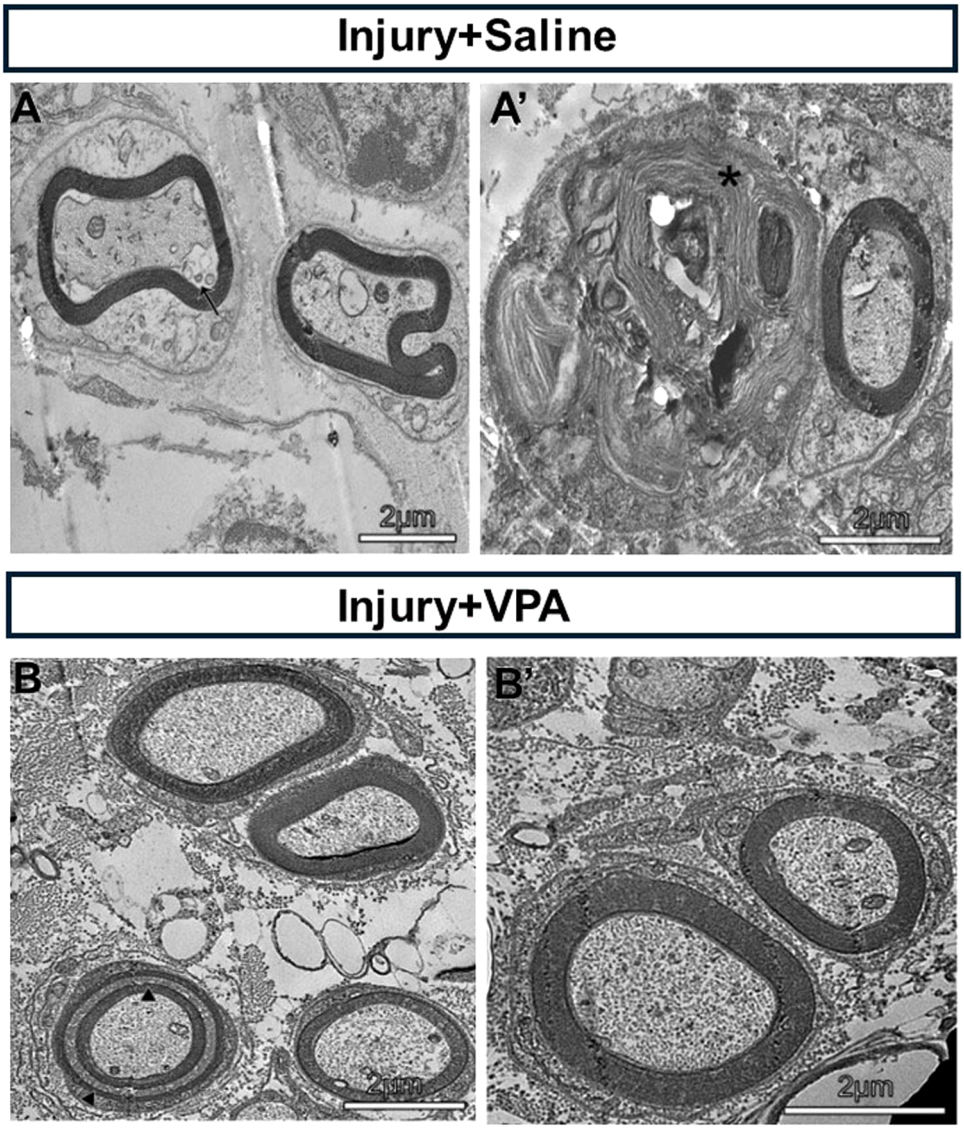

Fig. 3. Ultra-thin cross sections of injured nerves from Injury+saline or Injury+VPA experimental groups. (A) The injury+saline group contains more tortuous myelin fibers, with portions of axonal degeneration evidenced by cytoskeleton disintegration (arrow), and (A') myelin ovoids (asterisk). (B) Injury+VPA group presented more rounded, more clustered myelinated fibers, with myelin lamellae with greater compaction (arrowheads) in the same fiber. Figure B' indicates healthy looking axoplasm.

sense, we hypothesized that myeloid cells, more specifically neutrophils, would be modulated by VPA treatment. To gain insight into this hypothesis, we performed the characterization of the acute immune response after ischiatic nerve crush and VPA treatment using flow cytometry. On the fourth day after ischiatic nerve crush, still in the acute inflammatory phase, mice were euthanized and the total cells of the peritoneal cavity were collected and phenotyped using the antibodies CD11B and Ly6G. It was possible to note that mice from the injury+VPA experimental group showed a significant increase in the $CD11B^{+}/Ly6G^{+}$ subset when compared to the other experimental groups sham, injury, injury + saline (Fig. 4A,B,C,D,E). On the other hand, the $CD11B^{+}/Ly6G^{-}$ subset did not show significant differences among experimental groups, injury, injury+saline and injury+VPA. Morphological analysis corroborates flow cytometry findings and we morphologically identified segmented neutrophils and neutrophils still in the ring shape, which are immature cells and still capable of responding to inflammatory stimuli (Fig. 4D,D', inset, asterisk). The same was not observed in the other experimental groups (Fig. 4 A',A'', B',B'',C',C'').

Altogether, these results suggest that VPA treatment may be involved in the modulation of the behavior of neutrophils after ischiatic nerve injury in the peritoneal cavity.

Thus, we conclude that VPA treatment displays an immunomodulatory capacity, focusing on the neutrophil subset in the peritoneal cavity, with a potential role on the sensory and motor recovery after ischiatic nerve injury.

4. Discussion

In this study, we induced a crush injury to the ischiatic nerve in mice with the goal of evaluating the regenerative potential of VPA in a murine experimental model. It has been shown that VPA, an antiepileptic drug also used as a mood stabilizer, demonstrated beneficial effects on the functional and histological regeneration of the ischiatic nerve in rats (Rao et al., 2014). The Pinprick test aiming to assess painful function provided evidence that VPA therapy shows a significant effect on painful function compared to the control group. The statistically significant difference observed in the first two weeks after injury suggests that VPA may act as a pain modulator, providing effective relief compared to the

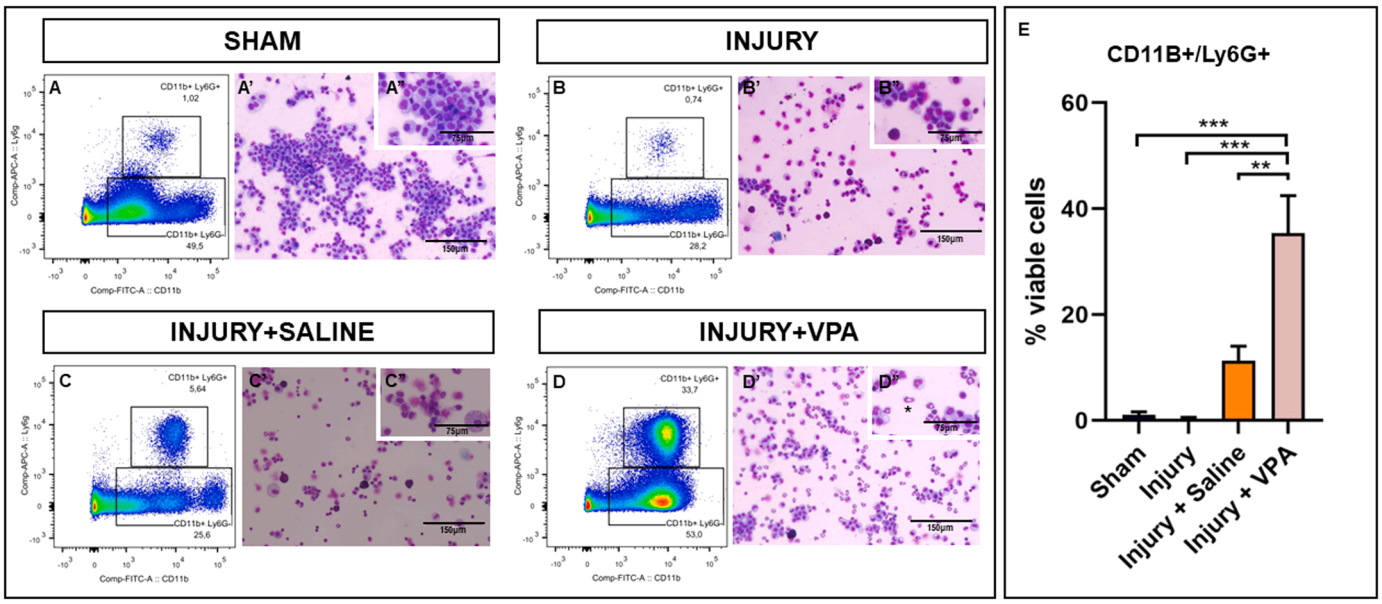

Fig. 4. VPA treatment promoted an immunomodulatory capacity on peritoneal neutrophils in ischiatic nerve injured mice. Characterization of the acute immune response in the peritoneal cavity by flow cytometry and morphology among experimental groups. (A) Sham group displayed 1.02 % (B) Injury group displayed 0.74 % (C) Injury+saline group displayed 5.64 % and (D) injury+VPA group displayed 33.7 % of CD11b+/Ly6G+ cells (neutrophils), respectively. (E) The differences observed in the of CD11b+/Ly6G+ subset were statistically significant among Injury+VPA and other experimental groups. The morphological analysis corroborated the flow cytometry phenotyping of Sham (A',A''), Injury (B',B''), Injury+saline (C',C'') and Injury+VPA (D',D'') experimental groups, highlighting the presence of immature neutrophils displaying ring-shaped nucleus only in the Injury+VPA group (Fig. 4 D', asterisk). N=4. One-way ANOVA followed by Tukey's post-test. Values are represented as mean and standard error of the mean (SEM). **p<0.01; ***p<0.001.

baseline condition. A relevant study corroborating these findings was conducted by Chen et al. (2018) and showed the effect of VPA on reducing the neuropathic pain sensitivity in rats by inhibiting neuronal excitability. In fact, previous work from the literature has evaluated the effects of VPA in animal models of inflammatory pain and showed that VPA reduced inflammation-induced pain by modulating signaling pathways involved in pain transmission (Guo et al., 2021). This was accomplished by modulating microglial function and inhibiting neuroinflammatory response (Guo et al., 2021). Thus we interpret that our findings are in agreement with the literature as the results obtained here argue in favor of a functional and histological regeneration of the ischiatic nerve upon VPA treatment.

Aiming to bridge functional and histological regeneration of the ischiatic nerve with motor function recovery, we performed functional analysis using IFI, which is an objective and quantitative measure to assess functional recovery after ischiatic nerve repair in murine models. IFI has proven to be an effective index for evaluating functional recovery after nerve injury and subsequent repair and has been widely used in experimental studies showing sensitivity to detect differences in nerve function over time after surgery (Inserra et al., 1998). In this sense, we observed a significant difference in the IFI in the first week post-surgery between VPA and other experimental groups, suggesting that ischiatic nerve repair initially affects functional recovery. In the second week post-surgery, significant differences were observed among VPA and sham groups, indicating that VPA treatment has a significant role in functional recovery. We interpret that VPA treatment might anticipate motor functional improvement. This interpretation is in agreement with previous studies demonstrating improved motor function in the VPA treated group in sciatic nerve transection models (Wu et al., 2008; Rao et al., 2014).

The analysis of the total number of myelinated nerve fibers revealed a significant increase in the injury+VPA. These findings suggest that the use of VPA may have a positive effect on promoting the growth and development of myelinated fibers. Previous studies have also reported similar results, showing that VPA can enhance myelination and the formation of myelinated fibers in animal models of nerve injury (Smith et al., 2019). It is believed that VPA exerts its effects through various pathways, including the increase in the expression of neural growth factors such as BDNF (Zhang et al., 2018).

Correlating the increase in the number of myelinated fibers with the improvement in sensitivity and motor function, as shown in our study, it is suggested that myelination plays a critical role on functional recovery upon nerve injury (Modrak et al., 2020). The quantification of the total number of blood vessels is also an important measure to assess angiogenesis and blood supply in specific tissues. In the present study, the injury+VPA treated group showed an equivalent number of blood vessels when compared to the sham group, showing that VPA may exert a pronounced effect on nerve vascularization in this specific study. It has been reported, mainly by works on cancer cells, that VPA and iHDACs in general act through inhibition of angiogenesis (Osuka et al., 2012). In this sense, our results support an alternate role for VPA in angiogenesis, probably regulating the behavior of immune cells, that in turn, may secrete pro-angiogenic factors that may lead to the observed phenotype. Further work will be necessary to better elucidate the effects of VPA on angiogenesis during sciatic nerve regeneration.

It is interesting to highlight that in other works VPA was delivered by local or oral administration (Wu et al., 2008; Rao et al., 2014), a relevant difference since in our work we delivered VPA intraperitoneally. It is important to highlight that the peritoneal cavity is an autonomous site displaying different cellular subsets of immune cells including monocytes, T lymphocytes, dendritic cells, natural killer cells, mast cells and neutrophils from the bone marrow (Mutsaers 2002). Thus, the delivery of VPA to the peritoneal cavity may modulate the behavior of these cells and influence the inflammatory response systemically since the transdiaphragmatic lymphatics vessels may make the cells and/or soluble factors, such as cytokines and chemokines, available for distant sites such as the ischiatic nerve. Although the effects of iHDACs on immune cells have been extensively explored in the literature, there is a lack of studies on the effects of these drugs on neutrophils biology and behavior. It has been shown that the treatment of neutrophils with VPA leads to disruptions in neutrophils behavior, such as an attenuation in phagocytosis and chemotaxis triggered by pathogens mostly due to the

increased expression of a benzodiazepine receptor (Caldiroli et al., 1998; Zhang et al., 2009). In agreement, additional work demonstrated a significant impairment in neutrophils chemotaxis upon VPA treatment in a model of intestinal ischaemia-reperfusion inflammation (Kim et al., 2012). These results are in line with the view that iHDACs act as anti-inflammatory modulators (Moran et al., 2023).

In this sense, we reasoned that VPA might play a role in improving the transition from the inflammatory to the resolutive phase and enhance tissue repair by regeneration. In our model, we hypothesized that VPA injection in the peritoneal cavity would modulate immune cells focusing in the neutrophil subset given that different works have shown a critical role for these immune cells during regeneration. For instance, in an *in vivo* study, Sas et al. (2020) characterized a $CD14^{+}/Ly6G^{low}$ subset of immature neutrophils after crush injury to the optic nerve in mice. In this study the authors characterized *in vivo* a new subset that stimulates the survival of neurons in the central nervous system and the restoration of axons. This subset of neutrophils displayed an enhanced expression of genes coding for type 2 cytokines and alternatively activated M2 macrophages, such as *Arg1, Mrc1, and Il4ra,* indicating that alternatively activated neutrophils play critical roles during tissue regeneration. In agreement with this hypothesis, *Arg1* and *VEGFα* expressing "N2" neutrophils were found in intratumor infiltrates and are associated with decreased anti-tumor immunity as well as tumor growth (Fridlender et al., 2009). In the present work, morphological analysis of peritoneal cells upon VPA treatment, showed neutrophils displaying ring-shape nucleus at 3 days post injury, suggesting that VPA might have a role in amplification as well as maintenance of immature neutrophils locally at the peritoneal cavity. Further studies are necessary to better characterize the neutrophil subset that is responsive to VPA treatment and elucidate whether their phenotypic and molecular features match with N2 neutrophils. This subset of innate immune cells might emerge as a putative target for therapeutic strategies aiming to improve neurological outcomes in individuals with central nervous system injuries.

A recent report revealed a new subset of immature innate immune cells, the neutrophils, with neuroprotective and regenerative properties in the peripheral nervous system upon tissue injuries (Balog et al., 2023). Another work has characterized zymosan modulation of neutrophils which displays neuroregenerative properties, providing insight into the heterogeneity of neutrophils and the unique subgroups that can be identified in different contexts (Jerome et al., 2022). In fact, the $CD14^{hi}$ subset of neutrophils improved axonal regeneration in injuries of both central and peripheral nervous systems, supporting the hypothesis that neutrophils have a beneficial effect in these injuries. In this sense, our results are in agreement with the main findings of the literature and showed for the first time a significant increase in the total number of the $CD11b^{+}/Ly6G^{+}$ subset in the peritoneal cavity of VPA treated mice. It is important to highlight that the peritoneal cavity is an autonomous site, covered by mesothelial surfaces, maintained by the continuous influx of myeloid and lymphoid cells from bone marrow, mesenteric milk spots and omentum, structures highly reactive to inflammatory stimuli composed by mesothelial cells which release cytokines (Mutsaers 2002). In this scenario, the inflammatory response in the peritoneal cavity could be locally or systemically controlled by the output and input of cells through transdiaphragmatic lymphatics vessels and by 'stomata', which are cavities between mesothelial cells, amplifying in this way, their repertoire, making inflammatory cells available to sites of tissue injury such as the ischiatic nerve. In this sense, further experiments will be necessary to track peritoneal cells at the injury site and corroborate the hypothesis that the peritoneal cavity would be a relevant source of inflammatory cells upon VPA injection.

Thus, our study offers a tractable epigenetic therapy to modulate the behavior of cellular innate immune subsets involved in nervous system regeneration focusing on neutrophils. Although VPA treatment showed positive impacts on pain control, functional recovery, and myelination, the significant increase of $CD11b^{+}/Ly6G^{+}$ neutrophils in the peritoneal cavity of the VPA treated mice still needs to be elucidated as a cellular mechanism that underlies the positive impacts observed in the functional recovery upon peripheral nerve injury.

5. Conclusion

Traumatic injuries of the PNS represent a significant clinical challenge, potentially causing damage to sensory and motor function. Despite considerable efforts to improve existing repair techniques and to develop new approaches, the percentage of patients achieving complete functional recovery, and the extent of that recovery, has not significantly increased in almost 70 years. In this work we investigated an epigenetic therapy strategy for ischiatic nerve injury based on VPA treatment. In fact, VPA treatment demonstrated a significant improvement in pain sensitivity and motor function associated with a significant increase in the number of myelinated fibers and blood vessels suggesting an acceleration in the regeneration process. In addition, a subset of CD11b+/Ly6G+ cells was significantly expanded in the peritoneal cavity of VPA treated mice indicating that VPA treatment exhibits immunemodulatory characteristics with significant regenerative potential. However, further studies are necessary to better address the physiological and molecular mechanisms involved in VPA treatment of ischiatic nerve injury and its potential translational effectiveness.

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CRediT authorship contribution statement

Kátia Carneiro: Writing – review & editing, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Thayse Pinheiro Da Costa:** Methodology. Marcia Cury El-Cheikh: Methodology. **Bruna Dos Santos Ramalho:** Methodology. **Greice Nascimento Pires:** Methodology. **Viviane de Oliveira Souza:** Writing – original draft, Methodology, Investigation. **Tiago Bastos Taboada:** Methodology. **Ana Maria Blanco Martinez:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: ANA M BLANCO MARTINEZ reports financial support by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). ANA M BLANCO MARTINEZ reports a relationship with Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). that includes: funding grants. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data Availability

Data will be made available on request.

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