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Remote Ischemic Post-conditioning Modulates Brain Expression of PIWI Proteins After Experimental Cerebral Ischemia

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Abstract

This study explores the involvement of PIWI family proteins in cerebral ischemia and their potential contribution to the neuroprotective effects of remote ischemic post-conditioning (RIC), a promising intervention for ischemic stroke whose underlying mechanisms remain to be fully elucidated.

Using a mouse model of transient middle cerebral artery occlusion (MCAo), we evaluated the effects of RIC induced by femoral artery occlusion (FAo) on brain injury, inflammation, and PIWI proteins expression in the brain. RIC significantly reduced infarct volume, cerebral edema, and neurological deficits, while attenuating the ischemia-induced upregulation of pro-inflammatory cytokines, including IL-1β and TNF-α, in the brain cortex. The ischemic insult induced a robust upregulation of PIWIL2 and PIWIL4 in the ipsilateral cortex, primarily in neurons, with PIWIL4 also detected in astrocytes and infiltrating myeloid cells. Notably, RIC suppressed this cortical upregulation, suggesting a potential link between PIWI proteins and ischemic damage. By contrast, in neurogenic regions of the hippocampus, RIC selectively enhanced PIWIL4 expression, particularly in neurons of the CA3 region and dentate gyrus. This increase was associated with elevated expression of the neurogenic and repair-related factors TGF-β1 and TREM2. Accordingly, in the subventricular zone of post-conditioned mice, PIWIL4 was localized in SOX2-positive neural progenitor cells that also express the proliferation marker Ki67, thus suggesting a role of PIWIL4 in RIC-induced neurogenesis.

These findings reveal a dual, region-specific role of PIWILs in ischemic stroke: contributing to injury in the cortex, while supporting regenerative processes in neurogenic regions. Given their involvement in cerebral ischemia, PIWI pathways emerge as promising targets for neuroprotection and recovery following stroke.

Introduction

Ischemic stroke remains a leading cause of mortality and long-term disability worldwide [1], with limited therapeutic options available for the acute phase, merely based on pharmacological and/or mechanical reperfusion [2]. Therefore, there is growing interest in elucidating the pathological mechanisms triggered by ischemia-reperfusion injury in order to identify novel therapeutic strategies to prevent tissue injury and/or to promote tissue repair and functional recovery.

Remote ischemic post-conditioning (RIC) consists in the application of brief, non-lethal ischemic episodes in a distal part of the body (typically a limb) after a major ischemic event in the brain. Recent meta-analyses have supported the evidence that RIC reduces infarct size and improves functional outcome in rodent models of stroke [3, 4]. Notably, the neuroprotective efficacy of RIC - induced by transient occlusion of the femoral artery in rodents subjected to transient focal cerebral ischemia - has recently been confirmed by a multi-centre preclinical trial, further underscoring its translational relevance [5–7]. Indeed, in patients, this intervention has emerged as a promising neuroprotective strategy for the treatment of ischemic stroke, both as a standalone treatment and in combination with conventional reperfusion therapies [8–11]. Various endogenous mechanisms have been shown to underlie brain

protection by RIC, including attenuation of blood-brain barrier damage, reduced inflammatory responses and neurovascular remodelling [12–18]. Nevertheless, the molecular pathways through which this intervention promotes post-ischemic recovery are poorly understood.

Proteins of the P-element-induced wimpy testis (PIWI) family are a subclass of Argonaute proteins that interact with PIWI-interacting small RNAs (piRNAs) and were first discovered for their crucial role in silencing transposable elements (TEs) in germline cells, thereby protecting genome integrity [19, 20]. However, research over the past decade has shown that PIWI proteins have broader functions in the regulation of gene expression [20, 21], beyond their canonical role in germline transposon silencing [22–24]. In fact, through the interaction with their piRNAs partners, PIWI proteins orchestrate post-transcriptional mRNA turnover [25], induce chromatin modifications [26] and sustain germline stem cell self-renewal [27].

In mice, PIWI proteins comprise three homologs, PIWIL1 (MIWI), PIWIL2 (MILI) and PIWIL4 (MIWI2), which show sequential and overlapping expression patterns during spermatogenic differentiation [28, 29]. Notably, PIWI proteins and piRNAs have also been identified in somatic tissues, including the brain, albeit at significantly lower levels than in the germline [30–32]. Growing evidence supports specific roles for PIWI proteins in neuronal polarization, cortical development and cell fate regulation of adult neural progenitors, both in vitro and in vivo [32–35]. Moreover, altered expression and aberrant localization of PIWI proteins were found in postmortem brain samples of patients with chronic neurodegenerative disorders, including amyotrophic lateral sclerosis, Alzheimer's and Parkinson's disease [36–38], highlighting their potential involvement in maladaptive brain responses.

Although altered piRNA profiles have been detected following ischemic stroke both in preclinical models [39] and in patients [40], their effector partners, the PIWI proteins, have not been systematically investigated in the context of brain ischemia. Therefore, here we aim at characterizing the expression dynamics and cellular localization of PIWI proteins in mice subjected to transient focal cerebral ischemia and their potential involvement in neuroprotection exerted by RIC.

Materials and methods

Animals

Adult male C57BL/6 mice (8–10 weeks of age, body weight: 26 ± 2 g) were obtained from Charles River Laboratories (Calco, Como, Italy) and maintained under controlled environmental conditions (i.e., ambient temperature of 22°C, relative humidity of approximately 65% and a 12h light/dark cycle). Food and water were provided ad libitum.

The in vivo procedures were conducted respecting the Italian Ministry of Health guidelines (DL 26/2014), in conformity with the 2010/63 European Directive, and the experimental protocol (n. 750/2022-PR) was approved by the local animal welfare organism (OBPA, University of Calabria) and by the Ministry of

Health at the National Institute of Health (Rome). Animal handling was conducted in compliance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) Guidelines 2.0 [41].

Sample size estimation was performed using an a priori power analysis to achieve 80% statistical power with a significance level of 0.05 (OpenEpi software version 3.01). Based on our previous experience with the middle cerebral artery occlusion (MCAo) model, we hypothesized an ischemic lesion volume difference of approximately 25 mm^3 - corresponding to an estimated 30% reduction in infarct size - between MCAo animals and those receiving a neuroprotective intervention. Assuming a standard deviation of 15 mm^3, the calculated minimum sample size was 6 animals per experimental group. Animals were randomly assigned to the following experimental groups:

(1) MCAo/FAo: mice were subjected to 1h MCAo followed (10 min after the beginning of reperfusion) by 10 min femoral artery occlusion (FAo), representing the post-conditioning stimulus;

(2) MCAo: mice were subjected to 1h MCAo followed, 10 min after the beginning of reperfusion, by a sham surgery at the level of the femoral artery;

(3) SHAM/FAo: mice underwent sham surgery (as a control for MCAo surgery) followed by 10 min FAo;

(4) SHAM: mice underwent only sham surgical procedures.

Animals were sacrificed 48 h after the onset of reperfusion following 1-h MCAo or the corresponding sham procedure.

Surgical procedure to induce focal cerebral ischemia and remote ischemic post-conditioning

Anaesthesia was induced by 5% isoflurane (Iso-Vet 1000 mg/g, Piramal Critical Care) vaporized in air and maintained throughout the surgical procedure at a lower concentration (1.5-2%). Body temperature was maintained at 37°C ± 0.5°C via a rectal probe connected with a feedback-controlled homeothermic blanket.

Focal cerebral ischemia was achieved by occluding the middle cerebral artery using a silicone-coated nylon filament (diameter: 0.23 mm, Doccol Corporation, Redlands, CA, USA). In accordance with the Longa method [42–44], the filament was introduced into the internal carotid artery (ICA) for 9–11 mm from the carotid bifurcation, until moderate resistance was felt, indicative of successful MCAo. Following a standardised protocol [6, 7], animals were classified as ischemic and thus included in the experimental analysis when they exhibited more than 3 of the following neurological signs (evaluated 45 minutes after MCAo): ellipsoidal palpebral fissure, lateral extension of one or both ears, asymmetric body bending, and/or lateral extension of the limbs. After 1h of MCAo, animals were re-anesthetized, and the filament was withdrawn to allow reperfusion. Sham-operated mice were subjected to the same surgical procedure, without filament insertion.

To induce RIC, after 10 minutes of reperfusion, the femoral artery was exposed and occluded for 10 min with a microsurgical clip. FAo was confirmed by visual inspection of the distal part of the femoral artery. To perform sham surgery, the femoral artery was exposed without vessel occlusion.

Assessment of neurological deficits and brain infarct quantification

Neurological deficits were assessed 48 h after MCAo or SHAM surgery using the dichotomized De Simoni composite neuroscore [45] that ranges from 0 (no detectable deficit) to 56 (the worst performance across all categories). Total scores represents the sum of 6 general deficits (fur [0–2], ears [0–2], eyes [0–4], posture [0–4], spontaneous activity [0–4], and epileptic behavior [0–12]) and 7 focal deficits (body asymmetry [0–4], gait [0–4], climbing [0–4], circling behavior [0–4], forelimb symmetry [0–4], compulsory circling [0–4], and whisker response [0–4]. Scoring was performed by an investigator blinded to experimental group allocation.

After the neurological assessment, mice were sacrificed under isoflurane anesthesia, and their brains were dissected and immediately frozen at -20°C. Fifteen (20 µm thick) coronal slices were cut at 0.5 mm intervals from the frontal pole using a cryostat, then mounted on glass slides and stained with cresyl violet. Images of the stained sections were acquired using a digital scanner and analysed with ImageJ (version 1.53, NIH, USA) analysis software to quantify infarct pale area and cerebral edema of each section. Infarct volume (mm^3) was calculated as the sum of infarct areas across all sections multiplied by the interval thickness between sections.

Real-time Polymerase Chain Reaction (PCR) in mouse brain samples

Quantitative real-time PCR (qPCR) analysis was performed in ipsilateral and contralateral cortical and hippocampal tissues, as well as in circulating leukocytes collected 48 h after 1-h MCAo or sham surgery [46]. Leukocytes were isolated from mouse blood collected in K_3EDTA-containing tubes and centrifuged at 300g for 10 min to remove plasma. The remaining pellet was washed in BD Pharm Lyse™ (BD Biosciences) until erythrocytes were completely lysed.

Total RNA was extracted using TRIzol™ Reagent (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions and resuspended in RNase-free water. RNA concentration and purity were determined using a BioTek Synergy H1 Hybrid Reader spectrophotometer (Agilent, USA) and complementary DNA (cDNA) was synthesized using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems, #4387406). Following a 1:2 dilution in RNase-free water, qPCR reactions were loaded onto MicroAmp™ Optical 96-well Reaction Plates sealed with MicroAmp™ Optical Adhesive Film (Applied Biosystems, Thermo Fisher Scientific). Plates were run on a QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific). The comparative cycle threshold method (Ct) was used to quantify the transcripts and values are expressed as -fold differences. All TaqMan® Gene Expression Assays were purchased from Thermo Fisher Scientific and included: Arg1 (Mm00475988_m1), Chil3

(Mm00657889_mH), interleukin-1β (IL-1β; Mm00434228_m1), PIWIL1 (Mm00451649_m1), PIWIL2 (Mm00502383_m1), PIWIL4 (Mm01144775_m1), Prdx1 (Mm01621996_s1), transforming growth factor-β1 (TGF-β1; Mm00441726_m1), tumor necrosis factor-α (TNF-α; Mm00443258_m1), TREM2 (Mm04209424_g1) and the reference housekeeping gene GAPDH (Mm99999915_g1).

Immunofluorescence

Paraformaldehyde (PFA)–fixed brains were cryoprotected, frozen and embedded in OCT compound, before being cut into coronal sections (40 µm thick) at defined bregma levels according to the Paxinos and Franklin mouse brain atlas [47]. Analysis of the subventricular zone (SVZ) was performed on coronal sections spanning approximately + 0.5 to 0.0 mm relative to bregma, whereas hippocampal analysis was conducted at approximately − 1.7 to − 2.1 mm relative to bregma, where CA1, CA2, and CA3 subfields are fully developed.

Sections were collected into 24-well plates containing PBS and processed free-floating, as previously described [48]. Non-specific binding was blocked by incubating brain slices for 30 min in 5% normal goat serum prepared in PBS containing 0.3% Triton X-100. Primary antibodies were diluted in PBS containing 0.5% bovine serum albumin (BSA) and 0.3% Triton X-100, before being added to the sections for overnight incubation at 4°C on an orbital shaker. The following primary antibodies were used: rabbit polyclonal anti-PIWIL2 (1:150; #ab36764, Abcam), rabbit polyclonal anti-PIWIL4 (1:100; #ab180867, Abcam), mouse monoclonal anti-NeuN (1:100; #MAB377, Merck Millipore), mouse monoclonal anti-SOX2 (1:200; #sc-365823, Santa Cruz), mouse polyclonal anti-ki67 (1:200; #28074-1-AP, Proteintech). At the end of the incubation with the primary antibody, the sections were exposed for 2 h at room temperature with the appropriate (anti-rabbit or anti-mouse) Alexa Fluor–conjugated secondary antibodies (Alexa Fluor 488 or Alexa Fluor 594; 1:200; Life Technologies, Monza, MB, Italy). Nuclear counterstaining was performed by incubating brain slices with 4',6-diamidino-2-phenylindole (DAPI; 1:500; Sigma-Aldrich, Milan, Italy) for 15 min at room temperature, protected from light. Finally, sections were mounted onto glass slides and coverslipped with Fluoromount mounting medium (Sigma-Aldrich, Milan, Italy). Immunofluorescence images were acquired using a confocal laser scanning microscope (Fluoview FV300, Olympus, Segrate, Milan, Italy) and analyzed with a dedicated software (cellSens version 4.1.1, Olympus).

Statistical analysis

Data are presented as scatter plots along with mean ± standard deviation (SD) for quantitative variables, or medians with interquartile range for categorical ordinal variables (i.e., neuroscore). Statistical analysis was performed using GraphPad Prism 8.0.2 (GraphPad Software, USA). The unpaired Student's t-test was used for comparisons between two experimental groups, while comparisons among multiple groups were performed using one-way or two-way ANOVA, followed by Tukey's or Bonferroni's post hoc tests, respectively. The neurological deficit scores, being ordinal in nature, were analyzed using the

Mann-Whitney test followed by Bonferroni's correction method for multiple comparisons. A p value < 0.05 was considered statistically significant.

Results

Remote ischemic post-conditioning affords neuroprotection and dampens neuroinflammation caused by focal cerebral ischemia

As shown in Fig. 1, RIC induced by 10 min FAo significantly reduced cerebral infarct volume and edema caused by 1h MCAo followed by 48h of reperfusion in mice. Accordingly, neurological deficits were attenuated by RIC, resulting in amelioration of both focal and total deficits caused by ischemia (Fig. 1E). In fact, 44% (11 out of 25 mice) of mice exposed to FAo achieved a favourable functional outcome (De Simoni score ≤ 20), compared with only 15% (3 out of 20 mice) in the MCAo group.

Neuroprotection by RIC was associated with attenuation of the neuroinflammatory response triggered by MCAo. Specifically, exposure to RIC (i.e., 10-min FAo) attenuated the elevation of IL-1β and TNF-α mRNA expression triggered by 1 h MCAo (followed by 48h of reperfusion) in the ipsilateral cortex (Fig. 1F and G).

Ischemia and remote ischemic post-conditioning modulate PIWI family proteins in the cortex

To investigate the effects of ischemia on the cerebral expression of PIWI family members, we analysed levels of PIWI proteins mRNA in the cerebral cortex of mice subjected to 1h MCAo followed by 48h of reperfusion. As shown in Fig. 2, transient focal ischemia (i.e., 1h MCAo followed by 48h of reperfusion) induced a significant upregulation of both *PIWIL2* and *PIWIL4* mRNA in the ipsilateral (ischemic) cortex of mice compared with the corresponding region of the contralateral hemisphere or sham-control. By contrast, *PIWIL1* transcript was unaffected by ischemia-reperfusion in the cerebral cortex (Fig. 2A). Interestingly, RIC produced by 10 min FAo attenuated *PIWIL2* and *PIWIL4* mRNA elevation caused by transient MCAo in the ischemic cortex (Fig. 2B and C). Although *PIWIL1* transcript was elevated by FAo in the cortex of sham-operated animals (SHAM/FAo), its levels were comparable to MCAo alone when FAo was preceded by MCAo (MCAo/FAo group) (Fig. 2A).

Immunofluorescence analysis (Figure 2D and E) revealed that PIWIL2 and PIWIL4 proteins were almost absent in the contralateral fronto-parietal cortex, while abundantly expressed in NeuN-immunopositive aberrantly shaped (i.e., damaged) neurons of the ipsilateral region of mice subjected to 1h MCAo followed by 48 h of reperfusion. Notably, labelling of neuronal PIWIL4 was more intense than PIWIL2, according to quantitative PCR (Figure 2) showing a more dramatic (approx. 6-fold) elevation of *PIWIL4* transcript as compared with PIWIL2 in the ischemic hemisphere.

While PIWIL2 was exclusively expressed in neurons, PIWIL4 was also observed in other cell types populating the ipsilateral hemisphere. In particular, in the penumbra cortex, PIWIL4 protein co-localized with some GFAP-positive astrocytes, lining the outer border of the ischemic lesion (Fig. 3A). However, in the ischemic core, PIWIL4 was also expressed by Ly6B.2-positive myeloid cells (i.e., neutrophils and monocytes/macrophages) likely infiltrating from the blood (Fig. 3B). Nevertheless, quantitative analysis of *PIWIL4* mRNA expression in circulating leukocytes revealed a significant downregulation of this transcript after cerebral ischemia compared with SHAM controls, which was unaffected by remote ischemic post-conditioning (Fig. 3C).

Post-conditioning selectively enhances PIWIL4 in neurogenic regions

Based on previous evidence reporting that, besides gonads, the highest expression of PIWI family members occurs in the hippocampus, whereby the piRNA pathway contributes to the maintenance of lifelong neurogenesis [30, 33], we analysed PIWI proteins in this region.

Similar to the evidence in the cortex, *PIWIL1* transcript levels were increased in the hippocampus following the post-conditioning stimulus alone (i.e., 10 min FAo) as compared with SHAM. However, this effect was abolished when FAo was preceded by MCAo (Fig. 4A). By contrast, *PIWIL2* mRNA expression remained largely unmodified across all experimental groups (Fig. 4B). Intriguingly, transient MCAo upregulated *PIWIL4* transcript levels in the ipsilateral hippocampus as compared with sham conditions, and this effect was potentiated by remote ischemic post-conditioning (MCAo/FAo) (Fig. 4C), as also demonstrated by immunofluorescence (Figs. 4D and 4E).

Moreover, immunofluorescence analysis revealed that PIWIL4 colocalizes with pyramidal neurons of the CA3 subfield (Fig. 4D) and with neurons of the dentate gyrus (Fig. 4E) in the ipsilateral hippocampus of ischemic post-conditioned mice.

Elevation of PIWIL4 in the ipsilateral hippocampus of ischemic mice subjected to 10-min FAo was coincident with an upregulation of *TGF-β1* and *TREM2* (Fig. 5A and B), two factors implicated in neuronal differentiation and repair-associated plasticity in the adult brain [49–51]. Specifically, Spearman rank correlation analysis revealed that PIWIL4 transcript was strongly and positively correlated with TGF-β1 (r_s = 0.81, p = 0.072) and TREM2 (r_s = 0.94, p = 0.017) levels.

Besides the hippocampus, the SVZ represents a critical neurogenic region following stroke injury, whence newly born cells migrate to differentiate into the phenotypes of neurons damaged by ischemic lesion [52–55]. Thus, immunofluorescence analysis of the SVZ showed that SOX2-immunopositive neural progenitor cells express PIWIL4 in post-conditioned mice (MCAo/FAo), but not in animals subjected to MCAo alone (Fig. 6A). Interestingly, in the SVZ, SOX2-immunopositive cells also co-localized with the proliferation marker Ki67 (Fig. 6B), indicating enhanced proliferation of neural progenitor cells.

Discussion

The present work originally demonstrates that PIWI family proteins are implicated in the pathobiology of cerebral ischemia and may contribute to neuroprotection exerted by RIC. In particular, PIWIL2 and PIWIL4 were upregulated in the ischemic hemisphere of mice subjected to 1h MCAo followed by 48 of reperfusion; whereby PIWIL2 was exclusively expressed in neurons, while PIWIL4 was also expressed in non-neuronal cells, namely astrocytes and myeloid cells. Interestingly, remote ischemic post-conditioning induced by 10-min occlusion of the femoral artery attenuated the elevation of both PIWIL2 and PIWIL4 in the ischemic cortex. Moreover, this neuroprotective paradigm caused a selective upregulation of PIWIL4 in hippocampal neurons of the CA3 and dentate gyrus of the ischemic hemisphere, which was coincident with upregulation of *TGF-β1* and *TREM2*, two factors implicated in neuronal differentiation and repair-associated plasticity. The co-localization of PIWIL4 with SOX2/Ki67-immunopositive cells in the SVZ further supports the putative engagement of PIWIL4 in neurogenesis prompted by RIC.

Previous work has largely focused on the characterization of piRNA expression profiles, but limited information is available on their key molecular partners, the PIWI proteins, particularly in the setting of ischemic stroke. Altered piRNA expression has been described in acute neurological insults such as subarachnoid haemorrhage [56], and, most notably, ischemic stroke [40], supporting the notion that piRNA-mediated regulatory mechanisms may play a role in cerebral injury. In line with clinical findings, we have demonstrated for the first time that both PIWIL2 and PIWIL4 are elevated in the ipsilateral ischemic cortex of mice subjected to transient MCAo. The fact that neuroprotection exerted by RIC attenuates this effect strongly suggests that PIWIL2- and PIWIL4-mediated piRNA pathways may play a detrimental role in the lesioned tissue. Notably, the piRNA pathway maintains genomic integrity by TEs, among which long interspersed nuclear element-1 (LINE-1) is the most active retrotransposon in the human genome [57]. Given that PIWIL2-deficient mice exhibit long interspersed nuclear element-1 (LINE-1) promoter hypomethylation in their brain [19, 58], it could be speculated that piRNA pathway-induced activation of LINE-1 may underlie tissue damage and inflammation in the ischemic cortex. Accordingly, LINE-1 activation has been associated with neurodegenerative conditions and with sterile inflammation in the brain [59–63]. This is consistent with our findings documenting elevated levels of inflammatory cytokines (i.e., IL1-β and TNF-α) in the ipsilateral ischemic cortex, an effect that was attenuated by RIC, thus demonstrating an expression pattern similar to that observed for PIWIL2 and PIWIL4.

Although the involvement of PIWIL2 in global cerebral ischemia has previously been reported [64], this is the first evidence documenting PIWI protein modulation following focal cerebral ischemia. More importantly, to our knowledge, our data represent the first demonstration of significant changes in PIWIL4 expression in response to remote ischemic post-conditioning. Albeit the PIWIL2 transcript was also altered following ischemia and RIC, PIWIL4 exhibited a more robust and dramatic change in the ischemic cortex 48 h after reperfusion, suggesting a potentially distinct role in the post-ischemic response. Additionally, while PIWIL2 expression appeared to be restricted to neurons, PIWIL4 was also detected in non-neuronal cell populations within the ischemic hemisphere, pointing to a broader

involvement in the cellular response to injury. The presence of PIWIL4 in peripheral myeloid cells and in astroglia has previously been reported [34, 65, 66]. Indeed, PIWIL4 may regulate not only the neurogenic genes that are essential for normal neurogenesis but also those implicated in modulating glioma cell activity [34]. Thus, the increased expression of PIWIL4 in astrocytes and myeloid cells of the ischemic hemisphere may likely underlie their enhanced proliferation after the insult, possibly contributing to the neuroinflammatory response [67, 68].

Interestingly, we found that PIWIL4 modulation in the hippocampus displays a different pattern as compared to the damaged cortex. In fact, although PIWIL4 expression was unaffected by MCAo in the ipsilateral hippocampus, it was dramatically elevated after exposure to RIC as compared to SHAM conditions. The latter evidence, associated with the neuroprotective effects of RIC, strongly suggests that – at variance with the cortex - hippocampal PIWIL4 may actually activate downstream protective mechanisms. More specifically, our findings strongly suggest that PIWIL4 may be implicated in neurogenesis, since its elevation was selectively observed in neurons of the dentate gyrus and CA3 sub-regions. Indeed, the dentate gyrus is a site of continued neurogenesis in the adult brain; in turn, its major projection area, the CA3, sends reciprocal projections back to the dentate gyrus exerting an indirect control over neurogenesis [69–71]. The putative role of PIWIL4 in neurogenesis is further supported by its co-localization with neuronal progenitor cells of the SVZ of mice subjected to MCAo followed by FAo. This is consistent with the evidence that ischemic or hypoxic post-conditioning have been shown to enhance neurogenesis in subgranular zone and SVZ of rodents subjected to MCAo [72–74]. Progenitor cells stimulated by the ischemic insult in neurogenic niche are able to migrate to injury sites were they differentiate into the phenotype of neurons [52, 53]. Although previous work has reported that PIWIL2 and piRNAs are highly enriched in neural progenitor cells within the postnatal mouse hippocampus and are required for adult hippocampal neurogenesis [33], we originally observed a selective elevation of PIWIL4 following remote ischemic post-conditioning in dentate gyrus and CA3.

The putative contribution of neurogenesis is underscored by the evidence that elevation of PIWIL4 in the ipsilateral hippocampus of ischemic mice subjected to remote ischemic conditioning was coincident with an upregulation of *TGF-β* and *TREM2*, two factors implicated in neuronal differentiation and repair-associated plasticity in the adult brain [49–51]. The crucial involvement of PIWIL4 in the activation of *TGF*-β signalling has also been reported in breast cancer cells [75].

In mouse germ cells, PIWIL2 and PIWIL4 have distinct, non-hierarchical roles in piRNA biogenesis, transposon silencing, and DNA methylation. PIWIL2 is necessary for loading piRNAs into PIWIL4, while PIWIL4 mainly operates in the nucleus to repress specific retrotransposons (LINE-1) through de novo DNA methylation [76]. Regarding their somatic role, PIWIL4, but not PIWIL2, has been shown to play a role in regulating cell differentiation in some somatic tissues, indicating unique functions outside of germline silencing [34, 77]. Previous in vitro work has demonstrated that PIWIL4, but not PIWIL2, is involved in neuronal differentiation [34].

The hypothesis that PIWI proteins may exert distinct roles depending on the stimulus and on the brain region sampled is also supported by the pattern of modulation of PIWIL1. Although we did not detect any modification of *PIWIL1* expression levels in the brain of mice subjected to MCAo, its mRNA levels resulted elevated after remote ischemic post-conditioning in the cortex and hippocampus of both ipsilateral and contralateral hemispheres. In rodents, PIWIL1 participates in polarization and radial migration of newborn neurons in the developing cerebral cortex by regulating the stability and the expression of microtubule-associated proteins (MAPs) [32]. Moreover, the neocortical circuitry is disrupted in *Piwil1* knock-out mice, compromising proper cerebral development [35]. Upregulation of Piwil1a in neurons has been reported to underlie neuroprotection conferred by intranasal administration of Wnt3a in rats subjected to transient global cerebral ischemia [78]. Thus, it is intriguing to speculate that PIWIL1 elevation triggered by post-conditioning may actually play protective functions. The beneficial effects of PIWIL1 are also suggested by previous evidence documenting its dysregulated expression in postmortem brain samples from sporadic amyotrophic lateral sclerosis or Alzheimer's disease patients [36, 37]. Accordingly, viral PIWIL1 overexpression protects midbrain neurons against oxidative stress damage in a mouse model of Parkinson's disease [79].

In conclusion, our data highlight the intriguing possibility that PIWI proteins may exert different effects, depending on the brain region (ischemic cortex vs peri-lesional hippocampus) considered and on the specific stimulus (MCAo and/or FAo) applied. In particular, PIWIL2 and PIWIL4 participate in cerebral injury in the ischemic cortex, whereas PIWIL1 and PIWIL4 may contribute to the protective responses elicited by remote ischemic post-conditioning. More specifically, PIWIL4 is expressed in neuronal progenitor cells in neurogenic niche of post-conditioned mice, strongly supporting its contribution to neurogenesis.

A major challenge of the present study arises from the infeasibility to pharmacologically modulate PIWIL4, due to its dual functional role: detrimental effects in the cortex, while likely promoting neurogenesis in neurogenic niches. Thus, the lack of strategies enabling region-specific modulation represents a significant constraint. Despite this limitation, the data obtained support the proposed hypothesis, suggesting the validity of the underlying theoretical framework. Nevertheless, further studies will be required to expand upon these findings, also including evidence in more representative contexts, such as aged animals, including both sexes and co-morbidities.

Collectively, these findings further extend the emerging role of PIWI proteins beyond germline biology, arguing for their involvement in ischemic brain injury and neuroprotective programs triggered by remote conditioning.

Declarations

Author contributions

D.A., G.P. and C.D.S. conceived and designed the study. C.D.S., M.P. and D.A. performed experiments and collected data. C.D.S., M.P. and D.A. analyzed and interpreted the data. G.P., F.L.C., C.C. and G.B.

contributed to data interpretation and critically revised the manuscript. C.D.S. drafted the manuscript. D.A., C.C. and G.P. supervised the study. G.B. and F.L.C. were responsible for funding acquisition. All authors reviewed the manuscript, provided intellectual input, and approved the final version.

Competing Interests

The authors have no competing interests to declare that are relevant to the content of this article.

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Data Availability

Data generated from the current study is available from the corresponding author upon reasonable request.

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Figures

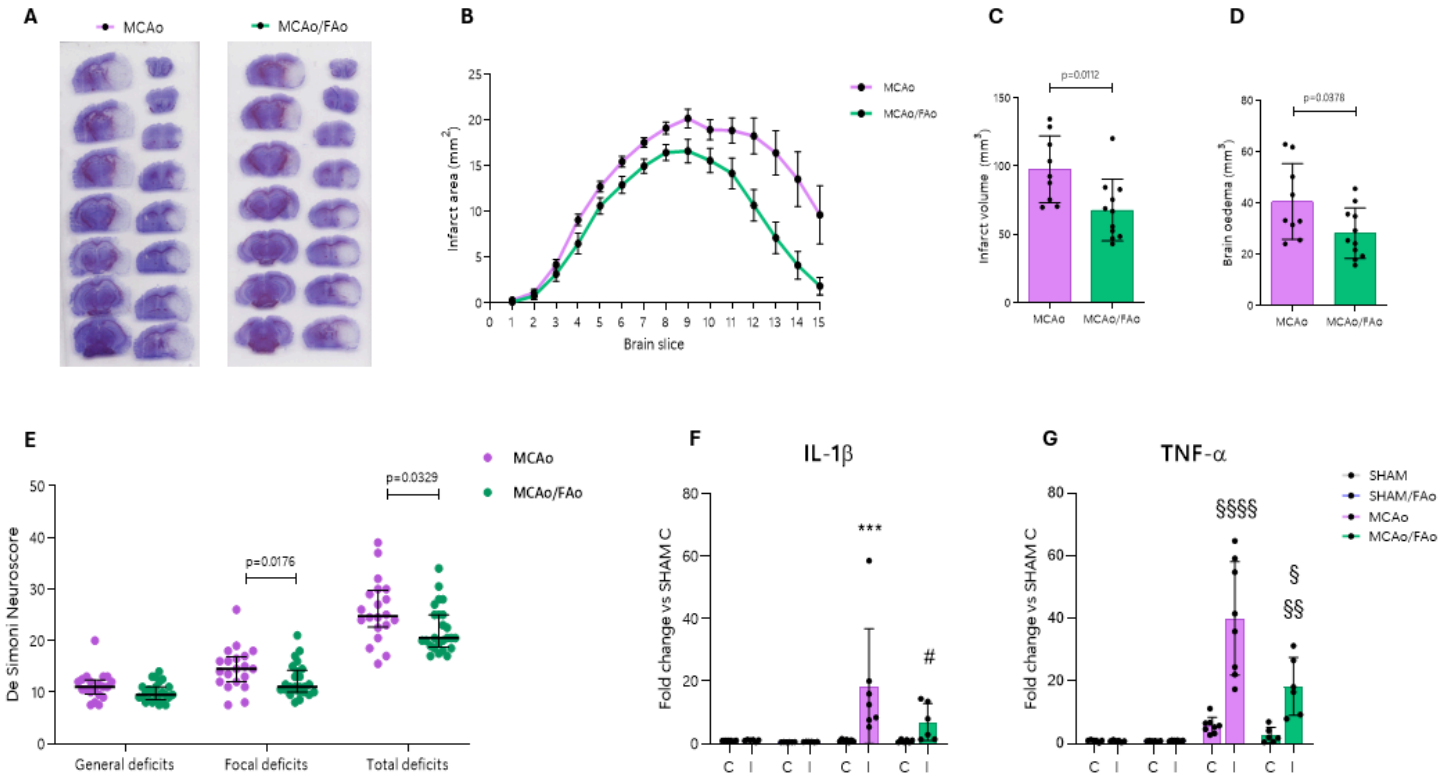


Figure 1

RIC provides neuroprotection and reduces inflammatory responses in the ischemic cortex of mice subjected to 1h MCAo followed by 48h of reperfusion. (A) Representative cresyl violet-stained coronal brain sections, (B) brain infarct areas, (C) volume and (D) edema in mice subjected to transient MCAo alone or exposed to RIC by occluding the femoral artery for 10 min (MCAo/FAo). Data were analysed using the unpaired Student's t-test and expressed as mean ± SD (n=9-11). (E) Neurological deficits caused in mice by MCAo or MCAo/FAo. Data were expressed as medians with interquartile ranges and analysed with the two-tailed Mann–Whitney test (n=20-25 mice per experimental group). mRNA expression of (F) *IL-1β*and (G) *TNF-α* was quantified in the contralateral (C) and ipsilateral (I) cortex of mice subjected to sham surgery (SHAM), 10 min FAo (SHAM/FAo), MCAo or MCAo/FAo. Data were analysed using two-way ANOVA followed by Bonferroni's post-test and expressed as mean ± SD (n=6-8 animals per experimental group). Significance is indicated as follows: *** P= 0.0009 vs corresponding C, p=0.0006 vs SHAM and p=0.0006 vs SHAM/FAo; # p= 0.0436 vs MCAo; §§§§ p<0.0001 vs corresponding C, SHAM and SHAM/FAo; §§ p=0.0074 vs corresponding C, p=0.0027 vs SHAM and p=0.003 vs SHAM/FAo; §=p<0.0001 vs MCAo

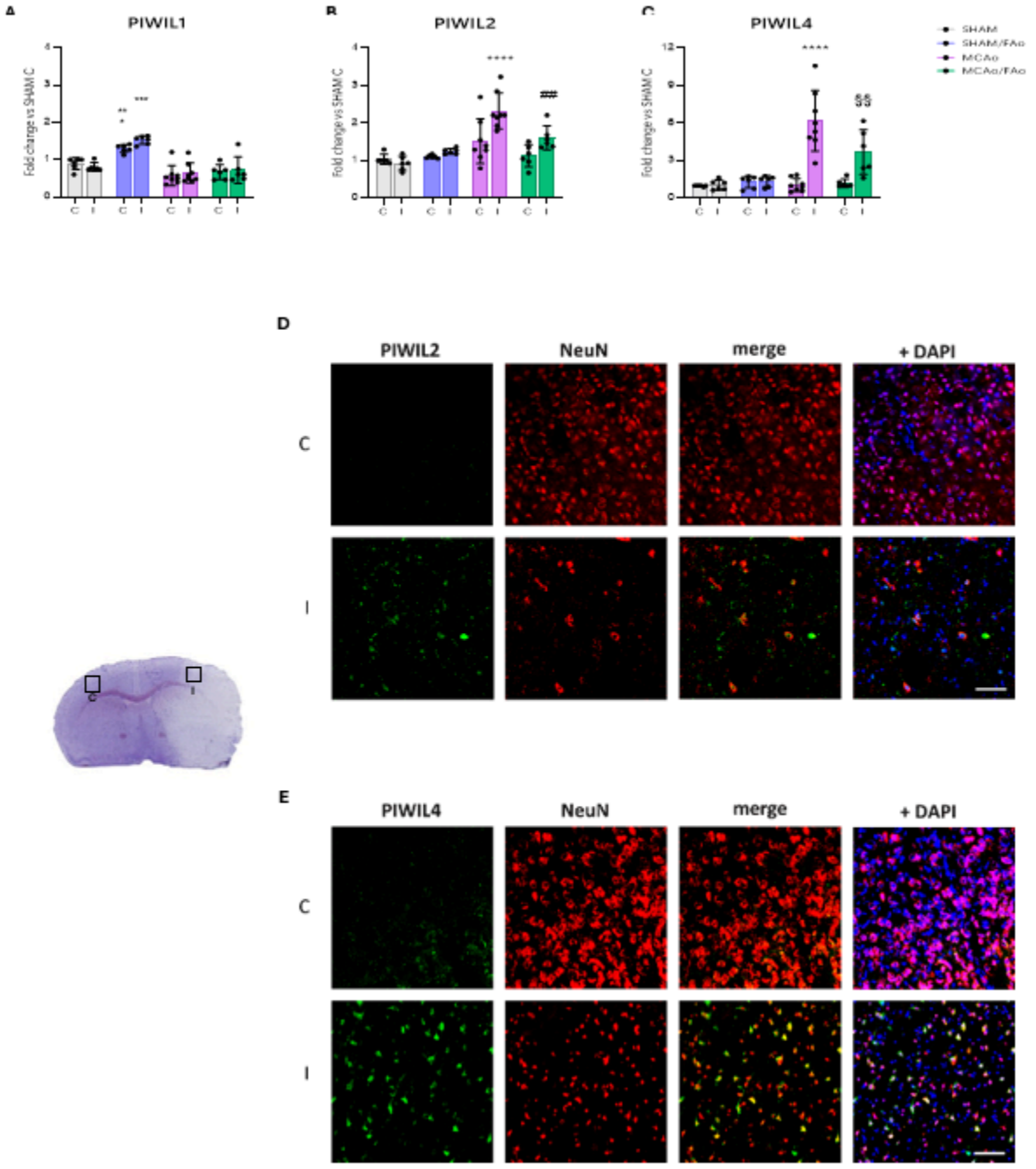


Figure 2

Elevation of PIWIL2 and PIWIL4 levels caused by transient MCAo in the ipsilateral cortex of mice is prevented by RIC. mRNA expression levels of (A) *PIWIL1*, (B) *PIWIL2*, and (C) *PIWIL4*were quantified in the contralateral (C) and ipsilateral (I) cortex of mice subjected to sham surgery (SHAM), 10 min femoral artery occlusion (SHAM/FAo), 1 h MCAo followed by 48 h reperfusion (MCAo), or MCAo combined with FAo (MCAo/FAo). Data were analysed by two-way ANOVA followed by Bonferroni's post-test and expressed as mean ± SD (n=6-8 mice per experimental group). Significance levels are indicated as follows: * p=0.0163 vs SHAM C; ** p<0.0001 vs MCAo/FAo C; *** p<0.0001 vs SHAM I and MCAo/FAo I; **** p<0.0001 vs corresponding C and SHAM I; ## p=0.0019 vs corresponding C, p=0.0087 vs SHAM I

and p=0.0027 vs MCAo I; §§ p=0.0081 vs corresponding C, p=0.0024 vs SHAM I, p=0.0091 vs SHAM/FAo I and p= 0.0019 vs MCAo I. Representative immunofluorescence images showing colocalization (yellow overlapping in merge panels) of (D) PIWIL2 or (E) PIWIL4 (green) with NeuN immunopositive neurons (red) in the contralateral (C) and ipsilateral (I) frontoparietal cortex of mice subjected to 1 h MCAo followed by 48 h of reperfusion. Nuclei were counterstained with DAPI (blue). Scale bar = 100 µm. The contralateral and ipsilateral regions sampled for immunofluorescence analysis are indicated in the square insets of the representative cresyl violet-stained coronal brain section in the left side of the figure

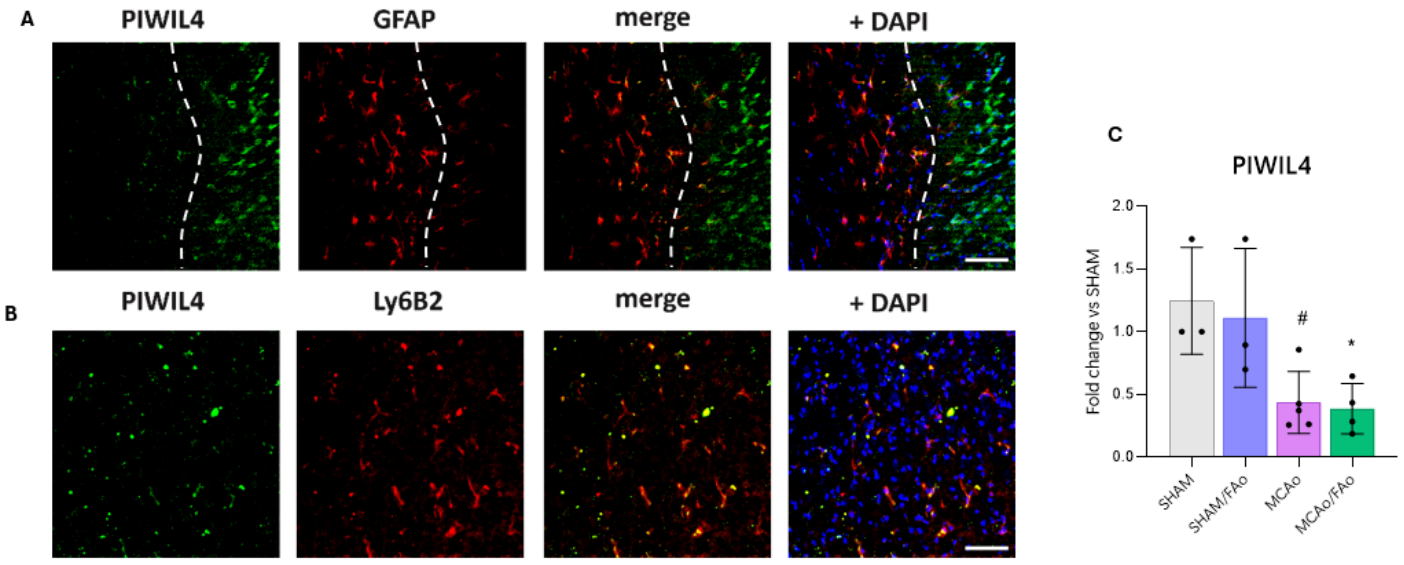


Figure 3

PIWIL4 protein is expressed by GFAP+ astrocytes and Ly6B2+ myeloid cells in the ischemic hemisphere, while its mRNA levels are reduced in leukocytes isolated from mice subjected to MCAo. Immunofluorescence images showing colocalization (yellow overlapping) of PIWIL4 (green) with (A) GFAP- or (B) Ly6B.2-immunopositive myeloid cells (red) in the ipsilateral cortex of mice subjected to 1 h MCAo followed by 48 h of reperfusion. Nuclei were counterstained with DAPI (blue). Scale bar = 100 µm. Dotted line indicates the border between the ischemic (right side) and peri-lesioned tissue (left side). (C) Quantitative real-time analysis of *PIWIL4*mRNA expression in leukocytes from mice subjected to SHAM surgery, 10 min FAo (SHAM/FAo), 1h MCAo followed by 48h of reperfusion (MCAo) or both (MCAo/FAo). Data were analysed using one-way ANOVA followed by Tukey's post-test and expressed as mean ± SD (n=6-10 mice per experimental group, each dot represents data obtained from blood samples pooled from two animals). Significance levels are denoted by # p=0.0375 vs SHAM and * p=0.0351 vs SHAM

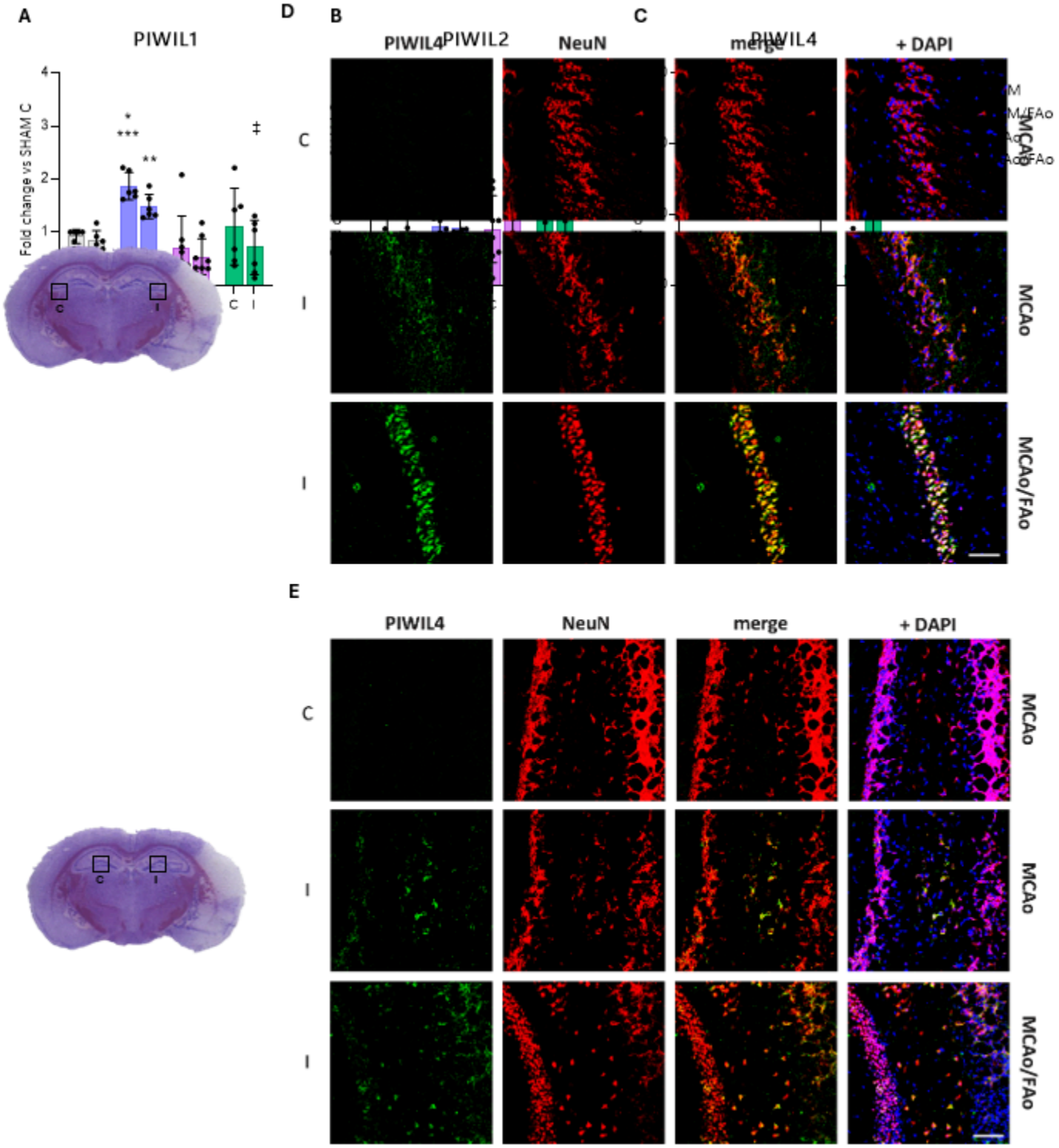


Figure 4

PIWIL4 is selectively increased in the ipsilateral hippocampus of ischemic post-conditioned mice. *PIWIL1* (panel A), *PIWIL2* (panel B) and *PIWIL4* (panel C) transcripts in the contralateral (C) and ipsilateral (I) hippocampi of mice subjected to SHAM surgery, 10 min FAo (SHAM/FAo), 1h MCAo followed by 48h of reperfusion (MCAo) or MCAo+FAo (MCAo/FAo). Data were analysed by two-way ANOVA followed by Bonferroni's post-test and expressed as mean ± SD (n=6-8 animals per experimental group). Significance levels are indicated as follows: *** p=0.0024 vs SHAM C; ** p= 0.0373 vs corresponding C and p=0.0205

vs MCAo/FAo I; * p=0.0211 vs MCAo/FAo C; ‡ = 0.0356 vs corresponding C; § p=0.0435 vs SHAM I; §§ p=0.0016 vs corresponding C and p=0.0031 vs SHAM/FAo I; §§§p=0.0006 vs SHAM I. Immunofluorescence images showing colocalization (yellow) in merge panel of PIWIL4 (green fluorescence) with NeuN immunopositive neurons (red fluorescence) in the ipsilateral (I) and contralateral (C) CA3 subfield (panel D) or dentate gyrus (panel E) of mice subjected to 1 h occlusion of the middle cerebral artery followed by 48 h of reperfusion alone (MCAo) or in combination with remote ischemic post-conditioning (MCAo/FAo). Nuclei were counterstained with DAPI (blue signal). Scale bar = 100 µm. The contralateral and ipsilateral regions sampled for immunofluorescence analysis are indicated in the square insets of the representative cresyl violet-stained coronal brain sections in the left side of the figure

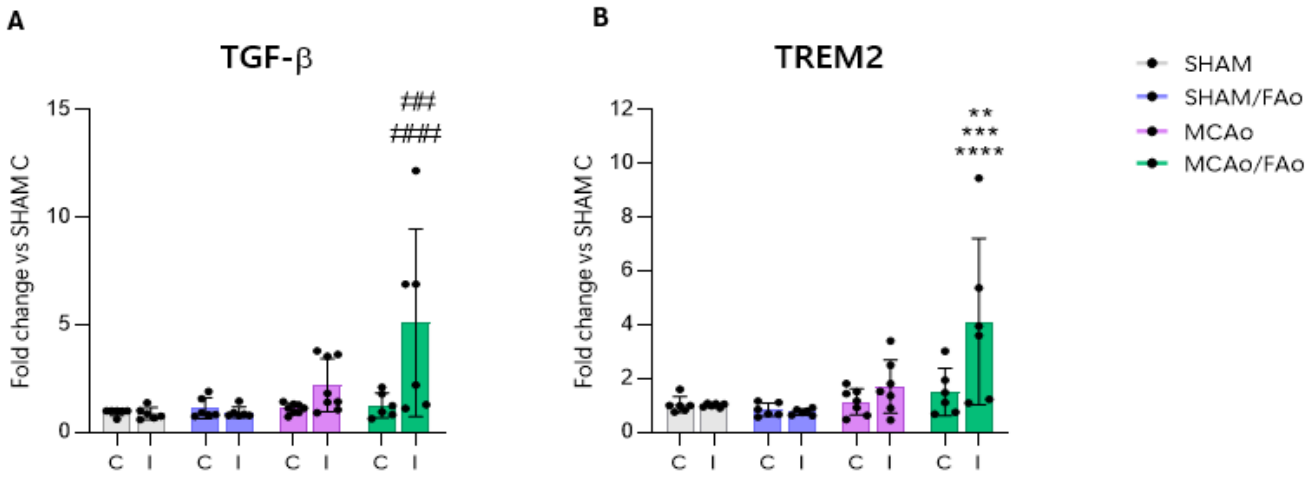


Figure 5

Remote post-conditioning by FAo results in transcriptional elevation of differentiation-associated factors in the ipsilateral hippocampus of ischemic mice. (A) *TGF-β* and (B) *TREM2*mRNA expression in the contralateral (C) and ipsilateral (I) hippocampi of mice subjected to SHAM surgery, 10 min FAo (SHAM/FAo), 1h MCAo followed by 48h of reperfusion (MCAo) or their combination (MCAo/FAo). Data were analysed by two-way ANOVA followed by Bonferroni's post-test and expressed as mean ± SD (n=6-8 animals per experimental group). Significance levels are indicated as follows: *** p=0.0001 vs corresponding C and p=0.0003 vs SHAM I; **** p<0.0001 vs SHAM/FAo I; ** p=0.0046 vs MCAo I; ### p=0.0003 vs corresponding C and p=0.0002 vs SHAM and SHAM/FAo I; ## p=0.0088 vs MCAo

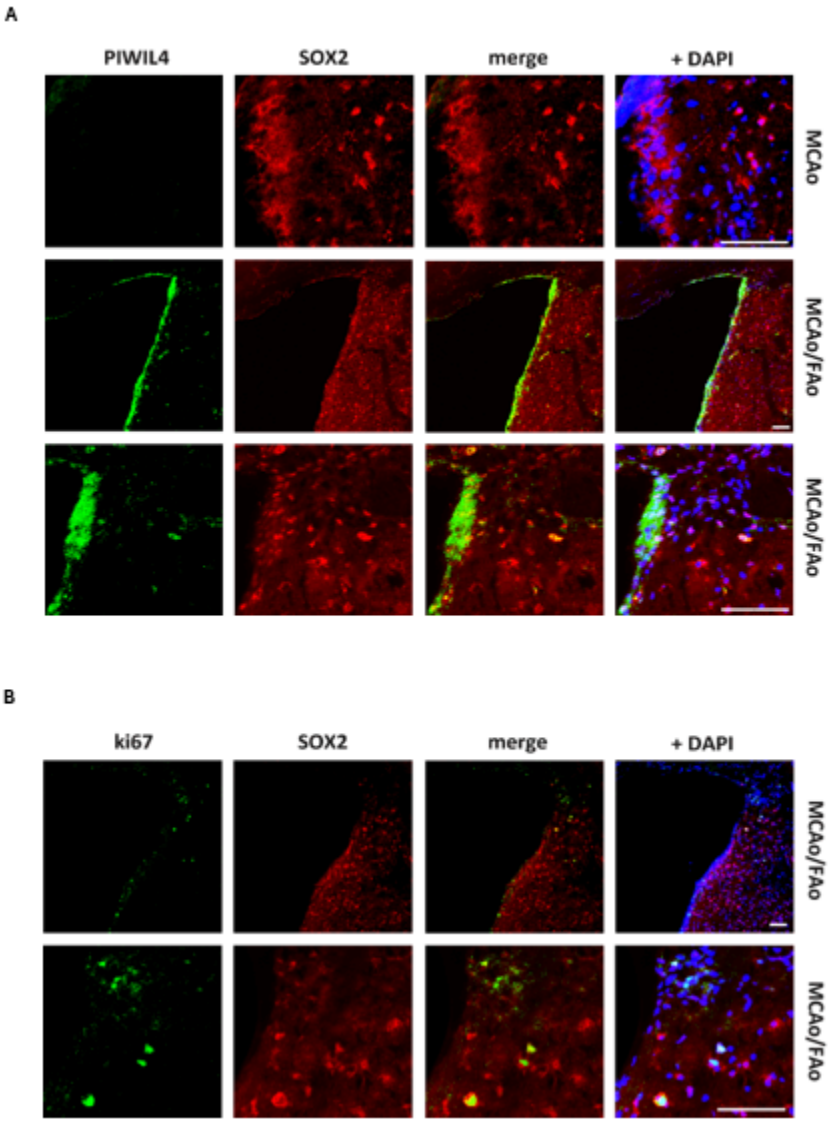


Figure 6

SOX2+ neural progenitor cells express PIWIL4 and the proliferation marker ki67 in the ipsilateral SVZ of ischemic post-conditioned mice. Immunofluorescence images showing colocalization (yellow in merge panel) of (A) PIWIL4 (green fluorescence) and (B) ki67 (green) with SOX2 stem/progenitor cell marker (red fluorescence) in the ipsilateral SVZ of mice subjected to 1 h MCAo followed by 48 h of reperfusion, followed or not by remote ischemic post-conditioning (FAo). Nuclei were counterstained with DAPI (blue signal). Scale bar = 100 µm