The Interleukin-8 (IL-8/CXCL8) Receptor Inhibitor Reparixin Improves Neurological Deficits and Reduces Long-term Inflammation in Permanent and Transient Cerebral Ischemia in Rats

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Leukocyte infiltration is viewed as a pharmacological target in cerebral ischemia. We previously reported that reparixin, a CXCL8 receptor blocker that inhibits neutrophil infiltration, and related molecules can reduce infarct size in a rat model of transient middle cerebral artery occlusion (MCAO). The study aims were to compare the effects of reparixin in transient and permanent MCAO using varied treatment schedules and therapeutic windows to evaluate effects on long-term neurological deficits and late inflammatory response. Reparixin, administered for 1 to 3 days, 3.5 to 6 h after MCAO, ameliorates neurological function recovery and inhibits long-term inflammation. The infarct size reduction at 24 h, evaluated by TTC staining, is more pronounced in transient MCAO. MRI analysis identified a decrease in the progression of infarct size by reparixin that was more evident at 48 h in permanent MCAO, and was associated with a significantly improved recovery from long-term neurological deficits.

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INTRODUCTION

Blockade of inflammation is considered a possible approach to the therapy of cerebral ischemia. Leukocytic infiltration, particularly of polymorphonuclear neutrophils (PMN) is a key aspect of the deleterious aspects of inflammation in stroke (1–3), and CXCL8 or related chemokines are induced in stroke in animal models (4) as well as in patients (5,6). Recently, we described reparixin (formerly termed repertaxin), a small molecular weight inhibitor of CXCR1 and CXCR2, the receptors for the CXCL8 family of chemokines implicated in the recruitment of PMN active in vivo (7), and the drug is now undergoing clinical trials for other indications. A preliminary study of reparixin in two models of cerebral ischemia in the rat indicated that it was more effective against transient ischemia than in permanent ischemia, where there was only a trend for reduction in infarct size (8), consistent with the hypothesis that PMN are mediators mainly in the reperfusion injury.

To better characterize the effect of reparixin in the two models of cerebral ischemia, and hence the role of CXCR1/2 ligands in neuroinflammation, we undertook a series of experiments aiming at investigating not only its effect on infarct size but also on long-term neurological outcome. In fact, infarct size only partially correlates with functional outcome in patients, and it is suggested it should only be used as a surrogate marker in clinical trials (9).

Transient cerebral ischemia was induced in rats by 1.5 h middle cerebral artery (MCA) occlusion (MCAO). In some experiments, we used a permanent ischemia model, often termed three-vessel occlusion, where the permanent occlusion of the right MCA and of the ipsilateral carotid and the temporary (1 h) occlusion of the contralateral carotid induce a damage with a penumbra surrounding the fixed lesions in the MCA territory (10,11). In these animals we measured the infarct volume 24 h after surgery, using triphenyltetrazolium hydrochloride (TTC) staining, quantified PMN infiltrate by measuring brain myeloperoxidase (MPO) or by immunochemistry, and performed behavioral testing including sensorimotor tests (De Ryck's (12), Bederson's (13), and foot-fault tests (14)) for up to 1 month to evaluate neurological deficits. As the results on reduction of infarct size

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Table 1. Effect of reparixin on cortical vs. subcortical damage 24 h after transient MCAO

	Total infarct	Cortex	Striatum
Experiment 1			
Saline (n = 6)	273 ± 24	181 ± 24	89 ± 9
Reparixin (n = 5)	68 ± 8 **	12 ± 4 **	56 ± 9 *
Experiment 2			
Saline (n = 8)	271 ± 25	193 ± 19	78 ± 8
Reparixin (n = 7)	143 ± 23 *	64 ± 18 **	79 ± 8

Results from 2 independent experiments are given. Data are expressed as the infarct volume in mm^3 and are the mean ± S.E; the number of rats per group is indicated in parenthesis. Experiment 1: reparixin was administered on the first day at 15 mg/kg i.v. starting 2 h after reperfusion followed by two 15 mg/kg s.c. doses at 2 h intervals. Experiment 2: reparixin was administered on the first day at 15 mg/kg i.v. starting at the time of ischemia followed by four 15 mg/kg s.c. doses at 2 h intervals. Infarct volumes are determined 24 h after MCAO. * $P < 0.05$ and ** $P < 0.01$ vs. saline (Student t-test).

in the permanent ischemia model were not conclusive, we used MRI to follow up infarct size progression in these rats.

These experiments used treatment schedules chosen according to previous studies with reparixin in various models of ischemia (7,8,15). However, in this study, we also characterized the drug in terms of therapeutic window and compared different injection schedules, either bolus or continuous infusion to gain information useful for future clinical trials.

Finally, because we show elsewhere (16) that the neuroprotective action of erythropoietin induces long-term functional improvement associated with a decrease in the late inflammatory response, we also evaluated the effect of reparixin on late inflammation in the ischemic brain by evaluating immunohistochemical markers of astroglial activation one month after ischemia.

The results indicate that reparixin reduces not only short-term PMN infiltration and infarct size, but also decreases long-term inflammation and improves long-term neurological outcome in both transient and permanent ischemia models.

MATERIALS AND METHODS

Animals

Male Crl:CD (SD)BR rats (Charles River, Calco, Italy) were used. Procedures involving animals and their care conformed to institutional guidelines that are in compliance with national (D.L. n.116, G.U. suppl. 40; February 18, 1992) and international laws and policies (EEC Council Directive 86/609, OJ L 358,1; December 12,1987; NIH Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996).

Drugs

Reparixin (as L-lysine salt) was from Dompé pha.r.ma. s.p.a., L'Aquila, Italy. The drug was dissolved in saline and administered as described in the text.

Transient Cerebral Ischemia

We used an intraluminal occlusion method with subsequent reperfusion (17). Overnight fasted rats (300-330 g) were anesthetized with 2-3% isoflurane in N_2O/O_2 (70%:30%) and a Stren nylon filament suture, blunted at the tip by heat to 0.35 mm diameter, was advanced through the right common carotid artery (CA) and the internal CA up to 19 mm from the bifurcation of the common CA and the external CA. Heparin (30U) was administered intravenously (i.v.) before insertion of the filament. Reperfusion began 90 min after MCA occlusion. The same surgery was performed in sham-operated rats but no ischemia was performed. Rectal temperature was monitored during ischemia and reperfusion period and, when it started rising above 37°C, the animals were placed in a cold room (10°C) and 70% alcohol was applied if there was a sudden rise (18). Adequate MCA occlusion was judged from neurological behavior, shown by gait disturbances with circling to the left (17).

Permanent Cerebral Ischemia

Fed rats (250-280 g) were anesthetized with chloral hydrate (400 mg/kg). The common CAs were visualized and the right one was occluded. A hole adjacent and rostral to the right orbit allowed visualization of the MCA, which was cauterized distal to the rhinal artery. To produce a penumbra around this fixed MCA lesion, the contralateral common CA was occluded for 1 h using traction with fine forceps (10). Body core temperature was held thermostatically at 37°C. In the present study we selected 24 h for evaluation of the injury because this time provides maximum infarction for both permanent and transient MCA occlusion (19).

Quantification of Ischemic Volume

To evaluate the extent of injury the rats were killed 24 h after ischemia and the brains were removed, transferred to cold saline and twelve serial 1-mm thick sections were cut through the entire brain. Six alternate sections were stained with triphenyltetrazolium chloride (TTC) (Sigma, St. Louis, MO, USA) as previously described (8,20). The extent of injury was quantified in six sections using a computerized image analysis system (AIS version 3.0 software, Imaging Research, St. Catherine's, ON, Canada). The other six sections were frozen on dry ice and stored at –80°C until MPO was measured.

Immunohistochemical and Histochemical Analyses

For immunohistochemical analyses, rats were perfused with paraformaledhyde and immunohistochemistry was performed on 30 μm free-floating sections using anti-CD11b (MRC OX-42) mouse monoclonal antibody (1:50; Serotec, UK) or anti-GFAP mouse mono-

clonal antibody (1:250; Immunological Sciences, Rome) as previously described (21). To quantitate the extent glial reaction, the area of GFAP and CD11b immunoreactivity in the ischemic hemisphere was measured using a digital image analyzer (Olympus DP Software). Glial spreading was calculated as the percentage of the GFAP- or CD11b-positive area over the total area of the ischemic hemisphere.

Histochemical determination of PMN was done on 5 μm paraffin sections by the naphthol AS-D chloroacetate technique for esterase that stains PMN in red (7,22). To quantitatively assess PMN infiltration, MPO activity in the tissue homogenate from the two hemispheres was quantified in six alternate sections for each animal as previously described (8). MPO activity is expressed as ΔA/min/mg protein and is the difference between the ipsilateral and the contralateral hemisphere.

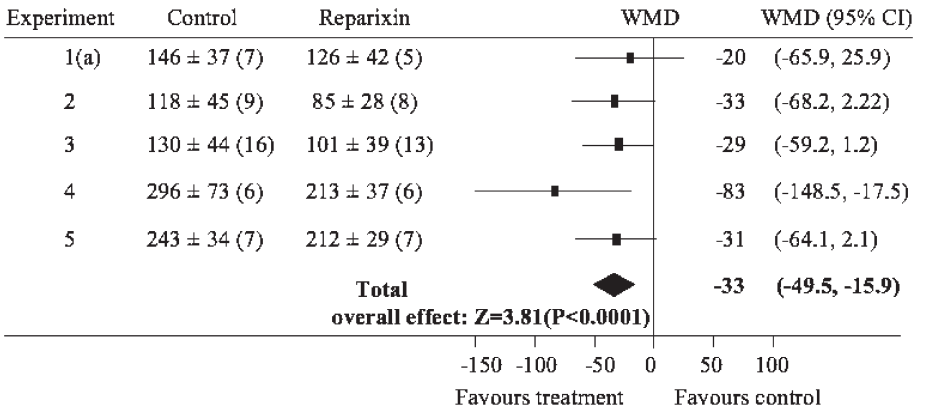

Figure 1. Effect of reparixin on infarct volume 24 h after permanent MCAO in five different experiments. Results of individual experiments are expressed as the infarct volume in mm^3 and are the mean ± S.D. The number of rats in each group is indicated in parenthesis. Reparixin (or saline) was administered with one of the following schedules: Experiments 1–3: 15 mg/kg i.v. at the time of MCAO followed by four 15 mg/kg s.c. doses at 2 h intervals; experiments 4-5: 15 mg/kg i.v. 1 h after MCAO, followed by three 15 mg/kg s.c.doses at 2 h intervals. Infarct volumes are determined 24 h after MCAO. Data meta-analysis was performed using Cochrane Collaboration's Review Manager software (RevMan Version 4.2.9 for Windows. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2003.). Experiment 1 is taken from ref (8).

MRI Evaluation

MRI measurements were taken 2, 24, and 48 h after ischemic insult using a 4.7T, vertical superwide bore magnet and a Bruker Advance II spectrometer with microimaging accessory. The ischemic volume was determined by trace of apparent diffusion coefficient maps (Tr(D)) computation as previously described (23,24). The progression of the ischemic damage was assessed over time using ANOVA for repeated measurements with lesion volume (evaluated by MRI) as dependent variable and the treatment as independent variable.

Neurological Deficits

Neurological deficits were evaluated using the foot fault (14), Bederson's (13), and De Ryck's (12) tests. In the postural reflex test of Bederson rats were scored as follows: grade 5, normal; grade 4, moderate (forelimb flexion and no other abnormality); grade 3, severe (reduced resistance to lateral push toward the paretic side, and forelimb flexion); grade 2, severe (same behavior as grade 3, with circling toward the paretic side when pulling the tail on the table); grade 1, severe (same behavior as grade 2, with spontaneous circling); grade 0, no activity. The limb-placing test developed by De Ryck examines sensorimotor integration in limb placing responses to visual, vibrissae, tactile, and proprioceptive stimuli. For each test, limb placing scores were 0, no placing; 1 incomplete and/or delayed (> 2 seconds); or 2, immediate and complete placing. For each body side the maximum limb placing score was 16.

The foot-fault test measures the ability of the animal to integrate motor responses. The rats were placed on a grid with 2 cm spaces between 0.3 cm diameter metal rods and were observed for 2 min. With each weight-bearing step, the paw may fall or slip between the wires and this is recorded as foot fault. The number of foot-faults for the paws contralateral and ipsilateral to the infarction was recorded with the number of successful steps and the foot-fault index was calculated as the percentage of contralateral limb foot-faults per limb step minus the percentage of ipsilateral limb foot-faults per limb step.

RESULTS

Effect of Reparixin on Ischemic Damage in Transient and Permanent Ischemia

We first confirmed the efficacy of reparixin in the model of transient cerebral ischemia used in our earlier work (8) and, additionally, investigated the regional selectivity of its protective action. Table 1 shows the results from two experiments where reparixin was administered on the first day at 15 mg/kg i.v. starting 2 h after reperfusion or at the time of ischemia followed by two or four 15 mg/kg s.c. doses at 2 h intervals. Reparixin prevented mainly, if not only, cortical damage. In experiment 1, reparixin reduced total infarct volume by 75%, and protection was much more evident in the cortex (–93%) than in the striatum (–37%). Of note, cortical damage contributed to 66% of the total damage in this experiment. In experiment 2, where cortical damage contributed to 71% of the total damage, reparixin reduced cortical damage by 67% but did not reduce striatal damage.

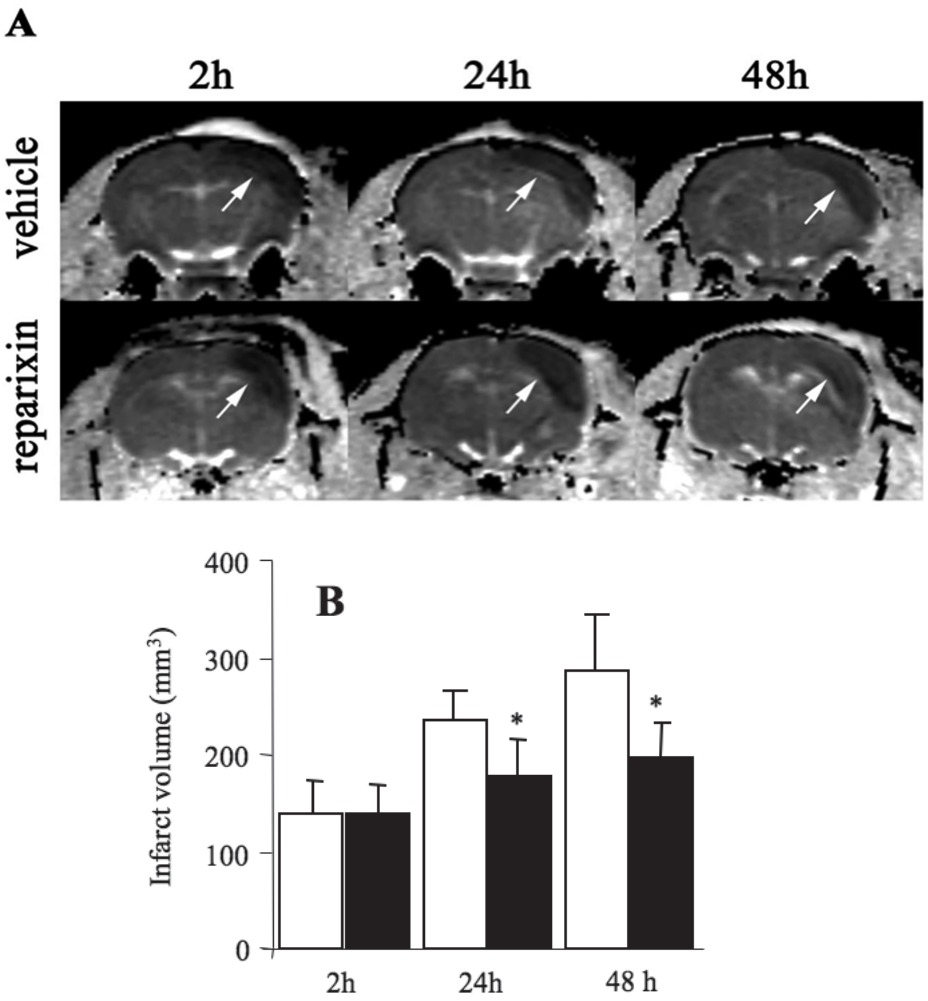

Figure 2. Evolution of cerebral ischemia in rats treated with vehicle or reparixin after permanent ischemia. Reparixin (15 mg/kg) was given 1 h after MCAO followed by three 15 mg/kg s.c. doses at 2 h intervals. A) Representative Tr(D) images of a coronal brain section taken 2, 24, and 48 h after the induction of ischemia in rats treated with vehicle (top) or reparixin (bottom). B) Infarct volume measured at different times in the Tr(D) images of vehicle (n = 8) or reparixin-treated rats (n = 8). *The evolution of the lesions with time, was significantly different from vehicle and drug treated groups, considering the whole time course of each group ($P < 0.05$).

We also performed several experiments with reparixin in a model of permanent MCAO. In a first set of experiments, we published that reparixin, even when given at the time of MCAO, resulted in only a trend (–15%, not significant) for a reduction in infarct volume (8) although it was as effective in reducing PMN infiltration as in transient MCAO. Five independent experiments, performed over a two-year period, where reparixin was tested in permanent MCAO showed a trend for a decreased infarct volume in the reparixin-treated group but this reached statistical significance in only one experiment. As shown in Figure 1, meta-analysis (25) was performed to compare the different experiments and provided the weighted mean difference (WMD) at 95% confidence interval. Heterogeneity between the experiments was calculated using the chi squared test. Because the test for heterogeneity was negative ($Chi^2 = 2.63$, $P = 0.62$), analyses were fit in a fixed effect model, but the results were the same when a random model was used. It can be seen that the overall effect of reparixin was highly significant ($P < 0.0001$).

To further confirm these results, we also evaluated by non-invasive MRI the evolution of cerebral infarct in permanent MCAO with or without reparixin (15 mg/kg, i.v. on the first day 1 h after MCAO followed by 3 s.c. doses at 2 h intervals).

As shown in Figure 2, panels A-B, the temporal evolution of the Tr(D)-derived average volume of the ischemic lesions differed significantly between controls and treated rats. The size of the Tr(D)-derived volume of the lesion at 2 h from the induction of ischemia was similar in vehicle and drug treated rats, but the lesions at 24 h significantly decreased in the reparixin group. While the lesion continued to enlarge over time in the vehicle-treated rats, lesion volume in the reparixin treated rats increased only slightly and the difference between the two groups were even greater at 48 h. The evolution of the lesions with time, was significantly different from vehicle and drug treated groups, considering the whole time course of each group ($P < 0.05$).

Effect of Reparixin on Long-term Neurological Deficits

We next investigated the effect of reparixin on long-term (up to 1 month) neurological recovery using the treatment schedules described above.

In transient ischemia, as shown in Figure 3, panels A-C, reparixin significantly improved sensorimotor recovery (De Ryck's test) after transient ischemia starting from 24 h after ischemia compared with saline controls. The improvement of neurological function by reparixin was maintained up to one month. A similar pattern was observed with Bederson's test and foot-fault test, although the differences for these tests were not statistically significant.

Also in permanent ischemia, reparixin significantly improved neurological func-

tions in rats as early as 1 day after MCAO (Figure 3, panels D-F).

Comparison of Different Treatment Schedules and Therapeutic Window in Transient MCAO

When reparixin was given for 3 days a significant rescue of neurological functions was observed for all the tests and throughout the observational period as shown for the De Ryck's (Figure 4, panels A–C). The better effectiveness of multiple doses of reparixin treatment to promote functional recovery after transient cerebral ischemia in rats, suggest that administration by continuous infusion could be the optimal treatment schedule, in view of a potential therapeutic use of the compound. Thus, to assess the efficacy of reparixin administered by continuous infusion on functional recovery from transient cerebral ischemia, the compound (15 mg/kg) was administered i.v. 2 h after reperfusion followed by s.c. infusion. The rate used was 10mg/h/kg in the following 48 h with the aim of achieving a plasma steady state concentration (Css) of 8μg/mL (30ng/mL of free drug taking into account protein binding (15)), a plasma Css superimposable to Css reached in human volunteers. Reparixin infusion significantly improved neurological functions starting from 1 day after ischemia and throughout the observational period (Figure 4, panels D-F). The improvement of sensorimotor recovery obtained with the De Ryck's test using the continuous infusion were confirmed by a significant effect with the Bederson's and the foot-fault tests.

To better define the therapeutic window, we also tested reparixin administered 6 h after ischemia (4.5 h after reperfusion). In these experiments, reparixin (15 mg/kg) was given i.v. 6 h after MCAO followed by s.c. infusion for 48 h at a rate of 10mg/h/kg, as shown in Figure 4, panels G-I. This led to a significant improvement of 24-h neurological function as evaluated by De Ryck's and Bederson's tests, while there was only a trend for the foot fault test.

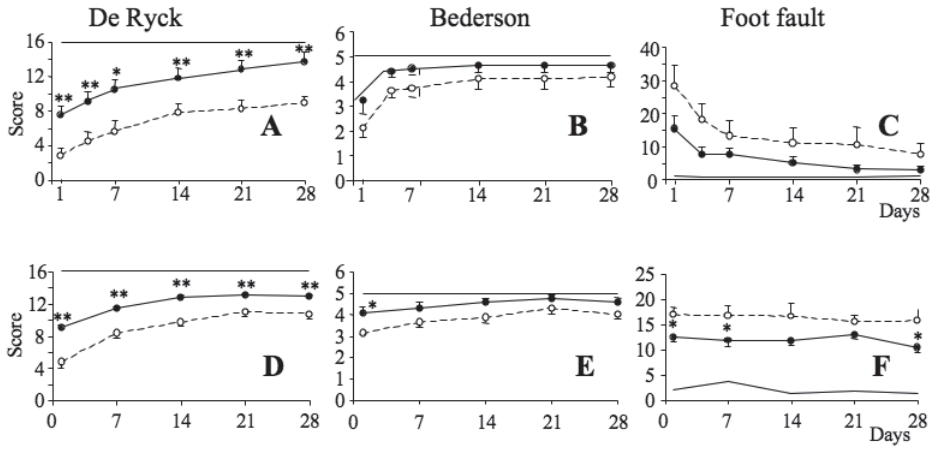

Figure 3. Effect of reparixin treatment on neurological deficits after transient (A–C) or permanent (D–F) MCAO. Transient MCAO (panels A-C): reparixin was administered 15 mg/kg, i.v. 2 h after reperfusion (i.e. 3.5 h after MCAO) followed by 2 15 mg/kg s.c. doses at 2 h intervals. Permanent MCAO (panels D-F): reparixin (15 mg/kg) was given 1 h after MCAO followed by 3 15 mg/kg s.c. doses at 2 h intervals. Neurological deficits were evaluated by De Ryck's (left panels), Bederson's (middle panels) and foot-fault tests (right panels) starting from 24 h after MCAO. Open circles, vehicle; closed circles, reparixin. For comparison the scores of sham-operated rats are also shown as a thin line. Data are mean ± S.E. of 7 rats. Statistical differences (Mann-Whitney test): $*P < 0.05$ and $**P < 0.01$ vs. saline.

Reparixin Reduces PMN Infiltrate and Long-term Inflammation in Ischemic Brain

Histochemical analysis in Figure 5 (A–D) shows that a significant PMN infiltration takes place 24 h after both transient MCAO and, to a lesser extent, permanent MCAO, and is reduced by reparixin given as described in Figures 1–3. Quantitative assessment of PMN infiltration using MPO (Figure 5E) show that reparixin significantly reduced 24-h PMN infiltrate by 70% and 40% in transient and permanent MCAO, respectively.

We evaluated the effect of reparixin on long-term inflammation after transient or permanent MCAO by immunohistochemical analysis for GFAP, a marker for hypertrophic astrocytes, or CD11b, a marker for the activated microglia. The effect of reparixin in reducing astrocyte hypertrophy after transient and permanent ischemia in representative rats is reported in Figure 6.

In transient ischemia, GFAP staining shows that, in contrast to coronal sections from control animals (A), ischemic rats present a stronger immunoreactivity (B) that is reduced and confined to a small subcortical region in reparixin-treated rats (C). The same anti-inflammatory effect of reparixin is detectable using anti-CD11b (D–F): while non-ischemic rats show little immunoreactivity (D), diffuse staining is present in the ipsilateral side of ischemic rats (E) and greatly diminished by reparixin either in terms of intensity or spreading (F).

Staining with GFAP in rats undergoing permanent ischemia show that, compared with control rats (G), ischemic rats show increased staining in the whole cortical and subcortical area corresponding to the side where the necrotic area is (H), while in reparixin-treated rats the staining is very low and limited to ischemic core (I). Of note, although permanent ischemia led to marked destruction of the tissue close to the focal zone, the preserved region showed GFAP immunoreactivity (H), and reparixin both reduced tissue loss and GFAP staining (I). As in the case of transient ischemia, CD11b immunoreactivity was strongly reduced in brains

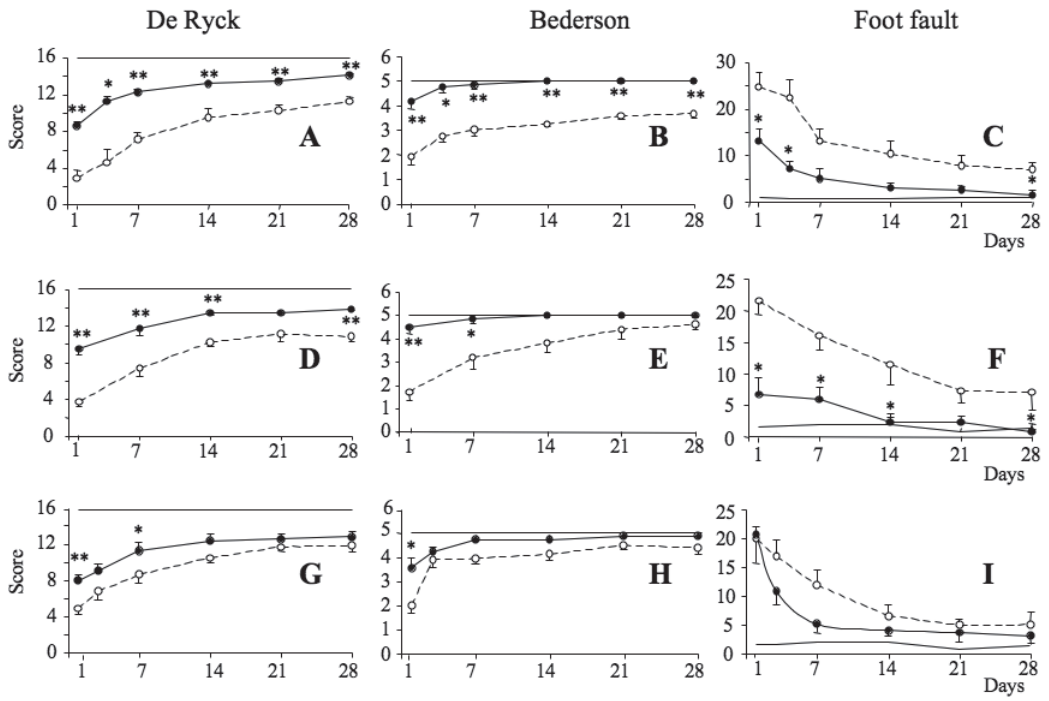

Figure 4. Effect of reparixin treatment under different schedules on neurological deficits after transient MCAO. Transient (1.5 h) MCAO was performed. Reparixin was administered as follows: Panels A-C: 15 mg/kg, i.v. 2 h after reperfusion (i.e. 3.5 h after MCAO) followed by two 15 mg/kg s.c. doses at 2 h intervals, then 3 times/day on days 2 and 3. Panels D-F: 15 mg/kg, i.v. 2 h after reperfusion (i.e. 3.5 h after MCAO) followed by s.c. infusion for 48 h at a rate of 10mg/h/kg. Panels G-I: 15 mg/kg, i.v. 4.5 h after reperfusion (6 h after MCAO) followed by s.c. infusion for 48 h at a rate of 10mg/h/kg. Neurological deficits were evaluated by De Ryck's (left panels), Bederson's (middle panels) and foot-fault (right panels) tests starting from 24 h after MCAO. Open circles, vehicle; closed circles, reparixin. For comparison, the scores of non-ischemic, sham-operated rats, i.e. "normal" values, are also shown as a thin line (normal values were: 16 for the De Ryck's test, 5 for the Bederson's test and 1–4 for the foot-fault test. Data are mean ± S.E. of 6–7. Statistical differences (Mann-Whitney): *P < 0.05 and **P < 0.01 vs. saline.

from rats undergoing permanent ischemia treated with reparixin (J-L).

Because loss of integrity close to the area of injury observed in ischemic brains can be considered indirect parameters of tissue damage (26), it is of interest to note that, in contrast to untreated ischemic rats, the sections obtained from reparixin-treated animals did not show any macroscopic feature of sufferance close to the injured area. Reparixin almost completely reduced long-term inflammation in the cortex, in agreement with the reduction in the infarct area where the cortical region is preferentially protected.

We also analyzed the morphology of the GFAP- and CD11b-positive cells in these samples. Analysis of higher magnification pictures (not shown) indicated that, in both models of MCAO, the morphology of GFAP-stained activated astrocytes does not seem different between the two groups. CD11b-positive cells show the typical morphology of activated microglia and, as described for GFAP, no difference in microglial morphology, at the single cell level, was detected by comparing the area of neuroinflammation of untreated and reparixin-treated mice.

The quantification of the GFAP and CD11b immunoreactivity further confirmed the beneficial effect of reparixin in reducing the spreading of astrocytes and microglia in cerebral area of rats after transient ischemia, as shown in Table 2. Of note, this quantitative assessment of GFAP and CD11b immunoreactivity was not performed in brains with permanent ischemia because of the loss of integrity close to the area of injury observed in untreated rats with permanent ischemia (as shown in Figure 6 H, K).

DISCUSSION

The protective action of reparixin demonstrates the pathogenic role of inflammation induced by chemokines, particularly PMN chemoattractants acting on CXCR1/2, in cerebral ischemia (27,28). This is in agreement with the notion that PMN infiltration is important in post-ischemic damage in the brain as suggested by several studies using anti-leukocyte strategies in experimental stroke (2).

We characterized the effect of reparixin, using different routes of administration, some of which, such as infusion, may better reproduce the clinical setting, in reducing infarct size in the model of

transient ischemia and could show for the first time that reduction in infarct size achieved by an anti-CXCR1/2 agent is accompanied by an improved long-term neurological recovery.

This observation strengthens the concept that inflammatory pathways may be important pharmacological targets in cerebral ischemia even looking at long-term effects that may better reflect the balance between neuronal injury and neuronal repair. While it has been postulated that inflammatory responses in the CNS may be beneficial in some contexts as potential sources for trophic factors (29), others have pointed out that inflammation can actually inhibit neurogenesis and the latter may be improved by anti-inflammatory drugs (30–33). The recently published observation that CXCL1, by acting on CXCR2, is an anti-apoptotic factor for rat astrocytes (34) is in agreement with ours showing that CXCR1/2 inhibition decreases astrocytosis, and supports the concept of a pathogenic, rather than protective, role of astrocytosis in our models. These findings indicate that inhibition of inflammation, or at least its CXCR1/2-mediated component, does not hamper long-term recovery from stroke.

The improvement of neurological functions reported here in the model of permanent MCAO deserves further discussion. In fact, in an early report, showing that reparixin reduced PMN infiltration but did not reduce infarct size significantly, we concluded that reparixin was not protective in permanent ischemia (8), but meta-analysis of several experiments clearly shows a protective effect of reparixin in this model as well. A protective effect was evident also when evaluating damage by either MRI or neurological tests. Clearly, protection by reparixin is less marked in permanent MCAO than in transient MCAO, and this could easily be explained by the fact that PMN infiltration, and thus its pathogenic contribution, is greater when reperfusion takes place. Furthermore, reperfusion might allow production of toxic oxygen species by the infiltrating leukocytes.

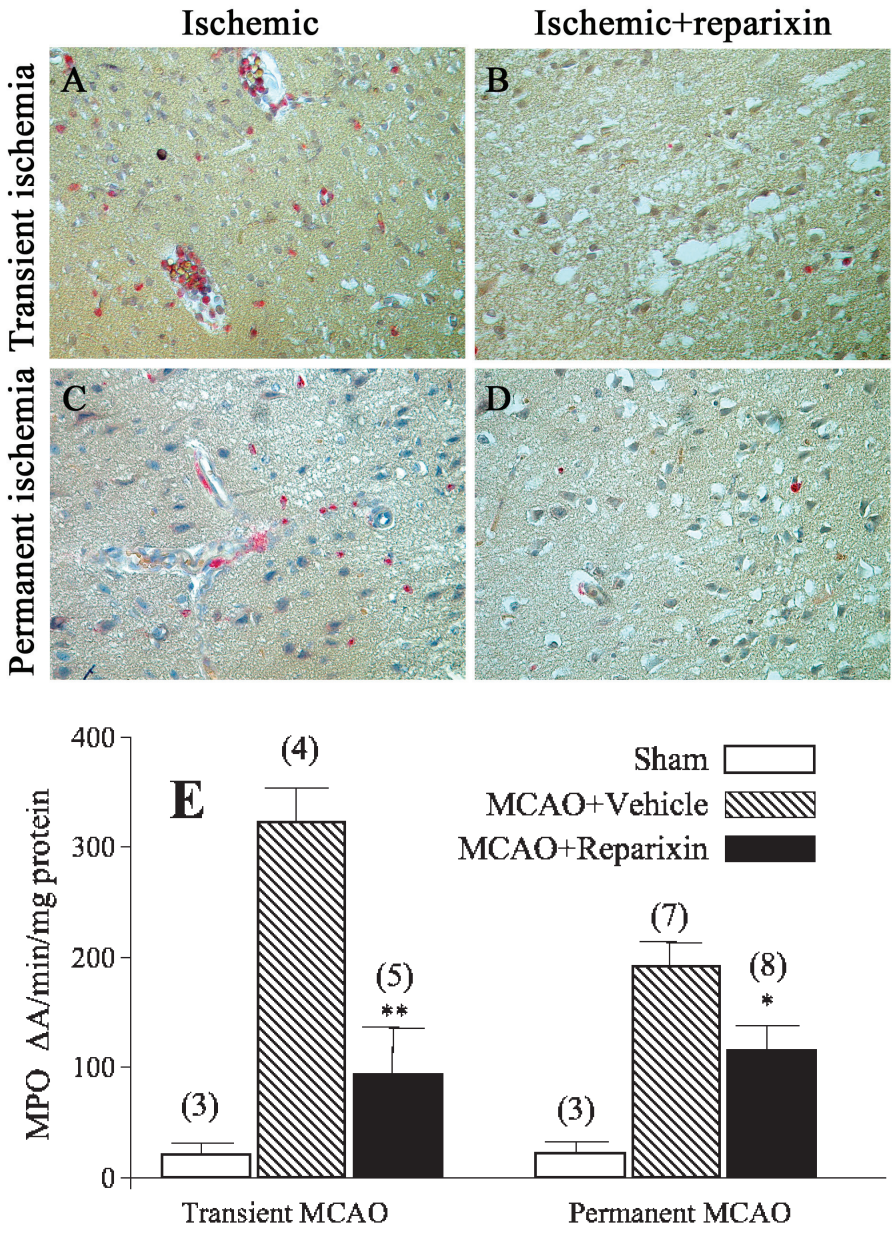

Figure 5. Reparixin decreases short-term (24 h) PMN infiltrate in the ischemic area. Representative histochemistry (400 ×) of the ischemic cortex 24 h after transient (A–B) or permanent (C-D) MCAO with vehicle (A, C) or reparixin (B, D). PMN are stained in red. No PMN was seen in sham-operated rats (not shown). Quantification of PMN by MPO activity is shown in panel E where data are expressed as ΔA/min/mg protein (difference between the ipsilateral and the contralateral hemisphere) and are the mean ± SE (number of rats in each group is indicated in parentheses). * $P < 0.05$, ** $P < 0.01$ vs. respective MCAO + saline by Student t-test.

Our observation that reparixin is more effective in reducing cortical infarction than subcortical infarction (Table 1), is compatible with the model of transient cerebral ischemia, where the MCA is occluded at the origin (at the junction of the anterior and middle cerebral arteries), producing an ischemic area in striatum and overlying cortex. During the ischemic time, the local cerebral blood

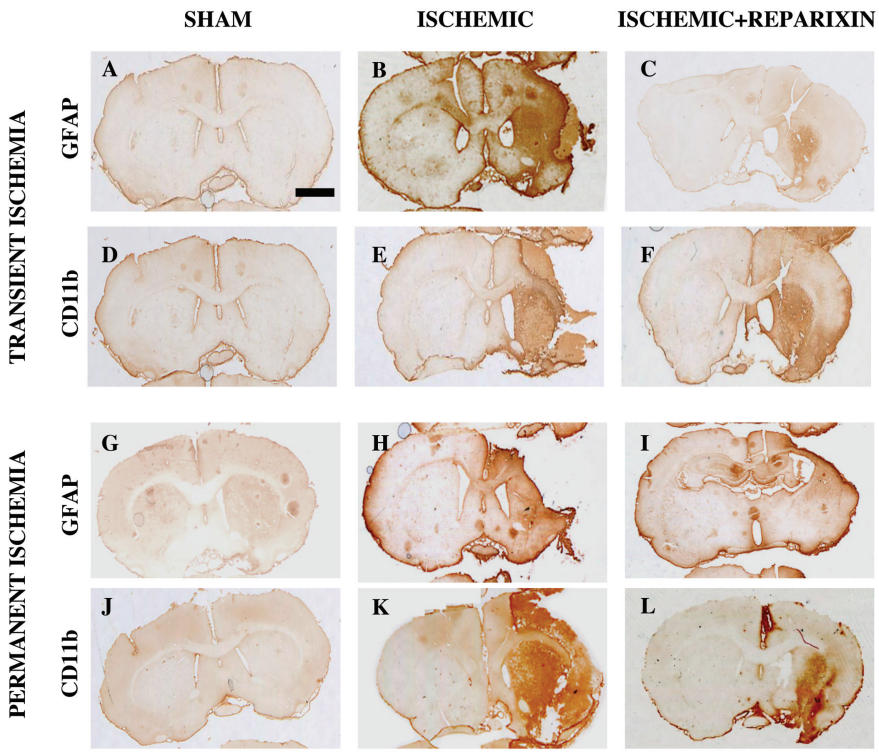

Figure 6. Effect of Reparixin on long-term reactive astrocytosis (GFAP staining) and microglia proliferation (CD11b staining) in transient (A–F) and permanent (G–L) ischemia. Rats were treated with reparixin as follows: transient ischemia, 15 mg/kg, i.v. 2 h after reperfusion (i.e. 3.5 h after MCAO) followed by two 15 mg/kg s.c. doses at 2 h intervals, then 3 times/day on days 2 and 3; permanent ischemia, 15 mg/kg i.v. 1 h after ischemia then s.c. every 2 h, 3 times. Analysis was performed 1 month after ischemia with anti-GFAP (A–C, G–I) or anti-CD11b (D–F, J–L).

flow of both areas is similarly reduced, but during reperfusion the cerebral blood flow remains depressed in the ischemic striatum and gradually recovers to its control value in the ischemic cortex (Takagi et al 1995). Consequently, as discussed in a recent review (Carmichael 2005), the striatum is the ischemic core zone and the infarction in this area is mostly necrotic and more resistant to most neuroprotective agents while cortical infarction is a region of delayed, progressive neuronal death or an ischemic penumbra more susceptible of neuroprotection. Interestingly, a similar pattern was reported with IL-1 receptor antagonist, another anti-inflammatory agent (35), and in a study showing that striatal injury by MCAO is not ameliorated by PMN depletion (36). Also the reduction of ischemic damage by erythropoietin, a neuroprotective agent that has a marked anti-inflammatory action in this context as well (16), is more pronounced in the cortical area than in the subcortical one (37). Of note, preliminary clinical trials with these two agents have indicated that they may be beneficial in the therapy of stroke (38,39).

In conclusion, the data from this study support the idea that CXCL8-mediated inflammation plays an important role in ischemic damage and provides a validation of the approach taking advantage of CXCR1/2 inhibitors.

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Table 2. Quantification of glial reaction in transient MCAO

Treatment	GFAP	CD11b
Vehicle	46.5 ± 3.0	59.2 ± 5.7
Reparixin	17.6 ± 1.6**	22.7 ± 1.0**

[a]Data are expressed as the percentage of immunopositive area (see Figure 6 for representative pictures and experimental details) in the ischemic hemisphere and are the mean ± SE of 3 rats for each group. Ten serial sections for each animal were analyzed for each marker. $^{**}P < 0.01$ by Student t-test.

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