METHYLPREDNISOLONE TREATMENT DELAYS REMOTE CELL DEATH AFTER FOCAL BRAIN LESION

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Abstract—Glucocorticoids have a prominent role in the treatment of CNS injuries. However, the cellular consequences of glucocorticoid treatment on remote degenerative responses after focal brain lesions have been poorly investigated. Here we examine the effectiveness of a high dose (50 mg/kg) of methylprednisolone sodium succinate (MPSS) in reducing neuronal loss, glial response and glial-derived inflammatory mediators in inferior olive and pontine nuclei after lesion of the contralateral cerebellar hemisphere using immunohistochemistry and Western blot techniques.

Quantitative analysis demonstrated that MPSS treatment significantly improved the survival of neurons in remote pre-cereellar stations. This survival was accompanied by reduction in the postlesional activation of microglia, astrocytes and interleukin-1 beta (IL-1β). Cell death resumed after suspension of MPSS treatment and this delayed wave of cell loss was paralleled by reactivation of the inflammatory markers analyzed.

The present study confirms the importance of inflammatory events in inducing remote cell death and that this type of degeneration can be delayed by MPSS treatment. Furthermore, the sustained effect of MPSS treatment, up to 28 days postlesion, and the reactivation of the degenerative phenomena after its suspension, support the hypothesis that glucocorticoid treatment, although capable of delaying cell death mechanisms, is not effective in blocking the cascade of remote degenerative events started by the primary lesion. © 2008 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: glucocorticoids, inferior olive, pontine nuclei, glia, inflammation, remote degeneration.

Glucocorticoids are well known for their anti-inflammatory and immunosuppressant properties (Marx, 1995; Ryu et al., 2006; Wilcens and De Rijck, 1997; Barnes, 2006) and are widely used to treat many CNS injuries (Hall, 1993, 1985; Giannotta et al., 1984; Leyvold et al., 2007; Anderson and Goodkin, 1998; Sloka and Stefanelli, 2005). Nevertheless, the efficacy of glucocorticoids in reducing or in promoting neurological recovery is presently being questioned (Pointillart et al., 2000; Short et al., 2000; Kamano, 2000; Gomes et al., 2005; Coleman et al., 2000). Beneficial effects of glucocorticoid treatment have been associated with reduced macrophages accumulation (Sekiya et al., 2001), down-regulation of inducible nitric oxide (NO) synthase transcription (Yu et al., 2004) and inhibition of apoptosis (Vaquero et al., 2006; Yu et al., 2004). However, there are also conditions in which glucocorticoids do not have anti-inflammatory effects and augmented inflammation and exacerbated neuronal death have been reported (Sapolsky, 1985; MacPherson et al., 2005; Dinkel et al., 2003).

After focal brain damage, neuronal death is present also in regions remote to the primary site of lesion. Remote cell death has been used to indicate different degenerative phenomena. In the present study, following the term used by Block et al. (2005), we will refer to “remote cell death” as those phenomena occurring in regions without spatial continuity, but functionally linked, with the primary lesion site. These remote effects have been linked to antero- and retrograde degeneration and have been considered an important outcome predictor (Binkofski et al., 1996). Further, sustained immune responses have been shown in regions far from the primary insult (Block et al., 2005). They are characterized by activation of resident brain glial cells, astrocytes and microglia, as well as by blood leucocyte accumulation. These cells, in concert, secrete soluble cytokines, adhesion molecules and other inflammatory mediators (Block et al., 2005; Dirmagi et al., 1999; Barone and Feuerstein, 1999). Although glucocorticoid treatment has been extensively proposed for the treatment of different CNS pathologies (Zhang et al., 2007; Oudega et al., 1999; Sekiya et al., 2001; Heiduschka and Thanos, 2006), its use in controlling remote cell death, as defined above, has never been evaluated.

Following cerebellar damage both regressive and re-active neuronal modifications have been reported in pre-cereellar neurons (Rossi et al., 1997; Strata et al., 1997; Viscomi et al., 2004). Remote regressive changes have been described in two waves of neuronal death, one acute and one delayed, that is, still present after 1 month. Remote delayed cell death has been described in detail both quantitatively and morphologically, and the involvement of neuronal nitric oxide synthetase (nNOS) enzyme and purinergic receptors has been clearly demonstrated (Flo-
renzano et al., 2002; Viscomi et al., 2004). Remote cell death presents a time course characterized by an early first massive wave of neuronal loss followed by a less intense although prolonged second wave. The high reproducibility and the well known course of remote cell death in precerebellar neurons after lesion of one cerebellar hemisphere make this experimental model useful for investigating damage-inflammation-related phenomena in regions far from the primary site of insult.

The present study addresses the effects of a synthetic glucocorticoid (methylprednisolone sodium succinate: MPSS) treatment on remote damage after cerebellar lesion by analyzing cell survival and glial and inflammatory indexes in precerebellar nuclei.

**EXPERIMENTAL PROCEDURES**

**Animals and surgery**

Experiments were performed using 100 male adult Wistar rats (body weight 200–250 g; Harlan San Pietro al Natisone, Udine, Italy): 89 were used for cerebellar lesion, four for sham surgery and seven as unlesioned controls. Animals were group-housed in standard cages and kept under a 12-h light/dark cycle in an air-conditioned facility. The experimental protocol was approved by the Italian Ministry of Health in agreement with the guidelines of the European Communities Council Directive of November 24, 1986 (86/609/EEC) for the care and use of laboratory animals. All efforts were made to minimize the number of animals used and their suffering. For surgical procedures, the rats were deeply anesthetized by i.p. injections of chloral hydrate (400 mg/kg) and then positioned in a stereotaxic apparatus. The skin of the skull was incised and the occipital bone was drilled and removed. Subsequently, the dura was incised to expose the cerebellum and the right cerebellar hemisphere was removed by suction. Then, the wound was sutured and the animals were returned to their cages. For the sham group surgery was interrupted after the dura lesion and, after suturing, animals were returned to their cages.

**Treatment**

Taking into account our previous data supporting the presence of different waves of neuronal cell death in precerebellar neurons after hemicerebellectomy (HCb) we decided to apply a protocol of continuous MPSS treatment up to 28 days. This schedule was chosen because at this time point the number of surviving neurons tends to be stabilized on a value around 20% of the pre-lesional values. We then added a further time point, 7 days after suspension of the treatment, to ascertain if the possible treatment effects were still present. Thus, after Hcb, rats were randomly divided into two groups: 1) Hcb MPSS continuous treatment (HCb-CT n=35 rats); 2) Hcb with saline treatment (HCb-S n=35 rats). Two further groups of animals were later added to evaluate the effects of short MPSS treatment: one group with Hcb and an acute MPSS treatment for 7 days followed by a further survival of 14 days (HCb-AT n=4 rats) and one group with Hcb and a continuous MPSS treatment for 28 days followed by a further survival of 7 days (HCb-IT n=7 rats). In Hcb-CT group, animals received a high dose of MPSS (Solu-medrol®, Pharmacia, Nerviano, Italy; 50 mg/kg i.p.) once daily until kill. In Hcb-S group, animals received an equivolume of saline once daily until kill. In Hcb-AT group, animals received the same dose of MPSS used in Hcb-CT group for 28 days and were killed 35 days after Hcb.

Taking into account the relatively high MPSS dose employed, the effects of a lower dose was evaluated at 7 and 14 days after Hcb in Hcb-CT group. In this group, after Hcb, animals (n=4 for each group) received 30 mg/kg i.p. of MPSS daily for 7 or 14 days and were killed at the end of treatment. The experimental procedures are summarized in Fig. 1.

During treatment body weight was monitored weekly in all Hcb animals. A weight loss, around 10%, was observed after the first week. This loss was rapidly recovered in the subsequent week. No differences were observed between MPSS high dose, MPSS low dose and saline-treated animals.

**Histology and histochemistry**

Animals were perfused transcardially with 250 ml of saline followed by 250 ml of 4% paraformaldehyde in a phosphate buffer (PB; 0.1 M; pH 7.4) under anesthesia induced by i.p. injections of sodium pentobarbital (60 mg/kg). Each brain was removed immediately, post-fixed in the same fixative for 2 h and, after three washes in PB, transferred to 30% sucrose in PB solution at 4 °C until it sank. Brainstem and cerebellum were cut into four series of 40 μm-thick transverse sections using a freezing microtome and collected in PB. Every fourth section was processed for NADPH-d (Saxon and Beitz, 1996) histochemistry and processed for Nissl counterstaining (NADPH-d/Nissl), as described previously (Viscomi et al., 2004). Finally, sections were mounted on chrome-alum-coated slides, air dried, dehydrated with ethanol, cleared in xylene and coverslipped. Bright field images were taken using a light microscope (Zeiss, Axioskop 2; Jena, Germany) equipped with a digital camera (Nikon, Coolpix 990).
**Immunohistochemistry**

Immunohistochemical procedures were performed at 4 °C on free-floating sections. PB 0.1 M was used for both chemical dilution and rinses. Before incubation with primary antibodies sections were incubated in a blocking solution (5% normal donkey serum, 0.3% Triton X-100, PB) for 2 h at room temperature. All primary antibody solutions were prepared in PB and 0.3% Triton X-100 and incubated overnight. Each incubation step was followed by three, 5-min rinses in PB.

Single immunofluorescence was performed to investigate microglial and astrocytic activations at all survival times considered. Sections were incubated with the following primary antibodies: mouse anti-nNOS (1:500; Sigma, St. Louis, MO, USA) to visualize microglial cells, or mouse anti-GFAP (1:500; Sigma, St. Louis, MO, USA) to visualize astrocytes. Subsequently, sections were incubated for 2 h at RT in a solution of Cy3-conjugated donkey anti-mouse IgG. Before the last rinse, sections were DAPI counterstained (1:1000; Sigma). Following incubation with the mix solution of primary antibodies, the sections were incubated for 2 h at RT in a mixture of secondary antibodies. These included Cy2-conjugated donkey anti-mouse IgG and Cy3-conjugated donkey anti-rabbit IgG (1:100; Jackson Immunoresearch Laboratories, West Grove, PA, USA). Further, to assess production of the glial noxious mediators IL-1β after HCb, a double immunofluorescence for nNOS and interleukin-1 beta (IL-1β) was carried out in all experimental groups at each time point considered. Sections were incubated with the following primary antibodies: mouse anti-nNOS (1:500; Sigma) and rabbit anti-IL-1β (1:200; Alomone, Jerusalem, Israel). Following incubation with the mix solution of primary antibodies, the sections were incubated for 2 h at RT in a mixture of secondary antibodies. IL-1β primary antibody was verified by preadsorption with its own specific target peptide (R&D Systems, Inc.). Preadsorption was carried out by mixing primary antisera, at the same concentration used previously, with recombinant rat IL-1β (10 μg/ml) for 2 h before incubation. Successively, sections were incubated in the secondary antibody mixture and processed as above.

To investigate the glial population responsible for the production of IL-1β, a triple immunofluorescence was performed. Sections were incubated in a solution of goat anti-IL-1β (1:1000), mouse anti-IL-1β (1:200) and rabbit anti-IL-1β (1:500). Following incubation with the mix solution of primary antibodies, sections were subsequently incubated for 2 h at RT in a mixture of secondary antibodies including Cy3-conjugated donkey anti-mouse IgG and Cy2-conjugated donkey anti-goat IgG (1:100; Jackson Immunoresearch Laboratories). The specificity of the IL-1β primary antibody was verified by preadsorption with its own specific target peptide (R&D Systems, Inc.). Preadsorption was carried out by mixing primary antisera, at the same concentration used previously, with recombinant rat IL-1β (10 μg/ml) for 2 h before incubation. Successively, sections were incubated in the secondary antibody mixture and processed as above.

**Quantitative and statistical analyses**

Qualitative and quantitative observations were limited to IO and Pn of the experimental side, i.e. projecting to the lesioned hemicerebellum. Cell counting of surviving neurons was performed on NADPH-d/Nissl-stained sections to assess the total number of neurons, as described previously (Viscomi et al., 2004). The immunoperoxidase material was examined under a light-transmission microscope (Zeiss, Axiostop 2) equipped with a CCD camera (ProgRes C10 plus, Zeiss). Single, double and triple immunofluorescence was examined under a confocal laser scanning microscope (Leica SP5; Leica Microsystems, Wetzlar, Germany) equipped with four laser lines: violet diode emitting at 405 nm (for DAPI), argon emitting at 488 nm and helium/neon emitting at 543 nm and 633 nm. To assess microglial and astrocytic activation within IO and Pn, quantitative analyses were performed off-line on confocal images acquired through the 20× objective at the 0.7 zoom factor. For IO, all labeled cells in two rectangular boxes (100 μm width × 200 μm length) randomly positioned in five regularly spaced sections were counted. Because of differences in the characteristics of the nuclei, for Pn quantitative data were obtained by adopting a different sampling strategy. Three digital square frames (200 × 200 μm) were placed at a regular distance to sample the entire medio-lateral extent of Pn. Only immunolabeled cells with a distinct nucleus (DAPI-positive) in the focal plane were counted. OX-42 and GFAP positive cells were digitally marked and recorded, and data were stored in an archive. All quantitative analyses were conducted blind to the animal's experimental group assignment. All data were expressed as mean ± standard deviation. Significances were tested using a two-way ANOVA (time × treatment) with Bonferroni post hoc test. In some cases, statistical analysis was performed with Student’s t-test. All statistical analyses were performed using Prism-4 software with the significance set at P < 0.01. For Western blot analysis densities of NeuN (46, 48 kDa) and β-actin (42 kDa) protein bands were measured using Kodak Image Station (KDS IS440CF) in control (CTRL; n = 3), in HCb-S (n = 3), in HCb-CBT (n = 3); the mean density ratio of NeuN 46 kDa/actin and NeuN 48 kDa/actin was calculated for each group. Data are the mean ratio between NeuN and actin and are presented as percentage of CTRL brains values. Statistical comparisons between HCb-S and HCb-CBT groups were made by using a two-way ANOVA with Bonferroni post hoc test. Significance was set at P < 0.01.

**RESULTS**

**Lesion features**

The extent of the cerebellar lesion was assessed by histological observation of the injury site in NADPH-d/Nissl-stained sections. All HCb animals considered in this work received a complete lesion of the right cerebellar hemisphere, with ablation of deep cerebellar nuclei and cerebellar peduncles (Fig. 2). In the sham surgery group no...
neurons of HCb-S and HCb-CT groups, showed an overall post hoc comparison performed on the number of surviving neurons in HCb-S, HCb-CT30 and HCb-CT groups at day 14 showed an overall significant effect for treatment (IO: \( F = 60.70 \), \( P < 0.0001 \); Pn: \( F = 94.39 \), \( P < 0.0001 \)). Post hoc comparisons demonstrated that HCb-CT data were significantly different from HCb-S (IO: \( P < 0.001 \); Pn: \( P < 0.001 \) and HCb-CT30 ones (IO: \( P < 0.001 \); Pn: \( P < 0.001 \)). No significant differences were present between HCb-S and HCb-CT30 groups (\( P > 0.05 \)) (Fig. 3E).

Data from HCb-AT and HCb-IT groups were used to address the delayed effect of MPSS treatment after its suspension. At day 21, HCb-AT rats presented approximately the same number of surviving neurons of the HCb-S rats (Fig. 3A, D) both in IO (393.3 ± 16.68 vs. 365 ± 20.45; \( P > 0.05 \)) and Pn (389.3 ± 12.69 vs. 353.3 ± 11.48; \( P > 0.05 \)) and, thus, fewer than the HCb-CT rats (Fig. 3C, D) (IO: 393.3 ± 16.68 vs. 667.3 ± 7.45; Pn: 389.3 ± 12.69 vs. 518.5 ± 13.96; \( P < 0.001 \)) (Fig. 3E day 21). At day 35 (Fig. 3E, day 35), the number of surviving neurons in HCb-IT was significantly lower than in HCb-S rats (IO: 418.8 ± 7.7 vs. 553.3 ± 22.45, \( P < 0.0001 \); Pn: 256 ± 6.6 vs. 334 ± 24.6, \( P < 0.0001 \)) but still significantly higher than in HCb-S rats (IO: 418.8 ± 7.7 vs. 236 ± 13.4, \( P < 0.001 \); Pn 256 ± 6.6 vs. 155.3 ± 6.3, \( P < 0.0001 \)). The rebound effect of the suspension of the MPSS treatment is also indicated by the comparison between data immediately after 7 days of treatment (HCb-CT at 7 days) and data obtained 14 days after suspension of the 7 day treatment (HCb-AT). A Student’s \( t \)-test clearly demonstrated the significant difference between the two groups (\( P < 0.0001 \)). Furthermore, comparison between HCb-CT at 28 days and HCb-IT demonstrated that suspension of a MPSS prolonged treatment is associated with a renewal of neuronal degeneration. Also in this case a Student’s \( t \)-test clearly demonstrated the significant difference between the two groups (\( P < 0.0001 \)) (Fig. 3E).

Quantification of NeuN protein in brain extracts has been related to neuronal cell density and differences in NeuN protein levels are considered an index of neuronal cell loss (Collombet et al., 2006). Therefore, NeuN protein was quantified using Western blot analysis in homogenates of IO plus Pn of CTRL, HCb-S and HCb-CT animals. As expected (Collombet et al., 2006; Unal-Cevik et al., 2004), the NeuN antibody revealed two bands at 46 and 48 kDa and another weak band at 66 kDa (Fig. 4A). As shown in Fig. 4A the treatment highly affected NeuN values. NeuN levels were evidently lower in HCb-CT and HCb-S preparations in comparison to control values. At each time point considered, HCb-S values were significantly inferior to HCb-CT ones for band 1 (Fig. 4B). Band 2 data were not so clear cut. Significant differences between HCb-S and HCb-CT data were present only at later time points (Fig. 4B, day 21 and 35). A two-way ANOVA followed by Bonferroni post hoc was performed on NeuN/actin densities.

MPSS treatment delays secondary cell death

The lesion of the right hemicerebellum induced retrograde cell death in the two main contralateral precerebellar stations, namely, the IO and Pn. In HCb-S animals the intensity of retrograde degeneration and the time course of cell death were comparable to those reported previously (Viscomi et al., 2004). The evaluation of the number of surviving neurons after HCb in precerebellar nuclei in HCb-CT and HCb-S animals presented self-evident differences at all time points considered. In both IO and Pn, cell density was evidently higher in the HCb-CT than in the HCb-S group (Fig. 3A, B). Highly significant differences between HCb-S and HCb-CT were already evident at day 7 after the lesion and were maintained throughout the MPSS treatment (Fig. 3E). A two-way ANOVA followed by Bonferroni post hoc comparison performed on the number of surviving neurons of HCb-S and HCb-CT groups, showed an overall significant effect for treatment (IO: \( F = 1450.0 \), \( P < 0.0001 \); Pn: \( F = 600.1 \), \( P < 0.0001 \)) and for time (IO: \( F = 999.1 \), \( P < 0.0001 \); Pn: \( F = 1932.0 \), \( P < 0.0001 \)) and also interaction was significant (IO: \( F = 60.6 \), \( P < 0.0001 \); Pn: \( F = 27.45 \), \( P < 0.0001 \)). The effect of 30 mg/kg daily injection of MPSS treatment was evaluated at 7 and at 14 days after HCb by comparing the number of Nissl stained neurons in IO and Pn of HCb-S, HCb-CT and HCb-CT30 animals. The number of surviving neurons in HCb-CT30 group was almost coincident with that recorded in HCb-S group at both time points (Fig. 3A, C). One-way ANOVA followed by Bonferroni post hoc comparison performed on the number of surviving neurons in HCb-S, HCb-CT and HCb-CT30 groups at day 14 showed an overall significant effect for treatment (IO: \( F = 60.70 \), \( P < 0.0001 \); Pn: \( F = 94.39 \), \( P < 0.0001 \)). Post hoc comparisons demonstrated that HCb-CT data were significantly different from HCb-S (IO: \( P < 0.001 \); Pn: \( P < 0.001 \) and HCb-CT30 ones (IO: \( P < 0.001 \); Pn: \( P < 0.001 \)). No significant differences were present between HCb-S and HCb-CT30 groups (\( P > 0.05 \)) (Fig. 3E).

Comparisons between unlesioned rats (CTRL) and rats killed 21 days after dura lesion (Sham) of the total number of neurons and of the number of neurons expressing nNOS demonstrated the total lack of effects of sham procedures on the survival of IO and Pn neurons (Figs. 3E; 5A–D). Sham procedures lightly influenced the number of astrocytes and microglial cells (Fig. 7A–D).

![Fig. 2. Bright field image of Nissl-stained section of the cerebellar lesion in a representative case. Note the complete absence of the hemisphere and deep nuclei of the right hemicerebellum, with complete sparing of surrounding structures. Scale bar=800 μm.](Image)
Fig. 3. MPSS treatment delays remote neuronal loss after Hcb. (A–D) Bright field images of Nissl-stained sections from IO of a saline treated rat (HCb-S; A), continuously MPSS 50 mg/kg treated rat (HCb-CT; B), continuously MPSS 30 mg/kg treated rat (HCb-CT30; C), and acute treated rat (HCb-AT; D) 21 (A; B; D) or 14 (C) days after Hcb. Note the higher density of neurons in B compared with A and C and D. Scale bars=45 μm. (E) Total number of neurons in IO and Pn in control (CTRL; empty rhombi), Sham (Sham; filled rhombi), saline treated (HCb-S; empty circles), MPSS 50 mg/kg treated (HCb-CT; filled circles), MPSS 30 mg/kg treated (HCb-CT30; filled squares), MPSS 50 mg/kg acutely treated (HCb-AT; empty squares) and MPSS 50 mg/kg interrupted treatment (HCb-IT; empty triangles) groups at different time points. *** $P<0.001$. 

mean ratio expressed as percentage of control values comparing HCb-S, and HCb-CT groups. These analyses demonstrated an overall significant effect for treatment (band 1: $F_{1,1005} = 51.8; P < 0.0001$; band 2: $F_{1,1005} = 22.9; P < 0.0001$) and for time (band 1: $F_{1,1005} = 176.6, P < 0.0001$; band 2: $F_{1,1005} = 22.5, P < 0.0001$) and also interaction was significant (band 1: $F_{1,1005} = 6.5, P = 0.01$; band 2: $F_{1,1005} = 82.8, P < 0.0001$).

**MPSS treatment has a slight effect on lesion-induced NADPH-d expression**

A role for NO in mediating remote effects of cerebellar damage was suggested previously and related to cell resistance to axotomy-induced cell death (Viscomi et al., 2004). To assess the effects of MPSS treatment on NO-mediated cell survival, we used NADPH-d histochemistry to evaluate the number of nNOS expressing cells in IO and Pn of the three experimental groups at the different time points.

After cerebellar lesion, both in HCb-S and HCb-CT the number of NADPH-d positive neurons showed the already reported biphasic trend, characterized by an increase until day 21 and a progressive reduction at the latest time points considered (Viscomi et al., 2004). This trend was present in both HCb-S and HCb-CT animals. A two-way ANOVA followed by Bonferroni post hoc comparison performed on the number of NADPH-d positive neurons of HCb-S and HCb-CT groups, showed an overall significant effect for treatment only in IO ($F_{1,1005} = 4.7, P = 0.01$) and in both nuclei for time (IO: $F_{1,1005} = 367.7, P < 0.0001$; Pn: $F_{1,1005} = 594.3, P < 0.0001$). Interaction was significant in both nuclei (IO: $F_{1,1005} = 7.5, P < 0.001$; Pn: $F_{1,1005} = 5.3, P < 0.001$).

Significant differences between HCb-S and HCb-CT rats were present at day 14 in IO (164 ± 12.80 vs. 133 ± 7.43; $P < 0.001$) and at day 21 both in IO (194.3 ± 8.77 vs. 172 ± 9.83, $P < 0.01$) and Pn (251.1 ± 4.41 vs. 233.8 ± 1.76; $P < 0.01$) (Fig. 5A, B) and at day 35 both in IO...
The effect of 30 mg/kg daily injection of MPSS treatment was evaluated at 7 and at 14 days after HCb by comparing the number of NADPH-d-stained neurons in IO and Pn of HCb-S, HCb-CT and HCb-CT30 animals. The number of NADPH-d positive neurons in both IO and Pn was not different in HCb-S and HCb-CT animals with the exception of days 14, 21 and 35 in IO and of days 21 and 35 in Pn. *** P<0.001; ** P<0.01. (C, D) Percentages of Nissl neurons expressing NADPH-d in IO and Pn of CTRL (empty rhombi), sham (filled rhombi), saline treated (HCb-S; empty circles), MPSS 50 mg/kg treated (HCb-CT; filled circles), MPSS 30 mg/kg treated (HCb-CT30; filled squares), MPSS 50 mg/kg acutely treated (HCb-AT; empty squares) or MPSS 50 mg/kg interrupted treatment (HCb-IT; empty triangles) rats. At each time point, the percentage was lower in HCb-CT than in HCb-S-treated animals.

(95.2±8.3 vs. 118.0±16.6, P<0.01) and Pn (101.3±12.1 vs. 124.3±14.8, P<0.01).

Further information on the relation between nNOS expression and neuron survival, was obtained by plotting NADPH-d stained neurons as percentages of IO and Pn surviving neurons (NADPH-d/Nissl ratio Fig. 5C, D). In MPSS-treated rats, the increment in the number of surviving neurons was not accompanied by a parallel increment in nNOS neurons and thus the NADPH-d/Nissl ratio was significantly different between HCb-CT and HCb-S groups.

Suspension of treatment affected nNOS induction. At day 21, after 14 days suspension, in the HCb-AT group the number of nNOS expressing neurons was similar to that recorded in HCb-C group at both time points to that recorded in HCb-S and HCb-CT groups. One-way ANOVA followed by Bonferroni post hoc comparison performed on the number of NADPH-d neurons in HCb-S, HCb-CT30 and HCb-CT groups at day 14 was not significant.

Suspension of treatment affected nNOS induction. At day 21, after 14 days suspension, in the HCb-AT group the number of nNOS expressing neurons was similar to that of HCb-S rats (IO: 84.0±5.8 vs. 95.2±8.3, P=0.27; Pn: 88±3.8 vs. 101.3±12.18, P=0.11), but was significantly lower than that of HCb-CT rats (IO: 84.0±5.8 vs. 118.0±8.3, P<0.01; Pn: 88±3.8 vs. 124.3±7.4, P<0.01) (Fig. 5A, B).

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and for time (IO: F = 238.1, P < 0.0001; Pn: F = 406.7, P < 0.0001) and also interaction was significant (IO: F = 30.9, P < 0.0001; Pn: F = 23.6, P < 0.0001).

MPSS treatment does not modify lesion-induced nNOS and P2X1 co-activation

Ionotropic purinergic receptors (P2XR) are ATP-gated cationic channels composed of seven known subunits (P2X1–7) and involved in different functions in neural tissue (Seitz et al., 1999). After cerebellar lesion, nNOS and P2X1,2 are induced in precerebellar neurons. This evidence has been interpreted as indicating functional interrelations between purinergic and nitrergic systems (Florenzano et al., 2006) and it has been linked to cell survival (Florenzano et al., 2002, 2008; Viscomi et al., 2004). To assess whether MPSS treatment is able to influence postlesional co-activation of nNOS and P2X1, we compared quantitative P2X1/nNOS double labeling data obtained in HCb-S, HCb-CT and HCb-AT groups at day 21 after axotomy. Data are presented in Fig. 6. They demonstrate a substantial similarity in the percentages of nNOS and P2X1 co-expression in the different groups.

MPSS treatment reduces glial activation in regions remote to the primary lesion

There is increasing evidence that brain injury is accompanied by a marked inflammatory reaction characterized by macrophage infiltration and glial cell activation (Moran and Graeber, 2004; Hurley and Coleman, 2003). To characterize interrelations between MPSS treatment and glial responses in regions remote to the primary focal damage, glial cell populations were evaluated by immunolabeling.

**Microglia response.** Starting on day 7, a large number of densely stained and ramified OX-42 immunopositive activated microglial cells were found in precerebellar nuclei of HCb-S animals. In HCb-S rats, a clear increment in OX-42-immunopositive cells at later time points, up to day 21, followed by a rapid decline was observed (Fig. 7A–B). Chronic high dose MPSS treatment had a notable effect on the number of OX-42 positive cells (Fig. 8A–D). In fact, at each time point analyzed (7–35 days), in HCb-CT animals the number of microglial activated cells was significantly lower than in HCb-S rats (Fig. 7A, B). A two-way ANOVA followed by Bonferroni rats post hoc comparison performed on the number of microglial cells of HCb-S and HCb-CT groups, showed an overall significant effect for treatment (IO: F = 195.4, P < 0.0001; Pn: F = 445.4, P < 0.0001) and for time (IO: F = 125.3, P < 0.0001; F = 303.8, P < 0.0001) and also interaction was significant (IO: F = 11.2, P < 0.0001; P = 24.5, P < 0.0001) (Fig. 7A, B).

The effect of 30 mg/kg of MPSS treatment was evaluated at 7 and 14 days after HCb by comparing the number of OX-42 positive cells in IO and Pn of HCb-S, HCb-CT and HCb-CT30 animals. At both time points, the number of OX-42 positive cells in HCb-CT30 group was lower than that present in HCb-S animals but higher than that observed in HCb-CT ones. One-way ANOVA followed by Bonferroni post hoc comparison performed on the number of OX-42 positive cells in HCb-S, HCb-CT30 and HCb-CT groups at day 14 showed an overall significant effect for treatment (IO: F = 24.58, P < 0.001; Pn: F = 96.86, P < 0.0001). Post hoc comparisons demonstrated that HCb-S data were significantly different from HCb-CT (IO: P < 0.001; Pn: P < 0.001) and HCb-CT30 ones (IO: P < 0.01; Pn: P < 0.001). No significant differences were present between HCb-CT and HCb-CT30 groups (P > 0.05) (Fig. 7A, B).

Suspension the MPSS treatment induced a dramatic increase in microglial cell activation. At day 21 in the HCb-AT group, the number of microglial cells was comparable to that observed in the HCb-S group both in IO (37.45 ± 2.21 vs. 40.75 ± 3.60; P > 0.05) and Pn (78.52 ± 3.25 vs. 83.93 ± 4.14; P > 0.05) but significantly higher than that of HCb-CT rats (P < 0.001) (Fig. 7A, B). At day 35, 7 days after the end of the MPSS treatment, in the HCb-IT animals the number of microglial cells was significantly higher than both HCb-S (compare Fig. 8I, E) (IO: 33.23 ± 1.2 vs. 17.38 ± 2.1, P < 0.0001; Pn 76.15 ± 2.1 vs. 46.53 ± 2.6, P < 0.0001) and HCb-CT animals (compare

![Fig. 6. P2X1 and nNOS co-localization. Histograms of the percentage of P2X1 and nNOS co-localization 21 days after HCb in IO and Pn of saline-treated (HCb-S), MPSS continuously (HCb-CT) or acutely (HCb-AT) treated rats. Each bar represents the mean value of P2X1/nNOS and nNOS/P2X1 co-localization. No significant differences among percentages of nNOS and P2X1 co-localization were observed.](image-url)
Astrocytic response. After damage, an intense astrocytic response was observed in HCb-S animals. Starting from day 7, in HCb-S animals a large number of densely stained and hypertrophic GFAP-immunopositive cells with long and ramified processes was found (Fig. 9A–B). The progression of GFAP induction in both nuclei was similar to microglial activation. Chronic high dose MPSS treatment had a notable effect on the number of GFAP positive cells (Fig. 9A–D). Starting from day 7 until day 35 in HCb-CT animals the number of GFAP-positive astrocytes was significantly lower than in HCb-S rats (Fig. 7C, D). A two-way ANOVA followed by Bonferroni post hoc comparison performed on the number of astrocytes of HCb-S and HCb-CT groups, showed an overall significant effect for treatment (IO: $F=177.9$, $P<0.0001$; Pn: $F=510.9$, $P<0.0001$) and for time (IO: $F=170.0$, $P<0.0001$; Pn: $F=474.3$, $P<0.0001$) and also interaction was significant (IO: $F=11.4$, $P<0.0001$; Pn: $F=27.2$, $P<0.0001$) (Fig. 7C, D).

The effect of 30 mg/kg of MPSS treatment was evaluated at 7 and 14 days after HCb by comparing the number of GFAP positive cells in IO and Pn of HCb-S, HCb-CT and HCb-CT30 animals. The number of GFAP positive cells in HCb-CT30 group was lower than that present in HCb-S animals but higher than that observed in HCb-CT ones. One-way ANOVA followed by Bonferroni post hoc comparison performed on the number of GFAP-positive cells in HCb-S, HCb-CT30 and HCb-CT groups showed an overall significant effect for treatment (IO: $F=25.78$, $P<0.001$; Pn: $F=51.01$, $P<0.0001$). Post hoc comparisons demonstrated that HCb-S data were significantly different from HCb-CT (IO: $P<0.001$; Pn: $P<0.001$) and HCb-CT30 ones (IO: $P<0.01$; Pn: $P<0.01$). No significant differences were present between HCB-CT and HCb-CT30 groups ($P>0.05$).

As observed for microglial cells, suspension of MPSS treatment reactivated the astrocytic response. At day 21, in HCb-AT rats the number of GFAP-positive astrocytes was significantly higher than in HCb-CT animals (IO: 41.40 ± 3.30 vs. 24.65 ± 1.60, $P<0.001$; Pn: 116.1 ± 5.63 vs. 74.23 ± 6.29, $P<0.0001$), and similar to that of HCb-S animals (IO: 41.40 ± 3.30 vs. 43.28 ± 4.64, $P>0.05$; Pn: 116.1 ± 5.63 vs. 124.2 ± 5.13, $P>0.05$) (Fig. 7C, D; day 21). Similarly, at day 35 the astrocytic response in HCb-IT animals was so intense as to exceed that observed in HCb-S (IO: 33.88 ± 1.7 vs. 21.45 ± 1.7, $P<0.001$; Pn: 100.9 ± 1.3 vs. 75.6 ± 1.3, $P<0.0001$) and HCb-CT rats (compare Fig. 9I, G) (IO: 33.88 ± 1.7 vs. 14.9 ± 1.9, $P<0.001$; Pn: 100.9 ± 1.3 vs. 48.8 ± 4.3, $P<0.0001$ (Fig. 7C, D).
MPSS inhibits production of glial-derived deleterious mediators

It is well known that the glial response induced by CNS damage is associated with release of inflammatory mediators (Stoll et al., 1998, 2002). In the present model we used immunohistochemistry to investigate the expression of the pro-inflammatory cytokine IL-1β and its response to MPSS treatment. No IL-1β immunopositive somata or processes were observed in precerebellar nuclei of control (Fig. 10A) and sham-operated rats. In HCB-S animals, from day 14 to day 28 a clear IL-1β labeling was observed in precerebellar nuclei (Fig. 10B). Double labeling with nNOS clearly indicated that IL-1β was present around...
surviving neurons expressing nNOS (Fig. 10B, D, E). IL-1β expression remained high until day 28 and then disappeared quite abruptly. In Hcb-CT animals, no IL-1β immunopositive cells were detected as long as the treatment lasted: 7-35-day time window (Fig. 10C). After suspension of MPSS treatment, i.e. Hcb-IT rats day 35, IL-1β was expressed in both IO and Pn. Similar expression of IL-1β after suspension of MPSS treatment was observed in the Hcb-AT group (Fig. 10D). The effect of 30 mg/kg of MPSS treatment on IL-1β expression was evaluated at 7 and 14 days after Hcb. In Hcb-CT 30 animals no IL-1β immunopositive somata or processes were observed in precerebellar

Fig. 10. IL-1β expression. Double nNOS/IL-1β immunofluorescence confocal images from the IO of control (CTRL; A), saline-treated (Hcb-S; B), MPSS 50 mg/kg treated (Hcb-CT; C), MPSS 50 mg/kg acutely treated (Hcb-AT; D) and MPSS 30 mg/kg treated (Hcb-CT 30; E) rats, 21 (B, C, D) or 14 (E) days after Hcb. Note the robust and massive IL-1β induction in B and D, its lightness in E and the lack of immunoreactivity in A and C. Scale bars = 90 μm.
nuclei at day 7 (data not shown) while a light expression was observed at day 14 (Fig. 10E). No quantitative evaluation of IL-1β/H9252 expression was made. IL-1β/H9252 and GFAP or IL-1β/H9252 and OX-42 multiple labeling experiments were performed to identify the cellular source of IL-1β. In all stages presenting IL-1β activation, double labeling experiments demonstrated IL-1β-positive staining as GFAP positive (Fig. 11A–C) and as OX-42 negative (Fig. 11D–F).

DISCUSSION

The data presented here highlight different aspects of remote cell death associated with focal damage in the adult rat brain: i) remote cell death is accompanied by marked glial reaction and IL-1β expression; ii) MPSS treatment delays cell death, reduces microglial and astrocytic activations as well as IL-1β postlesional expression; iii) MPSS treatment suspension causes the resumption of cell death and reactivates microglial and astrocytic reactions as well as IL-1β expression.

Cell death in regions remote from the primary site of damage has been recognized as important in determining the clinical picture in a variety of neurological pathologies, such as stroke and traumatic brain or spinal cord injuries, and it has been associated with glial activation and expression of pro-inflammatory cytokines (Block et al., 2005). The present data, in line with these findings, demonstrate that retrograde degenerative phenomena in precerebellar nuclei after focal surgical lesion of the contralateral cerebellar hemisphere are also associated with marked, sustained astrocytic and microglial activations as well as expression of IL-1β. Glial activation was observed as early as 7 days after the cerebellar lesion and tended to peak about 3 weeks after the lesion, followed by a progressive decrement. Interestingly, day 21 after the lesion corresponds to the peak of the second wave of neuronal death in target-deprived precerebellar nuclei (Viscomi et al., 2004), supporting the relation between glial activation and delayed cell death.

Comparison between the rate of cell death and nNOS expression demonstrates that, after HCb, two different death phenomena are present. An early intense wave of cell death not associated with nNOS expression followed by later expression of nNOS associated with a less intense and more prolonged phase of cell death. nNOS expression starts around day 7 after HCb and the percentage of nNOS neurons progresses until day 21. Afterward, the number of nNOS neurons declines. No clear differences in the time course of astrocytic and microglial proliferations were observed. Finally, multiple labeling experiments demonstrated that IL-1β is produced by astrocytes and not by microglial cells.

In different experimental models, both astrocytes and microglia have been shown to sustain pro-inflammatory mechanisms and, in particular, to be able to produce IL-1β (Markiewicz and Lukomska, 2006). However, the two glial populations may respond differently in different CNS-re-
lated diseases. It has been recently stressed that micro-environmental features may explain differences in the glial reaction in various conditions of neuronal damage (Cao et al., 2007). The present data indicate a prevalent role of astrocytes in supporting the production of the potentially harmful cytokine IL-1β in a model of remote induced cell death. Cell death after target deprivation has been mainly studied in thalamo-cortical (Martin et al., 2003) and olivo-cerebellar (Buffo et al., 1998) circuits. Apoptosis (Waldmeier, 2003) and inflammation (Block et al., 2005) are considered to be key mechanisms sustaining delayed cell death in regions distant from the site of the primary lesion. This study represents the first attempt to address the role of MPSS treatment in preventing remote cell death after focal CNS lesions.

In recent years, glucocorticoid efficacy in cases of CNS damage was widely investigated (Kanellopoulos et al., 1997; Kaptanoglu et al., 2000; Sekiya et al., 2001; Vaquero et al., 2006; Heiduschka and Thanos, 2006) and its clinical use following CNS injury suggested (Short et al., 2000; Pointillart et al., 2000; Leybold et al., 2007). Glucocorticoid effects include inhibition of apoptosis (Kanellopoulos et al., 1997; Vaquero et al., 2006), reduction of inflammatory responses and inhibition of key pro-inflammatory cytokines such as IL-1β and TNF-alpha (Uz et al., 1999). The effects of corticosteroids on the brain are not straightforward (Herbert et al., 2006); pro-inflammatory effects have also been observed in experimental conditions (MacPherson et al., 2005) and the use of corticosteroids in trauma-related pathologies has been questioned (Short et al., 2000; Gorio et al., 2005). The present data indicate that high-dose MPSS chronic treatment is effective in reducing neuronal death in regions remote to the primary site of damage. This finding is supported by various observations. First, comparisons between cell counts in IO and Pn from Nissl/NADPH-d stained sections of Hcb-saline-treated and Hcb-MPSS-treated animals clearly indicate the effectiveness of glucocorticoid treatment in delaying neuronal cell death at all time points analyzed. Although data are semi-quantitative, they are reliable as indicated by the low variability and high statistical significance of the comparisons between neuronal counts in MPSS- and saline-treated animals (Fig. 3E). Furthermore, the effects of MPSS on cell loss were confirmed by Western blot analysis of the NeuN proteins (Fig. 4A–B). This latter technique is reliable in indicating neuronal density and has been successfully employed to evaluate neuronal loss in different experimental models (Collombet et al., 2006).

Insight on possible mechanisms of action of MPSS treatment on neuronal survival derives from Hcb-CT30 data. The employment of 30 mg/kg MPSS treatment did not prevent neuronal degeneration. Conversely, a significant reduction in both astrocytic and microglial responses, similar to that observed with 50 mg/kg, was observed. Interestingly, in the lower dose group the high glial response was associated with a reduced expression of the potentially harmful cytokine IL-1β (Fig. 10E). Thus, low doses, apt to reduce or even block glial inflammatory responses, are not effective in neuroprotection. This evidence opens up the possibility that low and high MPSS doses act on different mechanisms. This hypothesis is in line with evidence indicating that MPSS is effective in neuroprotection only at high doses through receptor independent pathways by inhibiting oxygen-free radical-induced lipid peroxidation (Demopoulos et al., 1982).

Inflammatory changes in areas far from the primary lesion have been linked to secondary cell death phenomena (Block et al., 2005; Williams et al., 2007). Recently, the importance of inflammatory mechanisms in inducing remote cell death, but not in affecting primary vascular lesions, has been elegantly demonstrated in a mouse model (Schroeter et al., 2006). Although the clinical importance of remote cell death is increasingly recognized (Binkofski et al., 1996; Hervé et al., 2005), very few proposals of therapeutic approaches for treating this aspect of brain damage have been made (Martin et al., 2003). The efficacy of glucocorticoid therapy in focal brain lesions is extremely controversial (Gomes et al., 2005). To our knowledge, the present study is the first attempt to evaluate the efficacy of MPSS in influencing remote cell death in focal CNS injuries. The present results, showing a MPSS-induced delay in longstanding cell death phenomena, provide further evidence of the complex interconnections between inflammatory and degenerative signals in the CNS (Zipp and Aktas, 2006).

Glial cells are suspected to play a key role in secondary neuronal degeneration by producing toxic mediators such as pro-inflammatory cytokines, NO, glutamate and free radicals (Stoll et al., 1998, 2002; Willison et al., 2002), and different studies have reported the efficacy of glucocorticoids in down-regulating the microglial response (Sekiya et al., 2001; Tanaka et al., 1997; Oudega et al., 1999). Many in vivo and in vitro studies have shown that the application of glucocorticoids can modulate astrocytic responses in both pathological and physiological conditions (Laping et al., 1994; Crossin et al., 1997; Garcia-Segura and Melcangi, 2006). Systemic administration of glucocorticoids leads to a decrease in GFAP at both mRNA and protein levels in the hippocampus (Laping et al., 1994), while an enhancement of GFAP expression has been reported in the hypothalamus after corticosterone treatment (Maurel et al., 2000). In pathological conditions, steroids can regulate astrocytic responses and the expression of astrocytes-related substances (Garcia-Segura and Melcangi, 2006). Of particular interest is the reported influence of steroids on astrocytic and regenerative responses in the CNS after axotomy of facial, spinal and pudendal motor neurons (Jones et al., 2001). Finally, steroid treatment has been proved to influence the astrocytic reaction also after central axotomy of rubro-spinal neurons (Storer and Jones, 2003). In line with the above mentioned studies, we observed that MPSS treatment significantly reduced the glial action in both IO and Pn after cerebellar damage (Fig. 7A–D). The lesion-induced microglial reaction, as evaluated by OX-42 immunohistochemistry, was still present during MPSS treatment, but it was about half of the reaction intensity observed in the lesioned, saline-treated rats. Similarly, MPSS treatment was able to reduce...
significantly the number of GFAP-positive astrocytes present after focal cerebellar lesion in both IO and Pn (Fig. 9). In line with the MPSS effect on the astrocytic response is the MPSS-related inhibition of IL-1β expression in pre-cerebellar nuclei. Taken together, these data point to astrocytes as the key mediators in remote cell death, possibly through the release of the harmful cytokine IL-1β.

The cytokine IL-1β plays a leading part in exacerbating neuronal damage (Loddick and Rothwell, 1996) and in inducing the proliferation of glial cells, mainly astrocytes (Giulian et al., 1988). The role for IL-1β in maintaining and increasing the evolution of astrocytic response was supported by a study demonstrating that IL-1β can stimulate extensive astrogliosis when microinjected at the site of cortical stab wound injury in neonatal mice (Balasingam et al., 1994). Further, astrocytic reaction was found to be attenuated in the brain of IL-1β knockout mice following CNS damage (Herx and Yong, 2001).

Suspension of the MPSS treatment reactivated microglial and astrocytic responses as well as IL-1β expression (Figs. 7, 10). The effects of the suspension of the MPSS treatment were evaluated in two different protocols. In the first one, rats were treated for 28 days and killed 7 days after the end of a protracted and continuous MPSS-treatment. In this condition, an acceleration of neuronal cell death was observed; however, comparison with the saline-treated group was still significant, indicating a persisting effect on neuronal survival. On the other hand, a significant glial response was observed. At day 35, the number of astrocytes and microglial cells in the HCb-IT group was significantly higher than that observed in HCb-S animals. Taken together, the data suggest a transient effect of MPSS treatment on neuronal survival possibly through modulation of the production of cytokine IL-1β by astrocytes. As long as high levels of glucocorticoids are present, the remote inflammation-related response is controlled but not stopped. When the MPSS treatment ends, the glial reaction resumes with intensity comparable to that observed immediately after the lesion, and neuronal degeneration increases. This interpretation is also supported by data from the second protocol (HCb-AT) of the MPSS treatment (Fig. 1). In this latter protocol, hemicerebellectomized rats underwent MPSS treatment for 7 days and precerebellar nuclei were analyzed 14 days after the end of treatment. In this case, comparison with lesioned saline-treated rats showed no difference in either number of surviving neurons or in entity of the astrocytic and microglial reactions. Moreover, the expression of IL-1β was similar to that observed in the lesioned saline-treated animals.

In the cerebellar model of remote cell death it was proposed that NO might be involved in mediating cell death (Loddick and Rothwell, 1996). Further, astrocytic reaction was found to be extensive astrogliosis when microinjected at the site of neuronal damage (Herx and Yong, 2001). The cytokine IL-1β was shown to stimulate severe astrogliosis and neuron/neuron or glial/neuron signaling in different pathological conditions and the possibility of purinergic/nitrergic functional interactions has been advanced (Viscomi et al., 2004; Florenzano et al., 2006; Yukawa et al., 2005). In particular, NO/ATP interactions have been considered to play a role in the control of delayed cell death in precerebellar neurons (Viscomi et al., 2004). To test this hypothesis, P2X1 and nNOS co-activation was evaluated at day 21, the highest peak of nNOS and P2X1, expression, in both MPSS-treated and saline-treated lesioned rats. The degree of co-activation observed in HCb-MPSS-treated rats perfectly matched that previously reported in HCb-saline-treated animals (Viscomi et al., 2004). Suspension of MPSS treatment (HCb-AT group Fig. 6) did not change the percentage of nNOS/P2X1 co-localization in either IO or Pn. This finding supports the idea of a functional NO/ATP link and is against the possible involvement of purinergic transmission in MPSS-induced resistance to axotomy.

CONCLUSION

In conclusion, the present data demonstrate that MPSS treatment stops/delays cell death in a model of remote neuronal degeneration after focal cerebellar lesion. This protective effect lasts only as the treatment lasts. Treatment interruption resumes cell death at a rate comparable to saline-treated animals. Remote cell death is paralleled by activation of different inflammatory indexes. High cell loss rate is associated with microglia and astrocystic activation, conversely high cell survival is associated with reduced inflammatory indexes. In synthesis, evidences here reported support the link between inflammation and remote cell death providing data for the search of an inflammation related drug, effective to counteract neuronal loss after focal brain damage.

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