Flupirtine as Neuroprotective Add-On Therapy in Autoimmune Optic Neuritis

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Multiple sclerosis (MS) is a common inflammatory disease of the central nervous system that results in persistent impairment in young adults. During chronic progressive disease stages, there is a strong correlation between neurodegeneration and disability. Current therapies fail to prevent progression of neurological impairment during these disease stages. Flupirtine, a drug approved for oral use in patients suffering from chronic pain, was used in a rat model of autoimmune optic neuritis and significantly increased the survival of retinal ganglion cells, the neurons that form the axons of the optic nerve. When flupirtine was combined with interferon- β , an established immunomodulatory therapy for MS, visual functions of the animals were improved during the acute phase of optic neuritis. Furthermore, flupirtine protected retinal ganglion cells from degeneration in a noninflammatory animal model of optic nerve transection. Although flupirtine was shown previously to increase neuronal survival by Bcl-2 up-regulation, this mechanism does not appear to play a role in flupirtine-mediated protection of retinal ganglion cells either *in vitro* or *in vivo*. Instead, we showed through patch-clamp investigations that the activation of inwardly rectifying potassium channels is involved in flupirtine-mediated neuroprotection. Considering the few side effects reported in patients who receive long-term flupirtine treatment for chronic pain, our results indicate that this drug is an interesting candidate for further evaluation of its neuroprotective potential in MS. (*Am J Pathol* 2008, 173:1496–1507; DOI: 10.2353/ajpath.2008.080491)

Flupirtine is a nonopioid analgesic, approved for long-term use in patients.¹ Neuroprotective properties of flupirtine have been previously demonstrated in models of

cerebral ischemia.^{2,3} In human brain slices a reduction of tumor necrosis factor-related apoptosis-inducing-ligand-induced neurodegeneration was described for this analgesic compound.⁴ As one of the mechanisms underlying its neuroprotective properties, an up-regulation of the anti-apoptotic protein Bcl-2 was identified.⁵

In an animal model of myelin oligodendrocyte glycoprotein (MOG)-induced optic neuritis we previously observed a down-regulation of Bcl-2 to be one of the signaling events involved in the degeneration of retinal ganglion cells (RGCs), the neurons that form the axons of the optic nerve (ON).^{6,7} In contrast to other models of experimental autoimmune encephalomyelitis (EAE), MOG-induced EAE in rats produces an encephalitogenic T-cell activation in parallel with a demyelinating autoantibody response.^{8,9} Additionally, the extent of axonal and neuronal injury is similar to that of the human disease and begins shortly after immunization.^{6,10}

Current therapies for multiple sclerosis (MS) mainly target the inflammatory infiltration.¹¹ However, in chronic progressive disease stages the present treatment strategies are insufficient, as neuronal and axonal degeneration continues to progress, finally leading to persisting neurological impairments.^{12,13} As of yet, no approved therapy targeting the neurodegenerative aspect of this disease is available. The possibility of an oral application and the involvement of Bcl-2 in the degeneration of RGCs in MOG-induced optic neuritis, led us to investigate the neuroprotective properties of flupirtine in our animal model.

Materials and Methods

Induction and Evaluation of EAE

All animal protocols were approved by the local authorities in Braunschweig, Germany. Female BN rats 8 to 10

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weeks of age were obtained from Charles River (Sulzfeld, Germany). Recombinant rat MOG^{19d}, corresponding to the N-terminal sequence of rat MOG (amino acids 1 to 125) was used to induce MOG-EAE. The rats were anesthetized by inhalation of diethyl ether and were then injected intradermally at the base of the tail with a total volume of 200 μ l of inoculum, containing 50 μ g of MOG kindly provided by Doron Merkler (Department of Neuropathology, University of Göttingen, Göttingen, Germany) in saline emulsified (1:1) with complete Freund's adjuvant (Sigma, St. Louis, MO) containing 200 μ g of heat-inactivated *Mycobacterium tuberculosis* (strain H 37 RA; Difco Laboratories, Detroit, MI).

The rats were scored for clinical signs of EAE either until day 8 after clinical manifestation or, if they developed no clinical signs of EAE, until day 25 after immunization. The signs were scored as follows: grade 0.5, distal paresis of the tail; grade 1, complete tail paralysis; grade 1.5, paresis of the tail and mild hind leg paresis; grade 2.0, unilateral severe hind leg paresis; grade 2.5, bilateral severe hind limb paresis; grade 3.0, complete bilateral hind limb paralysis; grade 3.5, complete bilateral hind limb paralysis and paresis of one front limb; grade 4, complete paralysis (tetraplegia), moribund state, or death. This score reflects the amount of spinal cord lesions and does not include visual symptoms.

Recordings of visual evoked potentials (VEPs) were performed as described previously.¹⁴ In six animals of each group, the mean visual acuity was calculated from the smallest size of alternating bars for which specific VEP potentials were recordable at day 1 of the clinically apparent disease or, if the animal developed no clinical signs of EAE, at day 19 after immunization with MOG.⁶ A second session of VEP recordings was performed in the same animals at day 8 after clinical manifestation of EAE or, if the animal developed no clinical signs of EAE, at day 25 after immunization. At the end of the experiment, the rats received an overdose of chloral hydrate and were perfused via the aorta with 4% paraformaldehyde. Data analysis of the VEP recordings was performed by an investigator blinded to the treatment applied.

Drug Administration

The rats were randomly allocated to four different groups: interferon (IFN)- β 1a monotherapy, flupirtine monotherapy, combination of flupirtine and IFN- β 1a, and vehicle treatment. To avoid direct interference with the immunization, all treatments were started on day 2 after immunization with MOG. In both the IFN- β 1a monotherapy group ($n = 6$) and the combination therapy group ($n = 6$), animals were treated three times per week with subcutaneous applications of 300,000 U (1.1 μ g) IFN- β 1a (Serono, Unterschleißheim, Germany) in 150 μ l of 0.9% sodium chloride. The control group received vehicle ($n = 12$). In the flupirtine monotherapy group ($n = 12$) and the combination therapy group, pelleted rat chow incorporating 0.6125 g/kg flupirtine (Astra-Zeneca, Frankfurt, Germany) was fed to the rats. Considering a daily food uptake of 20 g per rat, this concentration in the

food leads to a daily flupirtine uptake of 2.45 mg/kg body weight. This dosage is in a similar range as the daily uptake of a standard dosage in patients, in which 300 mg are routinely applied, resulting in 3.75 mg/kg body weight. The vehicle-treated rat groups were fed the identical standard rat chow not containing flupirtine. For intraocular injection of 2 mmol/L barium (Ba), animals were anesthetized with diethyl ether. By means of a glass microelectrode, 2 μ l of the solution were injected into the vitreous space of each eye, puncturing the eye at the cornea-sclera junction. The injections were performed at days 5, 10, 15, and 20 after immunization.

Measurement of Flupirtine Plasma Level

To determine the plasma level of flupirtine in rats treated with pelleted rat chow incorporating 0.6125 g/kg flupirtine, blood was collected 48 hours after initiating flupirtine application by sublingual puncture. The plasma sample was processed using a liquid/liquid extraction method. To 250 μ l of the plasma test samples, QC samples, and calibration standards, 20 μ l NaOH (1 mol/L) and 2 ml diethyl ether were added. After centrifuging at $2500 \times g$ for 5 minutes the samples were kept for 20 minutes at -40°C . The organic phase was then decanted from the frozen aqueous phase and transferred to new polypropylene vials that contained 500 μ l of n-hexane and 500 μ l of HCl (0.01 mol/L). After further centrifugation, the upper organic phase was discarded and the vials were placed into a vacuum centrifuge for 10 minutes at room temperature. Five hundred μ l of the residual solution was transferred into high performance liquid chromatography vials and 50 μ l of each sample were then injected directly into the high performance liquid chromatography system for measuring the flupirtine concentration in the individual plasma samples.

Quantification of RGC Survival

One week before immunization, retrograde prelabeling of RGCs was performed after anesthetizing the rats with 10% ketamine (0.75 ml/kg; Atarost GmbH and Co., Twistringen, Germany) together with 2% xylazine (0.35 ml/kg; Albrecht, Aulendorf, Germany). The skin was incised mediosagittally, and holes were drilled into the skull above each superior colliculus (6.8 mm dorsal and 2 mm lateral from bregma). Two μ l of the fluorescent dye Fluorogold (FG) (5% in normal saline; Fluorochrome Inc., Englewood, CO) were injected stereotactically into both superior colliculi. Axonal transport of FG with consecutive labeling of RGCs takes place within the first 24 hours after FG injection so that RGCs are fully labeled at the time of EAE induction (our own previous observations).

At the end of the experiment, retinas were dissected, flat-mounted on glass-slides, and examined by fluorescence microscopy (Axioplan 2; Zeiss, Göttingen, Germany) using a 4,6-diamidino-2-phenylindole filter (315/395 nm). RGC densities were determined by counting FG-labeled cells in three areas (62,500 μm^2) per retinal quadrant at three different eccentricities of 1/6, 3/6, and

5/6 of the retinal radius in blinded samples. In sham-immunized controls we previously detected 2730 ± 145 RGCs per mm^2 (mean $\pm$ SEM).¹⁵

Unilateral ON Transection

Rats were anesthetized by an intraperitoneal injection of ketamine and xylazine as described above. A skin incision close to the superior orbital rim was performed, and the right orbita was opened. The lachrymal gland was resected subtotally. After spreading of the superior extraocular muscles, the ON was exposed by longitudinal incision of the perineurium. ON transection was performed 2 mm from the posterior pole of the eye without damaging retinal blood supply. Retrograde labeling of RGCs was achieved by placing a small sponge soaked in 5% FG at the ocular stump of the transected ON. RGC counts were evaluated as described above at day 14 after ON transection.

Histopathology

After perfusion of the rat with 4% paraformaldehyde, ONs were taken for histopathological evaluation and were paraffin-embedded. Histological evaluation was performed on 4- μm -thick slices. Luxol-fast blue staining was used to assess demyelination. Photos of vertical sections were taken using an Axiocam MR (Zeiss). The images were processed using Axiovision 4.2 software (Zeiss) to evaluate the demyelinated area as a percentage of the whole ON cross section.

Additionally, immunohistochemistry was performed on ON cross-sections. ED-1-positive macrophages/activated microglia (MCA341R, diluted 1:500; Serotec, Oxford, UK), CD-3-positive T-cells (BZL03543, diluted 1:500; Biozol, Eching, Germany), and β -amyloid precursor protein (APP)-positive axons (MAB348, diluted 1:3000; Chemicon, Ford, UK) were detected using biotin-avidin detection. Spleen sections served as a control for ED1 and CD3 stainings. The evaluation of ED-1- or CD-3-positive cells was performed according to the following score: 0, no labeled cells; 1, a few positive cells in at least one of three different ON levels; 2, 10 to 50% of at least one ON cross section infiltrated with labeled cells; and 3, more than 50% of the ON cross section infiltrated with labeled cells in at least one ON level. The number of β -APP-positive axons was counted per cross section. For each histopathological parameter three different levels of each ON were evaluated. The investigators who performed neuropathological examinations were blinded to the treatment applied.

Immunocytochemistry was performed on cultured RGCs maintained for 48 hours in normal conditions and in media lacking growth factors, with and without the presence of 200 $\mu\text{mol/L}$ flupirtine. Cells were fixed in 4% paraformaldehyde and permeabilized with 0.3% Triton X-100. After blocking with 5% normal goat serum, cells were incubated overnight at 4°C in mouse anti-Bcl-2 (sc-7382, 1:100 in blocking solution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After washing, cells were

incubated with an Alexa 488-conjugated secondary antibody (Molecular Probes, Eugene, OR), washed, and mounted (Vectashield; Vector Laboratories, Burlingame, CA).

Cell Culture

Primary RGCs were obtained from 6- to 8-day-old Wistar rats as described previously.¹⁶ After 24 hours, media containing forskolin, brain-derived neurotrophic factor, ciliary neurotrophic factor, and insulin was removed and replaced with media lacking these neurotrophic factors and containing flupirtine dissolved in 100% dimethyl sulfoxide (Astra Medica, Bad Homburg, Germany), at a concentration of 1 to 200 $\mu\text{mol/L}$. Cells were maintained in media lacking neurotrophic factors, and containing equivalent dimethyl sulfoxide levels, as controls. Previous studies demonstrated the most prominent effect of flupirtine in neuronal cell cultures at concentrations of 10 to 200 $\mu\text{mol/L}$.^{2,17,18} We assessed cell viability using a (3,4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay 48 hours after removal of neurotrophins. Viability was assessed by counting the number of surviving cells in six fields of view in each of three wells per concentration and was performed on three separate cell preparations to ensure reliability of the data. Results are expressed as a percentage of controls. After 24 hours in culture, primary RGCs were treated with increasing concentrations of buthionine sulfoximine (100 $\mu\text{mol/L}$ to 10 mmol/L) both with and without the presence of 200 $\mu\text{mol/L}$ flupirtine. Forty-eight hours later, cell viability was assessed using a MTT assay as before, and again repeated on three separate cell preparations.

Western Blot Analysis

For Western blot analysis, animals received an overdose of chloral hydrate 6 hours after the last application of IFN- β 1a or vehicle. The dissected retinas were homogenized and lysed (150 mmol/L NaCl, 50 mmol/L Tris, pH 8.0, 2 mmol/L ethylenediaminetetraacetic acid, and 1% Triton, containing 0.1 mmol/L phenylmethyl sulfonyl difluoride and 2 mg/ml pepstatin, leupeptin, and aprotinin) for 20 minutes on ice. Cell debris were then pelleted at $13,000 \times g$ for 15 minutes. The protein concentration of the supernatant was determined using the BCA reagent (Pierce, Rockford, IL). After separation by reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the lysates (20 mg protein per lane), proteins were transferred to a polyvinylidene difluoride membrane and blocked with 5% skim milk in 0.1% Tween 20 in PBS-T. The membranes were incubated with the primary antibody against phospho-Akt (9271, 1:1000 in 1% skim milk in PBS-T; New England Biolabs GmbH, Schwalbach, Germany), Akt (9272, 1:1000 in 5% skim milk; New England Biolabs GmbH), phospho-MAPK 1 and 2 (9106, 1:200 in 1% skim milk in PBS-T; New England Biolabs GmbH), or Bax (sc-526, 1:1000 in 5% skimmed milk in PBS-T; Santa Cruz Biotechnology, Inc.). Membranes were washed in PBS-T and then incubated with horse-

radish peroxidase-conjugated secondary antibodies against rabbit IgG (1:2500 in 1% skim milk, Santa Cruz Biotechnology). For Western blot analysis of Bcl-2 levels (sc-7382, 1:200; 5% skim milk; Santa Cruz Biotechnology), a horseradish peroxidase-conjugated secondary antibody against mouse IgG was used (1:2000 in 1% skim milk in PBS-T, Santa Cruz Biotechnology, Inc.). MAPK 1 and 2 protein levels were detected using a primary antibody (sc-93-G, Santa Cruz Biotechnology Inc.) diluted 1:500 in 1% skim milk in PBS-T, and a horseradish peroxidase-conjugated secondary antibody against goat IgG (1:10,000 in PBS-T, Santa Cruz Biotechnology Inc.). Labeled proteins were detected using the ECL-plus reagent (Amersham, Arlington Heights, IL). To estimate the relative expression levels of the different proteins, the expression patterns were analyzed in the same retinal protein lysate. At least four different retinal protein lysates were used to study each effect. In addition, lysates were prepared from primary RGC cultures grown for 48 hours in either normal conditions, without growth factors, or a combination of growth factor withdrawal and increasing concentrations of flupirtine (10 to 200 $\mu\text{mol/L}$). After cellular lysis, lysates and Western blots were prepared as described above.

Patch Clamp Electrophysiology

The whole-cell patch-clamp technique was used to measure membrane currents in primary RGCs.¹⁹ Cells were subjected to electrophysiological recordings after 3 to 8 days in culture. The culture dishes were placed on the stage of an inverted microscope (Axiovert 135; Zeiss, Oberkochen, Germany). RGCs were identified by their size, typical morphology, and current profile.²⁰ Single-cell recording was then performed at room temperature (20 to 25°C). All indicated solutions were applied by continuous perfusion of the culture dishes. The following drugs were applied to the extracellular solution by a perfusion system: flupirtine (200 $\mu\text{mol/L}$, dissolved in 100% dimethyl sulfoxide), Ba^{2+} (1 mmol/L), and rTertiapin-Q (50 nmol/L; Alomone Labs Ltd., Jerusalem, Israel), a blocker of Kir1.1 and Kir3 channels.^{21,22} An EPC-9 amplifier and the Pulse software (Heka, Lambrecht, Germany) were used to generate voltage jumps, inject constant currents and acquire data. A routine correction for leak currents and capacitive transients was performed using a P/n method. Only experiments with series resistances below 30 M Ω were used for evaluation. Series resistance errors were compensated in the range of 30 to 60% with a routine of the Pulse software. Data analysis was performed with the program PulseFit (Heka). Micropipettes were pulled from borosilicate glass capillaries (Harvard Apparatus Ltd., Edenbridge, UK) on a horizontal puller (Zeitz Instrumente, Augsburg, Germany). When filled with internal solution the pipette resistance ranged from 2 to 6 M Ω . Patch pipettes were filled with an intracellular solution containing (in mmol/L): 130 KCl, 10 NaCl, 2 MgCl_2 , 10 EGTA, and 10 Hepes. The external solution contained (mmol/L): 130 NaCl, 5 KCl, 2 MgCl_2 , 2 CaCl_2 , 10 Hepes, and 10 D-glucose. The external solution

for perfusion experiments contained (mmol/L): 104 NaCl, 50 KCl, 2 CaCl_2 , 2 MgCl_2 , 10 Hepes, and 10 D-glucose. Solutions were adjusted to pH 7.4. To measure Kir currents, we changed the external solution to one containing 50 mmol/L KCl in place of 50 mmol/L NaCl. Inwardly rectifying potassium (Kir) currents were distinguished from other potassium currents by their sensitivity to the application of Ba^{2+} and tertiapin.^{21–23} Inward currents were elicited by applying hyper- and depolarizing voltage steps from -150 mV to 0 mV in 10 mV steps starting from a holding potential of -20 mV.

Statistical Analyses

Data are presented as mean $\pm$ SEM. For multiple group comparisons, statistical significance was assessed using a Bonferroni-corrected one-way analysis of variance. Student's *t*-test was used to assess RGC densities *in vitro* as well as the results from the patch clamp experiments. A *P* value less than 0.05 was considered to be statistically significant.

Results

Flupirtine Plasma Levels and Clinical Disease Course

In rats with MOG-induced optic neuritis we analyzed the effects of flupirtine as an add-on therapy to IFN- β 1a, one of the immunomodulatory therapies commonly used in MS patients. Three hundred thousand units of IFN- β 1a were applied subcutaneously three times per week. Twenty-four hours after being fed with pelleted rat chow incorporating 0.6125 g/kg flupirtine, the rat plasma levels of flupirtine were within the therapeutic range achieved in patients taking a standard dosage of flupirtine (Figure 1A).²⁴ In comparison to vehicle-treated controls, the application of flupirtine had no effect on the clinical disease course of MOG-induced EAE. In contrast, application of IFN- β 1a with and without combining it with flupirtine significantly improved the functional deficits at each time point analyzed (Figure 1B). The general clinical score in our animal model is mainly determined by spinal cord lesions, visual functions are not included.²⁵

Combination of Flupirtine and IFN- β 1a Results in Improved Visual Function

We performed electrophysiological recordings of VEPs to examine the effects of flupirtine and IFN- β 1a treatment on visual functions. In an individual rat, an intact function of both, the neuronal cell bodies in the retina and their associated axons in the ON is essential to generate VEPs. VEPs represent the electrical response of the visual association cortex to a light stimulus presented to one eye. Pattern VEPs with different sizes of alternating black and white bars were used to estimate the animal's visual acuity. We have previously shown that healthy sham-immunized rats have visual acuity values of 1.31 ± 0.16

Figure 1. Flupirtine plasma levels and clinical disease course. **A:** Twenty-four hours after application of 0.6125 g/kg flupirtine incorporated in pelleted rat chow, plasma concentrations of 624 ± 86 ng/ml flupirtine were observed in healthy rats, whereas none of the vehicle-treated (veh) plasma probes showed relevant flupirtine levels, only background activity was detected (mean $\pm$ SEM; $n = 5$ for each group). **B:** Comparison of the clinical disease course in the four different treatment groups. **▲**The mean clinical score in the rat group receiving combination therapy of flupirtine and IFN- β 1a, **■**IFN- β 1a monotherapy, **●**flupirtine monotherapy, and **□**vehicle-treated controls. Data are demonstrated as mean $\pm$ SEM. In comparison to vehicle-treated controls, the mean clinical score was significantly reduced if IFN- β 1a monotherapy or IFN- β 1a together with flupirtine were applied (one-way analysis of variance followed by Bonferroni correction, $P < 0.05$).

cycles per degree.¹⁴ A severe decline of specific cortical potentials in response to pattern stimulation occurred at the day of clinical manifestation of the disease. At that time point none of the vehicle-treated controls had detectable visual acuity values (Figure 2, A–E). Rats treated with flupirtine together with IFN- β 1a showed significantly higher visual acuity values of 0.42 ± 0.11 cycles per degree (mean $\pm$ SEM, $n = 8$ eyes for each group; $P = 0.021$, if compared with vehicle-treated controls) (Figure 2A). In rats receiving flupirtine monotherapy we detected visual acuity values of 0.22 ± 0.13 cycles per degree (mean $\pm$ SEM). In the IFN- β 1a monotherapy group we observed a mean visual acuity of 0.10 ± 0.06 cycles per degree (mean $\pm$ SEM). It was demonstrated previously in this animal model that optic neuritis can also occur in the absence of clinical symptoms,²⁶ which is in accordance with our observations that IFN- β 1a monotherapy significantly improved the clinical score but not the visual functions of the rats.

We additionally analyzed VEP recordings at day 8 after clinical manifestation of MOG-EAE (Figure 2B). At that time point, the group of vehicle-treated controls showed some improvement in visual functions in comparison to

the earlier time point. As a result, we observed only a tendency for improved visual acuity in the combination therapy group that was not statistically significant (0.11 ± 0.07 cycles per degree in the vehicle group versus 0.38 ± 0.12 cycles per degree in the combination therapy group; mean $\pm$ SEM; $P = 0.0658$; $n = 8$ eyes per group).

ON Histopathology Is Unchanged by Flupirtine Monotherapy

In ON cross-sections we determined the mean percentage of demyelination by Luxol-fast blue staining. Representative examples are demonstrated in Figure 3. Immunohistochemistry was used to identify ED1⁺ macrophages/microglia and CD3⁺ T lymphocytes. At day 8 after clinical manifestation of the disease, we detected no major differences in demyelination and inflammatory infiltration between the rats treated with flupirtine or vehicle (Table 1; Figure 3, A–D). Furthermore, the comparison of IFN- β 1a monotherapy with those rats treated with IFN- β 1a in combination with flupirtine also resulted in no significant effect of flupirtine on ON histopathology. As we have described previously, the application of IFN- β 1a significantly reduced inflammatory infiltration and demyelination in our animal model (Table 1).¹⁶

To detect acute axonal damage within the ONs, β -APP immunohistochemistry was applied (Figure 3, E and F). Flupirtine did not influence the accumulation of β -APP-positive axons in ONs (Table 1). As demonstrated before,¹⁶ application of IFN- β 1a reduced the number of β -APP-positive axons per ON cross section at day 8 after clinical manifestation of MOG-EAE (Table 1). This effect was present with and without combining IFN- β 1a with flupirtine.

Flupirtine Protects RGCs under Inflammatory and Noninflammatory Conditions

To determine whether flupirtine protects RGCs from apoptosis under autoimmune inflammatory conditions, we compared the number of RGCs retrogradely labeled with FG 1 week before immunization with MOG in the four different treatment groups (Table 1). At day 8 after clinical manifestation of MOG-EAE, survival of prelabeled RGCs was significantly increased in the animal group receiving flupirtine monotherapy and in the combination therapy group (Figure 2, F and G). At this time point, IFN- β 1a had no statistically significant effect on RGC survival. In a second, independent experiment we observed 1456 ± 109 RGCs per mm² in flupirtine-treated rats and 962 ± 100 RGCs per mm² in vehicle-treated controls at day 8 after clinical manifestation of MOG-EAE (mean $\pm$ SEM, $n = 6$; $P = 0.00856$). Comparing all four different treatment strategies we observed no statistically significant correlation between RGC survival and the degree of optic neuritis determined either by demyelination or by infiltration with ED1⁺ macrophages/microglia. We assume that

Figure 2. Visual function and RGC survival in rats suffering from MOG-induced optic neuritis. **A:** Visual acuity determined by pattern VEP recordings at the day of clinical manifestation of MOG-EAE in comparison to sham-immunized controls (ctrl).¹⁴ Electrophysiological responses to pattern stimulation were not detectable in any of the vehicle-treated controls (veh), whereas in the flupirtine and IFN- β 1a monotherapy groups a response to pattern stimulation using broad alternating black and white bars was present. The combination therapy groups receiving flupirtine together with IFN- β 1a showed a significantly better electrophysiological response in pattern VEPs. Statistically significant when compared with vehicle-treated controls (* $P < 0.05$). **B:** Visual acuity determined by pattern VEP recordings at day 8 after clinical manifestation of EAE. Only a tendency for improved visual acuity was observed in the combination therapy group in comparison to vehicle-treated controls ($P = 0.0658$). **C:** Representative example of VEP recordings using flash stimulation in an IFN- β 1a-treated rat. **D** and **E:** Representative examples of VEP recordings at EAE onset using pattern stimulation with 12 alternating black and white bars in an animal receiving flupirtine together with IFN- β 1a (**D**) and a vehicle-treated control (**E**). **F:** Number of RGCs per mm² in retinal flat mounts at day 8 after clinical manifestation of MOG-EAE. Statistically significant when compared to vehicle-treated controls (* $P < 0.05$). **G:** Examples of retinal flat mounts after FG prelabeled in a rat receiving flupirtine together with IFN (flupirtine + IFN), IFN monotherapy (IFN), flupirtine monotherapy (flu), and a vehicle-treated control (veh). **H:** Number of RGCs per mm² in retinal flat mounts 2 weeks after surgical ON transection. A significantly higher density of retrogradely labeled RGCs was present in rats that had received flupirtine in comparison to vehicle-treated controls (veh, * $P < 0.05$). Data are given as mean $\pm$ SEM. One-way analysis of variance followed by Bonferroni correction was used for multiple group comparison. Scale bar = 50 μ m.

the main reason for a lacking correlation of inflammation and neurodegeneration lies in the different modes of action of the two drugs analyzed. IFN- β 1a showed anti-inflammatory effects without affecting RGC loss whereas flupirtine increased RGC survival without influencing ON histopathology. Furthermore, at the day of clinical manifestation of MOG-EAE the application of flupirtine showed a tendency toward a reduced number of TUNEL-positive RGCs (5.5 ± 1.04 TUNEL-positive RGCs per retinal section, if flupirtine was applied versus 10.1 ± 1.70 TUNEL-positive RGCs per retinal section, if vehicle was applied; mean $\pm$ SEM, $n = 8$, $P = 0.0597$).

In addition, we investigated whether flupirtine reduces apoptosis of RGCs after traumatic axonal injury. Whereas the mean RGC density in healthy control rats is 2730 ± 145 cells per mm² ($n = 9$),¹⁵ 2 weeks after unilateral surgical transection of the ON, we observed a mean density of 446 ± 27 RGCs per mm² (mean $\pm$ SEM, $n = 4$) in vehicle-treated controls. Rats that had received flupirtine from the day of ON transection onwards had a significantly higher density of RGCs (614 ± 30 RGCs per mm²; mean $\pm$ SEM, $n = 6$, $P = 0.025$) (Figure 2H).

Furthermore, flupirtine protects immunopurified postnatal RGCs *in vitro* from apoptosis induced by growth factor withdrawal (Figure 4A). After 24 hours in culture, neurotrophic factors were withdrawn and flupirtine was

applied. A comparison with the survival of RGCs continuously supplied with neurotrophic factors was performed. The application of 200 μ mol/L flupirtine resulted in a significantly higher survival of RGCs cultured under growth factor withdrawal [$89.5 \pm 4.0\%$ of RGCs survived, if 200 μ mol/L flupirtine was applied versus $63.7 \pm 6.2\%$ without application of flupirtine (mean $\pm$ SEM); $n = 3$, $P = 0.038$] (Figure 4A). In contrast to observations in cortical neurons,⁵ flupirtine does not prevent intracellular glutathione depletion in cultured primary RGCs (Figure 4B).

Bcl-2 Is Not Involved in Flupirtine-Mediated RGC Protection

A sixfold up-regulation of Bcl-2 was previously demonstrated to be relevant for the neuroprotective action of flupirtine in cultured cortical neurons under excitotoxic conditions.⁵ Western blots of retinal protein lysates obtained at the day of clinical manifestation of MOG-EAE revealed that flupirtine treatment of the rats resulted in unchanged protein levels of the anti-apoptotic protein Bcl-2 and the pro-apoptotic member of the Bcl-2 family, Bax (Figure 4C). In healthy rats, retinal Bcl-2 protein levels were substantially higher than in MOG-EAE, but

Figure 3. Effects of flupirtine application on ON histopathology at day 8 after clinical manifestation of MOG-EAE. **A** and **B**: Luxol-fast blue staining of ON cross-sections was used to determine the degree of demyelination. A similar degree of demyelination was observed in ON cross-sections of rats treated with flupirtine (**A**) in comparison to vehicle-treated controls (**B**). **C** and **D**: Inflammatory infiltration with ED1⁺ macrophages/microglia was unchanged with (**C**) and without (**D**) flupirtine treatment. **E** and **F**: Acute axonal damage was analyzed in β -APP-stained ON cross-sections of a flupirtine (**E**) and a vehicle-treated animal (**F**), and demonstrated no effect of flupirtine on the presence of β -APP-positive profiles (arrows). Scale bars: 100 μ m (**A–D**); 2 μ m (**E, F**).

also in healthy animals the application of flupirtine had no influence on Bcl-2 expression (data not shown). Further Western blot analyses of the phosphorylation levels of Akt and MAPK 1/2 revealed that the neuroprotective properties of flupirtine in our animal model are independent of these signaling cascades, which we previously identified to be involved in the degeneration of RGCs in MOG-induced optic neuritis.^{6,7}

We further analyzed the expression level of Bcl-2 in immunopurified postnatal RGCs *in vitro* after growth factor withdrawal. In accordance with our observations in MOG-induced optic neuritis, the application of flupirtine on cultured RGCs resulted in unchanged expression levels of Bcl-2 (Figure 4D). Additionally, we observed a similar expression of Bcl-2 by immunocytochemistry in

RGCs cultured with and without the presence of 200 μ mol/L flupirtine (data not shown).

Inwardly Rectifying K^+ Channels Are Activated by Flupirtine

Inwardly rectifying potassium (Kir) channels are widely distributed in the central nervous system and play important roles in controlling neuronal signaling and membrane excitability.²⁷ These channels are characterized by an increasing conductance under hyperpolarization and a decreasing conductance under depolarization. A previous study revealed that flupirtine increases Kir currents in hippocampal neurons.²⁸ In cultured primary RGCs we performed whole-cell patch-clamp experiments to analyze the effects of flupirtine on current characteristics and the resting membrane potential. The application of 200 μ mol/L flupirtine to cultured RGCs for 24 hours resulted in a statistically significant hyperpolarization of the resting membrane potential (49.83 ± 3.02 mV, $n = 12$, if flupirtine was applied versus 39.07 ± 1.73 mV, $n = 14$, under control conditions; mean $\pm$ SEM, $P = 0.0038$). In addition, Kir currents of dissociated RGCs were activated by voltage steps from -150 mV to 0 mV in 10 -mV steps, starting from a holding potential of -20 mV. Absolute values are normalized, so that the last value obtained before application is considered as 100%. Application of flupirtine (200 μ mol/L, $n = 11$) resulted in a statistically significant increase in potassium currents after 1 minute (0.7045 nA $\pm$ 0.0832 before flupirtine; 1.027 nA $\pm$ 0.0634 after 1 minute flupirtine; $P = 0.0009$; 45.78% increase) (Figure 5, A and B). Application of dimethyl sulfoxide alone had no effect on potassium current amplitudes (data not shown). Ba^{2+} was used to distinguish Kir from other potassium currents.²³ Application of Ba^{2+} after preincubation with flupirtine (1 mmol/L, $n = 5$) almost completely blocked flupirtine-induced inward currents to control levels (control 0.6040 nA $\pm$ 0.1365 ; after 1 minute flupirtine + Ba^{2+} : 0.7234 nA $\pm$ 0.1443 ; $P = 0.2848$) (Figure 5C), indicating that flupirtine increases Kir currents in cultured RGCs. Furthermore, application of tertiapin (50 nmol/L, $n = 6$), which selectively blocks Kir3.3 and Kir3.4 channel subunits,^{21,22} also reduced the flupirtine-mediated increase in Kir currents back to control levels (after 1 minute flupirtine: 1.097 nA $\pm$ 0.0456 ; after 2 minutes flupirtine + tertiapin: 0.8771 nA $\pm$ 0.0714 ; 20.6% decrease; $P = 0.0445$).

Table 1. Histopathology of ONs and RGC Survival

Animal group	Percent of demyelination	Infiltrating ED1 ⁺ cells (score)	Infiltrating CD3 ⁺ cells (score)	Number of β -APP ⁺ axons	Surviving RGCs per mm ²
Flu + IFN- β 1a	$9.34 \pm 8.3^*$	$0.92 \pm 0.31^*$	0.50 ± 0.29	$0.14 \pm 0.11^*$	$1438 \pm 107^{\dagger}$
IFN- β 1a	$9.23 \pm 8.2^*$	$1.17 \pm 0.37^*$	0.47 ± 0.29	$1.47 \pm 1.38^*$	1144 ± 64
Flu	41.27 ± 8.2	2.13 ± 0.23	1.26 ± 0.24	6.56 ± 2.52	$1335 \pm 60^*$
Veh	45.90 ± 8.0	2.19 ± 0.19	1.14 ± 0.22	9.50 ± 3.31	1054 ± 86

Application of IFN- β 1a significantly reduced demyelination, inflammatory infiltration, and the number of β -APP⁺ axons in ON cross sections. Application of flu significantly increased RGC survival in retinal flat mounts.

*Statistically significant, if compared to vehicle treated controls ($P < 0.05$).

[†]Statistically significant, if compared to vehicle or IFN- β 1a monotherapy ($P < 0.05$).

Figure 4. Neuroprotective effects of flupirtine on RGCs *in vitro* and *in vivo*. **A:** Survival of cultured RGCs after growth factor withdrawal (–GF) is significantly increased if flupirtine is applied at a 200 μmol/L concentration. Statistically significant if compared with control (**P* < 0.05). **B:** The percentage of RGC survival under glutathione depletion induced by buthionine sulfoximine is similar with (gray bars) and without (black bars) application of 200 μmol/L flupirtine. Buthionine sulfoximine induced a dose-dependent loss of cultured primary RGCs. **C:** Western blot analyses of retinal protein lysates obtained at the day of clinical manifestation of MOG-EAE in rats treated with flupirtine or vehicle (veh). Similar protein levels of pAkt, Akt, pMAPK1/2, MAPK1/2, Bcl-2, and Bax were observed. **D:** Western blot analysis of Bcl-2 expression in cultured primary RGCs. Unchanged protein level of Bcl-2 under growth factor withdrawal (–GF) with and without application of 10, 100, or 200 μmol/L flupirtine.

We further analyzed whether the flupirtine-mediated activation of Kir channels, which we observed in RGCs *in vitro*, is relevant for the survival promoting effects that flupirtine exerts on RGCs *in vivo*. Therefore, we combined

Figure 5. Activation of Kir by 200 μmol/L flupirtine in cultured RGCs. Currents were evoked under control conditions (**A**) and after application of 200 μmol/L flupirtine (**B**) by hyperpolarizing voltage steps throughout the range of –100 to 0 mV from a holding potential of –20 mV. Representative traces of one RGC are shown without flupirtine (**A**), 1 minute after application of flupirtine (**B**) and 2 minutes after application of 200 μmol/L flupirtine and 1 mmol/L Ba²⁺ (**C**). Blockade of flupirtine-induced currents by co-application with Ba²⁺ identified the currents as inwardly rectifying K⁺ currents. **D:** Number of RGCs per mm² in retinal flat mounts at day 8 after clinical manifestation of MOG-EAE in rats receiving flupirtine or vehicle (veh) monotherapy, or intravitreal applications of barium combined with flupirtine (flu + Ba) or vehicle (veh + Ba). Data are presented as mean ± SEM. Statistically significant when compared to the vehicle-treated group (**P* < 0.05).

in MOG-induced optic neuritis flupirtine treatment with intravitreal application of Ba²⁺. Surviving RGCs were identified by FG prelabeled. RGC densities in rats that had received flupirtine together with intravitreal injections of Ba²⁺ were similar to those in the control group that

received only Ba^{2+} (Figure 5D). At day 8 after clinical manifestation of MOG-EAE, 1072 ± 154 RGCs per mm^2 survived in the combination therapy group. RGCs (1149 ± 128 per mm^2) were present in the Ba^{2+} monotherapy group (mean $\pm$ SEM, $n = 8$ eyes per group; $P = 0.657$), indicating that Ba^{2+} application abolished the neuroprotective effect of flupirtine. Both rat groups had a similar degree of demyelination and inflammatory infiltration in ON cross-sections (data not shown). Comparing RGC densities of the Ba^{2+} monotherapy group with those rats that had received normal rat chow revealed no toxic effect of intravitreal Ba^{2+} application ($P = 0.119$).

Discussion

Our present study demonstrates that flupirtine has neuroprotective properties under autoimmune inflammatory conditions. In our rat model of MOG-induced optic neuritis we observed an increased survival of prelabeled RGCs, the neurons whose axons form the ON. Combining flupirtine with IFN- β 1a, one of the standard therapies for MS, resulted in a significant improvement of visual function during the acute phase of MOG-induced optic neuritis. However, flupirtine monotherapy did not affect inflammatory infiltration, demyelination, or axonal damage in the ON. Protective effects of flupirtine on RGCs were also observed under noninflammatory conditions *in vivo* as well as *in vitro*, indicating a general inflammation-independent mechanism of action.

Different molecular mechanisms have previously been described, which may account for flupirtine-mediated neuroprotection. Firstly, increased expression of Bcl-2 in cultured cortical neurons was observed under excitotoxic conditions.⁵ Secondly, anti-oxidative effects have been demonstrated in rat hippocampal slices.²⁹ Finally, flupirtine activates different types of potassium channels. A dose-dependent activation of Kir channels in hippocampal neurons was observed²⁸ as well as an increase of M-currents in visceral sensory neurons.³⁰ We can rule out an up-regulation of Bcl-2 or an influence on intracellular glutathione depletion to be relevant for the flupirtine-mediated protection of RGCs. Instead, we identified an activation of Kir channels as underlying mechanism for the improved RGC survival. Kir channels represent a family of potassium channels distinct from classical voltage-gated K^+ channels.³¹ They play an important role in maintaining the resting membrane potential, thereby controlling the excitability of neurons.³² In autoimmune inflammatory conditions a mismatch of energy demand and ATP production occurs, which contributes to the level of neurodegeneration. On demyelinated axons increased sodium channel expression was observed, resulting in a higher energy demand to maintain the resting membrane potential and the sodium concentration within the normal range, particularly if repetitive firing occurs.^{33,34} An increase of the sodium concentration results, via reverse action of the sodium/calcium exchanger, in a lethal calcium overload.³⁵ Membrane hyperpolarization, eg, by activation of Kir channels, might protect neurons from repetitive firing and, thereby, from

lethal calcium influx,^{36,37} a mechanism that is also known as neuronal cell silencing.³⁸ However, flupirtine also activates KCNQ2/3 channels that generate M-currents³⁰ and might also contribute to the neuroprotective effect in RGCs via stabilization of the resting membrane potential. In support of our hypothesis that flupirtine prevents lethal calcium influx by inhibiting repetitive firing, flupirtine prevented an increase of the intracellular calcium concentration in hippocampal neurons.^{28,39} Previous studies in MOG-EAE in mice have demonstrated that increased sodium and calcium influx contribute to axonal and neuronal degeneration.^{40–43} Therefore, Kir channel activation with consecutive membrane hyperpolarization, which we observed in cultured RGCs, might be a strategy of reducing activity-dependent neurodegeneration in neuroinflammatory conditions. This concept of neuroprotection induced by an activation of Kir channels is also supported by previous studies in cerebral ischemia in mice. Transgenic overexpression of Kir 6.2 channels reduced the infarct area in permanent focal cerebral ischemia, whereas mice lacking Kir 6.2 channels showed enhanced neuronal cell death.^{44,45}

The expression of various types of Kir channels was demonstrated by immunohistochemistry in rat RGCs.⁴⁶ However, the corresponding currents recorded in cultured RGCs were mainly mediated by Kir channels that are comprised of Kir3 subunits, so that we assume that flupirtine exerts its neuroprotective properties via this type of Kir channels. This hypothesis is strengthened by our observation that the activation of Kir currents by flupirtine can be prevented by the application of tertiapin, a Kir channel blocker preferably acting on Kir 3.3 and Kir 3.4 channels.^{21,22} The involvement of Kir channels in flupirtine-mediated protection of RGCs and the lacking effect of flupirtine on apoptotic signaling in our animal model further demonstrates that multiple pathways contribute to the apoptosis of RGCs in MOG-induced optic neuritis. In previous experiments, we observed a down-regulation of the anti-apoptotic proteins Bcl-2, phospho-Akt, and phospho-MAPK 1/2 as well as an up-regulation of the pro-apoptotic protein Bax to be involved in RGC loss in MOG-EAE.^{6,7} In our previous study, substantial loss of RGCs occurred already 1 week before major changes in pro-apoptotic signaling were present,⁶ so that we hypothesize that ion channel dysfunction resulting in increased sodium and calcium accumulation might contribute to early RGC loss. We propose that the Kir channel activation, which we observed by flupirtine application in cultured RGCs, counteracts these early changes in ion channel function. Flupirtine treatment was started shortly after immunization of the rats to enable protection also of those RGCs undergoing apoptosis before clinical manifestation of the disease.

In our animal model, erythropoietin,^{7,47} ciliary neurotrophic factor (CNTF),⁴⁸ and glatiramer acetate²⁶ protected RGCs from degeneration by antagonizing pro-apoptotic signaling. The respective pathways were either influenced directly via activation of the erythropoietin receptor or the CNTF receptor^{7,48} or indirectly via inducing the expression of brain-derived neurotrophic factor after treatment with glatiramer acetate.²⁶ Our present experi-

ments revealed that the flupirtine-mediated neuroprotection does not affect these signal transduction cascades, indicating that RGC loss in MOG-EAE can, at least in part, be prevented without acting on these classical pro-apoptotic mechanisms. Comparing the amount of RGCs protected from degeneration by different therapeutic approaches at an identical endpoint, day 8 after clinical manifestation of MOG-EAE, revealed that combining flupirtine with IFN- β 1a increased RGC survival by 23%. This is in a similar range as the effects of local CNTF application (19%)⁴⁸ or the combination therapy of erythropoietin and methylprednisolone (22%).⁴⁷ Only erythropoietin monotherapy further improved RGC survival, resulting in a neuroprotective effect on 36% of RGCs undergoing apoptosis in the corresponding control group.⁷ We hypothesize that the high susceptibility for neurodegeneration in Brown Norway rats immunized with MOG is the reason why we observed only a partial protection of RGCs by flupirtine application.⁴⁹

Without affecting ON histopathology flupirtine exerted neuroprotective effects in MOG-induced optic neuritis both in the presence and absence of IFN- β 1a. In contrast, as we have shown previously IFN- β exerts anti-inflammatory effects in MOG-EAE, but has only a minor effect on RGC survival.^{16,26} In these studies, a small and transient protective effect of IFN- β on RGCs occurred in parallel with a delayed onset of MOG-induced optic neuritis.¹⁶ Comparing RGC densities at a later time point resulted in no statistically significant effect of IFN- β on RGC survival.²⁶ Neuroprotective properties of IFN- β 1b have also been observed in a spinal cord lesion model in rats.⁵⁰ However, in our present study the anti-inflammatory effects of IFN- β 1a predominated, resulting in a significantly increased survival of RGCs only if flupirtine was additionally applied. Monotherapy with IFN- β 1a was not sufficient to protect RGCs and, as a consequence of neuronal loss, IFN- β 1a alone did not improve visual functions determined by VEP recordings. The reduction of inflammatory infiltration and demyelination that we observed in the ON in both animal groups receiving IFN- β is in accordance with our previous results.^{16,26} Also in other EAE models, anti-inflammatory properties of human and rat IFN- β have been demonstrated.^{51–53} *In vitro* experiments revealed a stabilization of the blood-brain barrier (BBB) because of interactions of IFN- β 1a with rat astrocytes and endothelial cells.^{54,55} Stabilization of the BBB is also observed in patients with relapsing remitting MS in which IFN- β 1a reduces the number of contrast-enhancing lesions detected by MRI.^{56–58} The immunomodulatory effects of IFN- β are mediated via a decline of T-cell activity accompanied by a reduced expression of pro-inflammatory cytokines and matrix metalloproteinases.^{59–61} However, in chronic progressive disease stages of MS, in which the accumulating impairments correlate best with axonal and neuronal degeneration, the therapeutic effects of IFN- β in reducing permanent neurological deficits are limited, underlining the necessity for a neuroprotective add-on therapy.⁶² We cannot exclude the possibility that flupirtine mainly delays RGC loss instead of permanently preventing it. Considering the continuously ongoing neurodegeneration in chronic

progressive stages of MS, we assume that in MS patients even a delay in neuronal loss could result in slowed progression of neurological impairment.

The possibility that flupirtine might also exert neuroprotective effects on human neurons can be concluded from a double-blind study in patients suffering from Creutzfeldt-Jakob's disease, which revealed that oral flupirtine applications significantly reduce the deterioration of cognition.⁶³ Additionally, flupirtine-mediated protection of neurons from tumor necrosis factor-related apoptosis-inducing-ligand-induced apoptosis was observed in human brain slices.⁴ Because flupirtine significantly improved survival and function of RGCs as an add-on therapy to IFN- β 1a in our animal model, we encourage the evaluation of this well-tolerated oral drug for promoting neuroprotection in MS.

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