J. Cell. Mol. Med. Vol 11, No 1, 2007 pp. 111-119

# Expression of RhoA by inflammatory macrophages and T cells in rat experimental autoimmune neuritis

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*Received: November 7, 2006; Accepted: November 30, 2006*

## Abstract

RhoA is one of the best-studied members of Rho GTPases. Experimental autoimmune neuritis (EAN), which is characterized by infiltration of T cells and macrophages into the peripheral nervous system, is an autoantigen-specific T-cell-mediated animal model of human Guillain-Barré Syndrome. In this study, RhoA expression has been investigated in the dorsal/ventral roots of EAN rats by immunohistochemistry. A significant accumulation of RhoA+ cells was observed on Day 12, with a maximum around Day 15, correlating to the clinical severity of EAN. In dorsal/ventral roots of EAN, RhoA+ cells were seen in peri-vascular areas but also in the parenchyma. Furthermore, double-labelling experiments showed that the major cellular sources of RhoA were reactive macrophages and T cells. In conclusion, this is the first demonstration of the presence of RhoA in the dorsal/ventral roots of EAN. The time courses and cellular sources of RhoA together with the functions of RhoA indicate that RhoA may function to facilitate macrophage and T-cell infiltration in EAN and therefore could be a potential therapeutic target.

**Keywords:** experimental autoimmune neuritis • RhoA • macrophages • T cells

## Introduction

Experimental autoimmune neuritis (EAN) is an autoantigen-specific T-cell-mediated animal model of human acute demyelinating inflammatory disease of the peripheral nervous system (PNS), Guillain-Barré Syndrome (GBS). EAN is induced by immunization with autoantigen or by adoptive transfer of neuritogenic T cells. EAN is a monophasic disease characterized by weight loss, ascending paraparesis/paralysis and spontaneous recovery. Histopathologically, EAN is characterized by blood-nerve barrier (BNB) breakdown, severe demyelination in the PNS and infiltration of PNS by activated T cells and macrophages [1–7].

In EAN, T cells are activated by autoantigen following immunization, then attach to the venular endothelium in the PNS and penetrate the BNB. The infiltrated T cells amplify the local inflammation by recruiting more T cells and macrophages *via* chemokines and cytokines [8–13]. Subsequently, the breakdown of the BNB allows the passage of the circulating autoantibodies that synergize with T cells and macrophages to produce demyelination [14, 15]. The macrophage is a major effector cell of demyelination and responsible for most of the neuropathological effects in EAN peripheral nervous system injury [16]. The recruited macrophages not only phagocytose myelin debris but also secrete a variety

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doi:10.1111/j.1582-4934.2007.00004.x

of cytotoxic agents, like inflammatory cytokines, reactive oxygen metabolites, to act on endothelium to produce breakdown of BNB, or act on Schwann cells to cause myelin degeneration, or directly affect axons [17–19]. Depletion of macrophages or inhibition of their activity has been shown to suppress the clinical development of EAN [20, 21]. Therefore, T cell and macrophage migration to PNS lesion are critical for the development of EAN.

Rho GTPases belong to the Ras superfamily and act as intracellular switches that control multiple signalling cascades by switching between biochemically active and inactive states [22]. Rho GTPases transduce extracellular signals to the actin cytoskeleton and play important roles in regulating cell motility, morphology and migration [22]. Cell migration is a multi-step process and Rho GTPases are involved in many steps of this complex process, including lamellipodium extension, new adhesion to surrounding matrix proteins, cell body contractions, tail retraction and secretion of proteases to remodel the environment [23]. Furthermore, Rho GTPases also play a role in induction of apoptosis and formation of free radicals in CNS injury [24, 25]. In EAN, inhibition of Ras attenuates the course of EAN [26].

RhoA is one of the best-known members of Rho GTPases [27]. The involvement of RhoA in the regulation of leukocyte morphology, motility and migration was observed [28–31]. In addition, RhoA also plays a role in mediating T-cell migration, chemotaxis and cytokine secretion [32, 33]. Furthermore, up-regulation of RhoA in infiltrated macrophages in CNS injury has been observed in our group [34–36], but its expression pattern has not been studied so far in EAN. Therefore, this investigation was performed to study the spatiotemporal expression of RhoA in ventral and dorsal root of EAN rats.

# Material and methods

## Animal experiments

Male Lewis rats (8–10 weeks of age, 170–200 g, Charles River, Sulzfeld, Germany) were housed with equal daily periods of light and dark and free access to food and water. All procedures were performed in accordance with the published International Health Guidelines under a protocol approved by the Administration District Official Committee. Rats were immunized by subcutaneous injection of 50 μg of synthetic neuritogenic P2 peptide as described previously [37]. Rats were scored daily for development of EAN. The severity of EAN was scored as follows: 0 = normal, 1 = reduced tonus of tail, 2 = limp tail, impaired righting, 3 = absent righting, 4 = gait ataxia, 5 = mild paresis of the hind limbs, 6 = moderate paraparesis, 7 = severe paraparesis or paraplegia of the hind limbs, 8 = tetraparesis, 9 = moribund, 10 = death.

Rats were killed on 0, 6, 10, 12, 15, 18, 24 or 48 days (n = 3 per group) after immunization. Rats were deeply anaesthetized with ether and perfused intracardially with 4°C 4% paraformaldehyde in PBS (phosphate buffered saline). Spinal cords including ventral and dorsal roots were quickly removed and post-fixed in 4% paraformaldehyde overnight at 4°C. Spinal cords with ventral and dorsal roots were divided into 8 mm segments and embedded in paraffin, serially sectioned (3 μm) and mounted on silan-covered slides.

## Immunohistochemistry

After dewaxing, sections were boiled (in a 600 W microwave oven) for 15 min in citrate buffer (2.1 g citric acid monohydrate/l, pH 6). Endogenous peroxidases were inhibited with 1% $H_2O_2$ in pure methanol for 15 min. Sections were incubated with 10% normal pig serum (Biochrom, Berlin, Germany) to block non-specific binding of immunoglobulins and then with a mouse monoclonal antibody against RhoA (sc-418, Santa Cruz Biotechnology; dilution 1:100). Antibody binding to tissue sections was visualized with a biotinylated rabbit anti-mouse IgG $F(ab)_2$ antibody fragment (DAKO, Hamburg, Germany). Subsequently, sections were incubated with a horseradish peroxidase-conjugated Streptavidin complex (DAKO, Hamburg, Germany) diluted 1:100 in Tris-BSA for another 30 min, followed by development with diaminobenzidine (DAB) substrate (Fluka, Neu-Ulm, Germany). Finally, sections were counterstained with Meyer's hemalaun. As negative controls, the primary antibodies were omitted.

## Double staining

In double-labelling experiments, spinal cord sections were pre-treated as described above and then incubated with the appropriate antibodies. Then slices were once more irradiated in a microwave for 15 min in citrate buffer and were immunolabelled as described above, but by omission of counterstaining with hematoxylin. Visualization was achieved by adding secondary antibody (biotinylated rabbit anti-mouse IgG) at a dilution of 1:400 in TBS-BSA for 30 min and alkaline phosphatase conjugated Avidin complex diluted 1:1400 in Tris-BSA for 30 min. Consecutively, immunostaining was developed with Fast Blue BB salt chromogen-substrate solution. The following antibodies were used: ED1 (1:100;

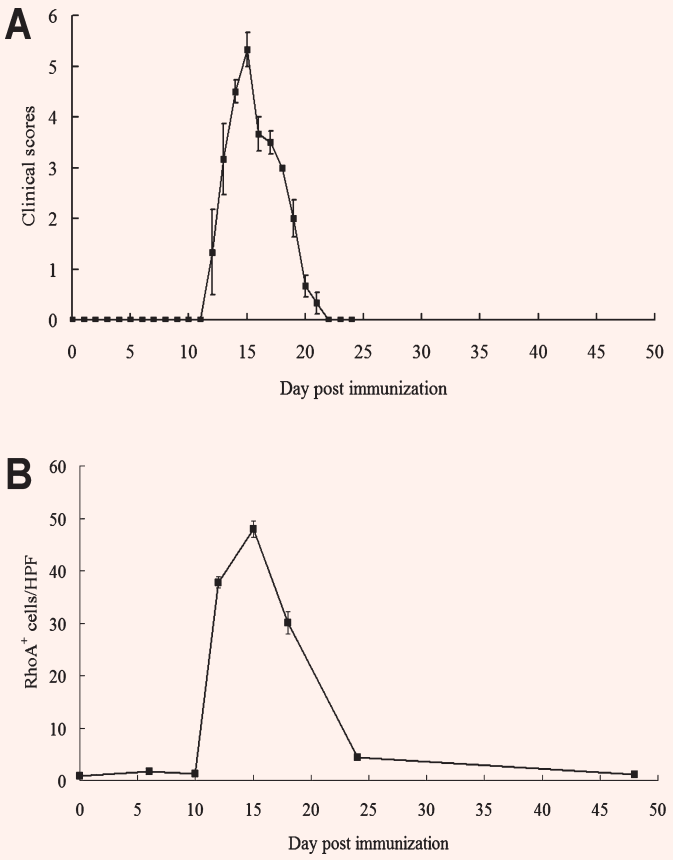


**Fig. 1** The time course of RhoA$^+$ cell accumulation in dorsal and ventral roots of EAN rats. (**A**) Clinical score of EAN severity. Rats (n = 6) were immunized with synthetic neuritogenic P2 peptide and monitored for development of EAN. Severity of disease was graded as follows: 0, normal; 1, reduced tonus of tail; 2, limp tail, impaired righting; 3, absent righting; 4, gait ataxia; 5, mild paralysis of the hind limbs; 6, moderate paraparesis; 7, severe paraparesis or paraplegia of the hind limbs; 8, tetraparesis; 9, moribund; 10, death. Results are given as mean clinical score ± SEM. (**B**) The time course of lesional RhoA$^+$ cell accumulation in dorsal and ventral roots of EAN rats. After immunostaining, the whole section was scanned at 40 times magnification for the ventral or dorsal roots of highest RhoA$^+$ cell accumulations (hot spots), followed by counting the number of RhoA$^+$ cells within a single high-power field (HPF, x400 magnification) in each hot spot. Five hot spots were counted for each section and the mean value was taken. In each field studied, only positive cells with the nucleus at the focal plane were counted. Results were given as arithmetic means of positive cells per HPF and SEM. Statistical analysis was performed by one-way ANOVA followed by Dunnett's Multiple Comparison test (Graph Pad Prism 4.0 software).

Serotec, Oxford, Great Britain) for activated microglia/macrophages, W3/13 (1:100; Serotec, Oxford, Great Britain) for pan T cells, rabbit polyclonal antibodies against P2X4 receptor (1:200; Alomone Labs, Jerusalem, Israel) or endothelial monocyte-activating polypeptide II (EMAPII, 1:100; BMA, Augst, Switzerland).

## Evaluation and statistical analysis

After immunostaining, sections were examined by light microscopy. Slides were evaluated and positive cells were quantified at the ventral or dorsal roots. Briefly, the whole section was scanned at 40 times magnification for the ventral or dorsal roots of highest RhoA$^+$ cell (hot spot), followed by counting the number of RhoA$^+$ cells within a single high-power field (HPF, x400 magnification) in each hot spot. Five hot spots were counted for each section and the mean value was taken. In each field studied, only positive cells with the nucleus at the focal plane were counted. Counting was done by observers unaware of the time-point of lesions. Results were given as arithmetic means of positive cells per HPF and standard errors of means (SEM). Statistical analysis was performed by one-way ANOVA followed by Dunnett's Multiple Comparison test (Graph Pad Prism 4.0 software). For all statistical analyses, significance levels were set at $P < 0.05$.

# Results

## Clinical course of EAN

Rats developed the first neurological signs of EAN (reduced tail tonicity) at Day 12 after immunization. Disease severity was maximal at Day 15 with rats showing mild to moderate paralysis of the hind limbs or paraparesis (mean clinical score of 5). Rats recovered fast from disease and by Day 22 neurological signs were no longer observed (Fig. 1A).

## RhoA expression in rat dorsal and ventral roots following EAN

RhoA expression in dorsal and ventral roots of normal rats and EAN rats was studied performed with immunohistochemistry. No immunoreactivity (IR) was detected for the negative control without primary antibody (Fig. 2A). In normal rats, IR of RhoA was occasionally observed in the dorsal and ventral roots ($0.8 \pm 0.2$ per HPF, Fig. 2B).

Following immunization, slight accumulation of $RhoA^+$ cells was observed already at Day 10 ($1.3 \pm 0.2$ per HPF, $P < 0.05$ *versus* normal control; Fig. 1B) and increased dramatically until Day 12 ($37.8 \pm 1.1$ per HPF, $n = 3$, $P < 0.05$ *versus* normal control; Fig. 1B). The accumulation of $RhoA^+$ cells reached a maximum at Day 15 ($48 \pm 1.5$ per HPF, $n = 3$, $P < 0.05$ *versus* normal control; Fig. 1B) and corresponded to the peak of disease severity. The accumulation of $RhoA^+$ cells remained significantly elevated until Day 18 ($30.1 \pm 2.1$ per HPF, $n = 3$, $P < 0.05$ *versus* normal control; Fig. 1B). At Day 24, a considerable decrease of $RhoA^+$ cell infiltration was observed (4.3 (0.4 per HPF, $n = 3$, $P < 0.05$ *versus* normal control; Fig. 1B). $RhoA^+$ cells were mainly located in the perivascular spaces corresponding to Virchow-Robin-like spaces, which represent the infiltrative route for blood-borne leukocytes, and in the surrounding parenchyma (Fig. 2C). However, $RhoA^+$ cells were also observed to distribute to the long-distant parenchyma (Fig. 2D).

## Double-labelling experiments

We further characterized the accumulated $RhoA^+$ cells by a double labelling with monoclonal antibodies directed against activated macrophages (ED1) or pan-T cells (W3/13). A double-staining experiment was performed in dorsal and ventral roots of Day 12 and 15 EAN rats (3 rats for each time-point). A co-expression of RhoA and ED1 was observed. Almost all $ED1^+$ macrophages expressed RhoA but only about 70% $RhoA^+$ cells expressed ED1 (Fig. 2F). T-cell infiltration is a major feature of EAN and almost all $W3/13^+$ T cells co-expressed RhoA (Fig. 2I). However, only about 30% $RhoA^+$ cells expressed W3/13. Therefore, the cellular sources for RhoA are T cells and activated macrophages in EAN.

EMAPII is a pro-inflammatory cytokine expressed by activated macrophages in the parenchyma of the CNS [38]. $P2X_4$ receptor (P2X4R), which is also a sensitive marker for reactive microglia/macrophages in the CNS, is an ATP gated cation channel and may play a role in modulating excitotoxic events following brain injury [39–41]. Similar to ED1, almost all $EMAPII^+$ or $P2X4R^+$ cells co-expressed RhoA and only about 70% $RhoA^+$ cells expressed EMAPII or P2X4R (Fig. 2G and H).

# Discussion

We have analysed the expression of RhoA in dorsal and ventral root of EAN rats. A significant $RhoA^+$ cell accumulation was observed 12 days after immunization with maximal levels at Day 15, which corresponded to disease severity. Further, double-labelling experiments confirmed that the major cellular sources for RhoA were T cells and reactive macrophages.

Thus RhoA-expression is seen in a migratory leukocytic cell population infiltrating PNS tissues prone to an inflammatory autoimmune attack. Interestingly, the involvement of RhoA in leukocyte trafficking appears most plausible, as RhoA regulates the actin cytoskeleton, cell polarity, microtubule dynamics and vesicular trafficking. Further, an up-regulation of RhoA has been seen in certain other central nervous system disorders associated with cell movement, like brain trauma [34, 35], spinal cord injury [42] and experimental autoimmune encephalomyelitis (EAE) [43–45]. In CNS trauma, RhoA up-regulation is mainly seen in mononuclear cells and is considered to be involved in causing neurotoxic effects and thus could be a therapeutic target [34, 35, 42]. In EAE models, RhoA is up-regulated in macrophages and endothelial cells of blood-brain barrier and considered to mediate migration of lymphocytes and monocytes to CNS [43–45]. Our study here provides data of up-regulation of RhoA in PNS of EAN. The accumulation of $RhoA^+$ cells correlates positively with the severity of EAN, indicating the importance of RhoA in the development of EAN.

RhoA expression is seen with two major inflammatory cell populations: macrophages and T cells. RhoA functions to regulate the organization of the actin

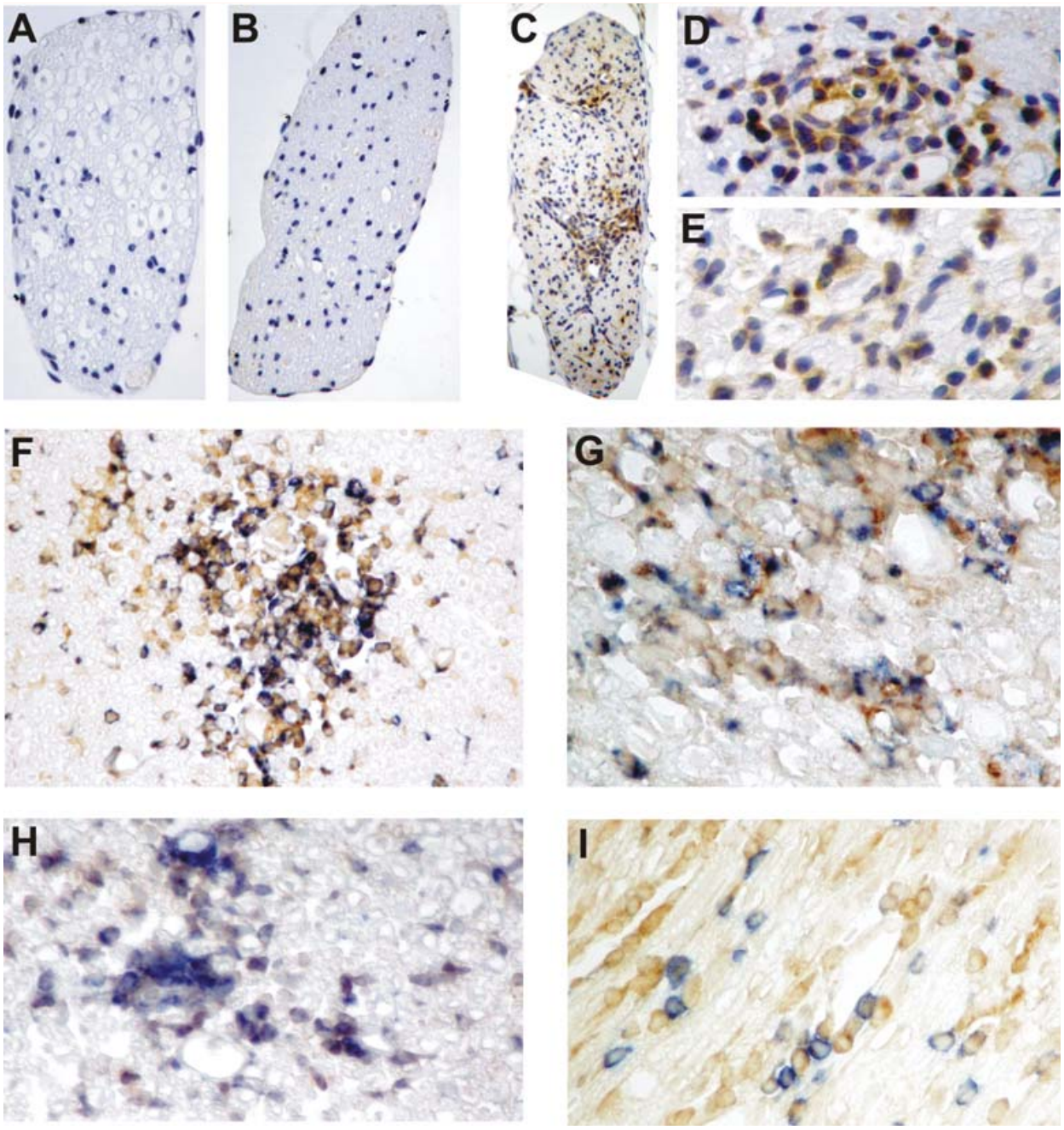


**Fig. 2** Immunohistochemical labelling of RhoA in spinal cord dorsal and ventral roots of normal and EAN rats. (**A**) Immunostaining of spinal cord dorsal and ventral roots without the primary antibody as a negative control. (**B**) RhoA expression was absent in dorsal/ventral roots of normal adult rats. (**C**) Accumulation of RhoA+ cells was seen in dorsal/ventral roots Day 15 after immunization in EAN. RhoA+ cells accumulated near blood vessels (**D**), but were also seen in the parenchyma (**E**). (**F**–**I**) RhoA double-labelling experiments. Fifteen days after immunization, most RhoA+ cells (brown) co-expressed reactive microglia/macrophages marker ED1 (**F**, blue), EMAPII (**G**, blue) or P2X4R (**H**, blue). The infiltration of W3/13+ T-lymphocytes (blue) could be detected and cells co-expressed RhoA (brown) (**I**). Original magnification: **A**–**I** x400.

cytoskeleton of cells and is essential for the regulation of cell shape, polarity, motility and adhesion [46, 47]. Activation of RhoA has opposing effects on cell migration: it decreases migration by enhancing adhesion, but promotes migration by inducing cell body contraction. In less adherent cells, like macrophages and T cells, RhoA does not affect adhesion but promotes cell body contraction [48]. In macrophages, the involvement of RhoA in the regulation of morphology, motility and migration is established [28–30]. RhoA is known to be important in the activation and recruitment of macrophages and inhibition of Rho reduces the migration and chemotaxis of macrophages [48]. RhoA is involved in transforming growth factor-β1 and granulocyte-macrophages colony-stimulating factor-mediated macrophage migration [49, 50]. Lipoxins, the potent mediators of monocyte activation, stimulate the reorganization of actin cytoskeleton in macrophages through RhoA [30]. BCL6 was observed to suppress RhoA activity and alters macrophage morphology and motility [29]. In T cells, RhoA also controls cell morphology and induces cell invasion [32, 33, 51, 52].

In addition to induce migration, RhoA plays a role to cause certain cytotoxic effects. RhoA is known to induce the formation of superoxides by macrophages [53]. RhoA is present in cytotoxic lymphocytes and is important for cell-mediated cytotoxicity [54]. In asthmatic patients, RhoA also helps the secretion of interferon γ and interleukin-4 [55]. Furthermore, RhoA is involved in other potential cytotoxic effects, like cellular calcium influx, release of excitatory amino acids and reactive oxygen species, inflammatory response, activation of intracellular proteases and apoptosis [24, 31, 33, 56, 57].

In our study, the accumulation of $RhoA^+$ macrophages and T cells is observed in dorsal/ventral roots of EAN in accordance with disease severity. The expression of RhoA in almost all infiltrated macrophages and T cells in dorsal/ventral roots of EAN rats together with the known function of RhoA indicates that RhoA may function to cause migration of macrophages and T cells to lesioned nerves and cause cytotoxic effects. Infiltration of T cells and macrophages is the major feature of EAN and is important for the initiation and development of EAN. Inhibiting accumulation of T cells and macrophages is sufficient to prevent development of EAN. Therefore, it is reasonable to speculate that the inhibition of RhoA activity in EAN may attenuate the course of disease. Inhibition of Ras proteins, the funding members of the large superfamily of GTPases that is divided into five branches: Ras, Rho, Rab, Arf and Ran, attenuates lymphocyte proliferation and the clinical course of EAN [26]. Although this study did not show whether the Ras protein antagonist inhibits lymphocyte migration, it did prove that a member of the Ras superfamily is involved in EAN development. More interestingly, several Rho inhibitors have been shown to influence RhoA activity and reduce inflammatory cell migration through brain endothelial cells in rat EAE, the central nervous system analogous disease of EAN and attenuate clinical signs of EAE [43–45].

Owing to their pleiotropic functions, Rho GTPases are considered promising target structures for novel therapeutic agents. Four types of Rho inhibitors have been developed with different mechanisms of action, including depletion of the cellular pool of isoprene precursors by statins, inhibition of transfer of isoprene moieties to Rho proteins by inhibitors of prenyl transferases, direct inhibition of the activity of Rho proteins, and inhibition of Rho specific effector molecules [58]. The lesional expression of RhoA described herein makes it a suitable target for pharmacological intervention in EAN. The up-regulation of RhoA in our study was detected irrespective of its active status. This is of interest because total RhoA, active and inactive forms, is accessible to pharmacological intervention with C3 exoenzyme from C botulinum by either inactivating the active GTP-bound forms or preventing inactive GDP-bound forms from becoming active. The C3 exoenzyme directly inactivates activity of Rho proteins by specifically adenosine diphosphate-ribosylating Rho at its effector domain (Asn41) [59, 60]. Thus, total RhoA expression pattern in EAN represents a substrate for pharmacologic intervention targeting Rho signal pathway.

In summary, we have first shown the accumulation of $RhoA^+$ macrophages and T cells to dorsal/ventral roots of EAN rats, which is in accordance with the severity of disease, and indicates that RhoA may be a future therapeutic target in EAN.

## Acknowledgement

This work has been supported in part by the BMBF (03134298). The authors are solely responsible for the content of this article.

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