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4-Aminopyridine ameliorates mobility but not disease course in an animal model of multiple sclerosis ☆

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ABSTRACT

Neuropathological changes following demyelination in multiple sclerosis (MS) lead to a reorganization of axolemmal channels that causes conduction changes including conduction failure. Pharmacological modulation of voltage-sensitive potassium channels (K_V) has been found to improve conduction in experimentally induced demyelination and produces symptomatic improvement in MS patients. Here we used an animal model of autoimmune inflammatory neurodegeneration, namely experimental autoimmune encephalomyelitis (EAE), to test the influence of the K_V-inhibitor 4-aminopyridine (4-AP) on various disease and immune parameters as well as mobility in MOG_{35-55} immunized C57Bl/6 mice. We challenged the hypothesis that 4-AP exerts relevant immunomodulatory or neuroprotective properties.

Neither prophylactic nor therapeutic treatment with 4-AP altered disease incidence or disease course of EAE. Histopathological signs of demyelination and neuronal damage as well as MRI imaging of brain volume changes were unaltered. While application of 4-AP significantly reduced the standing outward current of stimulated $CD4^+$ T cells compared to controls, it failed to impact intracellular calcium concentrations in these cells. Compatibly, K_V channel inhibition neither influenced $CD4^+$ T cell effector functions (proliferation, IL17 or IFNγ production). Importantly however, despite equal disease severity scores 4-AP treated animals showed improved mobility as assessed by 2 independent methods, 1) foot print and 2) rotarod analysis (0.332 ± 0.03, $n = 7$ versus 0.399 ± 0.08, $n = 14$, $p < 0.001$, respectively).

Our data suggest that 4-AP while having no apparent immunomodulatory or direct neuroprotective effects, significantly ameliorates conduction abnormalities thereby improving gait and coordination. Improvement of mobility in this experimental model supports trial data and clinical experience with 4-AP in the symptomatic treatment of MS.

Introduction

Infiltration of immune cells, demyelination, axonal damage and neuronal degeneration are all indicative of the impaired interplay between the immune and nervous systems during multiple sclerosis (MS) (Frohman et al., 2006). Demyelination leads to several pathologic ultrastructural features including i.) myelin vacuolation possibly associated with degeneration of oligodendrocytes which results in a splitting of the myelin sheath (Judge and Bever, 2006), ii.) loss of myelin by active stripping of the myelin sheath by macrophages and iii.) 'dying-back gliopathy' in which the inner tongue of the myelinating oligodendrocytes degenerates first. These demyelination-associated mechanisms cause exposure of paranodal and internodal voltage-sensitive potassium channels (K_V channels) that are distributed in the axonal membrane resulting in abnormal potassium outward currents associated with slow action potential conduction, conduction failure or changes in the axon's capacity for repetitive discharges (Dunn and Blight, 2011). Blockade of K_V channels exposed at demyelinated axons has been suggested as mechanism of action of 4-aminopyridine (4-AP) leading to improved action potential conduction (Sedehizadeh et al., 2012). This improvement in conduction translates into clinical benefit as measured by objectively and subjectively assessed walking abilities relative to placebo in a proportion of MS patients (Goodman et al., 2009, 2010). Consequently

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symptomatic treatment strategies with dalfampridine (extended-release formulation of 4-AP) were approved for MS patients with impaired mobility.

Expression of voltage-dependent and independent potassium channels on T lymphocytes (Ehling et al., 2011) is linked to impaired T cell effector functions (Bittner et al., 2010). Selective pharmacological inhibition of $K_V1.3$, a voltage-gated potassium channel has been shown to reduce proliferation and cytokine production of human and murine effector memory T cells (Vennekamp et al., 2004). Corroborating these findings $K_V1.3$ deficient animals showed markedly lower incidence and severity in an animal model of MS (Gocke et al., 2012). Although the mechanisms of K_V current blockade by 4-AP are complicated and depend on factors such as the frequency of stimulation and the kinetic state of the channel (Choquet and Korn, 1992; Judge and Bever, 2006; Kirsch et al., 1986; Yeh et al., 1976) the IC_{50} range of 4-AP mediated inhibition of $K_V1.3$ channels was determined from 200 to 1500 μM (Coetzee et al., 1999; Grissmer et al., 1994). Based on these results it can be hypothesized that 4-AP exerts immunomodulatory effects besides the symptomatic improvement of action potential conduction in models of experimental demyelination and MS patients. Furthermore, it is unknown but conceivable that 4-AP has direct effects within the CNS leading to attenuation of neuropathological damage in neuroinflammation.

We here investigate the hypothesis of 4-AP exerting relevant immunomodulatory or neuroprotective properties besides promoting improvement of gait parameters in an experimental model, $MOG_{35–55}$ induced experimental autoimmune encephalomyelitis (EAE). 4-AP mediated effects on the disease course, immune parameters, histopathological signs of demyelination and neuropathological damage as well as mobility measures were assessed.

Materials and methods

Electrophysiology and calcium imaging

$CD4^+$ T cells were isolated from spleen using the $CD4^+$ T cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) as described before (Gobel et al., 2011). All measurements were conducted in the whole-cell configuration of the patch-clamp technique. Recording pipettes were fabricated from borosilicate glass (GT150TF-10, Clark Electromedical Instruments, Pangbourne, UK; typical resistance was 4–7 MΩ) and filled with an intracellular solution containing (in mM): K-gluconate, 88; K_3-citrate, 20; phosphocreatine, 15; NaCl, 10; HEPES, 10; $MgCl_2$, 1; CaCl2, 0.5; BAPTA, 3; Mg-ATP, 3; and Na_3-GTP, 0.5. The internal solution was set to a pH of 7.25 with KOH and an osmolarity of 295 mOsm/kg. Extracellular solution contained (in mM): NaCl, 125; KCl, 2.5; NaH_2PO_4, 1.25; HEPES, 30; $MgSO_4$, 2; $CaCl_2$, 2; glucose, 10; pH 7.35 and osmolarity was set to 305 mOsm/kg. 4-AP (100 nM, Sigma-Adrich, Munich, Germany) was applied via the bath solution. Membrane currents were recorded using an EPC-10 amplifier, and digital analysis was done using Fitmaster software (HEKA Elektronik, Lamprecht, Germany (Meuth et al., 2005)).

For intracellular calcium imaging experiments $CD4^+$ T lymphocytes were isolated as described above. All measurements were performed in HEPES buffer containing (in mM): NaCl, 120; KCl, 2.5; NaH_2PO_4, 1.25; HEPES, 30; $MgSO_4$, 2; glucose, 10; pH 7.25 and osmolarity was set to 305 mOsm/kg. Cells were loaded with 5 μM Fura-2 AM (Invitrogen, Karlsruhe, Germany) for 30 min at 37 °C. Anti-mouse CD3 (clone 145-2C11; ebioscience, Frankfurt, Germany; 10 μg/ml) was added after 15 min and fluorescence was measured with a TECAN infinite M200Pro fluorimeter (Tecan Group Ltd., Männedorf, Switzerland). Excitation was alternated between 340 and 380 nm and emission was measured at 509 nm. 4-AP (100 nM) was added 10 min before starting the measurements and goat anti-Armenian hamster IgG(H + L) (Jackson ImmunoResearch, Newmarket, Great Britain; 10 μg/ml) was added after 3 min of measurement for T cell receptor crosslink.

T cell proliferation and cytokine production

Splenocytes from naïve mice were isolated, cultured and stimulated as described earlier (Schuhmann et al., 2010). In brief, 1×10^5 splenocytes were cultured in 1 ml DMEM containing 10 mM HEPES, 25 μg/ml gentamicin, 50 μM mercaptoethanol, 5% FCS, 2 mM glutamine, and 1% nonessential amino acids (Cambrex; Verviers, Belgium) for three days and stimulated with CD3/CD28 beads (cell to bead ratio 2:1; Dynal Biotech, Hamburg, Germany) and 4-AP (concentrations as indicated). ^3H thymidine (Amerham; Piscataway, NJ) was added for the final 14 h, and radioactivity was measured on a β-scintillation counter (TopCount NXT; PerkinElmer, Rodgau-Jügesheim, Germany).

For assessing $CD4^+$ and $CD8^+$ T cell proliferation under 4-AP treatment, splenocytes were labeled with the cell proliferation dye eFluor®670 (5 μM; Invitrogen) according to manufacturer's protocol. 2×105 eFluor®670-labeled splenocytes were stimulated with CD3/CD28 beads or MOG. After 96 h splenocytes were stained with the following antibodies CD3-FITC (clone 17A2; BioLegend), CD8a-PE (clone 53–7.3; BD Pharmingen) and CD4-Pacific blue (clone GK1.5; BioLegend) to distinguish between the $CD4^+$ and $CD8^+$ T cells within the splenocytes. Stained cells were assayed on a FACSGalliosTM flow cytometer using Kaluza software (Beckman Coulter) to evaluate the T cell proliferation.

Experiments were performed in quadruplicates. In another set of experiments splenocytes were isolated from naïve and immunized mice at the disease maximum and 50 days after EAE induction, stimulated with $MOG_{35–55}$ peptide (10 μg/ml) and analyzed for proliferation. Supernatants were assessed for IFNγ, TNFα, IL4, IL10, IL21 and IL17 protein levels by ELISA (R&D Systems; Wiesbaden, Germany) according to the manufacturer's instructions.

Immunophenotyping

For analysis of T cell subtype distribution flow cytometry was performed as previously described using appropriate antibodies or isotype controls: rat anti-mouse CD4-PerCP (BD Bioscience, no. 553052), rat anti-mouse CD8a-AlexaFluor700 (BioLegend, no. 100730), rat anti-mouse CD25-APC (BD Bioscience, no. 557192), rat anti-mouse CD44-FITC (BD Bioscience, no. 553133), rat anti-mouse CD62L-APC (BD Bioscience, no. 553152) and rat anti-mouse CD69-FITC (BD Bioscience, no. 557392).

In a subset of experiments cells were centrifuged and subjected to further analysis by flow cytometry (stainings for annexin V and propidium iodide).

Induction and evaluation of EAE

All experiments were approved by and conducted in accordance with the laws and regulations of the regulatory authorities for animal care and use in Germany (AZ: 87–51.04.2010.A325).

EAE was induced by immunization of 10–12 week old female C57Bl/6 mice with $MOG_{35–55}$ peptide (Charite, Berlin, Germany) as previously described (Gobel et al., 2011). Pharmacological modulation was performed using 4-AP by intraperitoneal (i.p.) injection (one or two times daily) or oral application via the drinking water.

All animals were kept under standard conditions and had access to water and food ad libitum. The clinical course of EAE was monitored by two blinded investigators using the following scoring system: grade 0, no abnormality; grade 1, limp tail tip; grade 2, limp tail; grade 3, moderate hind limb weakness; grade 4, complete hind limb weakness; grade 5, mild paraparesis; grade 6, paraparesis; grade 7, heavy paraparesis or paraplegia; grade 8, tetraparesis; grade 9, quadriplegia or premorbid state; and grade 10, death. Animals, with a score higher than 7 or a loss of >20% of their body weight, were euthanized and continued with the accordant score until the end of the experiment.