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Full-length Article

Ca^{2+}-activated K^+ channels modulate microglia affecting motor neuron survival in $hSOD1^{G93A}$ mice

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ABSTRACT

Recent studies described a critical role for microglia in amyotrophic lateral sclerosis (ALS), where these CNS-resident immune cells participate in the establishment of an inflammatory microenvironment that contributes to motor neuron degeneration. Understanding the mechanisms leading to microglia activation in ALS could help to identify specific molecular pathways which could be targeted to reduce or delay motor neuron degeneration and muscle paralysis in patients. The intermediate-conductance calcium-activated potassium channel KCa3.1 has been reported to modulate the "pro-inflammatory" phenotype of microglia in different pathological conditions. We here investigated the effects of blocking KCa3.1 activity in the $hSOD1^{G93A}$ALS mouse model, which recapitulates many features of the human disease. We report that treatment of $hSOD1^{G93A}$ mice with a selective KCa3.1 inhibitor, 1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole (TRAM-34), attenuates the "pro-inflammatory" phenotype of microglia in the spinal cord, reduces motor neuron death, delays onset of muscle weakness, and increases survival. Specifically, inhibition of KCa3.1 channels slowed muscle denervation, decreased the expression of the fetal acetylcholine receptor γ subunit and reduced neuromuscular junction damage. Taken together, these results demonstrate a key role for KCa3.1 in driving a pro-inflammatory microglia phenotype in ALS.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a multifactorial disease characterized by the progressive degeneration of motor neurons (MN) and muscle paralysis. Despite current treatments, patients survive < 3–5 years after the initial diagnosis. Most ALS cases are sporadic (sALS), and only 5–10% have a familial origin (fALS). Among the latter, about 20% express a dominant mutant form of the Cu, Zn superoxide dismutase (SOD1) (Rothstein, 2009). Transgenic mice expressing a mutant SOD1 develop MN pathology, with muscle denervation and weakness similar to ALS patients (Fischer et al., 2004). Many evidence demonstrate that ALS is non-cell autonomous, with multiple co-players involved in disease progression (Robberecht and Philips, 2013). In particular, signals from both glial cells and muscles initiate and sustain MN degeneration (Boillée et al., 2006; Dobrowolny et al., 2008). Neuroinflammation is often associated with ALS (Philips and Robberecht, 2011): microglial reactivity, astrogliosis and lymphocyte infiltration are common in patients and in experimental models of the disease (Hall et al., 1998; Mantovani et al., 2009; Turner et al., 2004). Microglia carrying mutant SOD1 express pro-inflammatory genes, such as *il-1β*, *tnf-α* and *inos* (Philips and Robberecht, 2011; Almer et al., 1999; Henkel et al., 2004). Nevertheless, the exact contribution of neuroinflammation to the pathology of ALS is not clear, possibly acting in concert with additional factors. The knowledge of the molecular mechanisms driving

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the inflammatory responses, and their impact on MN, would be of great importance to develop effective therapeutic treatments. Among the possible modulators of the inflammatory response in the CNS, plasma membrane ion channels are good candidates, regulating membrane potential and intracellular signaling in T cells, B cells and innate immune cells such as macrophages and microglia (Feske et al., 2015; Zierler et al., 2016). In this work, we investigated the role of the intermediate-conductance, Ca^{2+}-activated K^+ channel KCa3.1, in shaping the activation state of microglia in a mouse model of ALS, the $hSOD1^{G93A}$ mice, which recapitulates many features of the human disease. In the CNS, KCa3.1 channels are expressed by microglial cells, where they regulate cell migration and phagocytic activity in physiological and pathological conditions such as glioma, ischemia, spinal cord injury (SCI) and Alzheimer's disease (AD) (Chen et al., 2011; Maezawa et al., 2012; Bouhy et al., 2011; D'Alessandro et al., 2013; Grimaldi et al., 2016). In some conditions (SCI), the expression of KCa3.1 is also reported on astrocytes and neurons (Bouhy et al., 2011). In AD, microglial KCa3.1 potentiate the neurotoxicity induced by oligomeric amyloid-β and lipopolysaccharide (LPS) treatment (Maezawa et al., 2012; Kaushal et al., 2007); while blocking KCa3.1 activity has beneficial effects in rodent models of multiple sclerosis and ischemic stroke, reducing TNF-α and IFN-γ expression in the spinal cord (Reich et al., 2005) or the infarcted area (Chen et al., 2016). The KCa3.1 inhibitor *per se* is not directly neuroprotective in the absence of microglia (Maezawa et al., 2011; D'Alessandro et al., 2016).

In the current study, we treated $hSOD1^{G93A}$ mice with the selective KCa3.1 inhibitor TRAM-34 starting at the pre-symptomatic stage and analyzed the activation state of spinal cord microglia by measuring the expression levels of "pro" and "anti-inflammatory" genes and cell morphology. We found that the chronic inhibition of KCa3.1 activity in $hSOD1^{G93A}$ mice: i) restrained the pro-inflammatory phenotype of microglia; ii) increased the number of healthy MNs; iii) preserved the number of healthy neuromuscular junctions (NMJ) in the *tibialis anterior* muscle; iv) and their maturation level, as assessed by mRNA analysis of AChR γ and ε subunit expression and by current recording on isolated muscle fibres. Furthermore, TRAM-34 treatment delayed motor symptom appearance, as shown by prolonged muscle strength and motor coordination, and increased mice survival. Taken together, these data demonstrate a crucial role for microglia in modulating disease onset and progression, and provide prove-of-concept for the potential targeting of KCa3.1 to reduce ALS-associated neuroinflammation and to protect MNs from degeneration.

2. Materials and methods

2.1. Animal model

The study was conducted in accordance with the ARRIVE guidelines (Kilkenny et al., 2010). All experiments and procedures were approved by the Italian Ministry of Health (authorization n. 78/2017-PR) in accordance with the ethical guidelines on use of animals from the EC Council Directive 2010/63/EU and from the Italian D.Leg 26/2014. All possible efforts were made to minimize animal suffering, and to reduce the number of animals used per condition by calculating the necessary sample size before performing the experiments. hSOD1(G93A) transgenic mice, which express about 20 copies of mutant human $SOD1^{G93A}$ [B6.Cg-Tg(SOD1-G93A)1Gur/J line] were obtained from Jackson Laboratory (Bar Harbor, ME, USA) (RRID:IMSR_JAX:004435) (Charles River, Calco, Italy). B6.Cg-Tg(SOD1-G93A)1Gur/J were also maintained as hemizygotes by breeding transgenic males with wild-type C57BL/6J females from Charles River Laboratories, both maintained on C57BL/6J genetic background. Age-matched non-transgenic C57BL/6J mice were always used as control mice. Only male mice were used for the experiments to minimize gender-induced differences in motor impairment and survival (Choi et al., 2008). Transgenic mice were identified by PCR on DNA obtained from tail biopsies. Briefly, tail tips were digested (overnight, 58 °C) in a buffer containing 100 mM Tris–HCl pH 8, 0.1% SDS 20, 5 mM EDTA pH8, 200 mM NaCl and 20 mg/ml proteinase K (Ambion-Thermo Fisher, Germany, #2548) and the genomic DNA was amplified with SsoFast Eva Green Supermix (Bio-Rad, California, #172-5201) using the following primers: SOD1 forward 5′-CATCAGCCCTAATCCATCTGA-3′; SOD1 reverse 5′- CGCGACTAACA ATCAAAGTGA-3′. Animals were housed in regular polycarbonate cages (30 × 16 × 11 cm), 2–3 per cage, at constant temperature (22 ± 1 °C) and humidity (50%), and were kept on a 12-h light cycle (light 7 a.m.to 7p.m.). Housing comprised nesting objects, with bedding (sawdust) materials. Food (regular chow, containing 14% protein, 5% fat, 3041 kcal ME/kg) and water were freely available. Microbiological analyses were routinely (each 3–4 months) performed and defined endemic Norovirus and Helicobacter in our conventional animal facility. Transgenic animals were weighed two times a week, beginning at 7 weeks of age. Starting at 6 weeks of age mice were evaluated for motor deficits with a behavioral score system: 0 = Full extension of hind legs away from the lateral midline when the mouse is suspended by tail; the mouse must hold this position for 2 s, and is suspended 2–3 times; 1 = Collapse or partial collapse of leg extension towards lateral midline (weakness) or trembling of hind legs during tail suspension; 2 = Curling of the toes and dragging of at least one limb during walking; 3 = Rigid paralysis or minimal joint movement; foot not used for forward motion; 4 = Mouse cannot stand up in 20 s from either side, euthanasia.

Mice were always treated in blinded fashion.

2.2. TRAM-34 treatment and survival analysis

Male transgenic animals were weighed twice a week from 6 until 18 weeks of age. Then, the animal status and weight was monitored daily. Starting at 7 weeks of age, $hSOD1^{G93A}$ mice were randomly grouped (at least 5 mice per experimental group) for vehicle and TRAM-34 (1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole treatment. Mice were treated daily (early in the morning) with 120 mg/kg of TRAM-34 or the same amount of vehicle (50 µl, peanut oil, Sigma-Aldrich, St. Louis, MO USA, #P2144) by intraperitoneal injections. The treatment regimen was chosen to reach a CNS concentration of TRAM-34 that effectively inhibits KCa3.1 channels, as previously described (D'Alessandro et al., 2013). TRAM-34 was synthesized as described (Wulff et al., 2000). Animals were treated until the age described in the text or until sacrifice for the survival analysis experiments. Animals were sacrificed when unable to stand up within 20 s after being placed on either side.

2.3. Isolation of lumbar microglia cells

Adult microglia were isolated from the lumbar spinal cord tract of age-matched non-transgenic C57BL/6J wt mice (non-tg *wt*) and $hSOD1^{G93A}$ mice as described in (Yip et al., 2009) with minor modifications. Mice were deeply anesthetized with chloral hydrate (i.p., 400 mg/Kg, Carlo Erba Italy, #334085) before being transcardially perfused with phosphate buffered-saline (PBS tablet, Sigma-Aldrich, #P4417). Spinal cords were then flushed out from the spinal canal using a 20 ml syringe filled with PBS and digested with 30 units of papain (15–23 U/mg protein, Sigma-Aldrich, #P3125) for 30 min at 37 °C. Tissue was then triturated with a pipette to obtain single cell suspensions, which were applied to 70-µm/40-µm cell strainers and used for the experiments. Purity of isolated microglia was assessed by morphology and surface staining for $CD11b^+$, $CD45^{low+}$, $Ly6G^-$, $Ly6C^-$. mAbs directly conjugated to PE, PE-Cy7, APC, APC-H7, and PerCP-Cy5.5 fluorochromes and specific for the following antigens (clone name in parentheses) were used: CD45 (1 0 4), CD11b (M1/70), Ly6C (HK1.4), and Ly6G (1A8). Antibodies were from eBioscience (Thermo Fisher, Germany) and BioLegend (San Diego, CA). Immunostaining was performed with saturating amounts of Abs for 30 min