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Neuronal overexpression of IP_3 receptor 2 is detrimental in mutant SOD1 mice

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ABSTRACT

Amyotrophic Lateral Sclerosis (ALS) is a devastating neurodegenerative disease causing progressive paralysis of the patient followed by death on average 3–5 years after diagnosis. Disease pathology is multi-factorial including the process of excitotoxicity that induces cell death by cytosolic Ca^{2+} overload. In this study, we increased the neuronal expression of an endoplasmic reticulum (ER) Ca^{2+} release channel, inositol 1,4,5-trisphosphate receptor 2 (IP_3R2), to assess whether increased cytosolic Ca^{2+} originating from the ER is detrimental for neurons. Overexpression of IP_3R2 in N2a cells using a Thy1.2–IP_3R2 construct increases cytosolic Ca^{2+} concentrations evoked by bradykinin. In addition, mice generated from this construct have increased expression of IP_3R2 in the spinal cord and brain. This overexpression of IP_3R2 does not affect symptom onset, but decreases disease duration and shortens the lifespan of the ALS mice significantly. These data suggest that ER Ca^{2+} released by IP_3 receptors may be detrimental in ALS and that motor neurons are vulnerable to impaired Ca^{2+} metabolism.

1. Introduction

A tight control of intracellular Ca^{2+} concentration is crucial for cell survival [1]. Motor neurons are especially vulnerable to disturbances in Ca^{2+} dynamics as they combine a low concentration of Ca^{2+} binding proteins with a relatively high amount of Ca^{2+}-permeable AMPA-type glutamate receptors [2]. This could contribute to the selective vulnerability of the motor neurons during Amyotrophic Lateral Sclerosis (ALS). ALS is a progressive neurodegenerative disease, characterized by the selective loss of motor neurons and the denervation of muscle fibers, resulting in muscle weakness and paralysis. In Europe, the disease has an annual incidence of 2.7 cases per 100,000 people [3] and the disease duration post diagnosis is 3–5 years. In 10% of patients, ALS is a familial disease and 20% of these familial ALS patients contain mutations in the gene encoding superoxide dismutase 1 (SOD1). As the disease progression is indistinguishable between familial and sporadic ALS, common disease mechanisms are predicted. One of these mechanisms is excitotoxicity that causes neuronal death by overstimulation of the glutamate receptors [2]. The only effective treatment in ALS, riluzole, reduces the glutamatergic input [2]. In general, excitotoxicity induces an increased intracellular Ca^{2+} concentration, which is hazardous to the motor neurons. In addition, release of Ca^{2+} from the endoplasmic reticulum (ER) may further increase the intracellular Ca^{2+} concentration resulting in increased excitotoxicity [4].

In this study, we investigate the role of elevated expression of an ER Ca^{2+} release channel, inositol 1,4,5-trisphosphate receptor 2 (IP_3R2) and concomitant increased IP_3-induced ER Ca^{2+} release in ALS. To this end, we generated a mouse with a neuronal overexpression of IP_3R2 and crossbred these mice with a mouse model for ALS, in which excitotoxicity contributes to motor neuron death [2].

2. Materials and methods

2.1. Animal generation and housing

Mice overexpressing human $SOD1^{G93A}$ were purchased from The Jackson Laboratories (Bar Harbor, USA) and maintained on a C57BL/6 background. In order to create transgenic mice, murine *itpr2* cDNA (8.1 kb) was cloned into the XhoI restriction site of the Thy1.2 expression cassette (kind gift from Novartis Pharma). A 15 kb DNA fragment was excised from the Thy1.2–IP_3R2 vector with the restriction endonuclease Not1. The transgenic mice over-

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expressing *itpr2* (Thy1.2–IP_3R2) were created by pronuclear microinjections (Polygene, Rümlang, Switzerland). We obtained three different founder lines and after crossbreeding of the line with the highest expression in the spinal cord to a full C57BL/6 background for more than five generations, these mice were crossbred with $SOD1^{G93A}$ mice. Chow and water were provided *ad libitum* and mice were housed in the conventional animal facility of the KU Leuven under standard conditions according to the guidelines of the KU Leuven.

2.2. Animal behavioral analysis

The hanging wire test was used to determine disease onset by assessing the ability of the mice to hold their own weight for 60 s. The mouse is placed on a wire grid and this grid is turned over while the mouse is holding its own body weight upside-down. If a mouse fails (drops from the grid before 60 s) and in consecutive trials cannot hold its own weight for 60 s, it is defined as symptomatic. Symptom onset determined by hanging wire was subsequently used to calculate disease duration. Grip strength measurements of all limbs were performed using a dynamometer (Chatillon, Largo, USA) every 5 days from 80 days of age onwards at which grip strength was determined with the mice placed on a small grid. Relative grip strength was determined per mouse by normalizing the absolute grip strength values (N) to the average of each mouse from day 90 to day 105. End stage was defined as the age at which mice could no longer right themselves within 30 s when placed on their back. End stage is used as a measurement of survival and is the moment when mice are euthanized to prevent further suffering. All animal experiments were performed with the approval of the Animal Ethical Committee of KU Leuven (020/2010).

2.3. Quantitative PCR

Isolation of mRNA occurred by the TriPure (Roche, Basel, Switzerland) method and the RNeasy kit (Qiagen, Venlo, The Netherlands). Reverse transcriptase PCR used random hexamers (Life Technologies, Carlsbad, USA) and Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV RT; Invitrogen, Carlsbad, USA). Quantitative PCR was performed with the StepOnePlus (Life Technologies) with TaqMan Universal PCR Master Mix (Life Technologies). Gene expression assays were purchased from Life Technologies and IDT DNA (Coralville, USA).

2.4. Ca^{2+} measurements in intact N2a cells

For the cytosolic Ca^{2+} measurements in intact N2a cells (ATCC:CCL-131), the cells were seeded in 96-well plates (Greiner, Wemmel, Belgium) at a density of approximately 1.2×10^4 cells cm^{-2} and investigated two days after seeding. The cells were loaded for 30 min with 5 μM Fura-2-AM at 25 °C in modified Krebs solution containing 135 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 11.6 mM Hepes (pH 7.3), 11.5 mM glucose and 1.5 mM Ca^{2+}. They were then incubated for at least 30 min in the absence of Fura-2-AM. Fluorescence was monitored on a FlexStation 3 microplate reader (Molecular Devices, Sunnyvale, CA, USA) by alternately exciting the Ca^{2+} indicator at 340–380 nm and collecting emission fluorescence at 510 nm. After 30 s, cells were exposed to agonist. Three replicates per experiment were used. The data were plotted as F340/F380 ratio corrected for the starting value (R-R0).

2.5. Statistical analysis

Analysis was performed with the statistical software package Prism Origin (GraphPad Software, La Jolla, USA). Survival and symptom onset was analyzed by Log-Rank statistics. Differences between 2 groups were analyzed using a Student's t-test. Significance was assumed at $p \leqslant 0.05$. Error bars represent the standard error of the mean.

3. Results

3.1. IP_3R2 overexpression increases agonist-induced Ca^{2+} signals in N2a cells

To investigate whether increased ER Ca^{2+} release through the IP_3 receptor may be detrimental in ALS, we cloned the cDNA of the murine ITPR2 gene into the Thy1.2 expression cassette (Fig. 1A). The Thy1.2 expression cassette drives transgene expression starting at postnatal day 9 and especially in neurons [5]. IP_3R2 is the most sensitive isoform of the receptor to IP_3 [6,7]. In the nervous system IP_3R2 is mainly localized in astrocytes [8–13]. The functional expression of IP_3R2 in N2a cells using the Thy1.2–IP_3R2 construct was first assessed by measuring intracellular Ca^{2+} responses triggered by bradykinin, which provokes intracellular IP_3 production. The peak values of the bradykinin-induced intracellular Ca^{2+} transients are significantly higher in Thy1.2–IP_3R2 expressing cells in comparison to cells transfected with the empty construct (Fig. 1B). These data imply that the Thy1.2–IP_3R2 construct functions properly, leading to the functional expression of IP_3R2 and increased Ca^{2+} responses to extracellular agonists.

3.2. Neuronal tissues of Thy1.2–IP_3R2 mice show increased IP_3R2 expression

We created transgenic mice overexpressing IP_3R2 by zygote injection of a fragment of the Thy1.2–IP_3R2 construct. We assessed expression of IP_3R2 by quantitative PCR (qPCR) in adult Thy1.2–IP_3R2 mice and non-transgenic littermates. In spinal cord (Fig. 2A) and brain (Fig. 2B), a significant increase of murine IP_3R2 mRNA is detected compared to non-transgenic controls. These qPCR results indicate that the construct successfully increases the expression of IP_3R2 in the nervous system. The Thy1.2–IP_3R2 mice are born at a normal Mendelian ratio and do not develop a phenotype as assessed up to 2 years of age (data not shown).

3.3. IP_3R2 overexpression is detrimental in ALS mice

To investigate whether increased amounts of IP_3R2 affect ALS, Thy1.2–IP_3R2 mice were crossbred with $SOD1^{G93A}$ mice. Neuronal overexpression of IP_3R2 does not affect symptom onset (Fig. 3A). Interestingly, Thy1.2–$IP_3R2 \times SOD1^{G93A}$ mice have a decreased survival (Δ = 16.2 days; Fig. 3B) and shorter disease duration (Δ = 10.0 days) compared to the $SOD1^{G93A}$ mice (Fig. 3C). This is confirmed by a faster disease progression on basis of grip strength between $SOD1^{G93A}$ mice overexpressing IP_3R2 and $SOD1^{G93A}$ (Fig. 3D). These data imply that increased neuronal ER Ca^{2+} release by increasing IP_3R2 levels is detrimental in ALS.

4. Discussion

We studied the potential contribution of Ca^{2+} originating from intracellular stores and the role for ER Ca^{2+} release channels in neurons during ALS. A potential role of IP_3R2 was previously suggested by genome wide association of single nucleotide polymorphisms (SNPs) in the ITPR2 gene with sporadic ALS [14], although this association was not found in other populations [15,16]. Interestingly, an increase of IP_3R2 gene expression is detected in blood

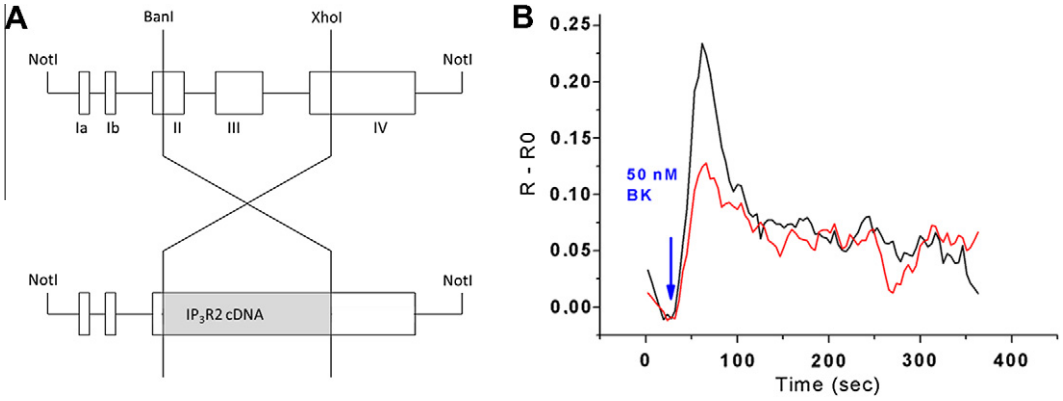

Fig. 1. The Thy1.2–IP_3R2 construct increases intracellular Ca^{2+} signals in transfected N2a cells. (A) The Thy1.2–IP_3R2 construct, and (B) average intracellular Ca^{2+} ratios in Thy1.2–IP_3R2 (black curve) or empty vector (red curve) transfected N2a cells as measured by the FlexStation when stimulated with bradykinin (BK 50 nM; $n = 3$; t-test on independent maximal values $p = 0.009$).

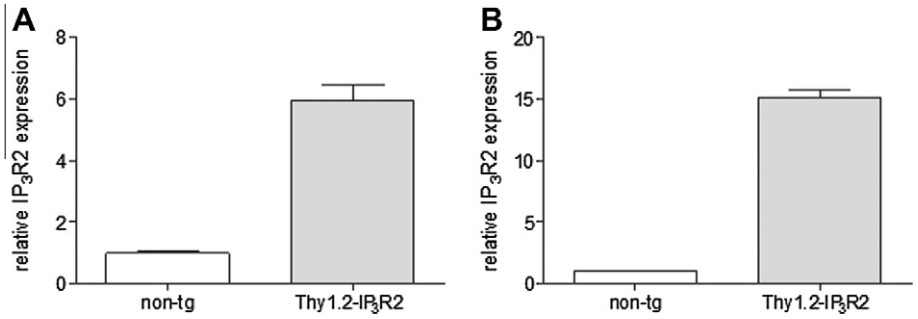

Fig. 2. Increased expression of IP_3R2 in the nervous system. Expression of IP_3R2 determined by qPCR of Thy1.2–IP_3R2 ($n = 3$) and non-transgenic littermates (non-tg; $n = 4$) in the spinal cord (A), and brain (B).

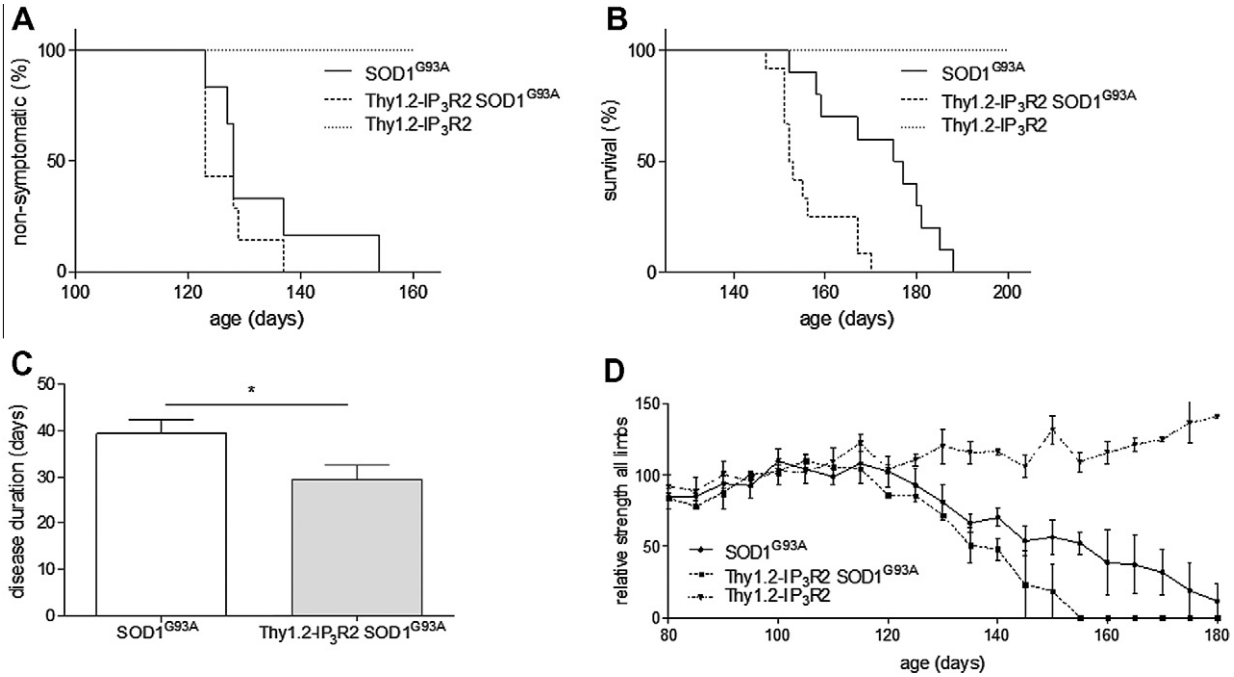

Fig. 3. IP_3R2 overexpression in neurons does not affect symptom onset but impairs survival in $SOD1^{G93A}$ mice. (A) Kaplan–Meier curve for symptom onset for Thy1.2–IP_3R2 $SOD1^{G93A}$ mice ($n = 7$, 126.6 ± 2.0 days) and $SOD1^{G93A}$ mice ($n = 6$, 132.8 ± 4.6 days, $p = 0.30$) as determined by the hanging wire test. (B) Survival analysis for Thy1.2–IP_3R2 $SOD1^{G93A}$ mice ($n = 12$, 156.0 ± 2.2 days) and $SOD1^{G93A}$ mice ($n = 10$, 172.2 ± 3.9 days, $p < 0.001$). (C) Disease duration of Thy1.2–IP_3R2 $SOD1^{G93A}$ mice ($n = 7$) and $SOD1^{G93A}$ mice ($n = 6$), and (D) relative grip strength analysis of all limbs of Thy1.2–IP_3R2 $SOD1^{G93A}$ mice ($n = 2$), $SOD1^{G93A}$ mice ($n = 3$) and Thy1.2–IP_3R2 mice ($n = 4$). $^*p < 0.05$.

samples of ALS patients [14]. Although IP_3R2 expression is low in neurons, an upregulation in the motor neurons of these patients could lead to a disturbance of Ca^{2+} dynamics in these cells and could contribute to the vulnerability of these patients to ALS. In order to investigate this possibility, we selectively overexpressed the IP_3R2 in postnatal neurons. This did not directly evoke a phenotype but crossbreeding one of these transgenic lines with the mutant $SOD1^{G93A}$ mice significantly decreased the disease duration and survival. A comparable negative effect on survival was also observed by genetically removing the GluR2 subunit which results in a higher Ca^{2+} permeability of the AMPA-type glutamate receptor [17]. Together, these data illustrate that genetic alterations disturbing the normal Ca^{2+} metabolism may worsen the disease process during ALS, at least in mutant $SOD1^{G93A}$ mice. We hypothesize that differences in Ca^{2+} handling could predispose patients to ALS.

From a therapeutic point of view, it may be of interest to prevent ER Ca^{2+} release in order to protect motor neurons. It is known that dantrolene that pharmacologically inhibits ER Ca^{2+} release via the ryanodine receptors can rescue cells *in vitro* [18–22]. Moreover, in rodent models of a number of neurodegenerative conditions including spinocerebellar ataxia type 2 [21] and type 3 [23], spinal cord injury [24] and cerebral ischemia [19] dantrolene is also protective. However, no such protection was observed in the mutant $SOD1^{G93A}$ mouse [22], indicating that the ryanodine receptor mediated Ca^{2+} release from the ER does not play an important role in this ALS model. In view of our new results, it would be interesting to develop specific IP_3R antagonists in an attempt to protect motor neurons.

We conclude that neuronal overexpression of IP_3R2 does not affect symptom onset, but decreases disease duration and shortens the lifespan of ALS mice. Our data imply that ER Ca^{2+} released through IP_3 receptors may be detrimental in ALS and confirm that a tight regulation of the intracellular Ca^{2+} concentration is crucial, especially in motor neurons.

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