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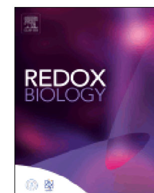

Research Paper

Real-time visualization of oxidative stress-mediated neurodegeneration of individual spinal motor neurons in vivo

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ABSTRACT

Generation of reactive oxygen species (ROS) has been shown to be important for many physiological processes, ranging from cell differentiation to apoptosis. With the development of the genetically encoded photosensitiser KillerRed (KR) it is now possible to efficiently produce ROS dose-dependently in a specific cell type upon green light illumination. Zebrafish are the ideal vertebrate animal model for these optogenetic methods because of their transparency and efficient transgenesis. Here we describe a zebrafish model that expresses membrane-targeted KR selectively in motor neurons. We show that KR-activated neurons in the spinal cord undergo stress and cell death after induction of ROS. Using single-cell resolution and time-lapse confocal imaging, we selectively induced neurodegeneration in KR-expressing neurons leading to characteristic signs of apoptosis and cell death. We furthermore illustrate a targeted microglia response to the induction site as part of a physiological response within the zebrafish spinal cord. Our data demonstrate the successful implementation of KR mediated ROS toxicity in motor neurons *in vivo* and has important implications for studying the effects of ROS in a variety of conditions within the central nervous system, including aging and age-related neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis.

1. Introduction

The generation of oxidative stress (OS) is an imbalance in the homeostasis of oxidation-reduction (redox) reactions and develops as a result of increased reactive oxygen species (ROS) in excess of available antioxidants. This disturbance of the normal redox state in favour of pro-oxidative factors is associated with numerous pathophysiological processes. While it is well-appreciated that ROS are physiologically important signalling molecules, excessive levels are associated with aging and the development of neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis (ALS) [1–6]. Pathogenic disruptions in ALS have been linked to OS via redox dysregulation including protein aggregation, hyper-excitability, mitochondrial dysfunction, and impaired axonal transport [7–10]. Motor neurons (MNs) seem particularly sensitive to these pathological effects and ROS have been demonstrated to result in DNA and tissue damage, inflammation and subsequent cellular apoptosis [11–16]. Overall ROS-mediated OS appears to be an important factor in the progression of neurodegenerative diseases.

The standard approach for experimental exposure of cells or organisms to ROS has been through the global application of ROS or ROS-generating reagents. New approaches have become available to selectively target ROS to individual cells or particular cellular structures [17–19]. Optogenetic approaches use genetically encoded, light-inducible, ROS-generating photosensitizers (RGP) that can be expressed in specific tissues and cell types (e.g. intestine or neurons), subcellular compartments (e.g. nucleus, cellular membrane or lysosome), or even fused to individual proteins. This transgenic approach allows for advanced temporal and spatial regulation of (sub-) lethal ROS production [20–22] that has not been possible previously. One RGP that has been successfully used to specifically deliver ROS under the optogenetic control is the phototoxic fluorescent protein KillerRed (KR) [23–25]. Upon prolonged green light excitation (absorption spectrum 540–580 nm) KR generates high levels of ROS along with photobleaching of the fluorophore. While photoexcitation of KR generates both singlet oxygen and superoxide radicals, singlet oxygen is considered the primary damaging agent produced by this photosensitizer [26]. KR-mediated cell death has been shown to occur via apoptosis and its phototoxic activity is directly related to level of excitation (light intensity), duration of excitation (illumination time) and expression

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level [26].

Zebrafish embryos provide an excellent model system for these optogenetic studies, as they are transparent and allow long-term live-imaging studies. Furthermore, increased efficiency of transposon-mediated transgenesis now makes it possible to generate transgenic zebrafish models expressing tissue-specific RGPs targeting single cells or whole organs *in vivo*. As a vertebrate model, 70% of human genes have a zebrafish orthologue, and 82% of genes known to be associated with human disease have a zebrafish counterpart [27]. KR has been successfully applied previously in cell culture, worms [*Caenorhabditis elegans*], and zebrafish [*Danio rerio*]. Kobayashi et al. (2013) utilised membrane-tagged KillerRed (memKR) to target chemosensory neurons in *C. elegans* [20]. Korzh et al. (2011) generated transgenic zebrafish lines expressing memKR to generate ROS in the hindbrain, the habenular commissure and the optic tectum [28]. Teh et al. (2014) described a memKR zebrafish model of light-induced cardiac deficiency i.e. changes in heart rate and heart contractility [25]. Here we utilised the advantages of the zebrafish system to investigate ROS-mediated OS and neuronal degeneration of individual MNs in the vertebrate spinal cord. Using single-cell resolution, real-time confocal live imaging of zebrafish spinal MNs we visualise for the first time the vulnerability of MN to ROS-induced OS, leading to their apoptotic degeneration. This approach provides a novel and important platform to investigate OS and its role in spinal cord MN degeneration *in vivo*.

2. Results

2.1. Selective expression of KillerRed in the zebrafish spinal cord

To express KillerRed (KR) in zebrafish spinal motor neurons (MNs), we generated transgenic zebrafish expressing KR targeted to the inner cell membrane via a neuromodulin N-terminal membrane localization signal (MLS) under the control of the MN-specific promoter *mnx1* (Fig. 1A). The *mnx1* gene encodes a homeobox transcription factor that has conserved functions in vertebrate MN differentiation [29] and is expressed primarily in post-mitotic spinal cord MNs and a small set of interneurons [30,31]. We used Tol2-mediated transgenesis as described previously [24] (Fig. 1B) to generate two stable transgenic zebrafish lines that either i) express KR in all spinal cord MNs (*Tg[mnx1:MLS-KillerRed]*) or ii) randomly integrate KR in a sub-set of spinal cord MNs (*Tg[4xnrUAS:MLS-KillerRed, cryaa: EGFP])*. Germ line transmission was confirmed by PCR analysis of genomic DNA from F1 generation embryos for the genetically encoded photosensitizer KR (Fig. 1C). *Tg [mnx1:MLS-KillerRed]* expresses KR in selected primary MNs (Fig. 1D, E). Mosaic expression of KR-positive MNs (KR + ve) and/or EGFP + ve MNs was achieved by crossing of *Tg[4xnrUAS:MLS-KillerRed, cryaa:EGFP]* and *Tg[met:GAL4,UAS:EGFP]* (ed6) [32] (Fig. 2).

2.2. Quantification of KillerRed-induced ROS production upon light illumination

Overall intracellular oxidant activity was measured in an unbiased approach using the cell-permeable chemical reporter CM-H2DCFDA. This innately non-fluorescent reporter yields a highly fluorescent adduct following oxidation [33], thereby becoming a sensitive oxidant sensor. Microplate reader quantification of the oxidized fluorescent reporter served as a proxy for intracellular reactive oxygen species (ROS) concentration *in vivo* [34]. The oxidative stressor hydrogen peroxide (H_2O_2) was used as positive control and induced strong ROS-mediated fluorescence in KR + ve control fish (Fig. 3A). At 3 days post fertilisation (dpf) light-illuminated KR + ve zebrafish demonstrated significantly increased fluorescence levels compared to non-illuminated KR + ve clutch mates (Fig. 3A).

We next investigated the impact of antioxidant treatment upon KR-mediated ROS generation. The potent ROS scavenging compound Nacetylcysteine (NAC) has been shown to successfully rescue overall cellular and oxidative stress [35–37]. Application of increasing H_2O_2 concentrations (1 mM, 2 mM, 5 mM, and 10 mM) led to significant and

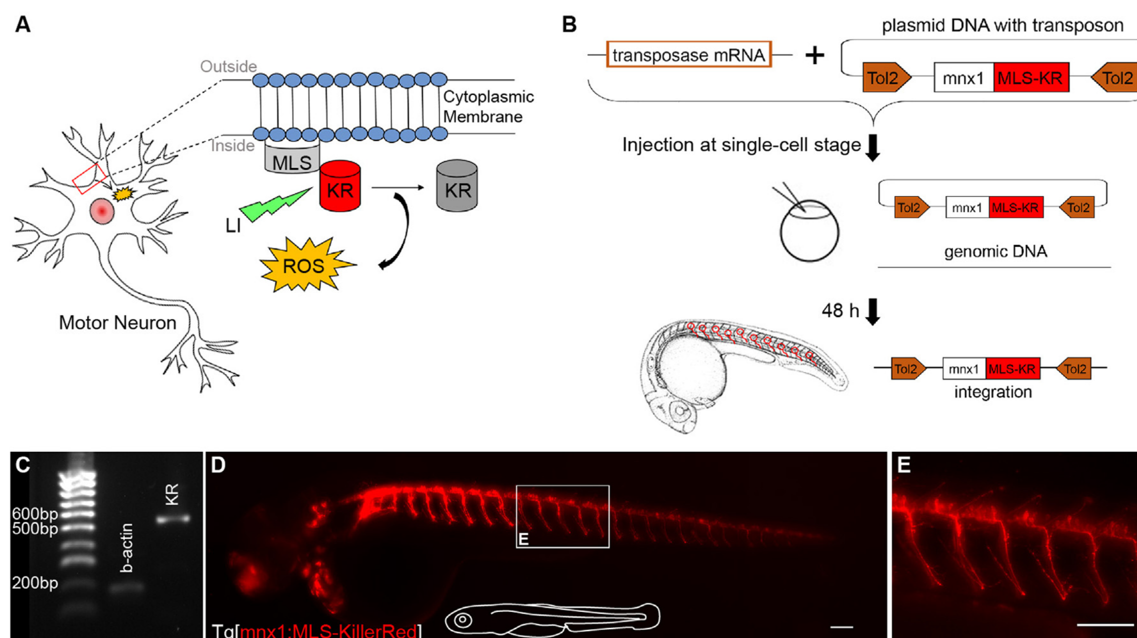

Fig. 1. Motor neuron (MN) specific expression of KillerRed (KR). (A) A membrane localization signal (MLS) targets the photosensitizer protein KillerRed (KR) to the intracellular cell membrane of MNs (*mnx1* promoter). Upon green light illumination (LI,), KR induces lipid oxidization generating reactive oxygen species (ROS) alongside photo-bleaching of KR. (B) Synthetic transposase mRNA and a Tol2 transposon plasmid DNA construct containing the Tol2 element, the *mnx1* promoter and the sequence encoding MLS-KR were co-injected into one cell stage zebrafish eggs. The Tol2 construct is excised from the plasmid DNA and integrated into the genomic DNA. Tol2 insertions in germ cells are transmitted to the F1 generation (modified after Kawakami et al., 2007). (C) PCR analysis of genomic DNA extracted from 24hpf F1 generation zebrafish embryos, confirmed germ line transmission of KR. Expected product size for MLS-KR was 531 bp, b-actin served as a positive control (housekeeping gene). (D-E) MN specific MLS-KR expression (red) at 3 dpf (*Tg[mnx1:MLS-KillerRed]*). Images are lateral views, anterior to the left, dorsal to the top. Scale bar 25 µm.

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