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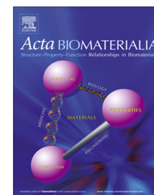

Full length article

Blue light potentiates neurogenesis induced by retinoic acid-loaded responsive nanoparticles

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ABSTRACT

Neurogenic niches constitute a powerful endogenous source of new neurons that can be used for brain repair strategies. Neuronal differentiation of these cells can be regulated by molecules such as retinoic acid (RA) or by mild levels of reactive oxygen species (ROS) that are also known to upregulate RA receptor alpha (RARα) levels.

Data showed that neural stem cells from the subventricular zone (SVZ) exposed to blue light (405 nm laser) transiently induced NADPH oxidase-dependent ROS, resulting in β-catenin activation and neuronal differentiation, and increased RARα levels. Additionally, the same blue light stimulation was capable of triggering the release of RA from light-responsive nanoparticles (LR-NP). The synergy between blue light and LR-NP led to amplified neurogenesis both *in vitro* and *in vivo*, while offering a temporal and spatial control of RA release. In conclusion, this combinatory treatment offers great advantages to potentiate neuronal differentiation, and provides an innovative and efficient application for brain regenerative therapies.

Statement of Significance

Controlling the differentiation of stem cells would support the development of promising brain regenerative therapies. Blue light transiently increased reactive oxygen species, resulting in neuronal differentiation and increased retinoic acid receptor (RARα) levels. Additionally, the same blue light stimulation was capable of triggering the release of RA from light-responsive nanoparticles (LR-NP). The synergy between blue light and LR-NP led to amplified neurogenesis, while offering a temporal and spatial control of RA release. In this sense, our approach relying on the modulation of endogenous stem cells for the generation of new neurons may support the development of novel clinical therapies.

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1. Introduction

The subventricular zone (SVZ), located between the ependymal layer of the lateral ventricles and the parenchyma of the *striatum*, is the largest neurogenic niche in the adult human and mammalian brain [1]. Within this region, self-renewing multipotent neural stem cells (NSCs) have the ability to differentiate into neurons, astrocytes, and oligodendrocytes [2,3]. The persistence of this germinal region throughout life supports the idea that new cells may be used to restore dysfunctional or damaged circuitries. Importantly, endogenous SVZ cells have been shown to proliferate, migrate and differentiate in response to brain injury, ultimately inducing functional repair. Indeed, the proliferation rate of SVZ cells is increased after seizures, ischemia, spinal cord transection, among other types of lesion [4]. The plasticity exhibited by the adult brain after injury suggests that pro-neurogenic factors may be good candidates for enhancing regeneration of the damaged brain [5]. In this context, a molecule of particular interest is

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retinoic acid (RA). RA is a metabolic product of retinol (vitamin A) that regulates cell proliferation, differentiation and apoptosis in the developing and adult mammalian brain [6,7]. The involvement of RA on adult neurogenesis is well documented. Depletion of RA in adult mice leads to decreased neuronal differentiation and cell survival within the subgranular zone of the hippocampus [8]. Similarly, administration of an inhibitor of RA synthesis (disulfiram) to neonatal mice decreased cell proliferation in the SVZ, and electroporation of dominant-negative RA receptors altered the morphology of neuronal progenitors and blocked neuroblast migration [9]. Importantly, defective RA signaling has been implicated in the onset of several brain diseases [10,11]. In the nucleus, RA signaling is transduced by RA receptor (RAR) and retinoid X receptor (RXR), activating gene transcription; Additionally, non-nuclear and non-transcriptional effects of RAR, namely on the activation of kinase cascades, were also reported [12]. Of note, it was recently reported that moderate ROS stabilize RARα, the most abundant RAR isotype [13]. These authors reported that direct exposure to low concentrations of hydrogen peroxide can increase RARα protein levels. With increased RARα levels, RA responsiveness is also expected to increase. In addition to this effect, moderate ROS are also capable of inducing NSC differentiation independently of RA signaling [14].

In the present work, we used two approaches that worked synergistically: blue light to induce ROS-mediated neurogenesis and RARα upregulation, and light-responsive nanoparticles (LR-NP) to release of RA (triggered by blue light) to potentiate neuronal differentiation of NSC. The use of light-responsive nanocarriers that can be remotely disassembled to release its content with spatial and temporal resolution are of high therapeutic value [15,16]. The successful development of this platform would provide innovative and efficient applications for brain regenerative therapies.

2. Materials and methods

All experiments were performed in accordance with European Community Council Directives (2010/63/EU) and the Portuguese law (DL n° 113/2013) for the care and use of laboratory animals.

2.1. SVZ cell cultures

SVZ cells were prepared from 1-3 day-old C57BL/6 mice as described previously [17].

2.2. Preparation of LR-NP

A solution of RA (24 μL, 50 mg mL^{-1}, in DMSO) and a solution of polyethylenimine and 4,5-dimethoxy-2-nitrobenzyl chloroformate (PEI-DMNC) (66.7 μL, 150 mg mL^{-1} respectively, in DMSO) were added simultaneously to an aqueous solution of dextran sulphate (DS; 5 mL, 0.4 mg mL^{-1}) and stirred for 5 min. Then, an aqueous solution of $ZnSO_4$ (120 μL, 1 M) was added and stirred for 30 min. The LR-NP suspension was dialyzed (Spectra/Por® 1 Regenerated Cellulose dialysis membrane, MWCO 6000–8000 Da, Spectrum) for 24 h, in the dark, against an aqueous solution of mannitol (5%, w/v), lyophilized and stored at 4 °C before use.

2.3. RA release studies

A solution of [^{3}H]RA in DMSO was used for the preparation of LR-NP, using a 1:20 ratio of labeled to unlabeled RA (1 nCi μg^{-1} RA). The initial RA cargo was quantified using 2/3 of the original LR-NP suspension (1 mg ml^{-1}). To quantify the controlled release of RA, a 10 μg mL^{-1} suspension of [^{3}H]LR-NP was irradiated with blue light (405 nm, 300 mW/cm^2). For each timepoint (0, 30, 60 and 600 s) the LR-NP suspension was centrifuged at 14,000g for 3 min, the supernatant collected and mixed with liquid scintillation fluid (1 mL; Ultima Gold, Packard Instrument SA, Rungis, France) and the scintillations counted in a TriCarb 2900 TR Scintillation analyser (Perkin Elmer, Buckinghamshire, UK).

2.4. In vitro cell treatments

Six-day-old neurospheres were adhered for 2 days onto poly-D-lysine (0.1 mg/mL)-coated 12-well μ-chamber slides (IBIDI, Germany) for all experiments except ROS quantification in which cells were adhered onto poly-D-lysine-coated 96-well plates, all in growth factor (EGF, FGF-2)-devoid medium. SVZ cells were then treated with LR-NP alone, laser alone, or LR-NP for 24 h followed by laser treatment (300 mW/cm^2, 405 nm; Z-Laser Optoelektronik Gmbh, Freiburg, Germany). Laser aperture was set to cover the area of one well (0.27 cm^2), with a fixed distance between laser source and cells (5 cm). During laser treatments, cell media was reduced to 80 μL/well to allow better light penetration and reduced dispersion. All the inhibitors used were added to cell media 1 h before treatments, namely apocynin (5 μM, Sigma, St. Louis, MO, USA), a broad NOX inhibitor, and IWR-1-endo (5 μM, Santa Cruz Biotechnology, Santa Cruz, CA, USA), a Wnt pathway inhibitor via proteasomal degradation of β-catenin. Controls including blank LR-NP (void nanoparticles) and DMSO (1:10000) were also performed.

2.5. Cell death

Propidium iodide (PI) is a cell death marker incorporated by necrotic and late-apoptotic cells. PI (3 μg mL^{-1}, Sigma) was added to cell media 10 min before the end of the 48 h treatments. Cells were fixed using 10% formalin solution, neutral buffered, rinsed with PBS, stained with Hoechst-33342 (2 μg mL^{-1}; Life Technologies), and mounted in Dako fluorescent medium (Dakocytomation Inc., Carpinteria, CA, USA). Images of PI uptake labeling were acquired using a fluorescent microscope (Axioskope 2 Plus; Carl Zeiss, Göttingen, Germany).

2.6. Intracellular ROS quantification

SVZ cells plated on 96-well plates were cultured in DMEM/F-12 medium devoid of phenol red and supplemented with 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 1% B27 supplement (all from Life Technologies). Cells were treated with laser alone for different time points; 10 min before the end of the experiment, 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) or MitoSOX red (both from Life Technologies) was added to cell media in order to reach a final concentration of 50 μM and 5 μM respectively. After 10 min, cells were washed with warm medium and emitted fluorescence was read in a microplate spectrophotometer plate reader at Ex/Em(DCFDA): 485/530 nm and Ex/Em(MitoSOX): 510/580 nm.

2.7. Immunocytochemistry

Cells were fixed with 10% formalin solution, neutral buffered. After washing three times with PBS, unspecific binding was blocked and cells permeabilized for 30 min, at room temperature (RT) with a solution containing 3% BSA, 0.3% Triton X-100. Cells were kept overnight at 4 °C in primary antibody solution, then washed with PBS and incubated for 1 h at RT with the corresponding secondary antibody. Primary antibodies were used as listed: goat polyclonal anti-sox2 (1:200; Santa Cruz Biotechnology), mouse monoclonal anti-Nestin (1:200; Abcam, Cambridge, UK), mouse monoclonal anti-GFAP (1:200; Cell Signaling Technology, Beverly, MA, USA), goat polyclonal anti-doublecortin (1:100; Santa Cruz), rabbit monoclonal anti-Ki67 (1:100; Abcam), mouse

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