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Glucocorticoids alter neuronal differentiation of human neuroepithelial-like cells by inducing long-lasting changes in the reactive oxygen species balance

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ABSTRACT

Prenatal exposure to excess glucocorticoid has been shown to have adverse effects on the developing nervous system that may lead to alterations of fetal and adult neurogenesis, resulting in behavioral changes. In addition, an imbalance of the redox state, with an increased susceptibility to oxidative stress, has been observed in rodent neural stem cells exposed to the synthetic glucocorticoid analog dexamethasone (Dex).

In the present study, we used the induced pluripotent stem cells (IPSC)-derived It-NES AF22 cell line, representative of the neuroepithelial stage in central nervous system development, to investigate the heritable effects of Dex on reactive oxygen species (ROS) balance and its impact on neuronal differentiation.

By analysing gene expression in daughter cells that were never directly exposed to Dex, we could observe a downregulation of four key antioxidant enzymes, namely *Catalase*, *superoxide dismutase 1, superoxide dismutase 2* and *glutathione peroxidase7*, along with an increased intracellular ROS concentration. The imbalance in the intracellular REDOX state was associated to a significant downregulation of major neuronal markers and a concomitant increase of glial cells. Interestingly, upon treatment with the antioxidant N-acetyl-cysteine (NAC), the misexpression of both neuronal and glial markers analyzed was recovered. These novel findings point to the increased ROS concentration playing a direct role in the heritable alterations of the differentiation potential induced by Dex exposure. Moreover, the data support the hypothesis that early insults may have detrimental long-lasting consequences on neurogenesis. Based on the positive effects exerted by NAC, it is conceivable that therapeutic strategies including antioxidants may be effective in the treatment of neuropsychiatric disorders that have been associated to increased ROS and impaired neurogenesis.

1. Introduction

The pathogenesis of a wide group of complex neurodevelopmental disorders has been linked to a variety of prenatal insults (Bellinger, 2013; Marco et al., 2011), including prenatal exposure to excess glucocorticoids (GCs) that exert adverse effects on the developing nervous system (COTTER, 2002; Seckl, 1998).

There are several conditions associated to prenatal high level of GCs, including placenta failure, genetic deficiency of 11β-Hydroxysteroid dehydrogenase Type 2 (11βHSD2), maternal infection or inflammation, severe maternal stress, or exogenous administration of GCs analogs (Cottrell and Seckl, 2009; Seckl and Holmes, 2007; Seckl and Meaney, 2004). For example, synthetic GCs, such as dexamethasone (Dex) have been commonly administered to pregnant women at risk of preterm delivery to induce lung maturation (Roberts and Dalziel, 2006). Several studies have linked fetal exposure to high levels of GCs to altered fetal and adult neurogenesis, as well as behavioral changes (Bose et al., 2010; Hauser et al., 2009; Ji et al., 2010; Khalife et al., 2013; Moors et al., 2012;

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Noorlander et al., 2014; Owen and Matthews, 2007). In particular, a new study by *Khalife* et al., has shown that prenatal exposure to synthetic GCs may increase the risk for children to develop later in life behavioral and emotional problems, including attention deficit hyperactivity disorder (ADHD) (Khalife et al., 2013). We previously showed that rat neurons and neural stem cells (NSCs) exposed to high levels of Dex exhibit an increase in intracellular reactive oxygen species (ROS) level and augmented susceptibility to oxidative stress (Ahlbom et al., 2000; Bose et al., 2010). Moreover, *in vitro* Dex exposure decreases NSCs proliferation and modifies the expression of genes playing a key role in cellular senescence and mitochondrial function (Bose et al., 2010).

In normal conditions, ROS modulate signaling pathways that affect gene expression, cell proliferation and differentiation (Ji et al., 2010; Rhee, 1999; Sauer et al., 2001; Tsatmali et al., 2006, 2005). The balance between the generation and elimination of ROS is maintained by the antioxidant defense system, including superoxide dismutase (SODs), Catalase and glutathione peroxidase (GPX). SODs are antioxidant enzymes converting O_2 into H_2O_2. This molecule is subsequently converted either into H_2O and O_2 by Catalase or into H_2O by the GPX enzyme. The balance control of intracellular ROS levels is a major strategy to regulate cellular growth and differentiation (Tsatmali et al., 2006, 2005). Consequently, misfunction of the antioxidant system and uncontrolled production of ROS impair cellular processes that can affect the development of the nervous system and may lead to neurodevelopmental/neuropsychiatric disorders (Yorbik et al., 2002).

The generation of human induced pluripotent stem cells (IPSCs) from adult somatic cells (Takahashi and Yamanaka, 2006) has opened a new avenue to understand the molecular basis of human neurodevelopment in normal and pathological conditions (Vaccarino et al., 2011). IPSCs, besides the ability to proliferate almost indefinitely and differentiate into any cell type, provide the unique possibility to derive disease-specific cells that can be used for *in vitro* disease modeling and *drug development* (Kim, 2014).

In the present study, we used long-term self-renewing neuroepithelial-like stem cells (lt-NES cells, AF22 cell line) derived from IPSCs (Falk et al., 2012) to investigate the long-lasting effects of Dex on ROS balance and the possible impact on neural differentiation. We applied the exposure paradigm that we have developed to study heritable effects *in vitro* (Bose et al., 2010).

Our results showed an increased intracellular ROS concentration and a concomintant downregulation of four key antioxidant enzymes, i.e.*Catalase*, *SOD1, SOD2* and *GPX7*, in daughter cells (never directly exposed) derived from Dex-exposed lt-NES. The alteration in the intracellular ROS balance was associated with a decreased neuronal differentiation and a significant downregulation of four major neuronal markers, namely the vesicular glutamate transporter 2 (*vGLUT2*), the glutamic acid decarboxylase 67 (*GAD67*), the microtubule-associated protein 2 (*MAP2*), and Doublecortin (*DCX*). Conversely, the glial fibrillary acidic protein gene (*GFAP*) was upregulated, pointing to heritable changes in the differentiation potential that favor glial differentiation. Remarkably, the altered gene expression pattern could be normalized upon treatment with the well-established antioxidant molecule N-acetyl-cysteine (NAC) (Dodd et al., 2015), suggesting a direct role played by the increased ROS concentration in the differentiation process impairment.

2. Materials and methods

2.1. Ethics statement

The donors of dermal fibroblasts used in this study gave a written informed consent concerning the sampling and the IPSC derived lt-NES AF22 cell line generation. Ethical permission for reprogramming human cells has been granted (Reprogrammeringavmänskligaceller, dnr 2012/208-31/3, addendum 2012/856-32).

2.2. Cell culture procedures and exposures

The human lt-NES AF22 cell line was generated from human IPSC as previously described (Falk et al., 2012). To promote the proliferation, lt-NES AF22 were plated at a minimum density of 40,000 cells/cm^2 on tissue culture plates coated with poly-L-Ornithine (0.1 mg/ml, Sigma-Aldrich) and laminin (5 μg/ml, Sigma-Aldrich). Cells were maintained in an undifferentiated and proliferative state in serum-free media containing DMEM/F12 (1:1) + L-Glutamine (+Pest) (Life Technologies™) supplemented with N2 (1:100) and B27 (1:500) (both from Life Technologies™) in the presence of EGF (10 ng/ml, Invitrogen) and FGF (10 ng/ml, R&D Systems) added every other day. Following visual confirmation of confluence, cells were passaged every third to fourth day, using TrypLE TM Express (1X + Phenol red) and Defined Trypsin Inhibitor (1X) (Gibco). To induce the differentiation, cells were plated in 12-multiwell plates (coated as described above) at a density of 60,000 cells/cm2. Growth factors were withdrawn and cells were maintained in DMEM/F12 (1:1) media supplemented with N2 (1:100), B27 (1:500), cyclic AMP (cAMP) (final concentration of 300 ng/ml, Sigma-Aldrich).

To study the heritable effects of Dex, proliferating lt-NES cells were exposed to 1 μM Dex (Sigma-Aldrich) diluted in DMSO, for 48 h (Fig. 1A). This non toxic concentration, used in several previous studies performed on both rodent and human NSCs (Bose et al., 2010; Moors et al., 2012; Sundberg et al., 2006), does not induce cell death, but inhibits cell proliferation also in our model (Fig. 1B). Control lt-NES cells were treated with DMSO. The parent cells (P; directly exposed to Dex) were kept in a proliferating state for two additional passages and the resulting daughter cells (D2; never directly exposed to Dex) were used for experimental purposes, as shown in Fig. 1A.

Depending on the experimental aim, D2 cells were allowed to differentiate for 1, 2 or 4 weeks and subsequently fixed for immunocytochemistry or harvested for qRT-PCR analysis. In some experiments, control and Dex-exposed cells were treated with NAC 25 μM (Sigma-Aldrich) during the differentiation phase.

2.3. Intracellular ROS level measurement

D2 lt-NES cells were plated on 96-multiwell plates, pre-coated as described above, at a concentration of 80,000 cells/cm2 and kept in proliferative media for 72 additional hours.

For ROS measurement in differentiated cells, D2 lt-NES cells were plated in 12-multiwell plates (coated as described above) at a density of 60 000 cells/cm2 and kept in differentiative medium for two weeks.

The intracellular ROS concentration in Dex-exposed and control D2 cells was determined by Image-iT ™ LIVE Green Reactive Oxygen Species Detection Kit (Invitrogen). Briefly, cells were washed gently with warm HBSS containing Ca^{2+} and Mg^{2+}, and incubated for 30 min with 25 μM of carboxy-H_2DCFDA, which is able to permeate live cells and become fluorescent in presence of non-specific ROS. Hoechst 33342 was added at a final concentration of 1.0 μM to the carboxy-H2DCFDA staining solution during the last 5 min of the incubation at 37 °C. Afterwards, the fluorescence intensity of each well was determined using a Fluoroskan Ascent FL 2.6 fluoroscope (Thermo Scientific). The excitation and emission wavelengths were respectively 485 nm and 538 nm for carboxy-H2DCFDA, and 355 nm and 460 nm respectively for Hoechst