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GLUTAMATE-INDUCED EPIGENETIC AND MORPHOLOGICAL CHANGES ALLOW RAT MÜLLER CELL DEDIFFERENTIATION BUT NOT FURTHER ACQUISITION OF A PHOTORECEPTOR PHENOTYPE

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Abstract—Müller cells are not only the main glial cell type in the retina but also latent progenitor/stem cells, which in pathological conditions can transdifferentiate to a neuronal phenotype and regenerate the neurons lost in a mature retina. Several signal transduction pathways can induce the dedifferentiation of mature Müller cells to a progenitor-like state, including that stimulated by glutamate. However, the precise molecular mechanisms by which terminally differentiated cells are initially primed to acquire multipotency remain unclear. In the present study, we have characterized early genetic and epigenetic events that occur immediately after glutamate-induced dedifferentiation of fully differentiated Müller cells is initiated. Using Müller cell-enriched cultures from postnatal rats, we demonstrate that glutamate triggers a rapid dedifferentiation response characterized by changes in cell morphology coupled to the induction of progenitor cell marker gene expression (e.g., nestin, lin28 and sox2) within 1 h. Dedifferentiation involved the activation of *N*-methyl-D-aspartate and type II metabotropic glutamate receptors, as well as global DNA demethylation (evident through the decrease in methyl-CpG-binding protein 2 immunoreactivity) and an increase in gadd45-β gene expression; although, early progenitor gene expression was only partially inhibited by pharmacological impairment of DNA methylation. Importantly, the expression of Müller glia identity genes (*i.e.*, glutamine synthetase; cellular retinaldehyde binding protein, CRALBP) is retained through the process. Dedifferentiated Müller cells held an early neuronal differentiation potential similar to that observed in retinal progenitor-enriched cultures but, contrary to the latter, dedifferentiated Müller cells failed to further differentiate into mature photoreceptor lineages. We speculate that, in spite of the initial triggering of the dedifferentiation pathways, these cells may exhibit a certain degree of epigenetic memory that precludes them from further differentiation. **© 2013 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: Müller glia, dedifferentiation, retinal progenitors, DNA demethylation, neuronal differentiation, epigenetic memory.

INTRODUCTION

Transdifferentiation is considered to be an uncommon but naturally occurring process by which terminally differentiated cells revert to a less differentiated state, from which they can switch lineages and differentiate into other cell types (Jopling et al., 2011). This process involves two steps: de-differentiation, whereby the cell reverts to a less differentiated form and then proliferates; and a differentiation program that results in the generation of new cells. Natural transdifferentiation occurs in the retina of various species in order to regenerate damaged tissue (Del Rio-Tsonis and Tsonis, 2003; Tsonis and Del Rio-Tsonis, 2004). While the sources of the cells involved in retinal regeneration varies between organisms, rodents and primates appear to rely on the capacity of retinal glia (Müller) cells to either dedifferentiate and transdifferentiate into new neurons (Ooto et al., 2004; Lawrence et al., 2007; Karl et al., 2008; Bhatia et al., 2011a) or to induce the acquisition of characteristics of neural stem cell from endogenous retinal progenitors (Simón et al., 2012). Although this suggests that Müller glia represent promising targets for therapeutic regeneration approaches in mammals, other authors have demonstrated that, despite an initial attempt to re-enter the cell cycle after damage in two different models of photoreceptor degeneration, Müller cells fail to overcome an endogenous blockade that impairs proliferation and transdifferentiation in rodents (Joly et al., 2011). The evidence highlights the importance of unraveling the molecular mechanisms that underlie the dedifferentiation and transdifferentiation capacity of these cells (Ahmad et al., 2011; Wohl et al., 2012).

Dedifferentiation implies that the specialized pattern of gene expression and the epigenetic codes of Müller glia are repressed, enabling a progenitor-like phenotype to be acquired (Powell et al., 2012). A limited number of genes and signaling pathways have been implicated in Müller cell dedifferentiation, proliferation and neural

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Abbreviations: cDNA, complementary DNA; DAPI, 4',6'-Diamino-2-Phenylindole; DNMT, DNA methyltransferase; DMEM, Dulbecco's minimal essential medium; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GS, glutamine synthetase; MeCP2, methyl-CpG-binding protein 2; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline; PN, post-natal day; PVDF, polyvinyldene difluoride; RT-PCR, reverse transcriptase polymerase chain reaction.

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differentiation in rodents (Das et al., 2006; Kohno et al., 2006; Wan et al., 2007; Takeda et al., 2008; Wohl et al., 2009, 2012; Abrahan et al., 2009; Besser et al., 2012) or humans (Bhatia et al., 2011b; Lawrence et al., 2007). Moreover, it was recently proposed that the dedifferentiation of Müller cells that precedes the regenerative response to injury in zebrafish was driven by changes in DNA methylation (Powell et al., 2012).

Glutamate is the main neurotransmitter of the vertical signaling pathway in the retina and it has been shown to induce the expression of progenitor cell markers in adult murine Müller glia, both *in vivo* and *in vitro* (Takeda et al., 2008), and to promote the proliferation of Müller-derived progenitor cells (Ramirez and Lamas, 2009). However, the precise molecular mechanisms by which terminally differentiated cells are initially primed to acquire multipotency remain unclear, such as the nature of the receptors or signal transduction pathways involved and the extent to which epigenetic mechanisms underlie the dedifferentiation process. Neuronal-induction cues such as all-trans retinoic acid and taurine have been reported to induce transdifferentiation of fully differentiated NIH/3T3 fibroblasts into photoreceptor gene-marker expressing cells (Wang et al., 2011).

In the present study, we have characterized the early genetic and epigenetic events that occur immediately after glutamate-induced de-differentiation of fully differentiated Müller cells and their capacity to transdifferentiate in response to retinoic acid and taurine. Better understanding these processes may enable us to manipulate Müller cell fate and enhance the pool of therapeutically useful retinal progenitors that can be derived from Müller glia.

EXPERIMENTAL PROCEDURES

Laboratory animals were treated and handled in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and the guidelines of the internal animal care committee (CICUAL-CINVESTAV).

Cell culture

The technique employed for Müller cell primary culture has been described previously (Hicks and Courtois, 1990; Das et al., 2006; Lamas et al., 2007). Briefly, enucleated eyes from 12-day-old Long-Evans rats were placed in Dulbecco's minimal essential medium (DMEM; Gibco BRL, Gaithersburg, MD, USA) with 10% fetal calf serum (FCS) and penicillin–streptomycin (1%), and they were stored overnight in the dark at room temperature. The eyes were then incubated for 30 min at 37 °C in DMEM containing 0.1% trypsin and 70 IU/ml collagenase (Sigma, Saint Louis, MO), after which they were transferred to 10% FCS–DMEM. After dissection of the retina, the cells were dissociated by trituration with a Pasteur pipette and they were seeded in 6-well Petri dishes in OPTIMEM (Gibco BRL, Gaithersburg, MD) containing 4% FCS. The cultures were maintained for 2 weeks or until they reached confluence, and a previous assessment of the purity of such Müller cell cultures demonstrated that over 95% of the cells express Müller cell markers and exhibit the typical asymmetrical morphology (Hicks and Courtois, 1990; Das et al., 2006; Lamas et al., 2007). Nevertheless, the presence of contaminating microglia in the monolayer culture that do not form neurospheres may be in the range of 2–4% (Das et al., 2006). To obtain neurospheres, a confluent Müller cell monolayer from post-natal day (PN) 6 rat retinas was cultured in Optimem/N2/FGF-2/EGF until the formation of neurospheres was observed as previously described (Ramirez et al., 2012). The neurospheres were recovered by sedimentation and dissociated to a single cell suspension with Accutase (Sigma), and re-cultured until formation of secondary or tertiary neurospheres.

Reagents and chemicals

Experimental cell plates were exposed to the following compounds: glutamate (50, 100 and 200 μM), the *N*-methyl-D-aspartate (NMDA) receptor antagonist MK801 (50 μM), curcumin (diferuloylmethane; 100 μM), and GABA (100 μM; all from Sigma Aldrich); and the type II metabotropic glutamate receptor agonist DCG-IV (1 μM; Tocris Bioscience, Ellisville, MO, USA). When cells were pretreated with curcumin or MK801, these compounds were applied to the medium 1 h before glutamate. To enhance photoreceptor marker expression the cells were cultured in DMEM/F12 with 1% FCS, penicillin–streptomycin (1:1000), brain-derived neurotrophic factor (BDNF) (10 ng/ml), supplemented with taurine (50 μmol/L) and retinoic acid (10 μmol/L; all from Sigma Aldrich) for 10 days.

Terminal deoxynucleotidyl transferase dUTP NIck-End labeling (TUNEL) assay

Apoptosis was analyzed after exposing the cells to 200 μM glutamate for 24 h using the In situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics, GmbH, Mannheim, Germany), according to the manufacturer's instructions. As a positive control, the cells were incubated with 200 μ/ml DNase I (Sigma Aldrich) for 10 min at room temperature.

Immunocytochemistry

Cells were cultured on poly-L-lysine-coated slides, fixed for 10 min with 4% paraformaldehyde, washed three times in phosphate-buffered saline (PBS) and blocked in 10% goat normal serum in PBS/0.1% Tween-20. Antibodies against nestin (1:200; Millipore, Billerica, MA, USA, MAB353), βIII-tubulin (1:1000; Millipore AB9354), methyl-CpG-binding protein 2 (MeCP2) (1:50; Abcam, Cambridge, UK, AB50005), glutamine synthetase (GS) (1:100; Abcam, AB16802) and rhodopsin (1:200; Millipore, MAB5356) were diluted in the blocking solution and allowed to bind to the slides overnight at 4 °C. After extensive washing, antibody binding was detected using Alexa 568 anti-mouse antibody and/or Alexa 488 anti-rabbit (1:500; Molecular Probes, Eugene,

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