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Neuroprotection of Retinal Ganglion Cells by Müller Glia *in vitro* in Health and Disease

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A mis padres

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Índice

Resumen 9

Abstract 11

1. Introducción 13

1.1 Retina: Estructura y función 15

1.2 Células ganglionares de la retina (RGCs) 18

1.2.1 Generalidades 18

1.2.2 Marcadores de RGCs 21

1.3 Células de Müller 22

1.3.1 Marcadores de las células de Müller 22

1.3.2 Funciones de las células de Müller 24

1.3.2.1 Mantenimiento de la integridad de la barrera hemato-retiniana. *24*

1.3.2.2 Regulación de la homeostasis de la retina *24*

1.3.2.3 Conducción de la luz *24*

1.3.2.4 Regulación de la actividad sináptica *25*

1.3.2.5 Metabolismo *25*

1.3.2.6 Respuesta inmune. *27*

1.3.2.7 Reparación y regeneración. *27*

1.3.2.8 Neuroprotección *27*

1.3.3 Heterogeneidad de las células de Müller 28

1.4 Interacción entre las Células de Müller y las RGCs en condiciones patológicas 30

1.4.1 Factores neuroprotectores secretados por las células de Müller. 30

1.4.2 Gliosis 32

1.5 Modelos de estudio 35

1.5.1 Animales 35

1.5.2 Cultivos celulares de células de la retina 36

1.6 Posibles aplicaciones de los modelos *in vitro* 38

1.6.1 Estudio de los lípidos de la retina 38

1.6.2 Estudios con células madre. 39

ANEXO 0 43

Glia-Neuron Interactions in the Mammalian Retina. *Prog Retin Eye Res.* 2016 Mar;51:1-40 43

2. Hipótesis y Objetivos / Hypotheses and Objectives 45

3. Material y Métodos 51

3.1 Animales 53

3.2 Extracción del tejido 53

3.3. Cultivos de células de la retina: RGCs, células de Müller y co-cultivos 54
3.4 Cultivos y diferenciación de células madre a RGCs 55
3.4.1 Cultivos de células madre de ratón (mESCs) 55
3.4.2 Diferenciación retinal de las células madre embrionarias de ratón mESCs 55
3.5 Inmunohistoquímica e inmunocitoquímica 56
3.6 Análisis de expresión mediante qPCR 57
3.7. Captura y análisis de imágenes 58
3.8. Análisis estadístico 58
3.9 Otras técnicas 59
3.9.1 Análisis de citometría de flujo de los cultivos de células de Müller 59
3.9.2 Análisis del ciclo células de las células de Müller por citometría de flujo. 60
3.9.3 Preparación de muestras para MALDI-IMS. 60
3.9.4 Ensayos de citoquinas multiplex 60

4. Resultados/Results 61
ANEXO 1 63
A Method to Isolate and Culture Adult Mammalian Müller Glia in order to Study Retinal Diseases. *Frontiers in Cellular Neuroscience*, 2019. (*Submitted*) 63
ANEXO 2 91
Comparative lipidomic analysis of mammalian retinal ganglion cells and Müller glia *in vivo* and *in vitro* using High-Resolution Imaging Mass Spectrometry. *Scientific Reports*. 2019. *(Submitted)* 91
ANEXO 3 113
Effect of Müller cells and their conditioned media on the survival of stem cell derived-RGCs. *(In preparation)* 113
ANEXO 4 127
Neuroprotective effect of Müller cells according to their localization in the retina *in vitro. (In preparation)* 127
ANEXO 5 143
Dexamethasone Protects Retinal Ganglion Cells but not Müller glia against Hyperglycemia *in vitro. PloS One*. 2018. 13(11):e0207913 143

5. Discusión 171

6. Conclusiones/Conclusions 185

7. Referencias 189

Resumen

Las células ganglionares de la retina (RGCs) son las únicas neuronas aferentes de la retina y transmiten la información visual al cerebro a través de sus axones, que forman el nervio óptico y su muerte causa una ceguera irreversible.

Las RGCs se encuentran en estrecho contacto con las células de Müller. Estudios previos del grupo de investigación en el que se ha realizado esta Tesis doctoral, han demostrado que las células de Müller, las células gliales más importantes de la retina, entre sus múltiples funciones, son capaces de neuroproteger a las RGCs **(Anexo 0).** Sin embargo, la heterogeneidad dentro de estas poblaciones de células y su capacidad de respuesta a diferentes estímulos puede cambiar en relación a la capacidad neuroprotectora de las células de Müller y, en consecuencia, influenciar en la supervivencia de las RGCs.

La presente tesis doctoral se basa en el estudio *in vitro* de las células ganglionares de la retina (RGCs) en interacción con las células de Müller en diferentes condiciones. Para estudiar los mecanismos por los cuales las células de Müller pueden contribuir a la neuroprotección de las RGCs y la relación entre estos dos tipos celulares tanto en condiciones fisiológicas como patológicas, hemos establecido cultivos primarios de células de Müller de mamíferos adultos. Debido a la importancia de estas células en la retina, hemos diseñado un protocolo fiable, rápido y sencillo para facilitar su estudio y la caracterización de estas células *in vitro* **(Anexo 1).**

El desarrollo de los cultivos de las células de Müller y los cultivos de RGCs previamente desarrollados por el grupo de investigación, nos ha permitido analizar la composición lipídica tanto de las RGCs como de las células de Müller, comparando por primera vez los resultados obtenidos tanto *in vitro* como *in vivo* por MALDI-IMS. Gracias a este estudio hemos identificado lípidos característicos de uno u otro tipo celular **(Anexo 2).**

Se ha comprobado el efecto de las células de Müller incrementando la supervivencia de las RGCs que proceden de células madre, así como un aumento de progenitores neuronales que se diferencian a RGCs. Los resultados obtenidos pueden ayudar a mejorar el desarrollo de terapias basadas en el uso de células madre para el tratamiento de enfermedades neurodegenerativas en las que se ven afectadas las RGCs **(Anexo 3).**

Otra característica importante de las células de Müller es la existencia de subpoblaciones que conlleva a una heterogeneidad que puede estar relacionada con su capacidad neuroprotectora. En este trabajo se ha analizado la capacidad neuroprotectora de las células de Müller según su localización de la retina, demostrando que la supervivencia de las RGCs que se co-cultivan con las células de Müller que proceden de las zonas más periféricas aumenta en comparación a las que proceden zonas centrales **(Anexo 4).**

Por último, se ha comprobado en un modelo *in vitro* de retinopatía diabética (condiciones hiperglicémicas), que la glucosa tiene un efecto perjudicial en la supervivencia tanto de las RGCs como de las células de Müller. Bajo esta condición las células de Müller aumentan la secreción de citoquinas pro-inflamatorias (IL-1β, IL-6 y TNFα) que provocan la muerte de las RGCs. La dexametasona revierte la acción de la glucosa, disminuyendo la secreción de estas proteínas pro-inflamatorias por parte de las células de Müller promoviendo la supervivencia de las RGCs **(Anexo 5).**

En conclusión, en la presente tesis doctoral se ha estudiado la capacidad neuroprotectora de la glía de Müller en diferentes situaciones tanto fisiológicas como patológicas. Un mayor conocimiento de los mecanismos de actuación de estas células en interacción con las RGCs nos puede ayudar al desarrollo de posibles tratamientos contra enfermedades neurodegenerativas en las que se afectan las células ganglionares de la retina.

Abstract

Retinal ganglion cells (RGCs) are the neurons that transfer visual information to the brain, making the eye the brain's portal to the world. Indeed, the importance of RGCs is clear when we consider that their death causes irreversible blindness. RGCs are in close contact with Müller cells, the main glial cell in the retina, and previous studies by the research group in which this doctoral thesis was carried out showed that Müller glia can offer neuroprotection to RGCs. However, the heterogeneity within these cell populations and their ability to respond to different stimuli may vary, altering the neuroprotective capacity of the Müller glia and, consequently, the survival of the RGCs **(Annex 0)**. To study the mechanisms by which Müller cells can contribute to the neuroprotection of RGCs and the relationship between these two cell types, we have established a method to obtain primary cultures of Müller cells from adult mammals. Due to the importance of these cells in the retina, we designed a reliable, fast and simple protocol to facilitate their study and the characterization of these cells *in vitro* **(Annex 1)**. Using these cultures, we could analyze the lipid composition of both RGCs and Müller glia, for the first time comparing the profiles obtained both *in vitro* and *in vivo* by MALDI-IMS. Thanks to this study, we have identified lipids that are characteristic of RGCs and Müller cells **(Annex 2)**.

In this doctoral thesis, Müller glia were seen to enhance the survival of stem cell derived-RGCs and to increase the neuronal progenitors that differentiate into RGCs. The results obtained may help improve the development of therapies based on the use of stem cells to treat neurodegenerative diseases in which the RGCs are affected **(Annex 3)**. Another important characteristic of Müller cells is the existence of heterogeneous subpopulations that may contribute in different ways to the neuroprotection of RGCs. Here, the neuroprotective capacity of Müller cells was assessed according to their retinal location, demonstrating that the survival of the RGCs is further enhanced when they are co-cultured with Müller cells that reside in the most peripheral areas than with those derived from central areas **(Annex 4)**.

Finally, in an *in vitro* model of diabetic retinopathy (hyperglycemic conditions) we show that glucose has a deleterious effect on the survival of both the RGCs and Müller cells. Under these conditions, Müller cells secrete larger amounts of the pro-

inflammatory cytokines (IL-1β, IL-6 and TNFα) that provoke RGC death. Dexamethasone reverses the action of glucose, dampening the secretion of these pro-inflammatory cytokines by Müller glia and thereby promoting the survival of the RGCs **(Annex 5)**.

In conclusion, the neuroprotective capacity of Müller glia has been studied in physiological and pathological situations. Better understanding the mechanisms of action of these cells and those that influence their interaction with RGCs may help us to develop novel therapies to combat and prevent neurodegenerative diseases in which RGCs are involved.

1. Introducción

1.1 Retina: Estructura y función

La retina es la capa de tejido sensible a la luz que se encuentra en la parte posterior globo ocular (Figura 1). Es considerada una extensión sensorial del sistema nervioso central (SNC) que recibe estimulación directa de las luces e imágenes del mundo exterior. Ésta es una tarea de gran envergadura que requiere una ingente funcionalidad neuronal y tal es así que aproximadamente la mitad de la corteza cerebral humana se dedica al análisis del mundo visual (Bear et al., 1998).

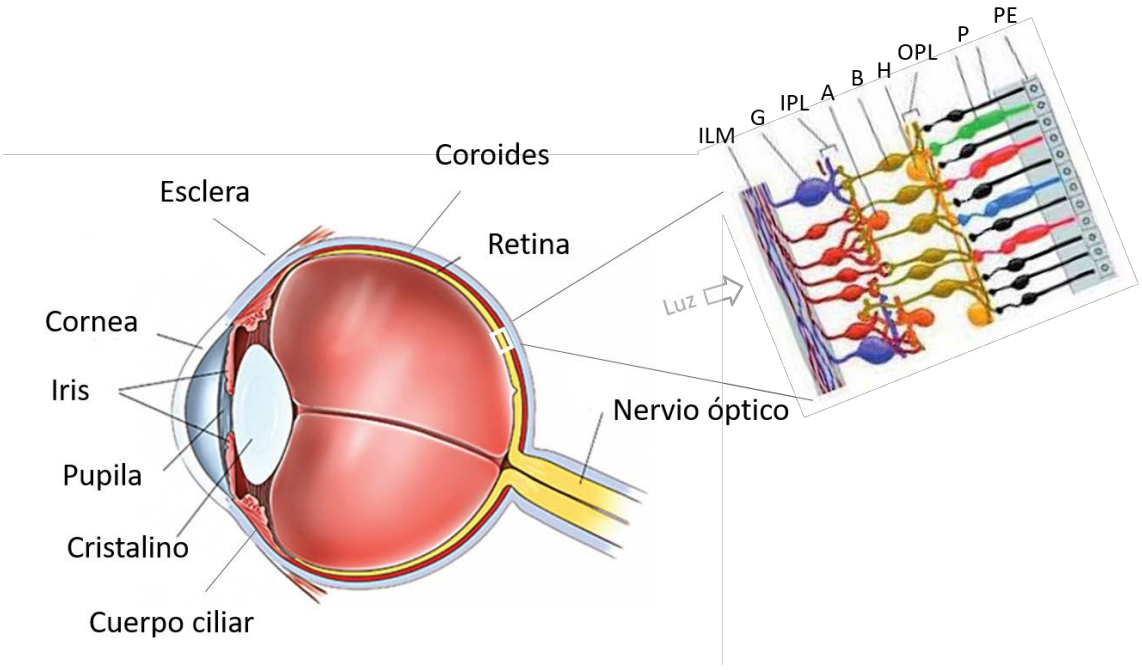

Figura. 1. Dibujo esquemático de los componentes del ojo y esquema aumentado de la retina en el que se observa su disposición en el ojo y las distintas capas de neuronas que la forman. Se representan: membrana limitante interna (ILM), células ganglionares (G), capa plexiforme interna (IPL), células amacrinas (A), células bipolares (B), células horizontales (H), capa plexiforme externa (OPL), fotorreceptores (P) y epitelio pigmentario (PE).

Comprender la organización de la retina de los vertebrados ha sido y sigue siendo el objetivo de muchos científicos a lo largo de los años. La retina se forma durante el desarrollo embrionario a partir de dos vesículas ópticas que nacen directamente del tubo neural (Mann, 1964). Ramon y Cajal en el siglo XIX presentó las primeras descripciones anatómicas completas de los tipos de células neuronales que constituyen la retina en varias especies de vertebrados.

La parte interna de la retina se encuentra en contacto con el vítreo y su porción más externa con la coroides. A su vez, la retina se extiende desde la cabeza del nervio óptico hasta la ora serrata. Las retinas de vertebrados están formadas por capas de cuerpos celulares y entre ellas, capas plexiformes caracterizadas por ser el lugar de las interacciones sinápticas. Todas ellas se encuentran organizadas estratégicamente en el tejido, gracias a lo cual se genera una estratificación de la retina en 10 niveles, los cuales se clasifican en capas nucleares y capas sinápticas.

Desde la parte más externa la más interna las capas de la retina se distribuyen de la siguiente manera: La coroides (1) en contacto con el epitelio pigmentario (2) que a su vez está en contacto con los segmentos externos de los fotorreceptores (3). Más hacia el interior se encuentra la capa nuclear externa (outer nuclear layer, ONL) (4), que es la capa formada por los cuerpos celulares de los fotorreceptores, es decir, de los conos y bastones. La siguiente, es la capa plexiforme externa (outer plexiform layer, OPL) (5) es la parte de la retina en la que contactan los fotorreceptores con las células bipolares y horizontales que son las células que forman la siguiente capa llamada capa nuclear interna (inner nuclear layer, INL) (6), junto con las células amacrinas y además los cuerpos celulares de las células de Müller, astrocitos y algunas células de la microglía. Las células amacrinas y las células bipolares contactan con las células ganglionares de la retina (retinal ganglion cell, RGC) en la capa plexiforme interna (inner plexiform layer, IPL) (7), estas a su vez forman la capa de las células ganglionares (ganglion cell layer, GCL) (8), en la que se encuentran los cuerpos de las RGCs además de astrocitos, algunas células de la microglía y los vasos y arteriolas con las células endoteliales correspondientes. Por último, la capa de fibras nerviosas (nerve fiber layer) (9), compuesta por los axones de las células ganglionares y en la zona más interna de la retina la membrana limitante interna (inner limiting membrane, ILM) (10) formada por astrocitos y las prolongaciones terminales de las células de Müller, que se extienden lateralmente (Polyak, 1941; Rodieck, 1973; Vecino et al., 2016) (Figura 2).

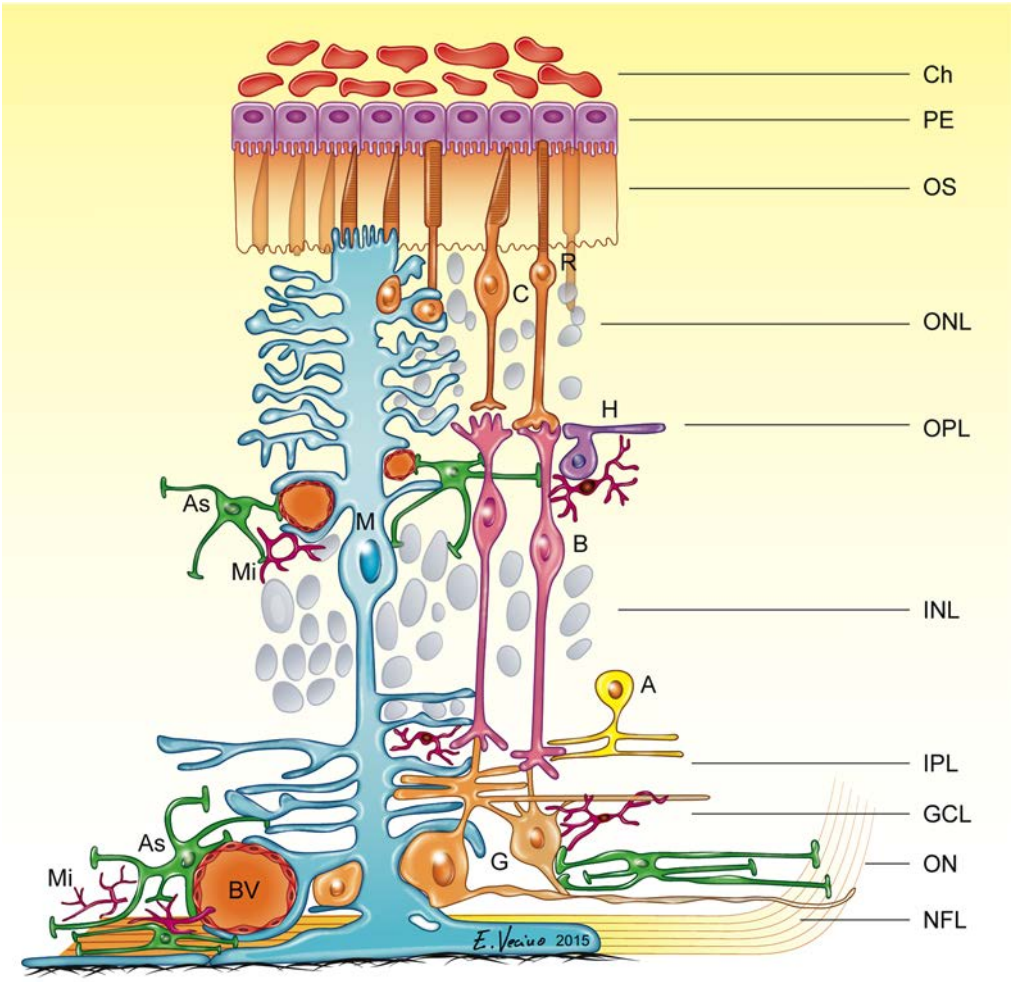

Figura 2. Dibujo esquemático de los componentes celulares de la retina: glía y neuronas. Disposición de los distintos tipos celulares en una retina de mamífero estándar. Nótese la localización de las distintas capas de la retina, desde la parte más externa a la más interna: coroides (Ch), epitelio pigmentario (PE), capa de segmentos externos (OS), capa nuclear externa (ONL), capa plexiforme externa (OPL), capa nuclear interna (INL), capa plexiforme interna (IPL), capa de células ganglionares (GCL), capa de fibras nerviosas (NFL), nervio óptico (ON). Nótese además la interacción entre las distintas células y con los vasos sanguíneos (BV). Células amacrinas (A), astrocitos (As), células bipolares (B), conos (C), células ganglionares (G), células horizontales (H), células de Müller (M), microglía (Mi), bastones (R) (Vecino et al., 2016).

Además, no toda la retina mantiene el mismo grosor en toda su extensión. La retina central cerca de la fóvea es considerablemente más gruesa que la retina periférica. Esto se debe a una mayor densidad celular tanto de los fotorreceptores, en particular los

conos, como de las células bipolares y RGCs en la retina más céntrica en comparación con la retina periférica (Reichenbach, 1987; Reichenbach and Bringmann, 2015).

Es en la retina donde comienza el procesamiento de las señales que darán lugar a la visión. La luz incidente en la retina, alcanza los segmentos externos de los fotorreceptores y asociado a ello, se desencadenan una serie de fenómenos químicos, conocidos como fototransducción, que transforman la energía lumínica en señales eléctricas o impulsos nerviosos. Estas señales se trasmiten a través de las neuronas hasta alcanzar finalmente los axones de las RGCs, los cuales se encargan de conducir la información hacia el cerebro, donde finaliza el procesamiento visual (Bear et al., 1998) a través de sus axones, que forman el nervio óptico (Nelson and Connaughton, 1995). Aunque la visión parece ser responsabilidad exclusiva de las neuronas (elementos excitables) de la vía visual, es necesaria la presencia de otra clase de células no neuronales (elementos no excitables) en este proceso, las células gliales o neuroglia como son las células de Müller, los astrocitos y la microglía.

1.2 Células ganglionares de la retina (RGCs)

1.2.1 Generalidades

Las células ganglionares son las neuronas responsables de transportar los mensajes eléctricos relativos a la señal visual desde la retina al cerebro mediante el nervio óptico que forman sus axones. Las células ganglionares son más grandes en promedio que el resto de las neuronas que componen la retina, tienen axones de gran diámetro capaces de pasar la señal eléctrica en forma de picos transitorios a las áreas receptoras del cerebro que están a centímetros de distancia de la retina. El nervio óptico recoge todos los axones de las células ganglionares en un haz de más de un millón de fibras (en humanos) que pasa la información a las zonas cerebrales que clasifican, integran y procesan la información (Kolb, 1995) principalmente en el núcleo geniculado lateral y en el colículo superior (Wassle and Boycott, 1991). Todas las RGCs comparten varias características: sus somas están ubicados en la capa de células ganglionares (GCL) (salvo raras excepciones), sus dendritas arborizan en la capa plexiforme interna, sus axones viajan a través del nervio óptico hasta el cerebro, reciben sinapsis en sus

dendritas, conducen potenciales de acción y liberan glutamato de sus terminales (Sanes and Masland, 2015).

Ramón y Cajal, clasificó por primera vez diferentes subtipos de células ganglionares según la forma (morfología dendrítica), la extensión (cuerpo celular y el tamaño del árbol dendrítico) y la cantidad de subcapas en las que se arborizan (niveles de estratificación en la capa plexiforme interna). Unos años más tarde, en la década de 1940, Polyak (Polyak, 1941) fue uno de los pioneros en la aplicación del método de Golgi a retinas de monos y chimpancés, que todavía sigue siendo muy útil en el estudio de retinas humanas, de gato, y mono. Desde entonces y gracias métodos más actuales como la microscopia electrónica, junto con las técnicas de inmunocitoquímica y las técnicas de registro electrofisiológico, actualmente, son varias las clasificaciones que se han hecho de las RGCs en diferentes tipos de animales. La subdivisión más utilizada surge dependiendo del lugar de la proyección de sus axones, por ejemplo, si transmiten señales a la capa magnocelular del núcleo geniculado lateral serían RGCs tipo M, y si, por el contrario, proyectan a la capa parvocelular serían tipo P (Polyak, 1941; Veiga-Crespo et al., 2013). A su vez, se pueden clasificar basándose en el tamaño del cuerpo celular, la morfología y la ramificación dendrítica o los niveles de estratificación en la capa plexiforme interna (Peichl and Wassle, 1983; Sanes and Masland, 2015; Veiga-Crespo et al., 2013). Una pequeña población de células (ipRGCs) son fotosensibles y expresan melanopsina. Se sabe que son cruciales para transmitir información de la luz desde la retina al cerebro y participan en el control del ritmo circadiano, el reflejo de luz pupilar y el sueño (Hattar et al., 2002; Masland, 2001; Schmidt et al., 2011). Además, en diferentes especies hay diferentes clasificaciones de RGCs debido a las diferentes morfologías encontradas, por ejemplo, en ratón, uno de los mamíferos más estudiados, se han encontrado 22 tipos diferentes de RGCs, y en cerdo las RGCs se pueden clasificar hasta 9 tipos (Roska and Meister, 2014; Veiga-Crespo et al., 2013; Volgyi et al., 2009).

Los axones de las RGCs no están mielinizados desde la retina a la lámina cribrosa, pero se mielinizan posteriormente. En los axones no mielinizados, los potenciales de acción se propagan por despolarización a lo largo de la membrana. Este proceso consume más energía que la conducción saltatoria característica de los axones mielinizados (Wang et al., 2003). Por lo tanto, como un proceso de adaptación al aumento de la necesidad de energía, los axones de las RGCs se caracterizan por estar llenos de mitocondrias (Wang

et al., 2003). Las RGCs tienen axones muy largos, lo que aumenta la vulnerabilidad de las células a diversos trastornos por ejemplo un estrés metabólico como la hipoxia, exposición a los radicales libres y la compresión mecánica (por ejemplo, en la lámina cribrosa). Estos daños inducen la muerte de las RGCs (Chidlow and Osborne, 2003; Schmidt et al., 2008). Para lidiar con estos factores estresantes, las RGCs tienen una alta capacidad antioxidante (atribuible a defensas antioxidantes endógenas, incluida la expresión de enzimas como la catalasa, la superóxido dismutasa, glutatión peroxidasa y peroxiredoxinas) en comparación con otras neuronas (Fatma et al., 2008; Kortuem et al., 2000). Sin embargo, estas células siguen siendo muy vulnerables frente a estímulos nocivos (Yu et al., 2013), lo que hace que el estudio de su neurodegeneración sea complejo cuando, además, cuando se diagnostica el trastorno, el daño ya ha comenzado.

Por todo ello, es de suma importancia el uso de modelos animales como por ejemplo, modelos animales de glaucoma, una neuropatía óptica caracterizada por la pérdida progresiva de las RGCs con la consecuente pérdida de visión gradual e irreversible. Estos modelos ayudan a estudiar el inicio y la progresión de la enfermedad, y además, facilitan el desarrollo de tratamientos (Urcola et al., 2006; Vecino, 2008; Vecino and Sharma, 2011). También han sido desarrollados modelos de isquemia retiniana, de retinopatía diabética o de degeneración macular asociada a la edad (Grigsby et al., 2014; Pinar-Sueiro et al., 2013; Tuo et al., 2007). En estos estudios se ha demostrado que existe una pérdida selectiva de RGCs. Esta heterogeneidad se manifestó en estudios en los que indicaron que las células ganglionares grandes (células ganglionares magnocelulares) y las fibras nerviosas se perdían selectivamente en modelos de glaucoma experimental en cerdos (Ruiz-Ederra et al., 2005) así como en primates no humanos y en pacientes humanos con glaucoma (Quigley et al., 1988). En apoyo a estas observaciones, otro estudio también encontró una pérdida selectiva del transporte axonal anterógrado a la capa magnocelular del núcleo dorsal geniculado lateral, que es la región que contiene los RGC más grandes (Dandona et al., 1991). Estos hallazgos sugieren que no todas las RGCs son igual de susceptibles al daño bajo las mismas condiciones patológicas.

También se han desarrollado en los últimos años modelos *in vitro* (figura 3). En estos estudios, ha sido demostrado que tras axotomía, las RGCs tienen una capacidad de regeneración limitada *in vitro* y que no todas ellas mueren a la vez, ya que, debido a su

heterogeneidad, hay células más sensibles que otras (Luo et al., 2001; Mey and Thanos, 1993).

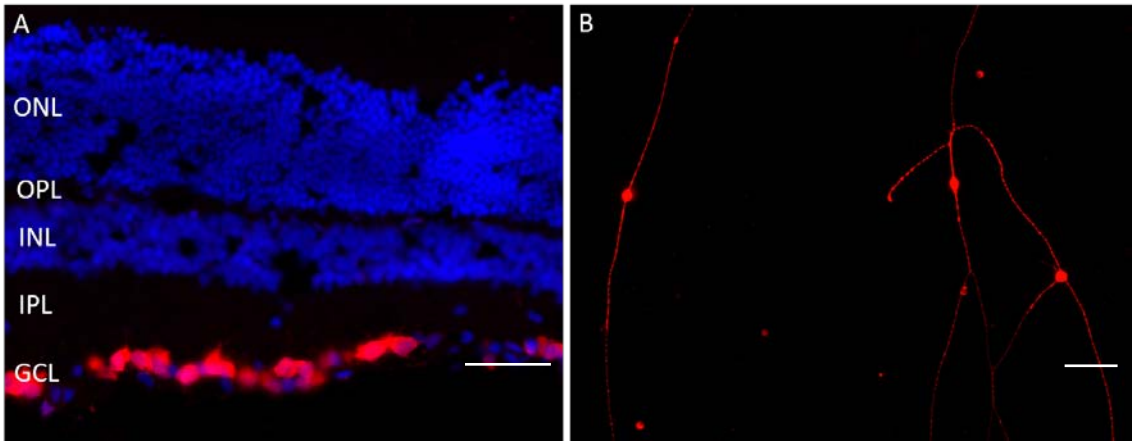

Figura 3. Células Ganglionares de la Retina (RGCs) *in vivo* e *in vitro.* (A) RGCs en un corte de retina de rata marcadas con un anticuerpo frente a RBPMS en rojo. (B) Cultivo primario de RGCs de rata. Las RGCs han sido marcadas con anticuerpo frente a βIII-Tubulina en rojo, y los núcleos celulares han sido teñidos con DAPI en azul. Barra de escala = 50 μm

1.2.2 Marcadores de RGCs

Para realizar estudios de cuantificación tanto del daño como de la posible protección de estas células, es necesario visualizar las RGCs con marcadores específicos. Para cultivos celulares de RGCs, anticuerpos frente a β-III-tubulina son una buena forma de marcar los microtúbulos de las RGCs para así poder visualizarlas, contabilizarlas y clasificarlas (Ruzafa and Vecino, 2015; Vecino et al., 2016) asi como el uso de neurofilamentos (Garcia et al., 2002). Otro marcador muy utilizado, es la proteína de superficie celular Thy1 (Barnstable and Drager, 1984). Los factores de transcripción Brn3 se expresan exclusivamente en las células ganglionares, e intervienen en la diferenciación neuronal y la supervivencia. Antes del uso Brn3, para cuantificar RGCs in vivo, era común el uso de Fluorogold, un marcador retrógrado que marca aproximadamente un 98% del total de RGCs, bien marcando retrógradamente desde el colículo superior en rata (Vecino et al., 2002) o bien desde el nervio óptico en cerdo (Garca et al., 2005).

El marcaje con anticuerpos contra Brn3a permite teñir el núcleo de un 92.2% de RGCs marcadas con Fluorogold que es el considerado el gold estándar por marcar un 100% (Nadal-Nicolas et al., 2009). El marcaje con anticuerpos frente a Brn3a, además de ser más fáciles de realizar, es importante ya que al ser nuclear permite el recuento de las células de forma automática al no solaparse el marcaje. Por otro lado, RBPMS es otro marcador específico para RGCs que fue descubierto recientemente. Esta proteína de unión a RNA con múltiples sitios de splicing (RBPMS) y con función aún no bien conocida, permite que anticuerpos contra ella marquen el citoplasma de RGCs en múltiples especies de mamíferos, esto dificulta en ocasiones el recuento automático de las células por el solapamiento del marcaje (Rodriguez et al., 2014).

1.3 Células de Müller

En 1851 el anatomista Heinrich Müller describió un nuevo tipo de célula en la retina, que describió como delgadas fibras que se extienden verticalmente a lo largo de la retina de los vertebrados (Müller, 1951) (H., M. (1851). Las células de Müller, que recibieron el nombre de su descubridor, es el tipo celular glial más importante de la retina. Destacan por su morfología radial única, que abarca todo el grosor de la retina y se extiende desde la membrana limitante interna (ILM) a la capa nuclear externa (ONL), lo que permite interacciones con todas las neuronas de la retina. Además, la glía de Müller se sitúa próxima al vítreo, los vasos sanguíneos y el espacio subretiniano y, por lo tanto, representan una conexión anatómica y funcional entre estos compartimentos y las neuronas de la retina. Las células de Müller interactúan con las neuronas de manera simbiótica y son responsables de su soporte funcional, metabólico y estructural. Por lo tanto, su particular localización en la retina le permiten realizar una variedad de funciones para mantener la homeostasis retiniana e iniciar una respuesta protectora en caso de un daño en la retina (Bringmann et al., 2006; Reichenbach and Bringmann, 2013).

1.3.1 Marcadores de las células de Müller

A pesar de que originariamente estas células fueron descritas a partir de tinciones argénticas que marcaban solo un porcentaje de las células (Müller, 1951), para llevar a

cabo estudios con estas células es necesario identificar las células de Müller por medio de marcadores inmunohistoquímicos (figura 4). Hay varias proteínas que están restringidas en su expresión a las células de Müller en la retina. Esto incluye proteínas estructurales que forman parte del citoesqueleto como son la vimentina, la proteína 4 asociada a los microtúbulos y la proteína ácida fibrilar glial (GFAP) (aunque esta es expresada también por los astrocitos). Otros tipos de marcadores de las células de Müller son las proteínas con funciones metabólicas específicas de la este tipo glial como son la proteína de unión al retinaldehído celular (CRALBP), el transportador de glutamato-aspartato (GLAST), la glutamina sintetasa (GS), canales de potasio del subtipo Kir4.1, acuaporina-4, piruvato carboxilasa, α-cristalina, la proteína de unión a GTP RhoB, la glutamato carboxipeptidasa II, anhidrasa carbónica C, y aunque no exclusiva de las células de Müller, el receptor de baja afinidad del NGF y el p75NTR, (Berger et al., 1999; Bunt-Milam and Saari, 1983; Garcia and Vecino, 2003; Linser and Moscona, 1981; Moscona et al., 1985; Parysek et al., 1985; Vecino et al., 2016).

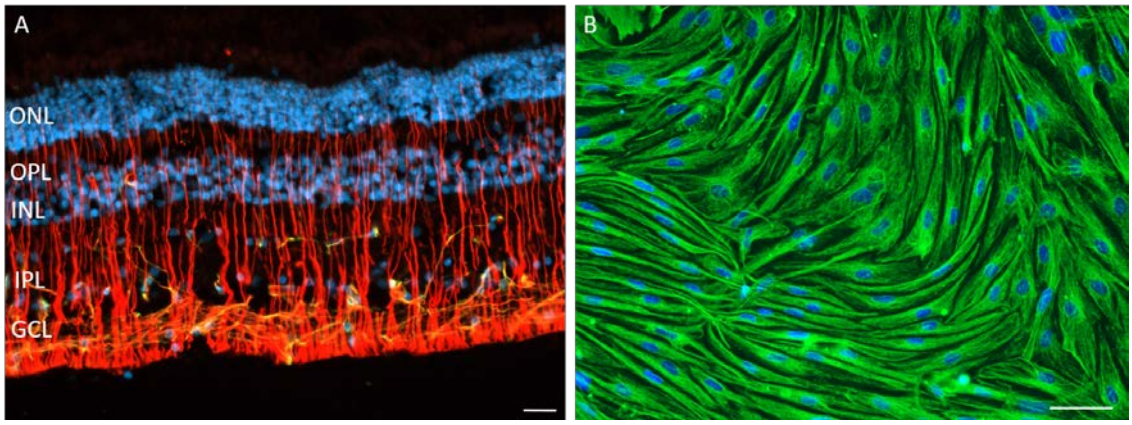

Figura 4. Células de Müller *in vivo* e *in vitro*. (A) Células de Müller en un corte de retina de cerdo marcadas con un anticuerpo frente a GFAP en verde y vimentina en tojo. (B) Cultivo primario de células de Müller de cerdo, las células han sido marcadas con un anticuerpo frente a vimentina en verde, y los núcleos han sido teñidos con DAPI en azul. Capa nuclear externa (ONL), capa plexiforme externa (OPL), capa nuclear interna (INL), capa plexiforme interna (IPL), y capa de células ganglionares (GCL). Barra de escala = 50 μm

1.3.2 Funciones de las células de Müller

Las células de Müller, llevan a cabo una gran variedad de funciones y casi todas son fundamentales y ayudan al soporte metabólico y estructural de las neuronas de la retina. A continuación, se describen algunas de las funciones más importantes de estas células.

1.3.2.1 Mantenimiento de la integridad de la barrera hemato-retiniana.

Las células de Müller participan en el establecimiento de la barrera hemato-retiniana, cuya integridad es esencial para el buen funcionamiento de la retina. La barrera hemato-retiniana ayuda restringir el movimiento de fluidos y moléculas entre la sangre y la retina e impiden la entrada de patógenos y otros agentes potencialmente dañinos en el tejido retiniano (Shen et al., 2012; Tout et al., 1993).

1.3.2.2 Regulación de la homeostasis de la retina

Además, las células de Müller están enriquecidas con numerosos canales iónicos para regular el equilibrio electrolítico. Durante la actividad neuronal, las neuronas liberan iones de potasio (K +) en los espacios sinápticos, que pueden ser captados por las células de Müller, que a su vez redistribuyen el exceso de K + en los espacios llenos de líquido fuera de la retina (es decir, la sangre, el humor vítreo y el espacio subretiniano), amortiguando así el desequilibrio de K + (Newman and Reichenbach, 1996; Newman, 1993; Reichenbach et al., 1992). Además de la acumulación de iones, las células de Müller también contribuyen a la homeostasis de la retina mediante de los canales de agua especializados acuaporina-4 (AQP4) situados en su membrana celular (Bringmann et al., 2005; Nagelhus et al., 1998).

1.3.2.3 Conducción de la luz

Las células de Müller son directamente responsables de la conducción de la luz en la retina. Dado que los fotorreceptores se encuentran en la capa más externa, la luz debe atravesar todas las capas de la retina antes de alcanzar su objetivo. Debido a este fenómeno, podría esperarse una pérdida masiva de intensidad de luz debido a la dispersión de la luz por las múltiples capas de células de la retina. Sin embargo, Franze et al. descubrió que la luz incidente es recogida por las células de Müller, que actúan

como fibras ópticas vivas que guían la luz a través del tejido retiniano hacia los fotorreceptores (Franze et al., 2007).

1.3.2.4 Regulación de la actividad sináptica

La estrecha conexión de las células de Müller con las neuronas que componen la retina, permite que las células de Müller participen en la actividad sináptica. Durante la neurotransmisión, las células de Müller son responsables de la eliminación rápida del glutamato en los espacios sinápticos, protegiendo así las neuronas contra la excitotoxicidad (Brew and Attwell, 1987; Bringmann et al., 2013). El glutamato en las células de Müller también se usa para la producción de glutation, un antioxidante crucial que protege la retina contra el estrés oxidativo. Cuando se produce estrés oxidativo, las células de Müller liberan rápidamente glutatión, una molécula que previene el daño neuronal al neutralizar las especies reactivas de oxígeno (ROS) (Huster et al., 2000). Además, estas células participan en la remodelación de los circuitos neuronales que promueven la formación de sinapsis y ayudan a mantener la función neuronal, proporcionando precursores de neurotransmisores (Pfrieger and Barres, 1996).

1.3.2.5 Metabolismo

Las células de Müller también tienen un papel crucial en el metabolismo de la retina, son el sitio principal de almacenamiento de glucógeno en la retina y, en momentos de necesidad, acceden a este almacenamiento para proporcionar glucosa a las neuronas. Las células de Müller se basan principalmente en la glucólisis anaeróbica, incluso cuando hay suficiente oxígeno presente. Esta función metabólica les permite ahorrar oxígeno para las neuronas de la retina y las hace menos susceptibles a la anoxia (Winkler et al., 2000). Además, a través de la degradación anaeróbica de su propia glucosa, las células de Müller producen grandes cantidades de lactato, que es captado por los fotorreceptores como fuente de energía alternativa (Poitry et al., 2000; Poitry-Yamate et al., 1995).

La relación metabólica descrita anteriormente, puede verse resumida en la Figura 5 (Vecino et al., 2016).

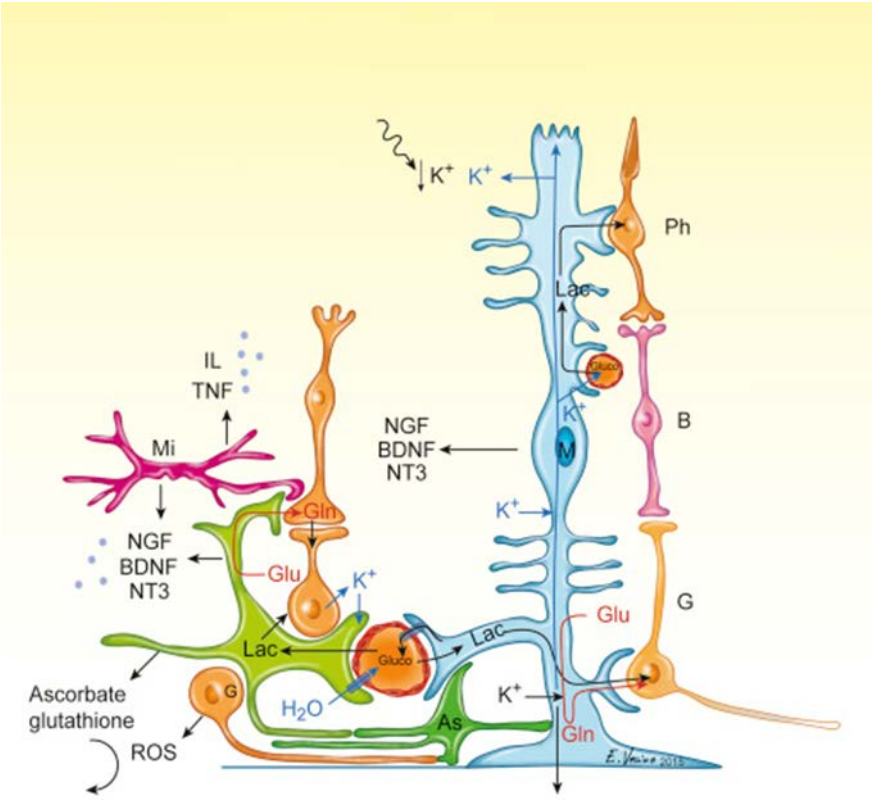

Figura 5. Esquema que muestra las principales interacciones de las células gliales con las neuronas en el metabolismo de la glucosa, la homeostasis de K^+, el H_2O, el glutamato (Glu) y el metabolismo de glutamina (Gln), la secreción de factores tróficos e interleucinas. As (astrocitos), B (células bipolares), G (células ganglionares), M (Células de Müller), Mi (microglia), Ph (fotorreceptores) (Vecino et al., 2016).

Además, las células de Müller también participan activamente en el metabolismo lipídico, sintetizando y transportando lípidos para satisfacer la necesidad de las neuronas (Mauch et al., 2001). Los lípidos son constituyentes fundamentales del sistema nervioso central (SNC) y el metabolismo de los lípidos defectuoso está relacionado con una serie de enfermedades del cerebro y del sistema nervioso periférico (SNP) (Trim et al., 2008). Hay muchos lípidos específicos de la retina que desempeñan un papel fundamental en la función y la enfermedad de la retina. Por ejemplo, el colesterol, el segundo lípido más abundante en la neuroretina después de los fosfolípidos (Bretillon et al., 2008), juega un papel clave en la modulación de los canales iónicos, el ciclo vesicular y el desarrollo de las dendritas de las neuronas de la retina (Dietschy and Turley, 2004; Gambert et al., 2017; Marquer et al., 2011), y la glía de Müller representa

la fuente principal para su producción y transporte (Fliesler and Bretillon, 2010; Jo et al., 2015). Además, se sabe que existe un traspaso de lípidos de las células de Müller a las neuronas para cubrir sus necesidades especialmente en relación con el mantenimiento y renovación de los axones de las RGCs y el buen funcionamiento de la sinapsis (Mauch et al., 2001; Reichenbach and Bringmann, 2010).

1.3.2.6 Respuesta inmune.

Mientras que las células microgliales son las principales responsables de la respuesta inmune en la retina, las células de Müller también colaboran en esta actividad. Estudios recientes han demostrado que las células de Müller participan activamente en la inmunidad innata de la retina, específicamente como respuesta a infecciones y también al inhibir la proliferación de las células T a través de moléculas unidas a la membrana. (Kumar et al., 2013).

1.3.2.7 Reparación y regeneración.

Aunque la glía de Müller de los mamíferos puede responder a una lesión, proliferar y expresar genes asociados con las células madre de la retina (Roesch et al., 2008), no pueden funcionan como progenitores de la retina *in vivo*. No obstante, estas características sugieren que, en las circunstancias adecuadas, la glía de Müller podría actuar como progenitores retinales que pueden usarse para la reparación. El potencial regenerativo de las células de Müller las convierte en candidatos ideales para terapias de reemplazo celulares para tratar enfermedades degenerativas de la retina, como la degeneración macular relacionada con la edad, la retinitis pigmentosa o el glaucoma, donde la pérdida de la visión se debe a la pérdida de las neuronas de la retina. Una estrategia regenerativa que movilice a los propios “progenitores” Müller de los pacientes para participar en la reparación de la retina tendría varias ventajas sobre las terapias de trasplante celular (Ruilin et al., 2014). *In vitro*, se ha demostrado que las células de Müller son capaces de generar neuronas y glía (Das et al., 2006).

1.3.2.8 Neuroprotección

Una de las funciones más importantes y en las que se basa la mayor parte de la presente tesis doctoral, es la capacidad de neuroprotección de las células de Müller. Las células de Müller están dotadas de la capacidad de sintetizar y segregar una gran cantidad de factores neurotróficos y, por lo tanto, representan un objetivo natural para la expresión

de estas proteínas. Los factores neurotróficos son una familia de factores de crecimiento que promueven el crecimiento, la supervivencia y la diferenciación de las neuronas. De hecho, la administración de factores neurotróficos podría detener o ralentizar la degeneración neuronal y ayudar a mantener función visual. Estudios previos del grupo de investigación demostraron la capacidad neuroprotectora que ejerce las células de Müller sobre los fotorrecptores (Del Rio et al., 2011)

La mayoría de los factores neurotróficos pertenecen a una de las tres familias clásicas: (i) neurotrofinas, incluidas los factores de crecimiento nervioso (NGF), el factor de crecimiento derivado del cerebro (BDNF), neurotrofina 3 (NT-3) y neurotrofina 4 (NT4). (2) ligandos de la familia GDNF, que incluyen GDNF, neurturina, artemina y persefina; y (3) familia de citoquinas, incluido el factor neurotrófico ciliar (CNTF) y el factor inhibidor de la leucemia (LIF). Otras proteínas que se han identificado como factores neurotróficos incluyen bFGF, IGF-1, VEGF, factor de crecimiento transformante β (TGF-β) y PEDF (Dawbarn and Allen, 2003; Kolomeyer and Zarbin, 2014; Pardue and Allen, 2018).

Debido a la gran repercusión de los estudios de los últimos años relacionados con esta función de las células de Müller y su posible uso terapéutico, los hitos más importantes relacionados con su interacción con las RGCs se explicarán con profundidad en el punto 1.4.1.

1.3.3 Heterogeneidad de las células de Müller

Debido a la gran cantidad de funciones en las que están involucradas las células de Müller es lógico pensar que puedan existir subpoblaciones más especializadas en unas funciones u otras. Poco se sabe sobre la heterogeneidad de las células de Müller pero en los últimos años, hay estudios que empiezan a evidenciar que no todas las células de Müller son iguales o tienen las mismas capacidades. Por ejemplo, solo el 30% de las células de Müller *in vitro* expresan el antígeno MHC de clase II, lo que sugiere que podrían estar involucradas en las reacciones inmunitarias de la retina (Roberge et al., 1985). En otro estudio, se analizó el transcriptoma de células de Müller individualizadas y se observó que ciertos genes se expresaban de manera heterogénea, incluidos los genes de mantenimiento comunes como la Beta-actina, la 2-microglobulina, la proteína de choque térmico 1b y la proteína de unión a la caja TATA (Roesch et al., 2008). Está

claro que existen diferencias moleculares en las células de Müller porque el homeodominio que contiene el factor de transcripción Chx10 se expresa solo en una subpoblación de ellas (Rowan and Cepko, 2004).

Las células aisladas *in vitro* pueden responder a ciertas condiciones cambiando rápidamente su expresión de proteínas y transformándose en un fenotipo proliferativo (Hauck et al., 2003). Mientras que marcadores como vimentina permanece estable en estas células, la expresión de GFAP, que probablemente se induce durante su aislamiento de la retina intacta, varía *in vitro* (Fischer et al., 2004). Otra evidencia de diversidad fenotípica en las células de Müller incluye la variación en la expresión de nestina en las células de Müller después de desprendimiento de retina en ratas adultas (Luna et al., 2010). Y además después de una lesión, se ha visto que poblaciones individuales de células de Müller pueden tener diferentes respuestas regenerativas (Roesch et al., 2012).

La retina de los anfibios y los peces es capaz de regenerarse completamente después de un daño, esto se debe gracias a la existencia de un remanente de la retina embrionaria, formando una zona germinal circunferencial que agrega continuamente nuevas neuronas a la retina (Hitchcock et al., 2004; Otteson and Hitchcock, 2003). En la retina de pollo en cambio, la capacidad de regeneración es mas limitada. En estos animales existe una región en la que se encuentran las células de Müller que tienen la capacidad de proliferar en respuesta a una lesión y esta, se limita a la periferia de la retina (Fischer and Reh, 2003). Además, aunque los mamíferos han perdido la capacidad de las células de Müller para proliferar y regenerar células *in vivo* como parte del sistema nervioso central, se ha demostrado que las células de Müller periféricas expresan proteínas características de células madre como CD44 (Too et al., 2017) y la proteína nestina después de un daño (Suga et al., 2014). Esta heterogeneidad entre las células de Müller de la periferia respecto a las el centro de la retina, pueden implicar una diferente capacidad de respuesta frente a un daño y en consecuencia tener diferentes capacidades neuroprotectoras. Por este motivo se estudiará la capacidad neuroprotectora de las células de Müller según su localización en la retina, comparando la supervivencia de las RGCs en co-cultivo con las células de Müller que proceden de la periferia de la retina con las que provienen del centro y de la banda visual.

1.4 Interacción entre las Células de Müller y las RGCs en condiciones patológicas

El estudio de la interacción de las células de Müller con las células ganglionares proporciona información de gran interés para el estudio de enfermedades neurodegenerativas en las que están implicadas las RGCs. Debido a la propiedad intrínseca de las células de Müller de neuroproteger y a su importante función en muchas enfermedades, el interés del papel de las células de Müller en relación con las RGCs bajo diferentes condiciones patológicas ha crecido exponencialmente en los últimos años.

1.4.1 Factores neuroprotectores secretados por las células de Müller.

Las neurotrofinas y los factores de crecimiento están involucrados en la supervivencia, diferenciación y desarrollo de las neuronas. Se ha estudiado que diferentes combinaciones de neurotrofinas en el ojo aumentan la supervivencia de RGC en diferentes situaciones experimentales. Las células de Müller son importantes para mantener la supervivencia de las neuronas y aunque los factores implicados en el desarrollo y la supervivencia en sí están menos definidos, se conoce que las células gliales, como las células de Müller, los astrocitos y la microglía sintetizan factores neurotróficos y pueden mediar directa o indirectamente acciones neurotróficas (Garcia and Vecino, 2003; Ruiz-Ederra et al., 2003; Vecino et al., 1998a; Vecino et al., 1998b).

De todos los factores neurotróficos, el CNTF es el más estudiado para la neuroprotección terapéutica de la retina. Una extensa investigación ha demostrado que el factor CNTF favorece la supervivencia de los fotorreceptores en casi todos los modelos animales de degeneración de la retina (Wen et al., 2012). Además, se ha demostrado que CNTF protege a las RGCs en varios modelos de diferentes enfermedades (Azadi et al., 2007; Cao et al., 1997; Fischer et al., 2004), y también promueve la regeneración axonal (Muller et al., 2009). A pesar de estos resultados, existen reticencias en el uso de CNTF como agente neuroprotector. Esta ambigüedad es causada porque dependiendo de la dosis se ha comprobado que puede resultar perjudicial para la correcta función de la retina (Zeiss et al., 2006).

Otro importante factor es el BDNF que destaca por su potente efecto neuroprotector, especialmente para las RGCs (Chen and Weber, 2001). El suministro exógeno de la proteína BDNF, así como aumentando su expresión, promueve la supervivencia de las RGCs después de la axotomía del nervio óptico (Mo et al., 2002; Sanchez-Migallon et al., 2016). Sin embargo, en la mayoría de los estudios, la neuroprotección de las RGCs fue solo a corto plazo (Guo et al., 2018). Este efecto de supervivencia transitoria de BDNF se atribuyó a una disminución de la expresión del receptor de BDNF (TrkB) en la superficie de las RGCs, posiblemente desencadenado por la exposición prolongada a BDNF o la propia axotomía. En respuesta a esta observación, se ha probado con éxito un enfoque combinado de la administración de proteínas BDNF y la terapia génica para aumentar la expresión de TrkB, aumentando considerablemente la supervivencia de las RGCs (Di Polo et al., 1998). Otros factores de interés incluyen PEDF y GDNF. Se ha informado que el aumento de la expresión de GDNF protege la retina del estrés oxidativo sin alterar la función retiniana normal (Koeberle and Ball, 1998) y también contribuye a la protección tanto de fotorreceptores como de RGCs en modelos animales de degeneración de la retina (Del Rio et al., 2011; Yan et al., 1999). Inicialmente el factor neurotrófico PEDF se atribuyó exclusivamente a las células del epitelio pigmentario de la retina (RPE) y se demostró que tenía efectos antiapoptóticos, antioxidantes y antiinflamatorios (Becerra et al., 2012; He et al., 2015). Más tarde se comprobó que también era secretado por las células de Müller y que además contribuía a la supervivencia de las RGCs después de un daño hipóxico/isquémico (Unterlauft et al., 2014).

Estudios previos del grupo de investigación en el que estoy integrada, han demostrado la capacidad de neuroprotección y regeneración de las células de Müller tanto en contacto con las RGCs como con del medio condicionado que secretan las células de Müller en cultivo (Garcia et al., 2002; Ruzafa et al., 2018). Además, un reciente estudio proteómico identificó nuevas proteínas implicadas en la neuroprotección de las RGCs como son la proteína SPP1 y la proteína basigina aunque ninguna es tan eficaz como el uso del medio condicionado directamente secretado por las células de Müller (Ruzafa et al., 2018). Elucidar que factor o factores son determinantes para la supervivencia de las RGCs después de un daño sigue siendo de gran interés tanto científico como clínico, siendo necesarias más investigaciones que estudien tanto las células de Müller y su medio secretado en interacción con las RGCs.

1.4.2 Gliosis

En el sistema nervioso central, la reactivación de los astrocitos (astrogliosis) se produce en respuesta a todas las formas de lesiones y enfermedades del sistema nervioso y tiene efectos protectores y perjudiciales (Sofroniew, 2005). Se cree que la gliosis representa un intento celular de proteger y preservar el tejido para evitar un daño mayor (Liberto et al., 2004). En la retina, las células de Müller son las células gliales principales y como se ha explicado anteriormente, desempeñan una gran cantidad de funciones cruciales para la correcta función neuronal (Bringmann et al., 2006). En respuesta a prácticamente todas las alteraciones patológicas de la retina, las células de Müller también se reactivan (Bringmann et al., 2006; Bringmann and Reichenbach, 2001). La gliosis reactiva consiste en cambios morfológicos, bioquímicos y fisiológicos de las células de Müller y estas alteraciones varían con el tipo y la severidad del daño, y aunque en un principio las alteraciones glióticas promueven la protección neuronal hay que tener en cuenta que las células de Müller también pueden contribuir a la degeneración neuronal, al desarrollo del edema en la retina enferma y a la formación de cicatrices gliales que impiden la reparación del tejido (Bringmann et al., 2006; Bringmann et al., 2004).

La gliosis de las células de Müller tiene efectos tanto citoprotectores como citotóxicos en las neuronas de la retina (Bringmann et al., 2006; Bringmann and Reichenbach, 2001). Después de la lesión, la gliosis es neuroprotectora, y se piensa que es un intento celular para limitar la extensión del daño tisular. Las respuestas protectoras de las células de Müller implican muchos mecanismos diferentes, incluido el amortiguamiento de los niveles elevados de potasio (Bringmann et al., 2006) y la regulación al alza de la proteína glutamina sintetasa, posiblemente como un mecanismo neuroprotector para limitar la neurotoxicidad del glutamato (Gorovits et al., 1997). Esto, aunque no es una interacción directa entre la glía y los RGC, representa un efecto importante de la glía sobre la supervivencia de RGC y la homeostasis retiniana (Bringmann et al., 2009b; Lucas and Newhouse, 1957). También la liberación de antioxidantes (Bringmann et al., 2006; Bringmann and Reichenbach, 2001; Garcia and Vecino, 2003) y la producción de factores neurotróficos, factores de crecimiento, citoquinas y eritropoyetina protegen a los fotorreceptores y neuronas muerte celular (Cao et al., 1997; Harada et al., 2000; Wen et al., 1995). Uno de los factores más relevantes liberados de las células de Müller

en condiciones hipóxicas es el factor de crecimiento endotelial vascular (VEGF) (Aiello et al., 1995; Eichler et al., 2004; Yafai et al., 2004). VEGF favorece la supervivencia de las células endoteliales y las neuronas retinales y puede restringir el daño inducido por el estrés oxidativo y la glucosa de los vasos retinianos (Yamada et al., 1999). Los efectos protectores del VEGF incluyen vasodilatación, revascularización, inflamación, proliferación de células gliales, neuroprotección y neurogénesis (Krum and Khaibullina, 2003; Yasuhara et al., 2004). Entre otros efectos positivos de la gliosis está la fagocitosis de sustancias exógenas, residuos celulares, proteínas séricas y hemoglobina (Egensperger et al., 1996; Ehrenberg et al., 1984; Inomata, 1975; Kaur et al., 2007; Mano and Puro, 1990; Rosenthal and Appleton, 1975; Stolzenburg et al., 1992). En respuesta a los estímulos patológicos, las células Müller también son capaces de desdiferenciarse a células que muestran características similares a las células progenitoras, estas células, pueden proliferar, migrar y se transdiferencian a células con fenotipo “neuronal” (Fischer and Reh, 2001; Ooto et al., 2004; Takeda et al., 2008; Vihtelic and Hyde, 2000). Esta transdiferenciación a células con fenotipo neuronal puede considerarse como un intento de regenerar el tejido.

Sin embargo, la gliosis podría contribuir a la neurodegeneración e impide la reparación tisular y la neuroregeneración. La desdiferenciación de las células de Müller también se asocia con un desacoplamiento funcional de las neuronas. Cuando el tiempo de gliosis es excesivo, se produce una regulación a la baja de las proteínas involucradas en funciones específicas de las células de Müller, como la glutamina sintetasa, anhidrasa carbónica, CRALBP y canales de potasio (Kir). Ademas, las acuaporinas interrumpen la interacción glio-neuronal y la regulación osmótica de la retina, contribuyendo al desarrollo del edema, la hiperexcitación neuronal y la toxicidad por glutamato (Bringmann et al., 2009a; Bringmann et al., 2006; Lewis et al., 1994; Lieth et al., 1998). Todo ello produce un deterioro de las funciones de apoyo de las células de Müller pudiendo incrementar la pérdida de neuronas, al aumentar la susceptibilidad a estímulos estresantes en la retina enferma. Además, las células de Müller activadas también tienen efectos citotóxicos más directos por la liberación de factores solubles, como las citoquinas pro-inflamatorias (Cotinet et al., 1997; de Kozak et al., 1997; Drescher and Whittum-Hudson, 1996).

En las formas más severas de gliosis reactiva, las células experimentan hipertrofia, proliferan y pierden su funcionalidad, formando cicatrices gliales que inhiben la

regeneración axonal y la supervivencia neuronal. La gliosis proliferativa que también se da en estadios avanzados de la retinopatía diabética, se asocia generalmente con la gliosis crónica y suele ser perjudicial (Sofroniew, 2009). Se pierde la integridad de la barrera de hemato-retiniana y, como resultado, los componentes del suero se filtran en el espacio perivascular, provocando la reentrada de las células de Müller en el ciclo celular y su proliferación (Bringmann et al., 2009a; Coorey et al., 2012).

Otro aspecto a resaltar es la liberación de VEGF por las células de Müller durante el proceso de gliosis es especialmente interesante, ya que actúa en dos direcciones. El VEGF es neuroprotector a bajas concentraciones como se ha explicado anteriormente, (Oosthuyse et al., 2001; Zheng et al., 2012) pero a altas concentraciones causa todo tipo de patologías vasculares, lo que sucede cuando se prolonga en daño y se continua con el proceso de gliosis (Tolentino et al., 2002).

Una de las enfermedades en la que la gliosis tiene un papel fundamental es la retinopatía diabética (RD) la principal causa de ceguera en la población de países desarrollados (Bringmann et al., 2006; Tezel and Wax, 2000). Durante el progreso de esta enfermad, se ha establecido que las células de Müller se activan expresando GFAP, un marcador común de gliosis reactiva, desde estadios tempranos de la enfermedad (Gerhardinger et al., 2005; Kusner et al., 2004; Puro, 2002). Durante muchos años, la RD se ha considerado una enfermedad microvascular, caracterizada por un aumento de la permeabilidad vascular debido a la ruptura de la barrera hemato-retiniana (BRB) (Madeira et al., 2015). Sin embargo, aunque los cambios vasculares son un sello clásico de este trastorno, varias observaciones sugieren que la microangiopatía es solo un aspecto más que contribuye a la disfunción de la retina (Coughlin et al., 2017) ya que también está asociada a la degeneración de las RGCs (Bloodworth, 1962; Santiago et al., 2007; Wolter, 1961). Es sabido que las células de Müller glióticas contribuyen al desarrollo y la progresión de la enfermedad al promover la vía de señalización de caspasa-1, la secreción de la citoquina pro-inflamatoria IL-1β y el estrés mitocondrial (Mohr et al., 2002; Vincent and Mohr, 2007; Vindeirinho et al., 2016). Sin embargo, el papel de estas células en la retinopatía diabética y en interacción con las RGCs, no está del todo claro, siendo necesarios más estudios tanto *in vivo* como *in vitro*, como el que aborda la presente tesis doctoral, en la que se ha estudiado el efecto de la glucosa en la supervivencia de las RGCs y el rol de las células de Müller en condiciones hiperglicémicas. En este estudio *in vitro* se someterán tanto a las RGCs solas como en

co-cultivo con las células de Müller a altas concentraciones de glucosa. Además, los actuales tratamientos de esta enfermedad incluyen el uso de glucocorticoides como la dexametasona, que puede apaciguar la secreción de citoquinas pro-inflamatorias características del proceso de gliosis (Fawcett et al., 1989). Por ello, también se evaluará el efecto de este glucorticoide en la supervivencia de las RGCs sometidas a altas concentraciones de glucosa.

1.5 Modelos de estudio

1.5.1 Animales

La presente Tesis Doctoral se ha llevado a cabo utilizando distintos modelos animales, como son el cerdo, la rata y el ratón. La retina porcina tiene un gran parecido a la retina humana lo que hace que sea un excelente modelo para el estudio del sistema visual. Se asemejan en tamaño forma y función, número y distribución de conos y bastones (Beauchemin, 1974; De Schaepdrijver et al., 1990; Ruiz-Ederra et al., 2004; Veiga-Crespo et al., 2013) y, además, su vasculatura es similar a la de los seres humanos (Galdos et al., 2012) lo que hace muy interesante su uso para el estudio de patologías humanas (Komaromy et al., 2003). Aunque los primates no-humanos se consideran el mejor sustituto de la retina humana debido a su gran parecido y a que son los únicos en poseer macula (Polyak, 1941; Volland et al., 2015), su alto coste y su implicación ética hacen difícil su uso. En la retina porcina, se han identificado diferentes regiones tras analizar la distribución de RGCs según la densidad celular y el tamaño de soma. En el centro de la retina se encuentra una banda horizontal de alta densidad de RGC, donde hay una alta proporción de RCGs con tamaño de soma pequeño. De la retina central a la más periférica, se observa una disminución de la densidad RGC, junto con una mayor presencia de RGCs con somas más grandes (Garca et al., 2005). Sin embargo, también han sido utilizadas otras especies como el ratón y la rata, ya tienen otras ventajas debido a su fácil manejo y estabulación, a que tienen menos restricciones éticas, son más económicos. En la retina de roedores la población de RGCs se distribuye también en gradiente de centro a periferia y algunos autores sugieren que existe una región con mayor densidad de RGC en la región temporal superior de la retina (Drager and Olsen, 1981; Dreher et al., 1992; Fukuda, 1977; Salinas-Navarro et al., 2009a).

1.5.2 Cultivos celulares de células de la retina.

Los últimos hallazgos sobre las células de Müller y las RGCs, se han derivado tanto de investigaciones en modelos animales de diferentes patologías, como de cultivos de células de Müller y RGCs. Estos cultivos, tanto de células en solitario como en co-cultivo (figura 6), proporcionan sistemas experimentales controlables para el examen de procesos fundamentales de retina, así como de la simulación de determinadas patologías y de la búsqueda de posibles tratamientos. Sin embargo, aunque existen varios protocolos para el aislamiento de estas células de diferentes especies de mamíferos, generalmente se basan en el uso de tejido de animales neonatales, probablemente debido a la pérdida de potencial de regeneración a medida que los mamíferos envejecen (Fischer et al., 2002; Schafer and Karl, 2017). Sin embargo, las enfermedades degenerativas de la retina son más frecuentes en adultos y, por consiguiente, los sistemas de modelos *in vitro* desarrollados con células adultas serían más útiles para investigar los eventos fisiológicos y patológicos que ocurren en la retina madura.

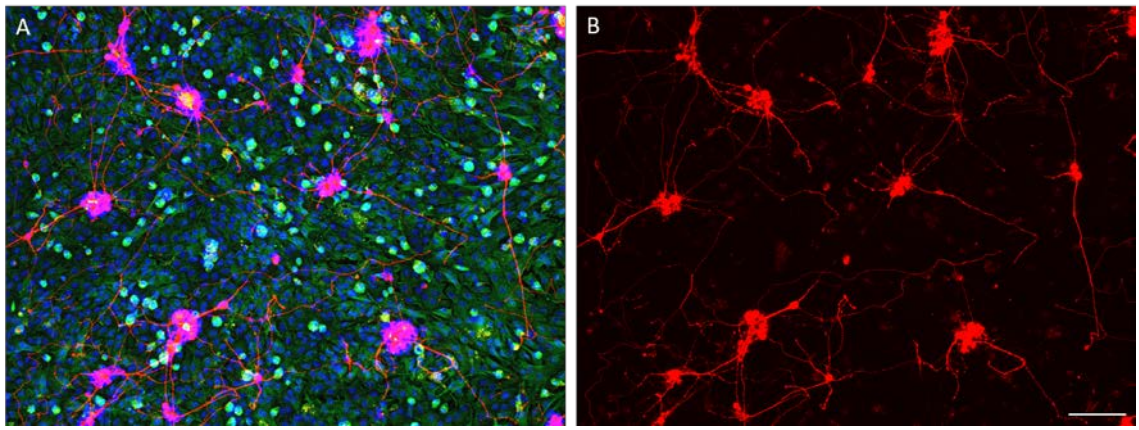

Figura 6. Co-cultivo de células de Müller y RGCs. Co-cultivo primario de células de Müller y RGCs de rata, las células han sido marcadas con un anticuerpo frente a vimentina, específico de las células de Müller en verde (A) y con un anticuerpo frente a Beta III tubulina (A y B), específico de las RGCs en rojo. Los núcleos han sido teñidos con DAPI en azul (A). Capa nuclear externa (ONL), capa plexiforme externa (OPL), capa nuclear interna (INL), capa plexiforme interna (IPL), y capa de células ganglionares (GCL). Barra de escala = 100 µm

La utilidad de los cultivos celulares primarios de Müller para el estudio de las funciones de estas células *in vitro* ha sido bastante limitada entre otras razones, por la dificultad de obtener poblaciones de células puras sin contaminación por astrocitos, microglía o

neuronas. En consecuencia, la mayoría de los estudios que se centran en estas células han utilizado líneas celulares permanentes obtenidas a través de la inmortalización de células primarias con oncogenes virales. Por ejemplo, la línea celular rMC-1 Müller de la retina de rata adulta (Sarthy et al., 1998), la primera línea celular permanente de Müller, así como la línea celular TR-MUL (Tomi et al., 2003) y la línea celular humana de Müller espontáneamente inmortalizada (MIO-M1) (Limb et al., 2002). Aunque el uso de estas líneas celulares es generalizado, algunas de ellas presentan un fenotipo no diferenciado y estas células han perdido la capacidad de dividirse más de un cierto número de veces. Esto puede deberse en parte a las mutaciones o alteraciones genéticas que llevan a su inmortalización o la transfección de los genes específicos para lograr ese estado (Alge et al., 2006; Pan et al., 2009).

Además, hay que tener en cuenta que algunos rasgos característicos de las células de Müller de cultivos primarios pueden alterarse o perderse después de 2 semanas de cultivo (Hauck et al., 2003; Merl et al., 2012). De hecho, se han detectado cambios en la expresión de proteínas que eran indicativos de la transdiferenciación hacia un fenotipo similar a fibroblastos entre el día 14 y el día 21 en cultivo (Limb et al., 2002). Estos cambios proteómicos pueden ser más acusados en las líneas celulares permanentes, por ejemplo, la línea MIO-M1 puede mostrar características progenitoras e incluso puede expresar marcadores de neuronas retinales postmitóticas como opsinas (Hollborn et al., 2011). Por lo tanto, muchos investigadores consideran que los cultivos primarios son más fisiológicamente similares a las células *in vivo*, lo que representa un modelo experimental más adecuado para su uso en la investigación de patologías retinales.

Los protocolos que existen actualmente para cultivar células de Müller ofrecen poca información para reproducirlos exitosamente en el laboratorio. Por lo tanto, en la presente tesis doctoral se ha desarrollado un protocolo más rápido, fácil y que se ha optimizado para tejido adulto de diferentes especies de mamíferos, proporcionando una herramienta valiosa para estudiar enfermedades en las que las células de Müller se ven involucradas.

1.6 Posibles aplicaciones de los modelos *in vitro*

El uso de sistemas de cultivos celulares *in vitro*, supone en la actualidad una plataforma tecnológica esencial para el desarrollo de múltiples investigaciones científicas. La evolución que ha sufrido esta metodología durante las últimas décadas, la ha convertido en una herramienta imprescindible en el intento de desentrañar los mecanismos moleculares y celulares que suceden en todo proceso biológico. Los cultivos celulares nos permiten estudiar las poblaciones de células de forma individual que pueden servir incluso para complementar estudios *in vivo*. Además, el uso de los cultivos no solo se limita a la investigación básica, en los últimos años, investigaciones con células madre y su diferenciación a células de la retina *in vitro* han abierto nuevas vías de actuación como por ejemplo trasplantes de RGCs para individuos con enfermedades neurodegenerativas como el glaucoma.

1.6.1 Estudio de los lípidos de la retina

Aunque los lípidos se consideran moléculas causales y de diagnóstico importantes para las enfermedades de la retina, se sabe poco acerca de los perfiles de los lípidos en la retina normal y las diferencias de los lípidos entre las células que componen la retina.

Gracias al continuo contacto de las células de Müller con las RGCs, permite a estas la manipulación del microentorno lipídico frente a la detección de diferentes estímulos, contribuyendo así patologías de la retina. Por ejemplo, es sabido que niveles alterados de colesterol subyacen enfermedades neurodegenerativas como los síndromes de Smith-Lemli-Opitz y Niemann-Pick, la retinopatía diabética, el glaucoma y la degeneración macular. Además, animales alimentados con dietas con exceso o deceso de colesterol sufren pérdidas neuronales (Fliesler and Bretillon, 2010; Fliesler et al., 2007; Gambert et al., 2017; Lakk et al., 2017; Omarova et al., 2012). Estos ejemplos muestran la importancia de estudiar los lípidos en la retina, y de forma específica, los de las células de Müller y las RGCs.

Hasta ahora, se han estudiado la distribución de lípidos en secciones de retinas de ratón (Hayasaka et al., 2008), salamandra (Roy et al., 2011), cerdo (Palmer et al., 2012) y humano (Zemski Berry et al., 2014) por MALDI-IMS. Sin embargo, en algunos casos, la homogeneización del tejido no permitió obtener información sobre la distribución

espacial de los lípidos en relación con los tipos de células en la retina. En los que sí se estudió la distribución espacial, la técnica MALDI-IMS permitió en algunos estudios, diferenciar entre capas de la retina, pero todavía no se ha podido concretar un perfil lipídico para cada tipo celular. Por lo tanto y por primera vez, en la presente tesis doctoral, se ha desarrollado un protocolo en el que la combinación de estudios *in vivo* e *in vitro* permiten mejorar la información obtenida y asociar un perfil lipídico a un tipo celular en concreto. Estos estudios pueden abrir un nuevo campo de estudio para comparar tejido sano con tejido patológico e identificar cambios lipídicos relacionados con la enfermedad y, por lo tanto, proporcionar más información sobre las implicaciones de los lípidos en las enfermedades de la retina, identificando nuevas dianas terapéuticas para retardar o prevenir la progresión de la enfermedad.

1.6.2 Estudios con células madre.

Algunas de las estrategias a seguir actualmente frente a enfermedades neurodegenerativas, son el diagnóstico temprano, la identificación de otros factores de riesgo modificables y la neuroprotección, sin embargo, ninguna de estas estrategias es aun efectiva para volver a regenerar las RGCs que se han perdido y revertir la pérdida de visión. Dado que la retina de los mamíferos no tiene capacidades regenerativas espontáneas, las únicas posibilidades de recuperar la pérdida de la visión después de la muerte de las RGCs, es el reemplazo celular mediante trasplantes o mediante reparación endógena a través de la capacidad de regeneración de las células de Müller.

Dado el gran número de personas que podrían beneficiarse de las terapias de trasplante de RGCs (Tham et al., 2014), es necesario establecer una fuente estable de RGCs antes de que los trasplantes puedan ser utilizados de manera viable en ensayos clínicos en humanos.

En las últimas décadas, los métodos de aislamiento y cultivo de células madre embrionarias de ratón y humano (mESCs y hESC) han evolucionado muy rápidamente (Evans and Kaufman, 1981; Thomson and Marshall, 1998) junto con las técnicas para reprogramar células somáticas en células madre pluripotentes inducidas (iPSCs) (Takahashi and Yamanaka, 2006; Yu et al., 2007). Hace más de una década, varios laboratorios desarrollaron protocolos básicos para diferenciar las ESC humanas y de ratón en neuronas de la retina (Eiraku et al., 2011; La Torre et al., 2012; Lamba et al.,

2006) y la mayoría de estos protocolos, fueron diseñados para imitar el desarrollo normal que sucede en un embrión.

En 2011, Yoshiki Sasai revolucionó las tecnologías para generar células de retina de células pluripotentes utilizando cultivos organoides tridimensionales (3D) (Eiraku and Sasai, 2011; Eiraku et al., 2011; Nakano et al., 2012). Este método se basa en colocar células pluripotentes disociadas en placas no adherentes en forma de U o V. Dado que las células no se pueden unir al fondo de la placa, se unen entre sí, formando agregados 3D llamados cuerpos embrioides (EBs). Usando sustancias concretas como medio de cultivo los EBs crecen y comienzan a expresar marcadores de células progenitoras de la retina, pero estos cultivos sufren cambios morfogenéticos para desarrollar primero estructuras como vesículas ópticas, copas ópticas y, finalmente, retinas totalmente laminadas que pueden contener todo el repertorio de tipos de células de la retina presentes en una retina normal.

Sin embargo, la mayoría de estos métodos aún son ineficientes para generar grandes cantidades de neuronas (exceptuando los fotorreceptores). Esto no es sorprendente, ya que los métodos de cultivo de células madre *in vitro* imitan lo que ocurre durante el desarrollo normal de la retina *in vivo*, y los fotorreceptores superan en número ampliamente a las RGCs tanto en retinas de ratones como humanas (Curcio and Allen, 1990; Jeon et al., 1998). De manera similar, se estima que el rendimiento promedio de las RGCs que provienen de células madre se encuentra entre el 0,1% y el 30% de las células en cultivo, que es similar al porcentaje normal de las RGCs en la retina en diferentes etapas de desarrollo. Además, se ha demostrado que el número de RGCs disminuye con el tiempo en los cultivos 3D (Aparicio et al., 2017). Esto también es esperable, ya que, durante el desarrollo normal, las RGCs recién “nacidas” experimentan dos oleadas de muerte celular programada a medida que se vuelven críticamente dependientes del soporte trófico de sus objetivos sinápticos (Isenmann et al., 2003). Dado que las células diana del cerebro no están presentes en los cultivos, la apoptosis mediada por Bax es probablemente la responsable de la disminución en el número de RGCs a lo largo del tiempo. Se ha sugerido que varios factores neurotróficos como BDNF, NT4 o CNTF, que son capaces de secretar las células de Müller, ayudan a mejorar la supervivencia de RGCs (Goldberg et al., 2002; Ma et al., 1998). Por lo tanto, debido a las propiedades neuroprotectoras de las células de Müller y sus factores secretados, y a la problemática planteada respecto al número de RGCs que se consigue a

partir de células madre, en la presente tesis doctoral se ha estudiado si las células de Müller y su medio secretado también pueden influir en supervivencia de las RGCs diferenciadas de células madre. Aumentando el número de RGCs, se ayudaría al progreso de las terapias de trasplantes, pudiéndose hacer realidad en un futuro próximo.

Conociendo las propiedades de las células de Müller, sus múltiples funciones para el correcto funcionamiento de la retina en general y de las RGCs en particular y su participación en numerosas patologías oculares, nos proponemos estudiar diferentes situaciones y modelos que nos ayuden a entender la interacción entre las células de Müller y las RGCs. Para ello, primero se optimizará y desarrollará un método de cultivo fiable de células de Müller, con el que se caracterizarán las células en cultivo y podremos estudiar su comportamiento en diferentes situaciones. Estos cultivos nos permitirán caracterizar por primera vez el lipidoma de las células de Müller y las RGCs comparando los resultados obtenidos por MALDI-IMS tanto *in vivo* como *in vitro*. Este estudio proporcionará un protocolo sencillo en el que poder estudiar los lípidos bajo diferentes condiciones y modelos patológicos.

Debido las propiedades neuroprotectoras intrínsecas de las células de Müller, los cultivos de células de Müller adultas se utilizarán también para evaluar el efecto de estas células en las RGCs diferenciadas de células madre. La difícil obtención de estas neuronas diferenciadas en cultivo y el requerimiento de un número mayor para sus posibles usos terapéuticos, hace que el uso las células de Müller adultas y su medio secretado sea una buena estrategia para aumentar la supervivencia de estas células.

Además, la heterogeneidad presente en el conjunto de las células de Müller, tanto *in vivo* como *in vitro* nos hace plantearnos si hay células de Müller más neuroprotectoras que otras. Por ello, se analizará la supervivencia de las RGCs en interacción con las células de Müller según su localización en la retina.

Por último, nos centraremos en analizar el efecto neuroprotector de las células de Müller en condiciones patológicas, en este caso en un modelo de retinopatía diabética. Para ello, se co-cultivarán células de Müller y RGCs y se someterán a altas concentraciones de glucosa. También se evaluará el efecto en la supervivencia neuronal del glucocorticoide dexametasona en los cultivos sometidos a altas concentraciones de glucosa, ya que es un fármaco ampliamente utilizado por los clínicos en pacientes con esta patología.

A continuación, en el ANEXO 0, se encuentran el resumen de una completa revisión realizada por este grupo sobre la interacción de las neuronas y las células de la glía en la retina de mamíferos. Además, cada artículo anexado en el apartado **4. Resultados** presenta una introducción propia del tema a tratar.

ANEXO 0

Glia-Neuron Interactions in the Mammalian Retina.

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Abstract

The mammalian retina provides an excellent opportunity to study glia-neuron interactions and the interactions of glia with blood vessels. Three main types of glial cells are found in the mammalian retina that serve to maintain retinal homeostasis: astrocytes, Müller cells and resident microglia. Müller cells, astrocytes and microglia not only provide structural support but they are also involved in metabolism, the phagocytosis of neuronal debris, the release of certain transmitters and trophic factors

and K(+) uptake. Astrocytes are mostly located in the nerve fibre layer and they accompany the blood vessels in the inner nuclear layer. Indeed, like Müller cells, astrocytic processes cover the blood vessels forming the retinal blood barrier and they fulfil a significant role in ion homeostasis. Among other activities, microglia can be stimulated to fulfil a macrophage function, as well as to interact with other glial cells and neurons by secreting growth factors. This review summarizes the main functional relationships between retinal glial cells and neurons, presenting a general picture of the retina recently modified based on experimental observations. The preferential involvement of the distinct glia cells in terms of the activity in the retina is discussed, for example, while Müller cells may serve as progenitors of retinal neurons, astrocytes and microglia are responsible for synaptic pruning. Since different types of glia participate together in certain activities in the retina, it is imperative to explore the order of redundancy and to explore the heterogeneity among these cells. Recent studies revealed the association of glia cell heterogeneity with specific functions. Finally, the neuroprotective effects of glia on photoreceptors and ganglion cells under normal and adverse conditions will also be explored.

2. Hipótesis y Objetivos / Hypotheses and Objectives

Una de las funciones principales de las células de la glía de Müller en la retina, es la neuroprotección, y su participación en la mayoría de procesos fisiológicos de la retina, tanto en condiciones normales como en patológicas. Estudios previos del grupo de investigación, han indicado que tienen un papel crucial en la supervivencia de las células ganglionares de la retina mediante factores secretados. Sin embargo, se sabe que no todas las RGCs son susceptibles a la muerte en una misma condición patológica. Por ello proponemos como hipótesis que existe una diferente capacidad de respuesta de las células de Müller a diferentes estímulos y una heterogeneidad en la población de estas células en la retina que pudiera estar relacionada con una distinta capacidad neuroprotectora. Por otra parte, pueden existir distintas poblaciones de RGCs que tengan distinta susceptibilidad a ser protegidas por las vecinas células de Müller. Por ello, proponemos que el estudio de la interacción entre RGCs y glía de Müller tanto en condiciones fisiológicas como patológicas, puede esclarecer los mecanismos moleculares por los cuales son capaces de neuroproteger a las células ganglionares.

Teniendo en cuenta los antecedentes descritos previamente y la hipótesis planteada, en el marco de la presente Tesis Doctoral, se ha desarrollado un objetivo general:

Estudiar el efecto neuroprotector de células de Müller y su interacción con las células ganglionares de la retina, tanto en condiciones fisiológicas, como patológicas.

Con el propósito de cumplimentar el objetivo global se han planteado 5 objetivos:

1- Establecer un método de cultivo primario de las células de Müller de cerdo, rata y ratón adulto.
2- Identificar y comparar *in vitro* e *in vivo* los lípidos presentes en células de Müller y las células ganglionares de la retina.
3- Analizar el efecto neuroprotector tanto de las células de Müller adultas como de su medio condicionado en las células ganglionares procedentes de la diferenciación de células madre.
4- Analizar el efecto neuroprotector tanto de las células de Müller como de su medio condicionado, en las células ganglionares de individuos adultos, atendiendo a la localización de las células de Müller en la retina, comparando células de Müller procedentes del centro, medio y periferia de la retina.

5- Estudiar el efecto tanto de las células de Müller como de la aplicación de la dexametasona en las células ganglionares de la retina, en un modelo *in vitro* de retinopatía diabética.

In addition to their participation in most of the physiological processes in the retina, under both normal and pathological conditions, neuroprotection is one of the main roles fulfilled by Müller glia cells in the retina. Previous studies indicated that the secretion of different factors by these glia cells is crucial for the survival of retinal ganglion cells (RGCs). However, not all RGCs are susceptible to death under the same pathological conditions and thus, we hypothesize that Müller glia may respond distinctly to different stimuli and that heterogeneity in this population of cells could be related to their neuroprotective capacities. Alternatively, distinct populations of RGCs may be susceptible to protection by their neighboring Müller cells to a different degree. Therefore, we proposed to study the interactions between RGCs and Müller glia in an attempt to clarify the molecular mechanisms by which the latter cells offer neuroprotection to RGCs.

In the light of what is currently known and the hypothesis postulated, the overall objective of this Doctoral Thesis was **to study the neuroprotective effect of Müller cells and their interaction with RGCs under physiological and pathological conditions.**

In order to address this global objective, the following partial goals were proposed:

1- To define a protocol to establish primary cultures of pig, rat and adult mouse Müller glia cells.
2- To identify and compare the lipids present in Müller glia and RGCs, both *in vitro* and *in vivo*.
3- To analyze the neuroprotective effect of both adult Müller glial cells and their conditioned medium on stem cell derived-RGCs.
4- To analyze the neuroprotective effect of both Müller glia and their conditioned medium on adult RGCs according to the location of the Müller cells within the retina.
5- To study the effect of both Müller glia and dexamethasone on RGCs in an *in vitro* model of diabetic retinopathy.

3. Material y Métodos

Todas las técnicas que se describen a continuación están explicadas con mayor detalle en los anexos correspondientes en el apartado 4. Resultados.

3.1 Animales

Este estudio fue realizado de acuerdo con la Resolución ARVO (Association for Research in Vision and Ophthalmology) para el uso de animales en experimentación. Los ojos de cerdo adultos se obtuvieron de un matadero local, y se transportaron al laboratorio en medio CO2 independiente (CO2-independent medium; Life Technologies, Carlsbad, CA, USA) más 0,1% de gentamicina (Life Technologies, Carlsbad, CA, USA) **(ANEXO 1, 2, 4).**

Se usaron ratas adultas Sprague-Dawley (200-250g) procedentes del animalario de la Facultad de Medicina de la Universidad del País Vasco **(ANEXO 1, 4, 5)**.

Los ratones adultos se obtuvieron tanto del animalario de la Facultad de Medicina de la Universidad del País Vasco como del animalario de la Universidad de California, Davis **(ANEXO 1, 3)**.

Los animales tuvieron acceso libre a alimentos y agua, con un ciclo de luz-oscuridad 12 horas y con una temperatura estable de 21°C. Las ratas fueron sacrificadas por exposición a CO2 y los ratones por dislocación cervical.

3.2 Extracción del tejido

Los ojos fueron enucleados y mantenidos en medio CO2 independiente para su uso en cultivos.

Para la realización de cortes en criostato en el **ANEXO 2**, la retina fue extraída y sin fijar, se dobló sobre sí misma encima de un papel para aportar rigidez y se congeló directamente a -20ºC. Una vez congelada, se puso sobre una cama de OCT dejando parte de la retina libre de este y se hicieron cortes a 30 μm de grosor. Los cortes se mantuvieron congelados a -80ºC en una atmósfera libre de oxígeno hasta su uso.

Para la realización de cortes en criostato para el **ANEXO 3**, los cuerpos embriónicos "embrionic bodies" (EBs) fueron fijados durante una noche en paraformaldheido (PFA) 4%, después fueron crioprotegidos durante una noche a 4°C en 30% de sacarosa en 0,1 M (Phosphate buffer) PB. Las muestras fueron integradas en medio de inclusión OCT Tissue-Tek (Sakura Finetek, Holanda), y se realizaron cortes de un espesor 14μm, que fueron almacenadas a -20°C hasta su uso.

3.3. Cultivos de células de la retina: RGCs, células de Müller y co-cultivos

Para la realización de los cultivos celulares, las retinas fueron extraídas en condiciones de asepsia. Se realizaron 3 tipos de cultivos: cultivos de RGCs usando medio Neurobasal-A (NBA) suplementado con B27 (Life Technologies, Carlsbad, CA, EUA); cultivos de Müller utilizando DMEM (Life Technologies, Carlsbad, CA, EE.UU.) con 10 o 20% de suero bovino fetal (FBS, Life Technologies, Carlsbad, CA, EE.UU.); y co-cultivos de RGCs y células Müller, utilizando medio NBA con 10% de FBS y suplementado con B27. A todos los medios se les añadió 1% de L-glutamina (2 mM) y gentamicina al 0,1% (50 mg/ml).

Dependiendo del animal, se usaron retinas enteras de rata o se cortaron trozos de retina de cerdo con un troquel de 8mm de diámetro. Estos fragmentos de retina fueron digeridos enzimáticamente con papaína y 10% de DNasa I (kit de disociación Worthington Papain Dissociation, Worthington Biochemical Lakewood, NJ, USA). La digestión para los cultivos de RGCs es de 90 minutos y para los de células de Müller y co-cultivos es de 30 minutos. La actividad enzimática de la papaína se detuvo mediante la adición de medio y el tejido fue desagregado mediante una suave trituración usando puntas de pipeta de diámetro decreciente. Las células disociadas se recogieron mediante centrifugación a 300g durante 5 minutos y fueron resuspendidas en su correspondiente medio. En el caso de las RGCs en medio NBA suplementado con B27 y en el caso de los cultivos de células de Müller en DMEM con distintas concentraciones de FBS dependiendo del experimento.

Para los cultivos de RGCs, se realizó además un gradiente siguiendo el protocolo del kit Worthington. Todas las células resuspendidas se sembraron en placas de 24 pocillos con

cubreobjetos de vidrio de 13 mm previamente revestidos con poli-L-lisina (Sigma, P4832, 100 µg/ml) y laminina (Sigma, L2020, 10 µg/ml). Las células se mantuvieron en un incubador humidificado a 37°C con una atmósfera de CO2 al 5%. En los cultivos de células Müller y co-cultivo, a día 1 se cambió todo el medio. Para mantener las células, la mitad del medio se cambia cada 3 días. Las células se fijaron con metanol -20ºC durante 10 minutos a día 6 para RGCs o 7 para células de Müller y co-cultivo **(ANEXO 1, 2, 3, 4 y 5)**.

3.4 Cultivos y diferenciación de células madre a RGCs

3.4.1 Cultivos de células madre de ratón (mESCs)

Las células mESC (R1) se mantuvieron en un medio con inhibidores de LIF + 2 (LIF + 2i) que consiste en: medio DMEM suplementado con un 10% FBS (Invitrogen, Grand Island, NY) aminoácidos no esenciales (NEAA) (Invitrogen, Grand Island, NY), piruvato de sodio (Invitrogen, Grand Island, NY), 0,1 mM de β-mercaptoetanol (Sigma Aldrich, Saint Louis, MO), 100 µl (10 millones de unidades/ml) de factor inhibidor de leucemia (LIF) (ESGRO Millipore, Billerica, MA), 3 µM de inhibidor de GSK3β (Stemgent, Cambridge, MA) y 0,4 µM de inhibidor de MEK (Stemgent Cambridge, MA). Las células se mantuvieron en placas recubiertas con Matrigel (Corning life science, Corning, NY).

3.4.2 Diferenciación retinal de las células madre embrionarias de ratón mESCs

Las colonias de células madre semiconfluentes se disociaron en una suspensión de células individuales mediante tratamiento enzimático TrypLE (Gibco, Rockville, MD) y una disociación mecánica suave. A continuación, se colocaron en placa 5000 células en placas de baja adherencia de 96 pocillos (Sbio, Hudson, NH) y se mantuvieron en medio de diferenciación retinal (RD) (Día 0): Medio esencial mínimo de Glasgow (GMEM) (Thermo Fisher, Waltham, EE. UU.) complementado con NEAA, piruvato de sodio, reemplazo de suero Knock-Out (KSR) (Thermo Fisher, Waltham, EE. UU.) y β-mercaptoetanol (Sigma, Steinheim, Alemania). Después de 12 horas las mESCs

flotantes forman espontáneamente agregados o cuerpos embrionarios (EBs). 24 horas más tarde (día 1), se añadió Matrigel (2%) a cada pocillo. En el día 4, las EBs se movieron a una placa de seis pocillos de adherencia ultra baja (Thermo Fisher Waltham, EE. UU) y el medio de cultivo se reemplazó por 1,5 ml de medio RD fresco y 0,5 de medio Tom: medio Neurobasal A (Thermo Fisher, Waltham, EE. UU.), Albúmina de suero bovino (BSA) (Invitrogen, Eugene, Oregón, EE. UU.), B27 (Life Technologies, Carlsbad, CA, EE. UU.), N2, NEAA, HEPES, piruvato de sodio y bicarbonato de sodio. En el día 5, el medio se reemplazó por 1 ml de medio RD y 1 ml de medio Tom. En el día 6, con 0,5 ml de RD y 1,5 ml del medio Tom y finalmente, en el día 7 todos los medios se reemplazaron con 100% del medio Tom. Las EBs se mantuvieron hasta el día 10 **(ANEXO 3)**.

3.5 Inmunohistoquímica e inmunocitoquímica

Para los cortes de criostato después del MALDI-IMS **(ANEXO 2)**, los cortes fueron lavados con metanol durante 1 minuto para retirar la matriz utilizada en el MALDI-IMS, fijados con 4% de PFA durante 2 minutos. Y los cortes obtenidos de las EBs **(ANEXO 3)** se descongelaron durante 15 minutos.

Después, todos los tipos de cortes fueron lavados dos veces en phosphate buffer saline-triton-100 (PBS-TX-100) durante 10 minutos, y se incubaron durante una noche con los anticuerpos primarios a 4ºC. Tras lavar dos veces en PBS, fueron incubados con el correspondiente anticuerpo secundario y DAPI diluido en PBS-Bovine Serum Albumin (BSA) (1%) durante 1 hora a temperatura ambiente. 4 ',6-diamidino-2-fenilindol (DAPI) fue utilizado como marcador nuclear a 1:10.000. Los cortes se lavaron dos veces con PBS durante 10 minutos y se montaron con un cubreobjetos en PBS-Glicerol (1:1).

Para los cultivos celulares, los cubreobjetos donde las células crecieron fueron bloqueados con tampón de bloqueo (BSA al 3% y Triton X-100 al 0,1% en PBS) durante 30 minutos a temperatura ambiente. A continuación, se incubaron con los anticuerpos primarios en tampón de bloqueo durante una noche a 4ºC. Tras lavar con PBS, se incubaron con los anticuerpos secundarios y DAPI en tampón de bloqueo durante 1 hora a temperatura ambiente. Para finalizar, se lavaron con PBS y fueron

montaron con FluorSave Reagent (Millipore Merk, Darmstadt, Alemania) **(ANEXO 1,2, 3, 4, 5)**.

Los anticuerpos primarios usados están descritos en la Tabla 1. Todos los anticuerpos secundarios usados fueron Alexa Fluor 568, 555 y 488 (Life Technologies, Eugene, Oregon, USA) y fueron usados a una dilución de 1:1000.

ANTICUERPO	MARCAJE	DILUCIÓN	REFERENCIA
Brn3a	RGCs	1:1.000	Santa Cruz Biotechnology
Beta III-Tubulin	RGCs	1:2.000	Abcam
GFAP	Astrocitos	1:1.000	Sigma
Glutamina Sintetasa	Células de Müller	1:10.000	Abcam
Vimentina	Células de Müller	1:10.000	Dako
P75NTR	Células de Müller	1:2.000	Abcam
α-SMA	Células de fenotipo fibroblástico	1:2.000	Sigma

Tabla 1. Anticuerpos primarios. Lista de anticuerpos primarios utilizados en este estudio, donde se indica la proteína que reconoce el anticuerpo, que tipo celular marca, en que dilución se utiliza y la casa comercial donde ha sido adquirido.

3.6 Análisis de expresión mediante qPCR

Para el análisis de la expresión de Brn3a y Math5, el ARN total se extrajo de los cuerpos embriónicos (Embronic bodies, EBs) obtenidos de células madre, mantenidos en cultivo con los medios condicionados obtenidos de los cultivos de células de Müller adultas de ratón, usando Trizol (Invitrogen) seguido de una extracción con cloroformo tal y como indican las instrucciones del fabricante. Después las muestras se trataron con ADNasa 1 (Qiagen, Hilden, Alemania) seguido de una purificación del ARN utilizando el kit de limpieza de ARN Qiagen mini. El cDNA se transcribió de manera inversa utilizando el kit Superscript III RT (Thermo-Fisher, San José, CA, EE. UU) según las instrucciones del fabricante. La PCR se realizó utilizando los cebadores para Brn3a (directo: 5 'CGT ACC ACA CGA TGA ACA GC 3' inverso: 5 'AGG AGA TGT GGT CCA GCA GA 3') y Math5 (directo: 5´CCC TAA ATT TGG GCA AGT GAA GA 3´ inverso 5´CAA AGC AAC TCA CGT GCA ATC3´) y los valores se normalizaron usando β-actina como control endógeno **(ANEXO 3).**

3.7. Captura y análisis de imágenes

Para la captura de imágenes en este estudio, se usó mayoritariamente un microscopio de fluorescencia (Zeiss, Jena, Germany) con cámara Zeiss Axiocam MRM (Zeiss, Jena, Germany) **(ANEXO 1-5)**.

Además del recuento manual de células **(ANEXO 3,4,5)**, se usaron distintos programas informáticos. Para cuantificar el número de núcleos de células de Müller en cultivos, se ha usado el software Zen (Zeiss, Jena, Germany) teneiendo en cuenta el tamaño del núcleo característico de estas células **(ANEXO 1,3,4,5)**.

3.8. Análisis estadístico

Los datos resultantes de las cuantificaciones de RGCs y células de Müller se han descrito como media y error estándar de la media. Para la facilidad de comprensión de los resultados, en muchos casos, estos valores han sido normalizados con el control. Y en otras ocasiones, el resultado ha sido expresado como el porcentaje de variación entre la situación control y la condición experimental o ha sido representados según el número total de células por cubre de 13mm que corresponde a un área de 132,66 mm^2 o por cubre de 12mm que corresponde a un área de 113,04 mm^2.

Los análisis estadísticos para realizar la comparativa entre las distintas condiciones experimentales se realizaron utilizando el software IBM SPSS Statistics v. 24.0. La homogeneidad de las varianzas se estudió mediante la prueba de Levene ($p < 0,05$). Para evaluar si existen diferencias significativas entre las distintas condiciones, en caso de comparar solo entre 2 medias se realizó un análisis no paramétrico U de Mann Whitney y en el caso en el que se querían comparar más de 2 medias, se realizó análisis ANOVA. En el caso de encontrar diferencias significativas, se realizó el test post-hoc Bonferri o Tukey y el test post-hoc Games-howell dependiendo de la homogeneidad de varianzas. Para todas las pruebas, el valor mínimo para establecer diferencias significativas se definió como $p < 0,05$ **(ANEXO 1-6)**.

Por otra parte, los espectros adquiridos del MALDI-IMS se alinearon para maximizar la correlación con el espectro promedio global y se normalizaron utilizando un software

específico (MSI Analyst, Noray Bioinformatics S.L.). Durante el análisis, el tamaño de los datos se redujo para eliminar todos los picos cuya intensidad era inferior al 0,5% del pico más alto del espectro, y los espectros se normalizaron utilizando un algoritmo de corriente iónica total. Los espectros también se alinearon utilizando el método Xiong y asumiendo una desalineación máxima de 0,02 a.m.u., lo cual es muy conservador para un analizador orbitrap. Para la representación gráfica, no se utilizaron algoritmos de interpolación o procedimientos de eliminación de ruido, siempre tratando de mantener el aspecto original de los datos.

El análisis estadístico para identificar las diferentes áreas en las secciones se realizó mediante la agrupación jerárquica divisiva a través de los análisis "Compete Rank" y k-medias. Después de determinar el grupo de células de Müller y las RGCs, se utilizó un análisis de componentes principales (PCA) y una ANOVA para identificar los lípidos específicos de cada tipo de célula tanto en las secciones de la retina y como en los microarrays **(ANEXO 2)**.

3.9 Otras técnicas

3.9.1 Análisis de citometría de flujo de los cultivos de células de Müller.

Para corroborar la pureza de los cultivos de células de Müller adultas se realizó un análisis de citometría de flujo para comprobar la expresión del marcador p75NTR. Después de siete días *in vitro*, las células de Müller se tripsinizaron y se lavaron con PBS, y después de una centrifugación a 1200 rpm durante 5 minutos, las células se resuspendieron en 1 ml de PBS, se fijaron y se permeabilizaron durante 1 hora a 4 ° C añadiendo etanol al 100% hasta alcanzar una concentración final de 70% de etanol. Luego, las células se lavaron y se resuspendieron en PBS + BSA (5 mg / ml) durante 30 minutos a temperatura ambiente para bloquear la unión no específica al antígeno, y luego se tiñeron durante la noche a 4 ° C con un anticuerpo anti-p75NTR en agitación. Después de lavar durante 1 hora a temperatura ambiente, se detectó el anticuerpo primario con un anticuerpo anti-conejo de cabra conjugado con Alexa Fluor 555 (Invitrogen, Eugene, Oregon, EE. UU.) Y las células se analizaron en un FACScan (Beckman Coulter Gallios, Brea, California, EE. UU.), para identificar las células positivas para p75NTR **(ANEXO 1)**.

3.9.2 Análisis del ciclo células de las células de Müller por citometría de flujo.

Los cultivos de células de Müller a 3 y 7 días *in vitro* se lavaron con PBS y EDTA al 0,02% y después se añadió tripsina al 0,025% durante 5 minutos a 37 ºC. Después de la centrifugación, las células se fijaron en etanol al 70% y se incubaron alícuotas de 1 x 10^6 células con yoduro de propidio (10 μg / ml) en presencia de RNasa A (250 μg / ml) durante 1 hora a 4 ° C. El ciclo celular se analizó luego en un FACScan (Beckman Coulter Gallios, Brea, California, EE. UU.) (**ANEXO 1**).

3.9.3 Preparación de muestras para MALDI-IMS.

Las secciones de la retina y los microarrays tanto de las células como de las membranas extraídas de los cultivos de las células de Müller y las RGCs, se descongelaron y se depositó sobre ellos DAN (1,5-diaminonaftaleno) utilizando un sublimador de vidrio (Ace Glass 8023). La matriz formó una capa delgada y uniforme que permite escanear las secciones de la retina y los microarrays en modo de ión negativo durante varias horas gracias a un analizador LTQ-Orbitrap XL (ThermoFisher, San José, CA, EE. UU.) equipado con un láser N2 (100 μJ de potencia máxima, tasa de repetición de 60 Hz) con una resolución espacial entre 15 y 25 μm **(ANEXO 2)**.

3.9.4 Ensayos de citoquinas multiplex

Para comprobar si las células de Müller eran capaces de secretar citoquinas en condiciones hiperglicémicas se cuantificaron las citoquinas presentes con un ensayo Q-Plex multiplex ligado a enzimas (Q-Plex Inflammation rat (9-Plex): Quansys Bioscience, Logan, UT, EE. UU.). Después de 6 días en cultivo, se recogió el medio condicionado (sobrenadante) para cada condición experimental, se filtró y se analizó la presencia de 9 citoquinas (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IFNγ y TNFα) en 150 μl de cada muestra se analizó en una placa Q-Plex de 96 pocillos de acuerdo con las instrucciones del fabricante. Los estándares se midieron por duplicado y las concentraciones de citoquinas se calcularon utilizando una curva estándar. Se evaluaron cuatro réplicas para cada muestra y se calcularon los promedios aritméticos. Los datos se normalizaron por 10.000 células **(ANEXO 5)**.

4. Resultados/Results

ANEXO 1

A Method to Isolate and Culture Adult Mammalian Müller Glia in order to Study Retinal Diseases. *Frontiers in Cellular Neuroscience*, 2019. (*Submitted*)

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Abstract

Müller cells are the predominant glial elements in the retina, extending vertically across this structure, and they fulfil a wealth support roles that are critical for neurons. Alterations to the behavior and phenotype of Müller glia are often seen in animal models of retinal degeneration and in retinal tissue from patients with a variety of retinal disorders. Thus, elucidating the mechanisms underlying the development of retinal diseases would help better understand the cellular processes involved in such pathological changes. Studies into Müller cell activity *in vitro* have been hindered by the difficulty in obtaining pure cell populations and the tendency of these cells to rapidly differentiate in culture. Most protocols currently used to isolate Müller glia use neonatal or embryonic tissue but here, we report an optimized devised protocol that facilitates the reliable and straightforward isolation and culture of Müller cells from adult pigs, rats and mice. In this protocol, the whole retina is digested with papain, and the homogenized cells are seeded in dishes coated with poly-L-lysine and laminin, to be cultured in DMEM with 10% FBS. Müller cells reach confluence after 7 days *in vitro*

and by immunostaining, we demonstrate that these cells expressed Müller cell-specific marker proteins like GFAP, glutamine synthetase, p75NTR and vimentin. The purity of the cultures was analyzed by flow cytometry and their proliferation kinetics were studied by Time-Lapse video. The protocol described here provides an efficient method for the rapid isolation of adult mammalian Müller cells, which represents a reliable platform to study therapeutic targets and to test the effects of drugs that might combat retinal diseases.

Introduction

The Müller cell is the predominant glial cell in the retina, representing 90% of the retinal glia. These cells are radially oriented cells that traverse the retina from its inner border to the distal end of the outer nuclear layer. They occupy an important position in the retina due to their role in connecting neurons and nerve fibers (Bringmann et al., 2006; Reichenbach and Robinson, 1995). Müller cells are born in the late stages of retinal histogenesis, when the majority of neuronal cell types have already been generated. This enables the Müller cells to establish an anatomical and functional link between retinal neurons and the compartments with which they exchange molecules, such as retinal blood vessels, the vitreous chamber and the sub-retinal space (Vecino et al., 2016).

The importance of Müller cells in the retina is such that in the last 20 years, research into these cells has increased substantially and they are now known to fulfil many crucial roles in the retina. Müller cells provide neurons with trophic substances and they remove metabolic waste, also playing a critical role in regulating the volume of the extracellular space, as well as maintaining ion and water homeostasis of the retina, and the blood-retinal barrier. They even release gliotransmitters and other neuroactive substances, regulating synaptic activity through neurotransmitter uptake and recycling, and providing neurons with neurotransmitter precursors. Müller cells are also important structural elements in the retina, buffering mechanical deformations in the tissue, and they guide light to the photoreceptors and (Reichenbach and Bringmann, 2010; Sarthy and Ripps, 2001). These cells modulate immune and inflammatory responses, and they are the first cells to respond to retinal insult, becoming rapidly activated in order to safeguard the retinal structure and immune privileges in the nervous tissue (Ash et al., 2014). Moreover, in response to infection and certain inflammatory scenarios, Müller cells undergo reactive gliosis. In addition, Müller glia offer neuroprotection to RGCs (Garcia et al., 2002; Ruzafa and Vecino, 2015), facilitating their sprouting and neurite regeneration through the secretion of specific factors (Ruzafa et al., 2018). They can also activate and protect photoreceptors (Del Rio et al., 2011), and if they cease to support retinal neurons, they contribute to neurodegeneration (Bringmann and Wiedemann, 2012; Pereiro et al., 2018). Müller cells are also considered to represent a potential source of adult stem/progenitor cells thus, they will have a major impact to

future cell-based therapeutic approaches in retinal degenerative diseases (Reichenbach and Bringmann, 2013; Vecino et al., 2016).

Although they are implicated in retinal diseases (e.g., glaucoma, diabetic retinopathy, age-related macular degeneration, retinitis pigmentosa, etc.) and neural regeneration, the heterogeneity between individual Müller cell types is not well understood. Much of the current information about Müller cells, their activity and dysfunction has been derived from animal models, and mainly from cultured Müller cells. However, while there are several protocols for the isolation of Müller cells from different mammalian species, these generally rely on the use of tissue from neonatal animals to get primary cell cultures (Table1), probably due to the loss of regeneration potential as mammals age (Fischer et al., 2002; Schafer and Karl, 2017). However, degenerative retinal diseases are more frequent in adults and consequently, *in vitro* model systems developed with adult cells would be more useful to investigate the physiological and pathological events that occur in the mature retina. Until recently, certain problems associated with primary Muller cell cultures limited their utility to study these cell's functions *in vitro*, not least the difficulty in obtaining pure cell populations free of contamination by astrocytes, microglia or neurons. Consequently, the majority of studies focusing on these cells have used permanent cell lines obtained through the immortalization of primary cells with viral oncogenes. Various Müller cell lines have been reported in the literature, including the rMC-1 Müller cell line from the adult rat retina (Sarthy et al., 1998), the first permanent Müller cell line, as well as the TR-MUL cell line (Tomi et al., 2003) and the spontaneously immortalized human Müller cell line (MIO-M1: (Limb et al., 2002). Although the use of these cell lines is widespread, some of them exhibit an undifferentiated phenotype and these cells may have lost the capacity to divide more than certain number of times. This may in part be due to the mutations or genetic alterations that lead to their immortalization or the transfection of the specific genes to achieve that state (Alge et al., 2006; Pan et al., 2009).

Some characteristic features of primary Müller cells may be altered or lost after 2 weeks in culture (Hauck et al., 2003; Merl et al., 2012). Indeed, changes in protein expression have been detected that were indicative of transdifferentiation toward a fibroblast-like phenotype between day 14 and day 21 in culture (Limb et al., 2002). These proteomic changes may be more dramatic in permanent cell lines and while the MIO-M1 line might exhibit progenitor characteristics, it may also express markers of post-mitotic

retinal neurons (Hollborn et al., 2011). Hence, primary cultures are considered by many researchers to be more physiologically similar to the cells *in vivo*, thereby representing a more suitable experimental model to reflect the *in vivo* state.

Species	Animal's age	Retina removed after digestion	Digestion Protocol	Substrate	Medium	Change of medium	Reference
Rabbit	N/S	No	Collagenase (4 mg/ml) + hyaluronidase 200 U/ml) + medium containing Ca^{2+}/Mg^{2+} 20´ + papain (26.4 U/ml, pH 6.5) 10´	N/S	N/S	N/S	(Trachtenberg and Packey, 1983)
Cat	Adult	No	Ca^{2+}/Mg^{2+}-free BSS + 0.5 mg ml Nagarse (Protease type XXVII) 37 °C, 30´.	0.1 mg/ml Poly-L-lysine.	DMEM with FCS	Every 5 days	(Lewis et al., 1988)
Rabbit	P6	No	0.125% trypsin/0.05% DNase room temperature 10´ and then 37 °C, 10´.	Poly-L-lysine (0.1 mg/ml) coated glass-coverslips	DMEM with FCS	N/S	(Scherer and Schnitzer, 1989)
Rat	P8-12	Yes	DMEM + 0.1% trypsin +70 UmL-collagenase, 37 °C for 60´	Untreated sterile glass coverslips	DMEM with 2 mM glutamine	After the first 6 days, replenished every 3-4 days	(Hicks and Courtois, 1990)
Human	19-88 years	No	CMF + 0.1% trypsin + 0,2% hyaluronidase + 4% chicken serum, 37 °C, 45´	N/S	DMEM and Ham´s F12 with FBS	Twice weekly	(Puro, 1991)
Rabbit	P3	No	Ca^{2+} free solution + 0.5 mg/ml papain, 35 °C, 35´	N/S	DMEM with 10% FBS	N/S	(Reichenbach et al., 1995)
Pig	N/S	No	10 ml L15 + 17 U/ml papain, 34 °C, 60´and L15 + 150 U/ml DNase, 34 °C, 30´	0.1 mg/ml monomeric type I Collagen	DMEM containing 20 mM Hepes and 10% FBS	N/S	(Guidry, 1996)
Rabbit	N/S	No	Papain (130 u/10 ml DMEM) + 1 mM EDTA+ 4.5 mg cysteine, 4 °C, 45´	N/S	DMEM with 10% FBS	Every 2-3 days	(McGillem et al., 1998)
Pig	Adult	No	10 ml L15 + 17 U/ml papain, 34 °C, 60´, L15 + 150 U/ml DNase, 34 °C, 30´ and Percoll density gradient.	poly-D-lysine (2 μg/cm^2) and laminin (1 μg/cm2)	DMEM with 10% FBS	N/S	(Garcia et al., 2002)
Pig	Adult	No	Papain (2.2 U) for 40 min at 37 °C and Percoll density gradient.	Plated directly onto cell culture plates (NUNC)	DMEM with 10% FCS	N/S	(Hauck et al., 2003)
Rat	P8-10	N/S	DMEM + 0.1% trypsin + 70 IU/ml collagenase, 37 °C, 30´	N/S	DMEM with 10% FBS	N/S	(Lamas et al., 2007)
Mouse	P12 and 4 weeks	No	Papain (180 units/mL) + DNase, 37 °C, 8–10 min.	N/S	NBA with 10% FBS, 1 mM L-glutamine, 1% N2 and EGF (100 ng/m)	N/S	(Ueki et al., 2012)
Mouse	P10 or P14	No	Papain for 45 min at 37 ºC	poly-D-lysine (50 mg/ml) and laminin (0.5 mg/ml).	DMEM/F12	N/S	(Schafer and Karl, 2017)

Table 1. Comparison of the Müller cells culture methods published for mammals. Abbreviations: FBS, fetal bovine serum; FCS, fetal calf serum; N/S, not stated; NBA, Neurobasal medium.

The protocols that currently exist to culture Müller cells offer little insight to allow their successful reproduction. Thus, we describe here a protocol that is fast, easy to reproduce, and that has been optimized for adult tissue from different mammalian species, thereby providing a valuable tool to study diseases involving Müller cells. Different enzymes for digestion, substrates and culture mediums have been compared in order to establish the optimal protocol. Using this culture method for adult Müller cells, their phenotypic characteristics can be readily characterized *in vitro*, including the specific Müller cell markers expressed. In conclusion, the protocol provides a useful method to culture adult mammal Müller cells, a model that can be used to analyze the behavior of these glial cells when subjected to certain stresses *in vitro*, mimicking certain retinal diseases with a view to understanding their etiology.

Materials and methods

Animals

Eyes from adult pigs were obtained from a local abattoir and transported to the laboratory in cold CO_2-independent Dulbecco's modified Eagle's medium (DMEM/-CO_2; Gibco-Life Technologies). Adult Sprague Dawley rat and adult BALB/c mouse eyes were obtained from animals reared at the University's animal house (University of the Basque Country, UPV/EHU). Animals were kept in standard housing conditions on a 12 hour light-dark cycle, with *ad libitum* access to food and water. Rats were humanely sacrificed by exposure to CO_2 and mice were sacrificed by cervical dislocation. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. The experimental protocol met European (2010/63/UE) and Spanish (RD53/2013) standards for the protection of experimental animals, and it was approved by the Ethical Committee for Animal Welfare of the University of Basque country.

Isolation and Culture of adult Müller Cells

Pig eyes were dissected within 1 to 2 hours of enucleation, and those of rats and mice immediately after enucleation. The retina was isolated in fresh DMEM/-CO_2 medium by circumferential section of the cornea and removal of the anterior chamber. Major

blood vessels were excised in the case of the pig retina and the retinas were then chopped up into small fragments. In order to establish the best protocol, different enzymes for digestion, substrates and culture media were tested. The retinas were incubated at 37 °C for 30 minutes in (1), a Sterile Earle´s Balanced Salt Solution (EBSS) containing Papain (20 U/mL) and DNase (2000 U/mL: Worthington, Lakewood, NJ, USA), or (2), a Trypsin-EDTA solution (0.25%: Life Technologies, Carlsbad, CA, USA). To stop the enzyme digestion, DMEM containing 10% FBS (fetal bovine serum) was added for 5 minutes at room temperature, and the tissue was then dissociated mechanically by careful homogenizing with pipettes of different tip sizes, recovering the cells by centrifugation at 1200 rpm for 5 minutes. The pelleted cells were re-suspended and cultured in three different media: (1) DMEM + 10% FBS; (2) DMEM-F12; and (3) Neurobasal A supplemented with B27 + 10% FBS (Life Technologies, Carlsbad, CA, USA).

The cells were seeded onto sterile 12 mm glass coverslips in 24 well plates, coated with poly-L-lysine (100 µg/ml: Sigma-Aldrich, St. Louis, MO, USA) alone or with laminin (10 µg/ml: Sigma-Aldrich, St. Louis, MO, USA), or left untreated .The amount of the cells seeded was: the circumference of a pig retina (8 mm diameter) per 4 wells; 1 rat retina per 8 wells and 1 mouse retina per well.

The cultures were maintained in a humidified incubator at 37 °C in an atmosphere of 5% CO_2, 95% O_2. The medium was totally replaced with fresh medium on day 1 of culture, by which time the Müller cells were mostly the cell type that had attached to the coverslips and thus, the other cell types were simply washed away with the debris facilitating the pure culture of Müller cells. Subsequently, the culture medium was changed every 3 days by replacing half the volume of the medium with fresh medium. These cultures reached confluency after 7 days in vitro (DIV).

Immunocytochemistry

After 7 days in vitro, the cells were washed in PBS (phosphate buffered saline, pH 7.0), fixed in methanol for 10 minutes at -20 °C and non-specific antigen binding was blocked for 30 minutes at room temperature with blocking buffer (0.1% Triton X-100 and 3% BSA -bovine serum albumin- in PBS). The primary antibodies used are shown in Table 2.

Table 2. Primary antibodies used.

Antigen	**Host**	**Dilution**	**Supplier**	**Ref.**
GFAP	Rabbit	1:1,000	Sigma-Aldrich, St. Louis, MO, USA	G9269
Glutamine Synthetase	Rabbit	1:10,000	Abcam, Cambridge, UK	Ab49873
p75NTR	Rabbit	1:2,000	Abcam, Cambridge, UK	Ab8877
Vimentin	Mouse	1:2,000	Dako, Glostrup, Denmark	M0725
CRALBP	Rabbit	1:2000	Abcam, Cambridge, UK	ab154898
Beta III-Tubulin	Rabbit	1:2000	Abcam, Cambridge, UK	Ab18207
Iba-1	Rabbit	1:1000	Wako, Richmond, VA, USA	016--20001

The antibodies were diluted in blocking buffer and incubated with the cultures overnight at 4 ºC. The cells were washed three times with PBS and the cultures were exposed for 1 hour at room temperature to the corresponding secondary antibodies at a dilution of 1:1,000 in PBS/BSA (1%): Alexa Fluor 488 and Alexa 555 conjugated goat anti-mouse and goat anti-rabbit antibodies (Invitrogen, Eugene, Oregon, USA). Following 3 washes of the cells, the coverslips were mounted with Fluor-save Reagent (Calbiochem, San Diego, CA, USA).

Flow cytometry analysis

After seven days *in vitro*, the cells were trypsinized and washed with PBS, and recovered by centrifugation at 1200 rpm for 5 minutes. The cells were resuspended in 1 ml of PBS, fixed and permeabilized for 1 hour at 4 °C by adding ethanol 100% to a final concentration of 70% ethanol. The cells were then washed and resuspended in PBS + BSA (5 mg/ml) for 30 minutes at room temperature to block non-specific antigen binding, and they were then stained overnight at 4 °C with an anti-p75NTR antibody with shaking (Table 2). After washing for 1 hour at room temperature, the primary antibody was detected with a secondary Alexa Fluor 555 conjugated goat anti-rabbit antibody (Invitrogen, Eugene, Oregon, USA) and the cells were analyzed on a FACScan (Beckman Coulter Gallios, Brea, California, USA) to identify those positive for p75NTR.

Flow cytometry analysis of cell cycle profiles

Müller cell cultures at 3 and 7 DIV were washed with PBS and 0.02% EDTA, adding 0.025% trypsin for 5 minutes at 37 ºC. After centrifugation, the cells were fixed in 70% ethanol and aliquots of 1×10^6 cells were incubated with propidium iodide (10 μg/mL) in presence of RNase A (250 μg/mL) for 1 hour at 4 °C. The cell cycle was then analyzed on a FACScan (Beckman Coulter Gallios, Brea, California, USA).

Time-lapse analysis of Müller cell cultures

Time-lapse analysis of the proliferation kinetics in pig and rat Müller cell cultures was performed, studying several variables: (1) the time that the same cell takes to divide again, the time between divisions; (2) the time from prophase to cytokinesis, considered as the time that Müller cells take to divide; and (3) the percentage of Müller cells that divide in the same field. We analyzed each field over 8 hours as this is the shortest time required for a cell to divide, thereby avoiding counting the same cell twice. The analysis was performed by taking one frame every 10 min, over 72 hours from the 3rd to 6th day in culture, using a 20X objective and a Zeiss Axio Observer (Zeiss, Jena, Germany) coupled to a digital camera (Zeiss Axiocam MRM, Zeiss, Jena, Germany) controlled by the Zen software (Zeiss, Jena, Germany). During imaging, the cells were maintained in a PM S1 incubator (Zeiss, Jena, Germany) at 37 °C under a humidified atmosphere of 5% CO_2 in air. At least eight different fields from three coverslips were analyzed for each experimental condition and from three independent experiments on pig, rat and mouse cultures.

Quantification and statistical analysis of Müller cells

Müller cells were analyzed on an epifluorescence microscope (Zeiss, Jena, Germany) coupled to a digital camera (Zeiss Axiocam MRM, Zeiss, Jena, Germany). A mosaic of the entire coverslip was obtained with a 10X objective and once the mosaic was defined, the coverslip surface area was calculated (132.73 mm^2). The semi-automatic Zen software (Zeiss, Jena, Germany) was used to count the total number of nuclei stained with DAPI, taking into consideration the limits of the axis of the nuclei of

Müller cells to achieve more accurate measurements. As such, we used a specific macro designed to measure the limits of the axes (10-40 μm), which was corrected manually for each image. At least three coverslips were analyzed for each experimental condition and from three independent experiments.

Müller cell density (cells per cm^2) and the parameters measured in time-lapse analysis were described as the mean and standard error of mean, and these parameters were compared between the different conditions. Statistical analyses were carried out using IBM SPSS Statistics software v.21-0 and the homogeneity of the variances was assayed with Levene´s test. A Mann–Whitney U test or ANOVA were used to assess whether there were significant differences between the groups. The minimum value of significance for both tests was defined as $p < 0.05$.

Results

Enzymes for digestion

In order to establish the best protocol to culture adult mammalian Müller cells, we evaluated different parameters to define the best conditions for Müller cell survival and proliferation. To initially digest the tissue, we evaluated the benefits of using two different enzymes, papain or trypsin. Following digestion of the tissue with either of these enzyme and mechanical dissociation, the total number of Müller cells in culture was analyzed after 7 DIV. Accordingly, the number of viable Müller cells in culture was seen to be significantly higher after digesting the retina with papain (30,233.67 ± 6,697.33 Müller cells/cm^2) than with trypsin (14,083.59 ± 2,635.36 Müller cells/cm^2: Figure 1).

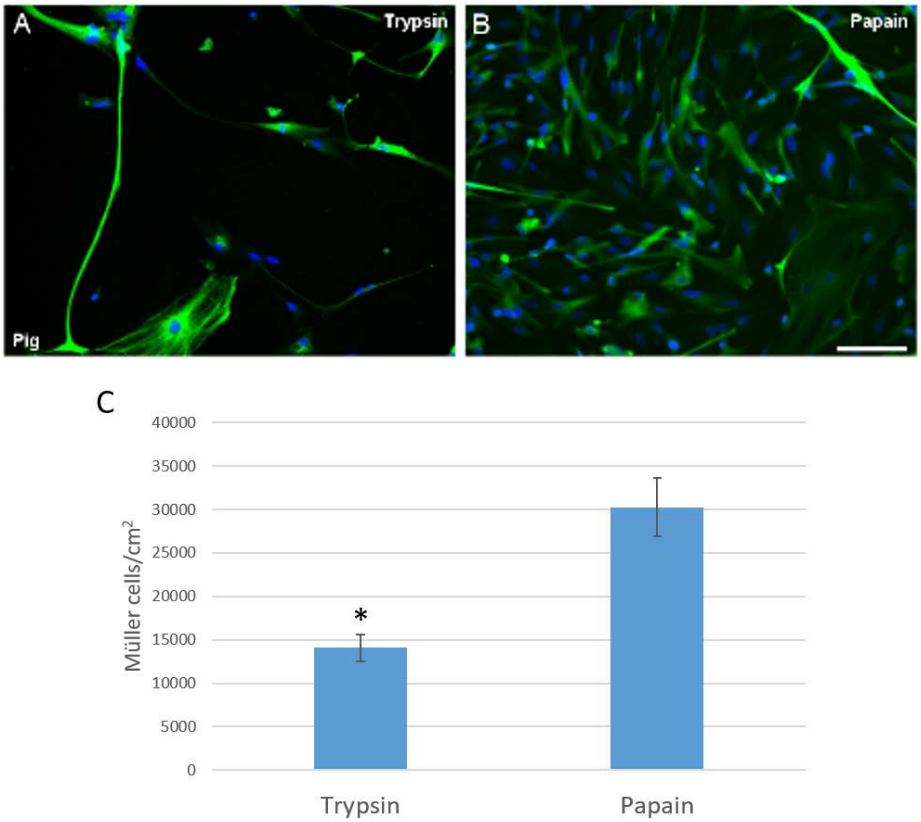


Figure 1. Analysis of Müller cell cultures derived from retinas digested with papain or trypsin. Images from Müller cell cultures derived from retinas digested with trypsin (A) or papain (B). Müller cells are labelled with an antibody against GFAP (green) and the nuclei are stained with DAPI (blue). The histogram represents the analysis of the number of Müller cells after 7 DIV (C). The survival of Müller cells after 7 DIV increased significantly when the retina is digested with papain: *p-value < 0.05. Scale bar, 100 µm.

Substrates

The effect of plating the cells on different substrates was also analyzed to select that which favored the survival and proliferation of Müller glia. Using a combination of poly-L-lysine and laminin as a substrate, the Müller cells obtained after 7 DIV was (30,166.96 ± 6,697.33 Müller cells/cm^2) as opposed to that obtained when poly-L-lysine alone was used as the substrate (9,177.25 ± 5,592.40 Müller cells/cm^2) or when the cells

were grown directly on the glass coverslips (1,968.36 ± 1,199.96 Müller cells/cm^2: Figure 2).

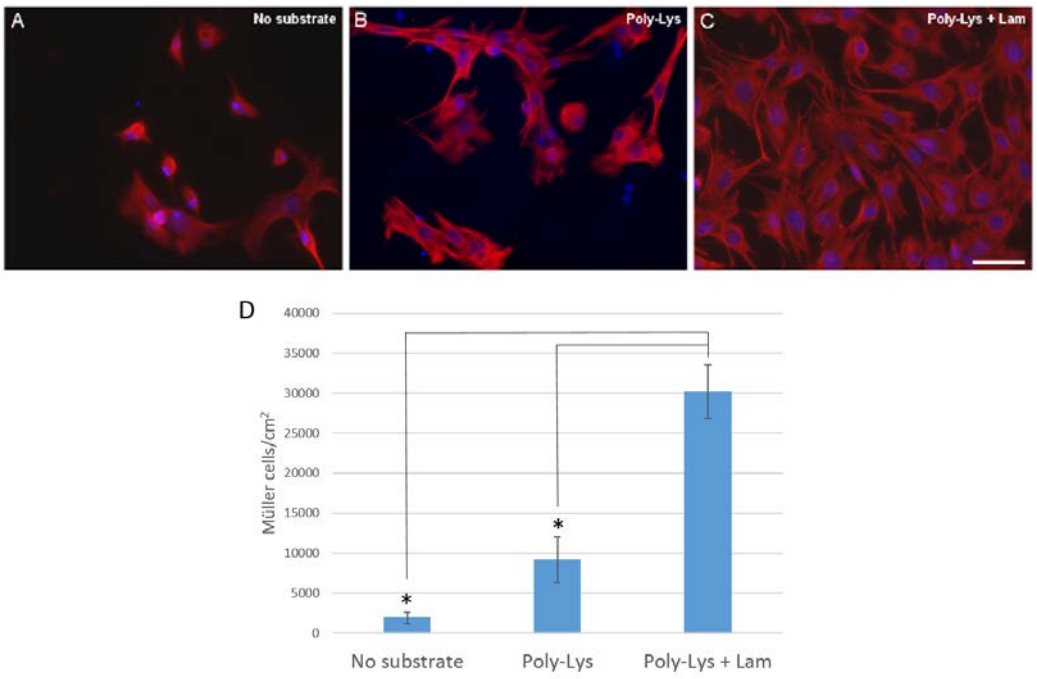


Figure 2. Analysis of the culture of Müller cells on different substrates: uncoated coverslips, poly-Lys (poly-L-lysine) and poly-Lys + Lam (laminin). Images of Müller cells growing on different substrates: uncoated coverslips (A), Poly-Lys (B) and Poly-Lys + Lam (C). Müller cells were labelled with an antibody against vimentin (red) and the nuclei were stained with DAPI (blue). Using poly-lysine and laminin as a substrate the Müller cell number increased significantly compared to the cells obtained on poly-L-lysine alone or when the cells were cultured on uncoated coverslips, as represented in the histogram (D): *p-value < 0.05. Scale bar, 50 µm.

Culture media

The purity of the cultures and the number of Müller cells was analyzed when the cells were maintained in three different media: 1) DMEM + 10% FBS; 2) DMEM-F12; and 3) NBA/B27 + 10% FBS. As a result, the Müller cells reached confluence after 7 DIV in DMEM + 10% FBS (30,233.67 ± 6,697.33 Müller cells/cm^2), whereas at the same time fewer cells were found when they were grown in DMEM-F12 medium (6,552.97 ± 964.21 Müller cells/cm^2). When cultured in NBA/B27 + 10% FBS the number of

Müller cells obtained was still significantly lower (16,759.31 ± 4,946.93 Müller cells/cm^2) and the cultures were not pure, since a significant number of neurons were evident, mainly RGCs (Figure 3).

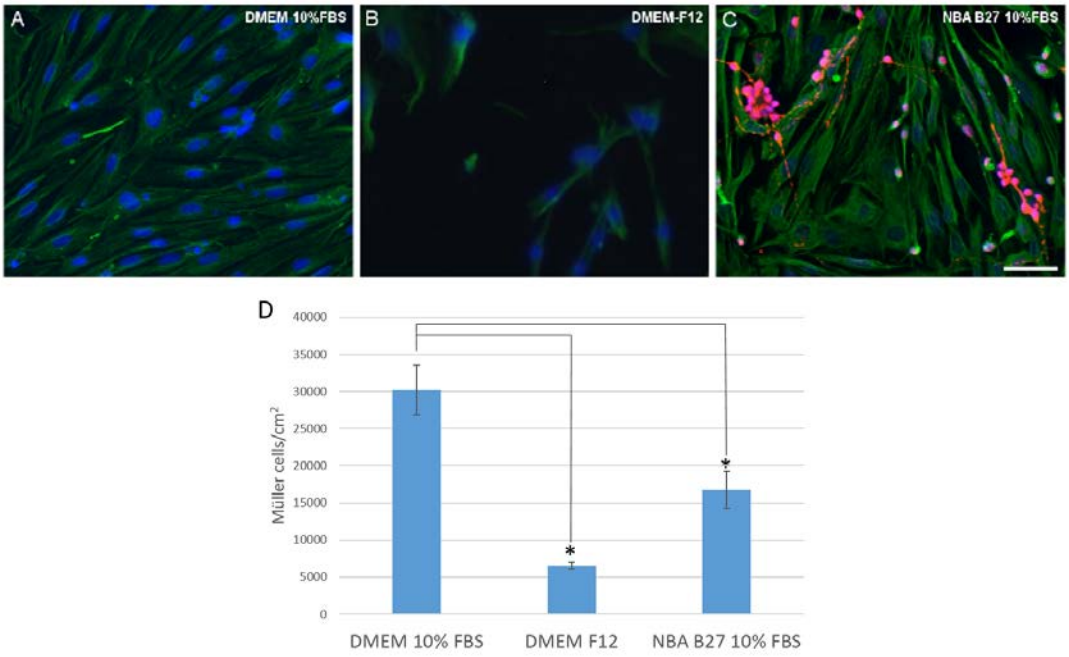


Figure 3. Analysis of Müller cells when cultured in different media: DMEM + 10% FBS, DMEM-F12, and NBA/B27 + 10% FBS. The purity and survival of the cells maintained in DMEM + 10% FBS (A), DMEM/F12 (B) or NBA B27 + 10% FBS (C) was analyzed. Neurons (RGCs) were labelled with an antibody against βIII-tubulin (red), Müller cells with an antibody against vimentin (green) and the nuclei were stained with DAPI. Note that the cultures maintained in NBA/B27 + 10% FBS were not pure (C). In DMEM + 10% FBS, Müller cells reached confluency more rapidly (A) and there were fewer Müller cells in the cultures grown in NBA + 10% FBS and DMEM-F12 at both time points, as seen in the histogram (D): *p-value < 0.05. Scale bar, 50 µm.

Having defined the optimal culture conditions, the expression of molecular markers of Müller cells was analyzed in the cultures of adult pig, rat and mouse retinas, specifically the expression of glutamine synthetase (GS), glial fibrillary acidic protein (GFAP), p75NTR and Vimentin (Figures 4, 5 and 6). The expression of the specific Müller glia markers was evident in each of the different animals after 7 DIV.

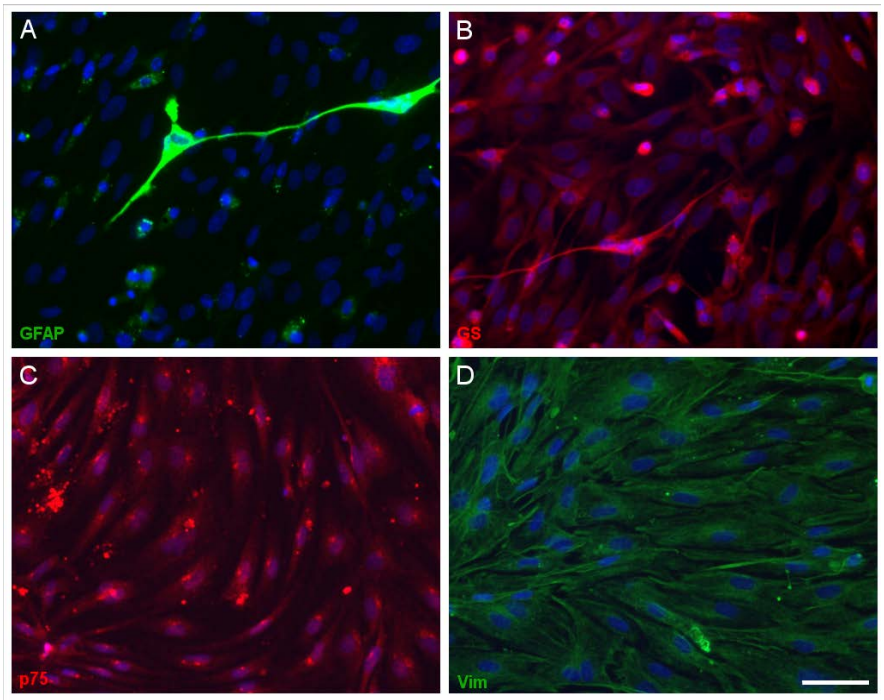


Figure 4. Expression of Müller cell markers in adult pig Müller cell cultures. Images of Müller cells labelled with antibodies raised against GFAP (green, A), glutamine synthetase (GS, red, B), p75NTR (red, C) and vimentin (green D): Scale bar, 50 µm.

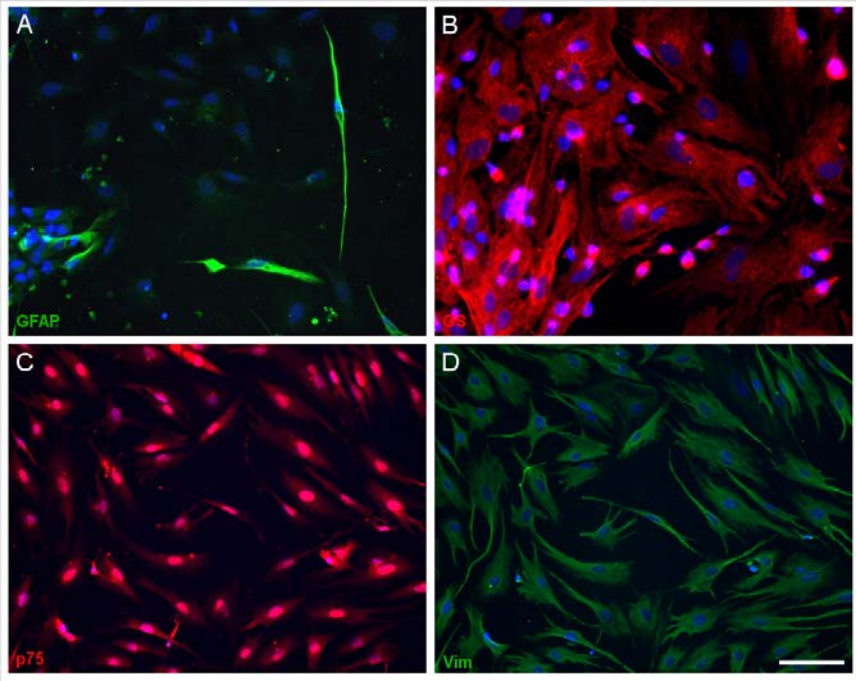


Figure 5. Expression of Müller cell markers in adult rat Müller cell cultures. Images of Müller cells labelled with antibodies against GFAP (green, A), glutamine synthetase (GS, red, B), p75NTR (red, C) and vimentin (green, D). Scale bar, 50 µm.

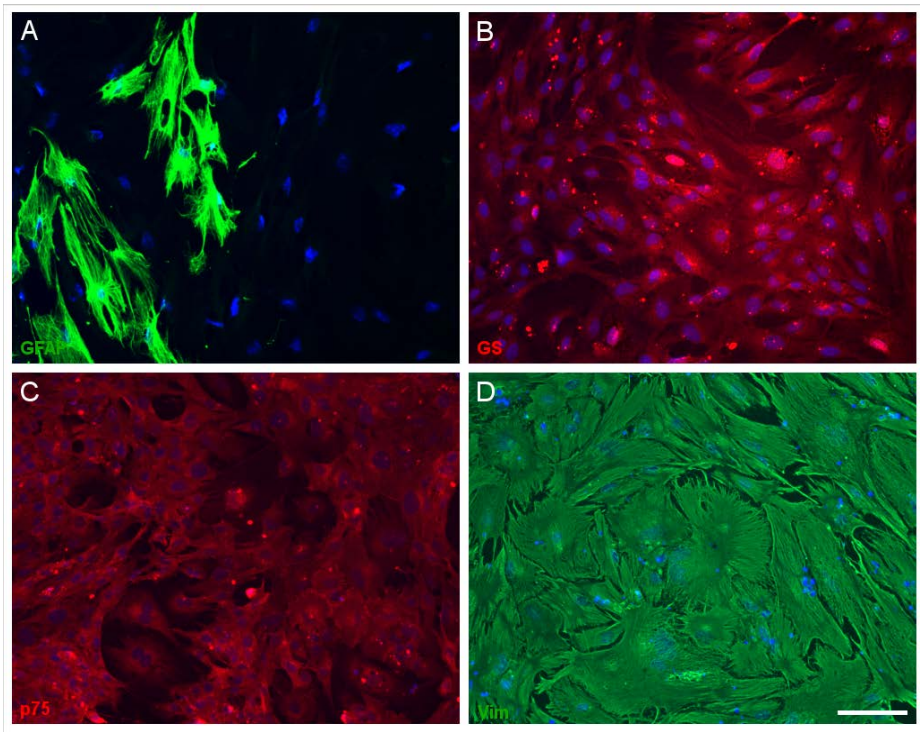
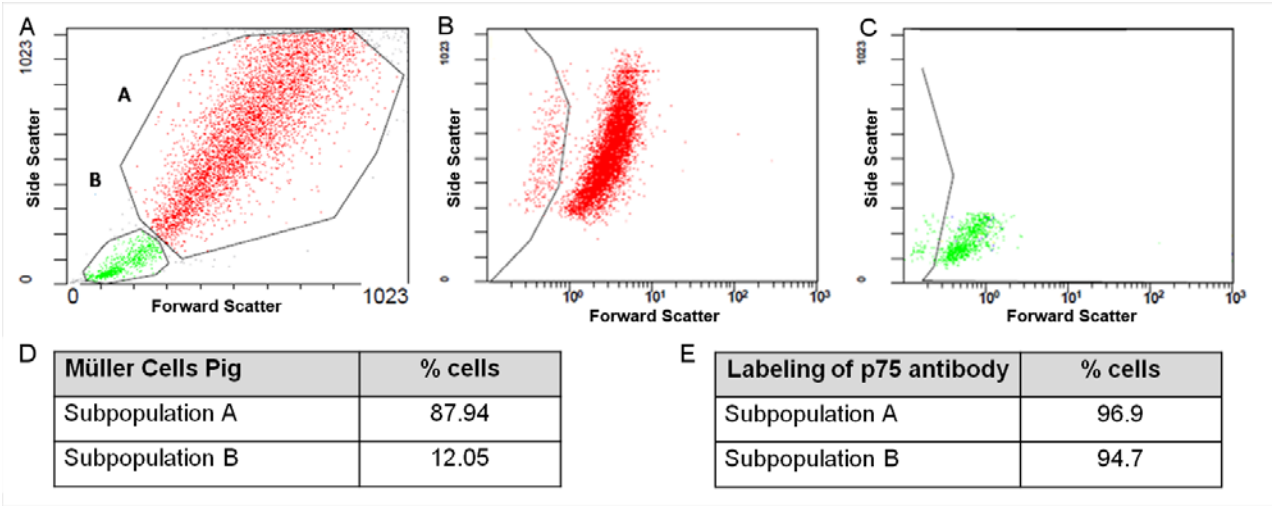


Figure 6. Expression of Müller cell markers in adult mouse Müller cell cultures. Images from Müller cells labelled with antibodies against GFAP (green, A), glutamine synthetase (GS, red, B), p75NTR (red, C) and vimentin (green, D). Scale bar, 50 µm.

To confirm the value of the protocol established to culture adult pig retinal Müller cells, the purity of these cultures was determined by flow cytometry. At 7 DIV, two subpopulations of Müller cells were evident (A and B) that were discriminated by their physical properties, since forward scatter can discriminate cells by size while the side scatter measurement provides information about their internal complexity (e.g., granularity: Figure 7 A, D). The purity of the cultures was assessed using an antibody against p75NTR, showing that 96.9% and 94.7% of the cells in subpopulations A and B were labelled for p75NTR, respectively (Figure 7 E).



D

Müller Cells Pig	% cells
Subpopulation A	87.94
Subpopulation B	12.05

E

Labeling of p75 antibody	% cells
Subpopulation A	96.9
Subpopulation B	94.7

Figure 7. Flow cytometry analysis of the purity of pig Müller cell cultures at 7 DIV. Note that two subpopulations of cells were identified based on their physical properties, A and B (A-C), with different proportions of cells in each subpopulation (D). Both the A and B subpopulations express p75NTR, confirming the purity of the cultures (E).

In addition, cell cycle FACS profiles of porcine Müller cell cultures were assessed after 3 and 7 DIV. The graphs illustrate the proportions of the cells in the different phases of the cell cycle, which correlates to the propidium iodide intensity. After 3 DIV, the cell cycle profile of the subpopulation A indicated that 75.8 % of the cells were in G0/G1, 11.1% in S phase and 7.7% in G2/M. In subpopulation B, 95.9% of the cells were in G0/G1, 0.8 % in S phase and 3.3% in G2/M at that time point. The cell cycle profile at 7 DIV showed that 91.3% of the cells in subpopulation A were in G0/G1, 3.5% in S phase and 5.1% in G2/M. Similarly, 95.8% of the cells in subpopulation B were in G0/G1, 0.7 % in S phase and 2.8% in G2/M (Figure 8, Table 3).

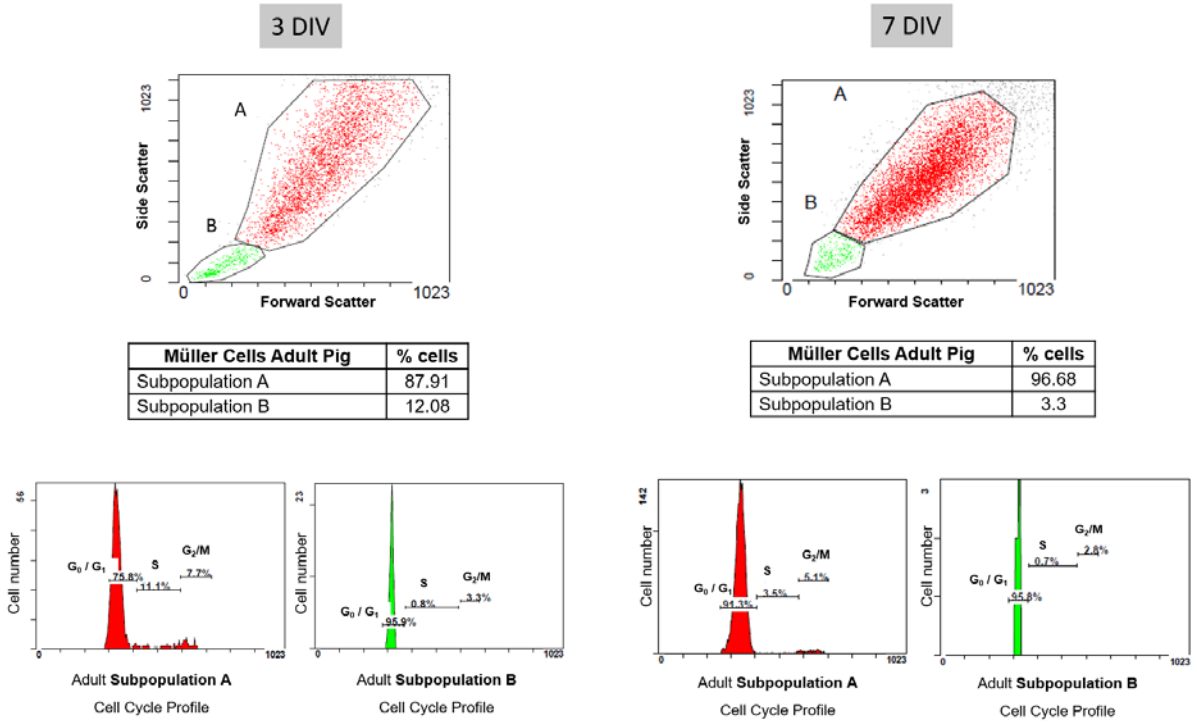


Figure 8. Cell cycle profile of pig Müller cells after 3 and 7 days in culture. The graphs illustrate the proportion of each subpopulation of Müller cells in the different phases of the cell cycle, reflected by the intensity of propidium iodide. Note that once the cells reached confluency (7 DIV), the cell cycle profile indicated there were fewer cells in S phase and G2/M in both subpopulations relative to the cultures at 3 DIV.

Table 3. Results from the cell cycle profile analysis of porcine Müller cell cultures.

	3DIV			7DIV		
	Total			Total		
A	87.91%			96.68%		
	G0/G1	S	G2/M	G0/G1	S	G2/M
	75.8	11.1	7.7	91.3	3,5	5.1
	Total			Total		
B	12%			3.3%		
	G0/G1	S	G2/M	G0/G1	S	G2/M
	95.9	0.8	3.3	95.8	0.7	2.8

A, subpopulation A; B, subpopulation B; DIV, Days in vitro.

The behavior of the Müller cells in culture was also analyzed using time-lapse video. In this time-lapse analysis of the proliferation kinetics of cultured pig, rat and mouse Müller cells, the time between divisions, the time that Müller cells take to divide and the number of Müller cells that divided per field was quantified. The time between divisions of Müller cells was 12.77 ± 3.14, 12.40 ± 2.78 and 13 ± 2.89 hours for pig, rat and mouse cultures, respectively. Regarding the time that Müller cells take to divide, this was quantified as 51.42 ± 11.08, 48.09 ± 12.49 and 42.38 ± 15.13 minutes in pig, rat and mouse cultures, respectively. Finally, the number of Müller cells that divided per field was analyzed every 8 hours, considering this to be the shortest time found between Müller cells divisions. The percentage of Müller cells that divided was 10.17 ± 3.22% for pig, 15.25 ± 7.9% for rat and 14.76 ± 5.75% for mouse Müller cells. Significant differences were not observed between these pig, rat and mouse Müller cell cultures (Figure 9).

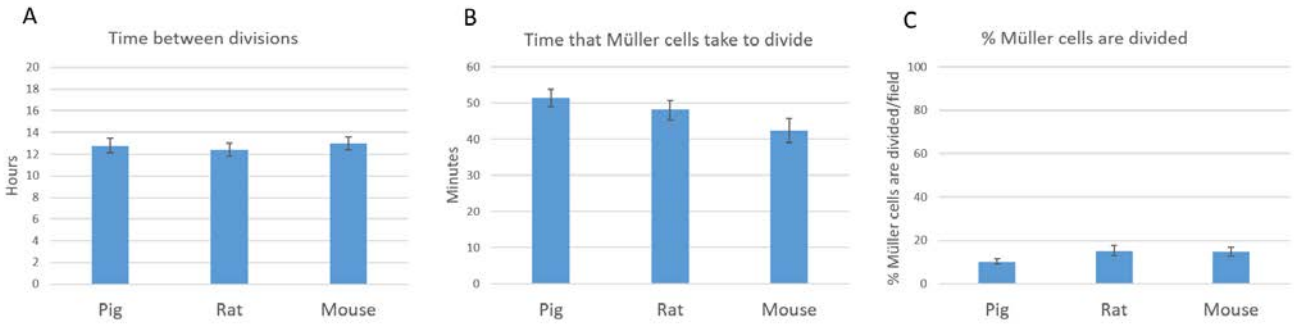


Figure 9. Time-lapse analysis of the proliferation kinetics of pig, rat and mouse Müller cell cultures. The time between divisions (A), the time that Müller cells take to divide (B) and the number of Müller cells that are dividing per field (C) were quantified and represented in graphs. No significant differences were observed between pig, rat and mouse Müller cell cultures: p-value > 0.05.

Discussion

We describe here a detailed protocol for the easy and reproducible isolation and culture of adult Müller cells. To find the best method to culture Müller cells, we systematically tested different elements in the protocol: the enzymes for digestion of the tissue, the substrates, and the culture medium. To date, the methods described in the literature focus on the isolation of the Müller cells from neonatal or very young animals. Here we focused our attention on obtaining pure primary adult Müller cell cultures, which we consider to be a more useful tool to study degenerative retinal diseases.

To determine the best method to culture adult Müller cells, we first assessed two different enzymes to digest the retinal tissue. As papain and trypsin have been commonly used in previous protocols (Table 1), both were tested over the same period of incubation. More Müller cells were obtained when retinas were digested with papain and these cultures reached confluence at 7 DIV. The cultures derived from trypsin digested retinas take longer to reach confluence, probably due to the lower number of cells that survived the digestion. Thus, we conclude that papain is a gentler enzyme for the digestion of the retinas than trypsin, consistent with reports that trypsin might be more toxic to central neurons than papain (Tabata et al., 2000).

Regarding the substrate for Müller cells, we cultured the cells on uncoated glass coverslips, or on coverslips coated with poly-L-lysine alone or in conjunction with laminin. As expected, the survival and proliferation of Müller cells was more optimal when they were grown on poly-L-lysine + laminin. Poly-L-lysine was tested as it is the most common substrate used in previous protocols (Table 1). Indeed, poly-L-lysine improves cell adherence due to the interaction between the positively charged polymer and negatively charged cells or proteins (Mazia et al., 1975). These data were consistent with our previous studies showing that poly-L-lysine and laminin is the best substrate for retinal cells, including Müller cells (Garcia et al., 2002; Ruzafa and Vecino, 2015; Vecino et al., 2015). Laminin exerts a variety of biological activities, not only mediating cell attachment but also, influencing cell proliferation, differentiation and motility (Paulsson, 1992). Indeed, the end-feet of Muller glial at the inner limiting membrane were found to be enriched in laminin-1 *in vivo* and *in vitro*, and laminin-1 promotes the motility of Muller glial cells (Mehes et al., 2002; Vecino and Kwok, 2016).

A crucial step in cell culture is the selection of the appropriate *in vitro* growth medium. A typical culture medium is composed of a complement of amino acids, vitamins, inorganic salts, glucose, and serum as a source of growth factors, hormones and attachment factors. Here we selected 3 different culture media based on those most commonly used in previous protocols: DMEM + 10% FBS; DMEM-F12; and NBA B27 + 10% FBS. DMEM-F12 is an extremely rich and complex medium that was designed to contain nutrients, growth factors and hormones instead of requiring a serum supplement (Mather et al., 1981) However, the number of Müller cells in the cultures at 7 DIV was significantly lower in this medium than when the cells were grown in DMEM + 10% FBS. It is known that the secretome of primary Müller cells in culture is influenced by the culture conditions (Ruzafa et al., 2018). Indeed, Müller cell proliferation is stimulated by numerous growth factors and cytokines derived from blood serum (Ikeda and Puro, 1994), which might explain the differences observed when serum-free DMEM-F12 was used as the medium. In the third media tested, NBA B27 + 10% FBS, pure cultures were not obtained due to the growth of neurons in the culture. In fact, Neurobasal-A is a basal medium that is designed to meet the specific requirements of neuronal cells in culture. Thus, it is clear of the media tested that DMEM + 10% FBS is the best option to obtain pure Müller cell cultures as this combination limits the appearance of neurons in the culture while encouraging the proliferation of Müller cells.

In order to confirm that Müller cells retain their characteristics after 7 DIV, we analyzed the expression of known Müller cell markers in pig, rat and mouse cultures: GFAP (Lewis et al., 1988), glutamine synthetase (Mack et al., 1998), p75NTR (Schatteman et al., 1988) and Vimentin (Davidson et al., 1990). We confirmed the expression of these molecular markers in our cultures of adult mammalian Müller cells, validating our protocol. However, while the presence of other glial cells like astrocytes cannot be completely ruled out, there were very few small DAPI stained nuclei in the cultures and nor was there any specific Iba-1 staining of microglia.

The purity of adult pig Müller cell cultures was confirmed by immunocytochemical and flow cytometry analysis. This easy, quick, robust and reliable technology serves to classify cell populations according to size, cytoplasmic complexity and the differential expression of surface, cytoplasmic and nuclear markers. Here we used the p75NTR marker detected by specific staining with fluorochrome-conjugated reagents (Othmer

and Zepp, 1992). Accordingly, two subpopulations of Müller cells were detected that were distinguished by their physical properties (A and B), although population (B) only represented 12.05% of the total Müller cell population on the 3rd DIV and 3.3% on the 7th DIV. These cells might correspond to cells that have just divided as they are smaller and less complex than the cells in subpopulation A, and most of them are in the G0/G1 phase of the cell cycle at both 3 and 7 DIV (95.9% and 95.8%). The heterogeneity of glial cells is widely known (Luna et al., 2010; Vecino et al., 2016) and hence, not all Müller cells in a retina may respond to a pathogenic stimulus in the same way. Indeed, these cells even have heterogeneous expression of proteins like GFAP, possibly due to the distinct roles they fulfill in the retina (Bringmann et al., 2006). Hence, it is not surprising to find different subpopulations of Müller cells distinguished on the basis of their physical properties. It is noteworthy that the proportion of the A subpopulation of Müller cells in G0/G1 increases from 75.8% at 3 DIV to 91.3% at 7 DIV, and the proportion of Müller cells in S phase and G2/M was reduced. This fact suggests that by day 7 the Müller cell culture is static as it has reached confluency, with only a small proportion of dividing cells.

It is important to note that there were no significant differences in the pig, rat and mouse cultures of Müller cells in terms of proliferation kinetics, including the time between divisions, the time that Müller cells take to divide and the number of Müller cells that are dividing in each field. As such, it appears that our protocol for Müller cell culture is reproducible in different mammalian species. In addition, the analysis of the Müller glia cell profile obtained from pig retinas, 11.1% of cells in S phase and 7.7 in G2/M phase, are consistent with the time-lapse results obtained, in which 10.17% of Müller cells were dividing per field in an 8 hour timeframe. Moreover, the cell cycle profile of adult pig Müller cell cultures adopted the typical characteristics of cells from control primary cell cultures (Pozarowski and Darzynkiewicz, 2004).

In summary, we present here a reliable method to obtain and culture for porcine Müller cells from adult retinas, using papain as the enzyme to digest the tissue, poly-L-Lysine and laminin as the substrate, and DMEM + 10% FBS as the culture medium (Figure 10). Compared to other published protocols, that presented here yields more cells and it is less time-consuming. Indeed, as this method avoids problems of rejection, the cells cultured can be used to study the behavior of Müller cells *in vitro* and *in vivo*. The protocol can easily be adapted to other mammalian species, as seen in rats and mice. As

such, this method will help test the effects of drugs on adult Müller cells, assisting researchers in their efforts to study therapeutic targets for retinal diseases like glaucoma, diabetic retinopathy, age-related macular degeneration and retinitis pigmentosa.

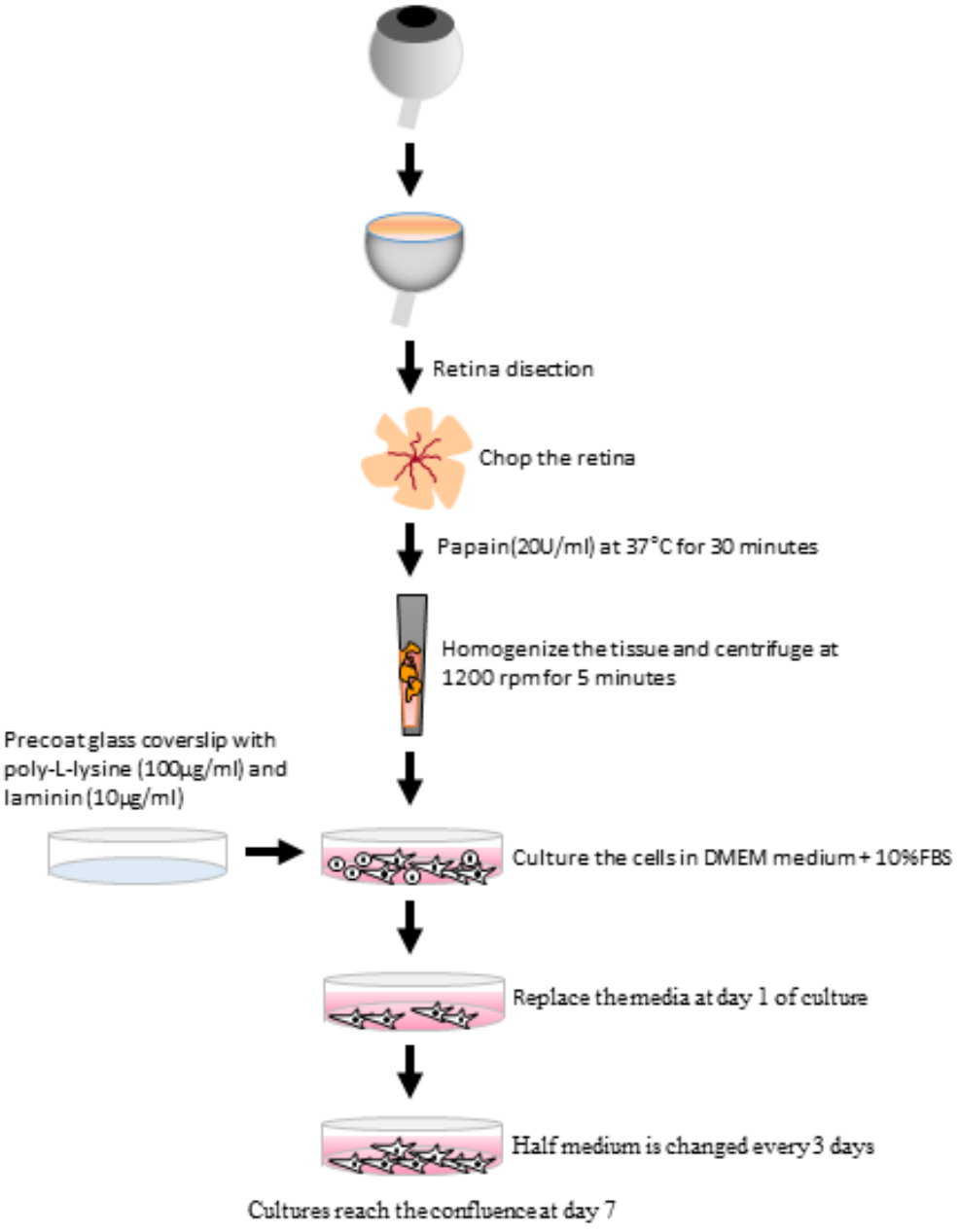


Figure 10. Scheme summarizing the main steps of the protocol to establish adult Müller cell cultures. The culture method for pig Müller cells from adult animals uses papain as the enzyme to digest the retina, poly-L-Lysine and laminin as the substrate and DMEM + 10% FBS as the culture medium.

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ANEXO 2

Comparative lipidomic analysis of mammalian retinal ganglion cells and Müller glia *in vivo* and *in vitro* using High-Resolution Imaging Mass Spectrometry. *Scientific Reports*. 2019. *(Submitted)*

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Abstract

PURPOSE: To establish the lipidomic bases to perform specific studies in ocular diseases by identifying the lipid signature of Müller glia and Retinal Ganglion Cells (RGCs) in retinal sections and primary cell cultures, as well as analyzing membrane microarrays established from these two cell types by Matrix Assisted Laser Desorption Ionization Imaging Mass Spectrometry (MALDI-IMS).

METHODS: Retinal sections, primary cultures of Müller glia and RGCs, and membranes extracted from these two cell types were studied. Isolated membranes and whole cells from primary cell cultures were printed onto glass slides using a non-contact

microarrayer (Nano Plotter). The LTQ-Orbitrap XL analyzer was used to scan the samples in negative ion mode and the RGCs and Müller cells were then identified immunohistochemically. The spectra acquired were aligned and normalized, and a statistical analysis was carried out to select the lipids specific to each cell type in the retinal sections and microarrays. The peaks of interest were identified by MS/MS analysis.

RESULTS: A cluster analysis of the MS spectra from the retinal sections identified regions containing RGCs and Müller glia, as confirmed by immunohistochemistry in the same sections. The relative density of certain lipids differed significantly (p-value $\leq$ 0.05) between the areas containing Müller glia and RGCs. Likewise, the *in vitro* analyses showed different densities of lipids between the RGC and Müller glia cultures. Finally, a comparative analysis of the lipid profiles in the retinal sections and microarrays suggests the presence of six peaks that correspond to a collection of 10 lipids characteristic of retinal cells. These lipids were identified by MS/MS.

CONCLUSIONS: The analyses performed on the RGC layer of the retina, on RGCs in culture and in cell membrane microarrays of RGCs indicate that the lipid composition of the retina *in vivo* is preserved in primary cell cultures. Specific lipid species were found in RGCs and Müller glia, allowing both cell types to be identified in assays using a lipid fingerprint. Further studies into these specific lipids and of their behavior in pathological conditions may well help identify novel therapeutic targets.

Introduction

The retina is a light-sensitive multi-layered tissue that lines the back of the eye and that is responsible for converting light into a nervous signal. Interactions between the neurons responsible for the communication between the eye and the brain, the retinal ganglion cells (RGCs), and the most abundant retinal glial cells, the Müller glia, are essential for the retina to remain functional. Müller cells span the entire thickness of the retina from the inner nerve fiber layer near the vitreous humor to the outer segment near the retinal pigment epithelium (RPE). These are specialized radial glial cells that constitute the physical and functional link between neurons and their environment, including blood vessels, the vitreous chamber and the sub-retinal space. This glia play a pivotal role in maintaining the structural integrity of the retina and they sustain retinal homeostasis by participating in essential processes like glucose metabolism, substrate exchange and vascular regulation. The RGCs are the output neurons in the retina, their axons constituting the optic nerve that carries the signals from the retina to the visual centers in the brain (Newman and Reichenbach, 1996; Vecino et al., 2016).

Lipids are fundamental constituents of the central nervous system (CNS) and defective lipid metabolism is related to a number of diseases of the brain and peripheral nervous system (PNS) (Trim et al., 2008). The activity of cells throughout the body is regulated by their plasma membrane, in which planar lipid microdomains known as “lipid rafts” (Singer and Nicolson, 1972) constitute dynamic platforms for multiple cell signaling events (Bevers et al., 1999), including the signaling that controls cell adhesion, cell migration, inflammation or immune reactions (Gambert et al., 2017). There are many unique lipids in the retina that play a fundamental role in retinal function and disease. For example, Polyunsaturated Fatty Acids (PUFAs) with Docosahexaenoic acid (DHA) (22:6, 22 carbons with 6 double bonds) are representative of the retina, accounting for approximately 50% of the fatty acids in photoreceptors(Fliesler and Anderson, 1983). This large amount of DHA results in highly fluid membranes, favoring efficient conformational changes during phototransduction. Moreover, DHA can be converted into neuroprotectin D1, which is involved in cell protective, anti-inflammatory and pro-survival repair signaling (Bazan et al., 2010; Bazan et al., 2011; Jastrzebska et al., 2011).

Another important lipid in the retina is cholesterol, the second most abundant lipid in the neuroretina after phospholipids (Bretillon et al., 2008). Free cholesterol plays key

roles in the modulation of ion channels, vesicle cycling and dendritic spine development (Dietschy and Turley, 2004; Gambert et al., 2017; Marquer et al., 2011), and in the retina, Müller glia represent the principal hub for the *de novo* production and transport of cholesterol (Fliesler and Bretillon, 2010; Jo et al., 2015). The access of Müller glia to the RGCs, astrocytes, pericytes and endothelial cells that form the neurogliovascular unit enables them to control the transport of ions, water, lipids and protein across the inner blood-retina barrier (Lakk et al., 2018; Reichenbach and Bringmann, 2010). Thus, manipulating the lipid microenvironment modulates the sensing of different stimuli by retinal Müller glia (chemical, osmotic and temperature), thereby contributing to retinal pathologies (Lakk et al., 2017). In fact, altered cholesterol levels underlie debilitating, blinding neurodegenerative diseases like Smith-Lemli-Opitz and Niemann-Pick Syndromes, diabetic retinopathy, glaucoma and macular degeneration, whereas animals fed cholesterol-deprived or cholesterol-enriched diets suffer a loss of neurons (Fliesler and Bretillon, 2010; Fliesler et al., 2007; Gambert et al., 2017; Lakk et al., 2018; Omarova et al., 2012). Moreover, it is known that there is a lipid shuttle from Müller cells to neurons in terms of covering the needs of neurons for lipids, especially in relation to the maintenance/renewal of the long projection axons of RGCs and for synaptogenesis (Mauch et al., 2001; Reichenbach and Bringmann, 2010). These examples show the importance of studying lipids in the retina, specifically those in Müller glia and RGCs.

Matrix Assisted Laser Desorption Ionization Imaging Mass Spectrometry (MALDI-IMS) is a technique that combines mass spectrometry and histology. MALDI-IMS technology has progressed rapidly over the past decade, with significant improvements in instrumentation (Chaurand et al., 2007; Jungmann et al., 2010), laser technology (Holle et al., 2006), sample preparation (Seeley et al., 2008; Yang and Caprioli, 2011) and bioinformatics analysis. These advances have increased the sensitivity of the technique, reduced the acquisition time and provided greater spatial resolution, thereby broadening the type of biological tissues and samples, and the features that can be analyzed (Zavalin et al., 2012).

The lipid distribution in retinal sections has been studied previously by MALDI-IMS on mouse (Hayasaka et al., 2008), salamander (Roy et al., 2011), pig tissues (Palmer et al., 2012) and human (Zemski Berry et al., 2014) tissue. Negative ion mode analysis of rat and human retinal tissue was performed using chloroform/methanol extraction of

homogenized tissue, followed by liquid chromatography–mass spectrometry (LC-MS) to identify retinal lipids (Ford et al., 2008). However, the homogenization of the tissue in this study meant no information on the spatial distribution of these lipids could be gained relative to the cell types in the retina. The spatial distribution and identities of lipid and retinoid metabolites are characteristic of specific retinal cell layers, as seen using high spatial resolution MALDI-IMS to analyze the Abca4$^{-/-}$mouse model of Stargardt disease. However, these studies mainly focused on the lipids in the photoreceptor layer in the retina and in the RPE (Anderson et al., 2014), leaving an important gap that this study attempts to fill.

Although lipids are considered important causative and diagnostic molecules for retinal diseases, there is little known about the lipid profiles in the normal retina and the lipid differences between the cells that compound the retina. To address this issue, we set out here to identify the lipid signature in sections of the porcine retina and Müller glia and RGCs primary cell cultures, using MALDI-IMS.

Here, we developed cultures of RGCs and Müller cells to generate whole cell and cell membrane microarrays, permitting multiple lipidomic studies to be performed on these samples (Anderson et al., 2014) and to compare the results achieved to the lipids obtained from retinal sections. The reproducibility of these microarrays is associated with the reduced noise and their sensitivity provides an accurate measure of the relative intensity of the lipids analyzed (Manuel et al., 2015). We use the porcine retina due to its similarity in size and structure to the human retina (Garca et al., 2005; Garcia et al., 2002; Veiga-Crespo et al., 2013), and since we have our model of glaucoma in pig displays the same pattern of neuron degeneration as humans (Ruiz-Ederra et al., 2005).

Material and Methods

Animals

Adult porcine eyes were obtained from a local abattoir and transported to the laboratory in cold CO_2-independent Dulbecco's modified Eagle's medium (DMEM-CO_2: Gibco-Life Technologies). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals from National Research Council (US). Moreover, all the experimental protocols met European (2010/63/UE) and Spanish (RD53/2013) regulations regarding the protection

of experimental animals, and they were approved by the Ethics Committee for Animal Welfare at the University of the Basque Country.

Retinal cultures

RGCs and Müller glia were isolated from adult pig eyes to establishing two types of culture: (1) RGC and (2) Müller cell cultures. The retinas were dissected out and dissociated enzymatically with papain (Worthington Papain Dissociation kit, Worthington Biochemical Lakewood, NJ, USA) for 90 min to obtain RGCs or for 30 min to obtain Müller cells, according to the manufacturer's instructions. The dissociated cells were recovered by centrifugation and the RGC cultures were prepared as described previously(Ruzafa and Vecino, 2015). Briefly, the dissociated cells were passed through an ovomucoid inhibitor-albumin gradient, where more RGCs than Müller cells pass due to their larger size (this step was excluded when preparing the Müller glia cultures). While this gradient does not purify RGCs to homogeneity, there is only minimal contamination of other cells. After purification, the cells were seeded in poly-L-lysine (10 µg/ml: Sigma–Aldrich, St. Louis, MO, USA) and laminin (10 µg/ml: Sigma–Aldrich, St. Louis, MO, USA) coated 6 well plates. The RGCs were seeded at 4 x 10^5 viable cells per well and the pure Müller cell cultures were established at 1.2 x 10^7 viable cells per well (as determined by trypan blue staining).

Different media were used for each culture type, containing 1% L-glutamine (2 mM: Life Technologies, Carlsbad, CA, USA) and 0.1% gentamicin (50 mg/ml: Life Technologies, Carlsbad, CA, USA): Neurobasal-A medium supplemented with 2% B27 (Life Technologies, Carlsbad, CA, USA) for RGC cultures; and DMEM with 10% FBS (Fetal Bovine Serum: Life Technologies, Carlsbad, CA, USA) for pure Müller cell cultures. The different conditions were employed from the start of the culture and the medium was changed every 48 hours. After 7 days in culture, the cells were trypsinized for 5 minutes at 37 ºC, centrifuged, resuspended in phosphate buffered saline (PBS, pH 7.4) and left at 4 ºC until use. At least 4 replicates of each culture type were performed in triplicate.

Immunocytochemistry of cultures

In order to check the quality of the cultures, at least 3 wells of each culture type were fixed in cold methanol and washed with PBS. After blocking non-specific antigens with

blocking buffer (3% BSA and 0.1% Triton X-100 in PBS), the cells were incubated with the following primary antibodies at a dilution of 1:2,000: a mouse anti-βIII-tubulin antibody (RRID:AB_430874, Promega Madison, WI, USA) as a specific RGC marker, and a rabbit anti-Vimentin antiserum (RRID:AB_1524552, Abcam, Cambridge, England) as a specific marker of Müller glia. After washing the cells, antibody binding was detected with an anti-mouse Alexa Fluor 488 and an anti-rabbit Alexa Fluor 555 (Life Technologies, Carlsbad, AC, USA) secondary antibody, diluted 1:1,000. In addition, the cell nuclei were labeled with DAPI (Life Technologies, Carlsbad, AC, USA) at a dilution of 1:10,000.

Cell membrane isolation

The cells isolated from pig Müller glia or RGCs cultures were homogenized using a Teflon-glass grinder (Heidolph RZR 2020) and a disperser (Ultra-Turrax® T10 basic, IKA) in 50 mM Tris buffer (TB) supplemented with 1 mM EGTA, 3 mM $MgCl_2$ and 250 mM sucrose. The crude homogenates were centrifuged at 40x g for 5 min and the resultant supernatants were centrifuged again at 18,000x g for 15 min (4 °C, Microfuge® 22R centrifuge, Beckman Coulter). The pellets were washed in 20 volumes of 50 mM TB and re-centrifuged under the same conditions. The protein concentration of each sample was measured using the Bradford method and aliquots of the homogenate were stored at -80 °C until use.

Microarray development

Microarrays were established from whole cells or membrane preparations printed onto glass slides using a non-contact microarrayer (Nano_plotter NP 2.1) and a printing solution(Rodriguez-Puertas and Barreda-Gomez, 2006). The piezoelectric tips dispense 4 nl per spot, printing each sample in triplicate. These microarrays were stored at -20 °C until use.

Tissue collection for retinal sections

Neonatal eyes were enucleated, removing the cornea, crystalline lens and vitreous humor. The retina was then carefully separated from the rest of the eye, cut into small rectangular pieces and folded back over itself like a “sandwich” before it was immediately frozen at -20 ºC. The retina “sandwich” was then partially embedded in

OCT medium to obtain cryosections of the aspect and not of the OCT embedded tissue (14 µm thick) that were stored at −80 °C in a N_2 atmosphere to protect them from oxygen and moisture degradation.

Sample preparation for IMS

Retinal sections and microarrays were thawed, and DAN (1,5-Diaminonaphthalene) (Thomas et al., 2012) was deposited on them using a glass sublimator (Ace Glass 8023) (Fernandez et al., 2014). The matrix formed a uniform thin layer that enables retinal sections and microarrays to be scanned in negative ion mode for several hours. A LTQ-Orbitrap XL analyzer (ThermoFisher, San Jose, CA, USA) equipped with an N2 laser (100 µJ max power, elliptical spot, 60 Hz repetition rate) scanned the material in negative ion mode at a spatial resolution between 15 and 25 µm. A mass resolution of 60,000 and 100,000 was used to record the data from the full scan spectra, and that of around 2,000 for MS/MS and MS^3 due to the higher sensitivity of the Ion Trap (IT). The scanning range in full scan spectra was 550-1,200.

Immunohistochemistry of retinal sections

The scanned sections were washed with methanol at room temperature (RT) for 5 minutes to remove the DAN and they were then fixed with 4% PFA (paraformaldehyde) at RT for 2 minutes. The sections were immunostained as described previously(Garcia et al., 2002), and after washing twice in PBS-Triton X-100 for 10 minutes, they were incubated overnight with the primary antibodies (both diluted 1:2,000): a mouse anti-βIII-tubulin antibody (Promega Madison, WI, USA) as a specific RGC marker, and a rabbit anti-Vimentin antiserum (Abcam, Cambridge, England) as a specific marker of Müller glia. After washing twice in PBS, antibody binding was detected for 1 hour with an Alexa Fluor 555 conjugated goat anti-mouse antibody (1:1,000, Invitrogen, Eugene, Oregon, USA) and an Alexa Fluor 488 conjugated goat anti-rabbit antibody (1:1,000, Invitrogen, Eugene, Oregon, USA) diluted in PBS-BSA (1%). Finally, the sections were washed twice with PBS for 10 minutes and mounted with a coverslip in PBS-Glycerol (1:1).

Data and statistical analysis

The spectra acquired were aligned to maximize the correlation with the overall averaged spectrum and normalized using dedicated software (MSI Analyst, Noray Bioinformatics S.L.). During parsing, the size of the data was reduced to eliminate all the peaks whose intensity was lower than 0.5% of the strongest peak on the spectrum, and the spectra were normalized using a total ion current algorithm (Deininger et al., 2008). Spectra were also aligned using the Xiong method (Xiong et al., 2012) and assuming a maximum misalignment of 0.02 a.m.u., which is very conservative for an orbitrap analyzer. For the graphical representation, no interpolation or smoothing algorithms, or de-noising procedures, were used, always trying to maintain the original aspect of the data. Re-scaling was required for some images of the distribution of the very weak peaks.

Statistical analysis to identify the different areas in the sections was carried out using divisive hierarchical clustering with Rank Compete (Cao et al., 2012) and k-means (Arthur and Vassilvitskii, 2007). After determining the cluster of Müller cells and RGCs, a principal component analysis (PCA) (Wold et al., 1987) and ANOVA were used to identify the specific lipids of each type of cell in the retinal sections and microarrays.

Peak assignment

The peaks of interest were identified by MALDI-MS/MS and MS^3 directly on the tissue samples to discriminate chemical variants of lipids with identical numbers of acylic carbons and double bonds. The assignment of lipid species was facilitated through a database created using tissue-dependent lipid composition. The experimental values of the precursor molecules and the fragments after MS/MS and MS^3 analysis were compared with this database, and with the Lipid MAPS (http://www.lipidmaps.org/) and Madison Metabolomics Consortium (http:mmcd.nmrfam.wisc.edu/) databases, using 0.005 Da as a tolerance window for the full scan precursors in order to identify each mass channel

Results

In order to identify the lipid patterns for RGCs and Müller cells, a strategy was developed that integrates *in vivo* and *in vitro* experiments within a lipidomics workflow (Figure 1).

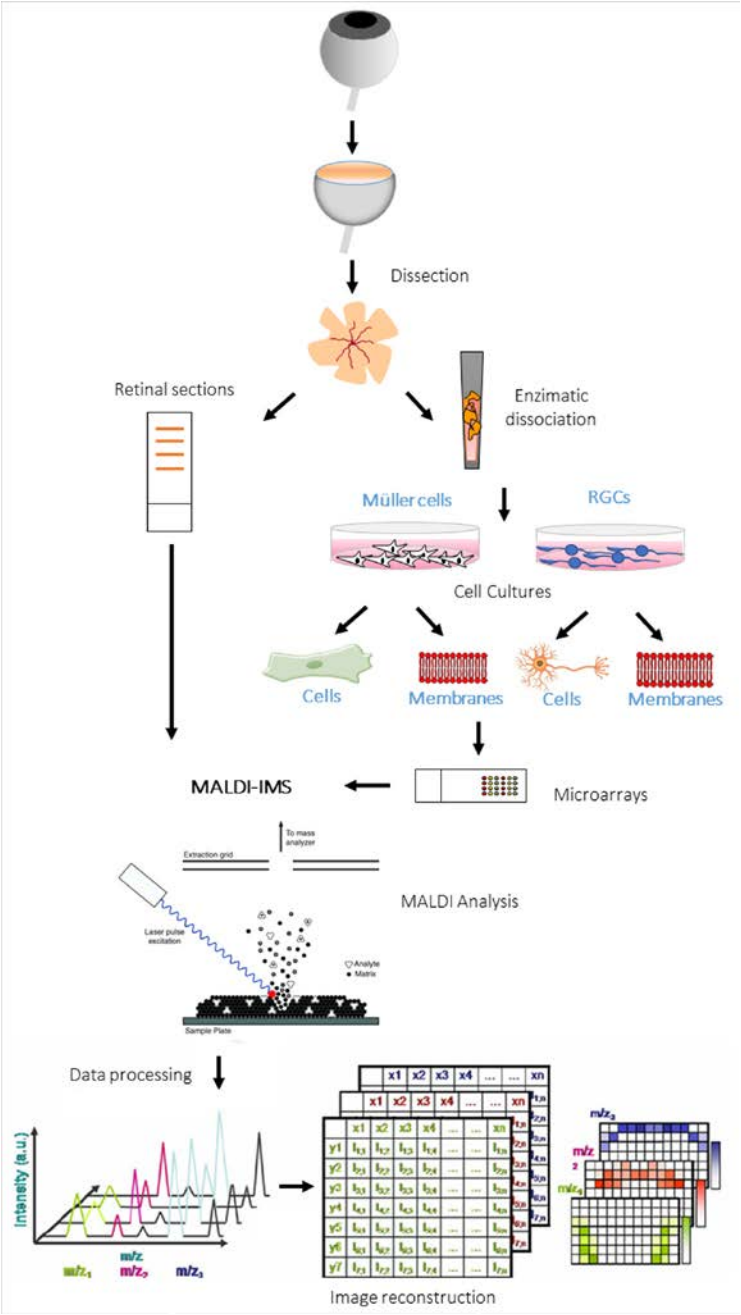

Figure 1: **Lipidomics work flow to identify the lipid signature of retinal sections, that of RGC and Müller cell cultures, and of cell membranes isolated from both cultures.**

The purity and quality of the cultures was verified prior to performing the lipidomic analysis by immunocytochemistry with antibodies that specifically recognized RGCs and Müller glia. After 7 days *in vitro* (div), Müller cell cultures were confluent, and RGCs had grown and regenerated their neurites (Figure 2A).

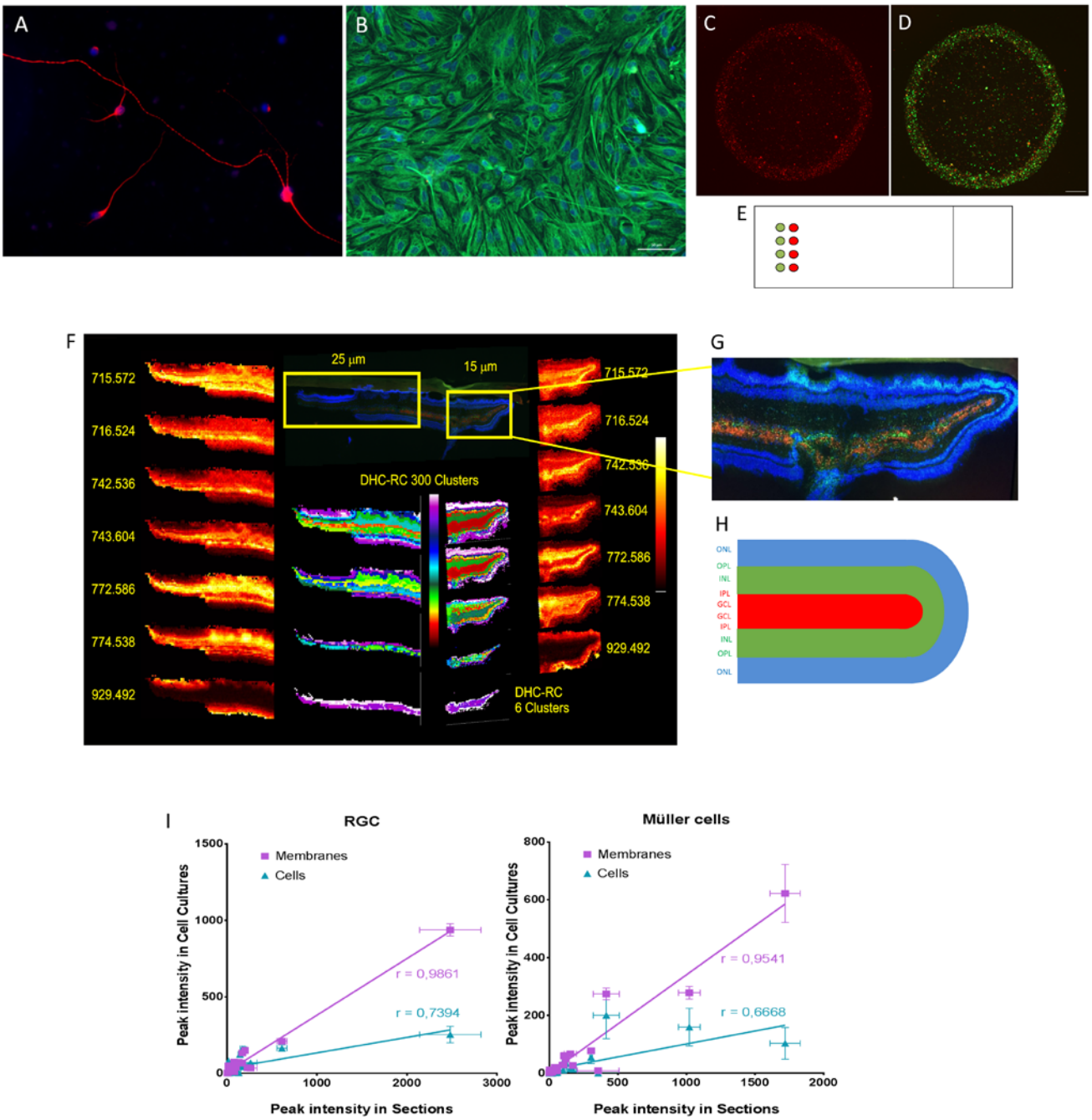

Figure 2: **Overview of different representative images for retinal cultures and sections and the correlation analysis obtained.** (A) Representative images of pig retina cultures, RGC stained with Beta III tubulin antibody (red) and (B) Pure Müller

cells culture stained with the vimentin antibody (green). Scale bar: 50μm. (C) Immunohistochemical analysis of cells, with RGCs stained with the Beta III tubulin antibody (red) and (D) Membranes from Müller cells culture stained with a p75 antibody (green). (E) Scheme of the microarrays. Scale bar: 100 μm. Representative images of pig retinal sections after MALDI-IMS analysis. (F) Cluster analysis of MS spectra showing several peaks corresponding to the area of RGCs (GCL and IPL) or Müller cells (INL and OPL). (G) Immunohistochemical analysis of RGCs labeled with the Beta III tubulin antibody (red), Müller cells labeled with the vimentin antibody (green) and nuclei stained in blue in a previously scanned retinal section. (H) Scheme showing the layer arrangement of the retinal sections. (I) Correlation analysis between the intensity of the peaks obtained from the cell cultures against those of the retinal sections, both for membranes and whole cells. RGCs (left) and Müller cells (right). Pearson correlation test using two-tailed P values. Nerve fibre layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL).

The data from the IMS analysis of the microarrays established from the Müller cell and RGC cultures were compared, highlighting significant differences in the relative lipid densities between these two cell types. Two different microarrays were prepared from the cultures for IMS analysis, one composed from collections of whole cells and the other from membrane preparations. The MALDI-IMS analysis highlighted the differential negative ions (m/z) and the normalized relative intensities obtained from whole cells or membrane preparations of cultured RGC and Müller cells (Table 1). Similar results were obtained for the whole cell and membrane preparations for each cell type, reflecting the consistency of the data.

Table 1. Summary of the differential negative ions (m/z) and the normalized relative intensity obtained after MALDI-IMS analysis of the microarrays prepared from whole cell or membrane preparations of RGC and Müller cell cultures.

	Membranes		Cells	
Ion	**Müller**	**RGC**	**Müller**	**RGC**
599,3243	9 ± 2	35 ± 4	1 ± 1	11 ± 3
700,5293	19 ± 3	41 ± 2	5 ± 3	21 ± 8
716,5261	49 ± 2	133 ± 4	34 ± 10	162 ± 16
722,5148	28 ± 5	69 ± 5	10 ± 10	43 ± 18
726,5467	5 ± 0	9 ± 1	1 ± 0	7 ± 0
738,5082	3 ± 1	15 ± 1	0 ± 1	10 ± 1
740,5246	7 ± 1	11 ± 2	2 ± 1	11 ± 2
742,5419	61 ± 6	150 ± 10	42 ± 17	158 ± 15
746,5735	67 ± 5	11 ± 2	15 ± 8	5 ± 1
764,5247	7 ± 2	70 ± 5	2 ± 2	57 ± 10
770,5726	59 ± 7	66 ± 4	45 ± 17	124 ± 13
771,6409	9 ± 2	11 ± 3	3 ± 0	14 ± 1
772,5878	274 ± 20	35 ± 2	200 ± 81	70 ± 11
774,5203	8 ± 2	3 ± 3	8 ± 0	7 ± 1
776,5616	26 ± 3	42 ± 3	13 ± 8	33 ± 11
790,5415	278 ± 22	211 ± 12	159 ± 65	165 ± 21
794,5725	77 ± 9	37 ± 1	54 ± 21	52 ± 6
797,6536	11 ± 1	49 ± 5	6 ± 1	81 ± 5
834,5309	8 ± 4	73 ± 7	0 ± 1	4 ± 1
836,5377	1 ± 0	20 ± 3	0 ± 1	4 ± 2
863,5688	4 ± 0	50 ± 4	0 ± 0	9 ± 0
885,5522	622 ± 100	938 ± 40	103 ± 55	254 ± 54
909,5504	20 ± 3	74 ± 5	3 ± 2	21 ± 4
913,5866	5 ± 1	14 ± 3	0 ± 1	4 ± 3

Following the MALDI-IMS analysis of retinal sections, a cluster analysis of the MS spectra identified regions that corresponded to the areas of RGCs and Müller glia, as confirmed by immunohistochemistry of the previously scanned sections (Figure 2F-G). The cluster analysis successfully identified the area that corresponded to RGCs (the ganglion cell layer, GCL) and the rest of the layers in the retina. Müller cells span the entire thickness of the retina, sharing their space with other cells and with RGCs. Therefore, the GCL area that corresponded to the RGC population (red) was compared with the area where Müller cells overlap with other cell bodies, the area that mostly corresponds to the inner plexiform layer (IPL, green). There was a significant difference in the relative density of certain lipids identified in the MS/MS analysis, (*p-value ≤ 0.05) between areas containing RGCs and those of Müller glia (Table 2).

Table 2. Summary of the differential negative ions (m/z) and the corresponding lipid species, together with the relative intensity, determined by MALDI-IMS analysis of the Müller glia and RGCs domains in retinal sections.

		Intensity	
Ion	Lipid Molecular Species	Müller	RGC
599,3243	Lyso-PI 18:0; PI O-16:0/2:0	47 ± 9	64 ± 9 *
700,5293	PE P-16:0/18:1; PE O-16:1/18:1	56 ± 10	99 ± 20 **
716,5261	PC 16:1/16:0; PC 16:0/16:1 PE 18:1/16:0; PE 16:0/18:1 PE 18:0/16:1	120 ± 14	171 ± 24 *
722,5148	PE P-16:0/20:4	100 ± 29	163 ± 42 *
726,5467	PE O-18:2/18:1; PE P-18:1/18:1	15 ± 5	33 ± 9 *
738,5082	PE 16:0/20:4	19 ± 4	35 ± 11 *
740,5246	PE 18:1/18:2	10 ± 3	19 ± 4 *
742,5419	PC 18:2/16:0; PE 18:1/18:1	110 ± 15	199 ± 19 ***
746,5735	PC 18:0/16:0; PE 18:0/18:0	153 ± 45	83 ± 21 *
764,5247	PE 18:1/20:4; PE 16:0/22:5	58 ± 5	122 ± 12 ****
770,5726	PC 18:1/18:1	108 ± 13	144 ± 19 *
771,6409	SM d22:0/18:1	7 ± 6	19 ± 4 *
772,5878	PC 18:1/18:0; PC 18:0/18:1; PC 20:1/16:0	415 ± 95	261 ± 72 *

774,5203	PS 17:1/18:0; PS 17:0/18:1	9 ± 2	5 ± 2 *
776,5616	PE O-18:1/22:5; PE P-18:0/22:5 PE O-18:0/22:6	171 ± 10	96 ± 10 ****
790,5415	PE 18:0/22:6	1021 ± 79	610 ± 55 ***
794,5725	PC 18:0/20:4; PC 16:0/22:4 PC 20:3/18:1	304 ± 10	233 ± 20 ***
797,6536	SM d42:2	3 ± 4	16 ± 3 **
834,5309	PS 18:0/22:6; PS 22:6/18:0	356 ± 152	131 ± 21 *
836,5377	PC [42:11]; PE [44:11]; PS O-[38:3] PS P-[38:2]	0 ± 0	22 ± 16 *
863,5688	PI 18:1/18:0; PI 18:0/18:1	21 ± 5	32 ± 7 *
885,5522	PI 18:0/20:4	1720 ± 110	2480 ± 341 **
909,5504	PI 18:0/22:6; PI 20:2/20:4	40 ± 12	78 ± 19 *
913,5866	PI 18:0/22:4; PI 22:4/18:0 PI 20:0/20:4	10 ± 7	26 ± 6 *

These data are the means ± S.E.M. of 4 retinal sections: ANOVA followed by Bonferroni's test or Kruskal–Wallis followed by Dunn's test.

To compare the results obtained from Müller cells and RGCs *in vivo* and *in vitro*, the correlation between the intensity of selected peaks obtained from retinal sections and their intensity in microarrays of whole cells or membrane preparations was performed. The intensities of the selected peaks from cultured RGCs and their membrane preparations were positively correlated (r = 0.7394 and r = 0.9861, respectively) with that from RGCs in the sections. In terms of the Müller glia in the sections and cultures, positive correlations were also found between the intensities of the selected peaks (r = 0.6668 whole cells and r = 0.9541 membrane preparations). These data confirm that the results obtained *in vivo* are similar to those from the *in vitro* experiments. It was notable that in both RGCs and Müller cells, the membrane correlation coefficient was stronger than that of the whole cells, such the peaks obtained from the membrane preparations are more similar to the peaks obtained from retinal sections (Figure 2I).

Finally, a comparison of the lipid profiles obtained from retinal sections and microarrays suggested a collection of six peaks (Table 3) characteristic of RGCs or

Müller glia in both retinal sections and cultures. These lipids were identified by MS/MS, detecting characteristic fragmentation spectra for all the peaks.

Table 3. MALDI-IMS analysis of retinal sections and cultures identifies selected lipids and the corresponding molecular lipid species that are more strongly represented in Müller cells or RGCs.

Ion	Molecular Lipid Species	Retinal sections		Retinal cultures				More intense in
		Intensity		Intensity				
		Müller	RGCs	Membranes		Cells		
				Müller	RGCs	Müller	RGCs	
794.5725	**PC 18:0/20:4; PC 16:0/22:4 PC 20:3/18:1**	304 ± 10	233 ± 20	77 ± 9	37 ± 1	54 ± 21	52 ± 6	Müller cells
790.5415	**PE 18:0/22:6**	1021 ± 79	610 ± 55	278 ± 22	211 ± 12	159 ± 65	165 ± 21	Müller cells
722.5148	**PE P-16:0/20:4**	100 ± 29	163 ± 42	28 ± 5	69 ± 5	10 ± 10	43 ± 18	RGCs
764.5247	**PE 18:1/20:4; PE 16:0/22:5**	58 ± 5	122 ± 12	7 ± 2	70 ± 5	2 ± 2	57 ± 10	RGCs
885.5522	**PI 18:0/20:4**	1720 ± 110	2480 ± 341	622 ± 100	938 ± 40	103 ± 55	254 ± 54	RGCs
909.5504	**PI 18:0/22:6 PI 20:2/20:4**	40 ± 12	78 ± 19	20 ± 3	74 ± 5	3 ± 2	21 ± 4	RGCs

Discussion

Lipid biochemistry in the human retina is remarkable from many points of view, not least for the specific accumulation of DHA (22:6) that is esterified to different phospholipid classes and molecular species within the retina. The lipids of retinal membranes have been studied extensively and much information has been obtained on the different lipid classes and on their fatty acid compositions (Fliesler and Anderson, 1983).

For the first time, this study combines *in vivo* and *in vitro* techniques to evaluate the specific lipids accumulated by Müller glia and RGCs. There is some evidence that certain lipids are specifically distributed in different retinal layers (Anderson et al., 2014; Zemski Berry et al., 2014). Given the importance of the differences between the different cells in the retina, defining the lipids that might be specific to individual cell

types, such as the RGCs and Müller glia studied here, may provide unique information regarding the homeostatic state of an organism and the development of diseased states. After a successful correlation analysis in which the results from *in vitro* experiments were easily extrapolated to *in vivo* results, a group of lipids were found in which four peaks were more strongly represented in RGCs and two that were more prevalent in Müller cells, both in sections and in microarrays.

The principal families of lipids in the retina are phosphatidylcholine (PC, ca. 40-50%), phosphatidylethanolamine (PE, ca. 30-35%), phosphatidylserine (PS, ca. 5-10%) and phosphatidylinositol (PI, ca. 3-6%), accounting for 85-90% of the total retinal phospholipids(Stinson et al., 1991). Therefore, it is not unusual that the peaks selected from the retinal samples belong to PCs, PEs and PIs. Phosphatidylcholines, are usually the most abundant glycerophospholipids (GPLs) and the key building blocks of membrane bilayers. They have a smaller head group to hydrogen bond through their ionizable amine group, which acts as a "chaperone" during membrane protein assembly to guide the folding of associated proteins. Using MALDI-IMS, four PCs were previously characterized in three distinct layers of a mouse retina section (PC 16:0/16:0, PC 16:0/18:1, PC 16:0/22:6 and PC 18:0/22:6) (Hayasaka et al., 2008), although none of them were specific to only one retinal layer. Our results found one mass channel (794.5754 Da), identified with MS/MS and MS^3 as a combination of three PCs, to be significantly more strongly represented in Müller cells than in RGCs in both the sections and cultures (**PC 18:0/20:4; PC 16:0/22:4 and PC 20:3/18:1).**

PEs are the second most abundant phospholipid in mammalian cells and they are extraordinarily abundant in the retina (Gugiu et al., 2006). PEs are enriched in the inner leaflet of membranes and they are particularly intense in the inner mitochondrial membrane. PEs are is also enriched in arachidonic acid (Martinez and Mougan, 1998) and they have quite remarkable activities: acting as a lipid chaperone that assists in the folding of certain membrane proteins; required for the activity of several respiratory complexes; and playing a key role in the initiation of autophagy (Patel and Witt, 2017). Our results confirm that certain PEs are more strongly represented in RGCs (**PE P-16:0/20:4, PE 18:1/20:4**; and **PE 16:0/22:5**). PE P-16:0/20:4 has been found in the mouse, rat and human brain(Han et al., 2001), it has been shown that individual GPL signatures in retinal cells, a subset of CNS cells, are similar to those of brain tissue (Hicks et al., 2006). PE 18:1/20:4 is present in both the optic nerve and retina, without

specifying cell type (Zemski Berry et al., 2014). Moreover, this lipid, with a high PE peak at 764 m/z corresponding to PE 18:1/20:4, might distinguish neural cells from non-neural cells (Li et al., 2007). According to our data and as found previously **PE 18:0/22:6** is more abundant in Müller glia, it is also specifically located in the outer retinal layers where Müller cells can be found (Zemski Berry et al., 2014).

PIs participate in essential metabolic processes, and they are primary sources of arachidonic acid and diacylglycerol. These second messengers serve as signaling molecules that regulate the activity of a group of related enzymes known collectively as protein kinase C, which in turn regulate many key cell functions like proliferation, differentiation, metabolism and apoptosis. We detected two peaks (*m/z* 885.55 and 909.55) that correspond to three PIs more strongly represented in RGCs, both in sections and microarrays. It is known that PIs are also important regulators of many ion channels and transporters, and that this regulation is central to neuronal excitability and synaptic transmission (Frere et al., 2012). Thus, these lipids more commonly represented in RGCs could be related to their neuronal activity. The distribution of the base peak at m/z 885.5 corresponded to **PI 18:0/20:4**, the former shown by MALDI-IMS in the nerve fiber/GC layer and inner nuclear layer of the mouse retina, spreading into the outer plexiform layer (Anderson et al., 2014), as well as in the optic nerve, retina and sclera (Zemski Berry et al., 2014). The peak *m/z* 909.5504 was identified as **PI 18:0/22:6** and **PI 20:2/20:4**, PIs that are more commonly found in RGCs. However, in literature these lipids are not as common as PI 18:0/20:4 to date, only **PI 18:0/22:6** has been found in the cod retina (Bell and Dick, 1990).

In summary, negative ion-mode imaging defines the spatial distribution of a number of lipid species, including PEs, PCs and PIs, representing the first comparative study between *in vivo* and *in vitro* assays. The combination of the techniques used provides high spatial resolution that enables lipid distributions to be identified, distinguishing specific retinal cell layers. The fact that there are lipids that are more characteristic of RGCs or Müller cells, belonging to some of the most relevant lipid families, suggests that they could fulfil a role in different cell activities. Interestingly, this technology could be used to compare healthy retinal tissue with pathological tissue in order to identify disease-related lipidomic changes, such as oxidation and age related glycation end products, and their spatial distributions. Thus, further studies will provide a more

information on the implications of lipids in retinal diseases, identifying new therapeutic targets to slow or prevent disease progression.

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ANEXO 3

Effect of Müller cells and their conditioned media on the survival of stem cell derived-RGCs. *(In preparation)*

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Abstract

Retinal neurons and particularly, retinal ganglion cells (RGCs), are susceptible to degeneration, either of an inherited cause or due to a wide variety of environmental insults, potentially leading to a loss of vision and blindness. Numerous strategies are being tested in different models of degeneration to try to replace the lost neurons and in recent years, stem cell technologies have opened promising avenues to obtain donor cells for retinal repair. Stem cell–based transplantation has most often been used in attempts to replace rod photoreceptors, yet the same tools could potentially be used for other retinal cell types, including RGCs. However, RGCs are not abundant in stem cell–derived cultures. Hence, to overcome this problem we have attempted to take advantage of the neuroprotective properties of Müller glia (the main glial cell type in the retina), and we have examined whether Müller glia and the factors they secrete could promote the differentiation of stem cells to RGCs. As such, stem cell derived-RGCs were co-cultured with adult Müller cells, or Müller cell conditioned media was added to these stem cells to evaluate its effects. RGC survival was substantially enhanced in both these cases, as witnessed by immunocytochemistry. The increase in the expression of Math5, a transcription factor expressed in retinal progenitors, was necessary for RGCs to develop, suggesting that Müller cells enhance the number of retinal progenitors that differentiate into RGCs. In conclusion, Müller cells and the factors they release promote stem cell derived-RGC survival and the differentiation of retinal progenitors into RGCs.

Introduction

Degenerative retinal diseases represent one of the main causes of irreversible vision loss. Indeed, retinal ganglion cell (RGC) death is a key aspect of several ocular diseases, including optic nerve injury, retinal ischemia and glaucoma, all of which can lead to loss of vision and blindness. It is therefore not surprising that the drive to find strategies for the functional replacement of retinal cells has intensified in recent years to combat these conditions.

Stem cells are functionally undifferentiated, immature cells, of a complex nature. These cells are capable of differentiating into different cell types and of generating populations these cell, indicating that they have the potential to repair tissue and restore function after lesion. Due to this potential, it is believed that stem cells may be able to either replace or repair damaged cells in the retina. Accordingly, the unique properties of stem cells has driven several studies into their potential therapeutic use in many diseases (Bennicelli and Bennett, 2013; Shintani et al., 2009; Siqueira, 2011; Zarbin, 2016).

In the past decade, the capacity to generate retinal cells from pluripotent stem cells using three-dimensional (3D) organoid cultures has become well established (Eiraku and Sasai, 2011; Eiraku et al., 2011; Nakano et al., 2012). This method is based on plating dissociated pluripotent cells onto special non-adherent U- or V-shaped plates. Since these cells cannot attach to the bottom of the plate, they attach to each other to form 3D aggregates called embryoid bodies (EBs). As this *in vitro* stem cell culture method mimics what occurs during normal retinal development *in vivo*, rod photoreceptors greatly outnumber the RGCs formed, as occurs in the mouse and human retina (Curcio and Allen, 1990). As such, the average yield of stem cell derived RGCs has been estimated to be between 0.1% and 30% of the cells in culture, similar to the normal percentage of RGCs in the retina at different developmental stages. In addition, the number of RGCs decreases over time in 3D cultures (Aparicio et al., 2017), as would also be expected given that newly born RGCs undergo two waves of programmed cell death during normal development as they become critically dependent on trophic support from their synaptic targets (Isenmann et al., 2003). Since the brain target of these cells (e.g., the lateral geniculate nucleus -LGN) is not present in the organoid cultures, Bax-mediated apoptosis is likely to be responsible for the temporal decline in RGC number.

Müller cells are the principal glial cells in the retina, and they can enhance the survival and neuritogenesis of RGCs in culture (Garcia et al., 2002; Kawasaki et al., 2000; Skytt et al., 2016). This support is preferentially activated by anatomic interactions, although it has been demonstrated that Müller cell conditioned medium (CM) can also significantly enhance the survival of cultured adult porcine RGCs (Garcia et al., 2002). Indeed, the neuroprotection afforded by Müller cell CM exceeds that observed in the retina itself (Reis et al., 2002) and accordingly, Müller cell-derived trophic factors appear to improve RGC survival and promote RGC neuritogenesis (Goldberg et al., 2002; Ruzafa and Vecino, 2015; Ruzafa et al., 2018).

Given the vast number of people who could benefit from RGC transplantation (Tham et al., 2014), it will be necessary to establish reliable and cost effective methods to obtain RGCs before their transplantation can be feasibly assessed in clinical trials. Thus, in an attempt to overcome the small number of stem cell derived-RGCs obtained from pluripotent cells and in light of the neuroprotection afforded to RGCs by Müller cells, this study evaluated the effects of co-culturing stem cell derived-RGCs and adult Müller glia, and how glial cell CM influences the survival of these stem cell derived-RGCs.

Materials and methods

Animals

Adult mice (*Mus* musculus, n=12) were used in these experiments, the animals having *ad libitum* access to food and water, and kept at a constant temperature of 21 °C on a 12 h light-dark cycle. The protocols for the use of animals were approved by the University of California Davis Institutional Animal Care and Use Committees.

Mouse ESC Culture

Mouse embryonic stem cells (mESCs, R1) were maintained in medium containing LIF+ 2 inhibitors (LIF+2i): ES qualified Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS: Invitrogen, Grand Island, NY), non-essential amino acids (NEAAs) (Invitrogen, Grand Island, NY), sodium pyruvate (Invitrogen, Grand Island, NY), 0.1 mM β-mercaptoethanol (Sigma Aldrich, Saint Louis, MO), 100 µl of leukemia inhibitory factor (LIF, 10 million units/ml: ESGRO, Millipore, Billerica, MA), 3 µM of a GSK3β inhibitor (Stemgent, Cambridge, MA) and

0.4 µM of a MEK inhibitor (Stemgent Cambridge, MA). All the cell lines were maintained in feeder-free conditions in growth factor-reduced Matrigel-coated plates.

Retinal Differentiation of mESCs

Retinal differentiation of mESCs was performed following a previously defined protocol, with minor variations (La Torre et al., 2012). Semi-confluent undifferentiated colonies were dissociated to a single cell suspension with TrypLE (Gibco, Rockville, MD) and gentle mechanical dissociation. Subsequently, 5,000 undifferentiated cells were plated in 96-well ultra-low attachment plates (Sbio, Hudson, NH) in retinal differentiation (RD) medium (Day 0): Glasgow minimum essential medium (GMEM: Thermo Fisher, Waltham, USA) supplemented with NEAAs, sodium pyruvate, Knock-Out Serum Replacement (KSR: Thermo Fisher, Waltham, USA) and β-mercaptoethanol (Sigma, Steinheim, Germany). In these conditions, floating mESCs spontaneously form aggregates or EBs in less than 12 hours. A further 24 hours later (Day 1), Growth Factor Reduced-Matrigel was added (2%) to each well and on day 4, the EBs were moved to ultra-low attachment six-well plates (Thermo Fisher, Waltham, USA) and the medium was replaced with 1.5 ml of fresh RD media and 0.5 of Tom´s media: Neurobasal A medium (Thermo Fisher, Waltham, USA), bovine serum albumin (BSA: Invitrogen, Eugene, Oregon, USA), B27 (Life Technologies, Carlsbad, CA, USA), N2, NEAAs, HEPES, sodium pyruvate and sodium bicarbonate. On day 5, the medium was replaced with 1 ml of RD medium and 1 ml of Tom´s medium, and on day 6, the medium was replaced with 0.5 of RD medium and 1.5 of Tom´s medium. Finally, on day 7 the medium was replaced with 100% Tom´s medium and the EBs were maintained until day 10.

Immunohistochemistry

In order to verify that RGCs were present 10 days after retinal differentiation, cryostat sections of whole EBs were immunostained overnight with a primary goat anti-Brn3a (1:1000, Santa Cruz, Dallas, USA) antibody and an anti-Beta III tubulin mouse monoclonal antibody (1:1,000, Sigma, Steinheim, Germany). After washing the sections twice in phosphate buffered saline (PBS), antibody binding was detected for 1 hour with an Alexa Fluor 555 conjugated anti-goat antibody (1:1,000, Invitrogen, Eugene, Oregon, USA) and an Alexa Fluor 647 conjugated goat anti-mouse antibody (1:1,000,

Invitrogen, Eugene, Oregon, USA) diluted in PBS-BSA (1%). The sections were again washed twice with PBS for 10 minutes and mounted in PBS-Glycerol (1:1).

Adult Müller cell cultures

Mice were sacrificed by cervical dislocation and their eyes were enucleated. The cornea, crystalline, lens and the vitreous were removed, and the retina was carefully extracted in fresh DMEM/-CO_2 medium. The retinas were then cut into small fragments and they were incubated at 37 °C for 30 minutes in a Sterile Earle´s Balanced Salt Solution (EBSS) containing Papain (20 U/mL) and DNase (2000 U/mL: Worthington, Lakewood, NJ, USA). Enzyme digestion was stopped by adding DMEM + 10% FBS for 5 minutes at room temperature and the tissue was then mechanically dissociated, recovering the cells by centrifugation at 1200 rpm for 5 minutes. The pelleted cells were re-suspended in DMEM + 10% FBS (Life Technologies, Carlsbad, CA, USA) seeded onto sterile 12 mm glass coverslips in 24 well plates, coated with poly-L-lysine (100 µg/ml: Sigma-Aldrich, St. Louis, MO, USA) and laminin (10 µg/ml: Sigma-Aldrich, St. Louis, MO, USA), and cultured in DMEM + 10% FBS (Life Technologies, Carlsbad, CA, USA). All the cells recovered from one mouse retina were seeded in each well. The cultures were maintained in a humidified incubator at 37 °C in an atmosphere of 5% CO_2 and 95% O_2. The medium was replaced with fresh medium on day 1 of culture and subsequently, every 3 days. These cultures reached confluence after 7 days in vitro (DIV).

Co-cultures of stem cell derived RGCs and Müller cells.

Day 10 EBs were collected and mechanically dissociated with accutase (Stem Cell Technologies), centrifuged at 1200 rpm for 2 min and resuspended in DMEM + 10% FBS. Dissociated EBs were seeded on a monolayer of Müller cells and maintained for 3 days in one of 3 different media: (1) NBA/B27, (2) NBA/B27 + 10% FBS, and (3) DMEM + 10% FBS. Dissociated EBs seeded onto sterile poly-L-lysine and laminin coated 12 mm glass coverslips in 24 well plates were used as controls.

Müller cell conditioned media collection

Conditioned media (CM) was collected when Müller cell cultures had reached confluence (day 7), first washing the wells three times with DMEM medium

supplemented with 1% L-glutamine and 0.1% gentamicin (Life Technologies, Carlsbad, CA, USA). DMEM was then added to each well and left for 3 h before the medium was changed to eliminate the rest of the FBS. Fresh DMEM was then added for 2 days before it was collected and sterilized by passing through a 0.22 μm filter. The CM was frozen in aliquots at −20 °C and the Müller cells that produced it were fixed for 10 min with methanol at −20 °C.

Application of conditioned media

EBs dissociated on day 10 were plated (10 EBS per well) on 13 mm poly-L-lysine and laminin coated glass coverslips in 24-well plates to test the activity of the CM. The cultures were maintained in a mixture of 50% NBA medium supplemented with 2% B27, 1% L-glutamine (2 mm: Life Technologies, Carlsbad, CA, USA) and 0.1% gentamicin, and 50% CM. The cells were cultured for 3 days at 37 °C in a humidified atmosphere containing 5% CO_2 and the medium was changed every 2 days. Cells maintained in NBA medium supplemented with 2% B27 and 1% L-glutamine were used as controls. The cells were fixed for 10 min with methanol −20 °C on day 3.

Undissociated 10 day EBs were also maintained in a mixture of 50% Tom´s media and 50% CM for 3 days, using EBs maintained in Tom´s medium alone as controls. The EBs were then collected for RNA extraction and at least three replicates of each culture were made, performing the procedure in triplicate.

Immunocytochemistry

After 3 days in co-culture or in the presence of CM, the cells were fixed in cold methanol (-20 ºC) and washed with PBS (pH 7.0). After blocking non-specific antigens with blocking buffer (3% BSA and 0.1% Triton X-100 in PBS), the cells were incubated with the following primary antibodies at a dilution of 1:2,000: a mouse anti-βIII-tubulin antibody (Abcam, USA) as a specific RGC marker, and a rabbit anti-Vimentin antiserum (Abcam, Cambridge, England) as a specific marker of Müller cells. After washing the cells, antibody binding was detected with anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 555 (Life Technologies, Carlsbad, AC, USA) secondary antibodies, diluted 1:1,000. In addition, the cell nuclei were labeled with DAPI at a dilution of 1:10,000 (Life Technologies, Carlsbad, AC, USA).

qPCR

Total RNA was extracted from the whole EBs maintained in CM using Trizol (Invitrogen) and chloroform extraction, according to the manufacturer's instructions. This RNA was digested with DNase-1 (Qiagen, Hilden, Germany), cleaned using the Qiagen RNA mini clean-up kit and reverse transcribed into cDNA using the Superscript III RT kit (Invitrogen) following the manufacturer's instructions. PCR was performed using the primers for Brn3a (forward 5′ CGT ACC ACA CGA TGA ACA GC 3′, reverse 5′ AGG AGA TGT GGT CCA GCA GA 3′) and Math5 (forward 5´ CCC TAA ATT TGG GCA AGT GAA GA 3´, reverse 5´ CAA AGC AAC TCA CGT GCA ATC 3´), normalizing the values to β-actin.

Quantification and statistical analysis of RGCs

RGCs and Müller cells were analyzed under an epifluorescence microscope (Leica Microsystems, Wetzlar, Germany) coupled to a digital camera (Leica Microsystems, Wetzlar, Germany). The total number of RGCs per coverslip area (113.04 mm^2) was quantified using the specific markers indicated. The RGCs and Müller cells in co-cultures were counted, and statistical analyses were carried out using the IBM SPSS Statistics software v.21-0 and the homogeneity of the variances was assayed with the Levene´s test. A Mann–Whitney U test or ANOVA were used to assess whether there were significant differences between the groups. The minimum value of significance for both tests was defined as $p < 0.05$. At least 4 complete coverslips and 3 independent experiments were analyzed for each experimental condition.

Results

Stem cell derived-RGCs were analyzed after EBs were collected on day 10. Upon differentiation, undifferentiated pluripotent stem cells can be directed toward a retinal cell fate following several steps that resemble normal developmental stages (Figure 1). These cells first develop into a neural epithelium that evaginates to form an optic vesicle–like structure and finally, an optic cup–like structure. Subsequently, the retinal progenitors can then differentiate into RGCs in these 10-day EBs.

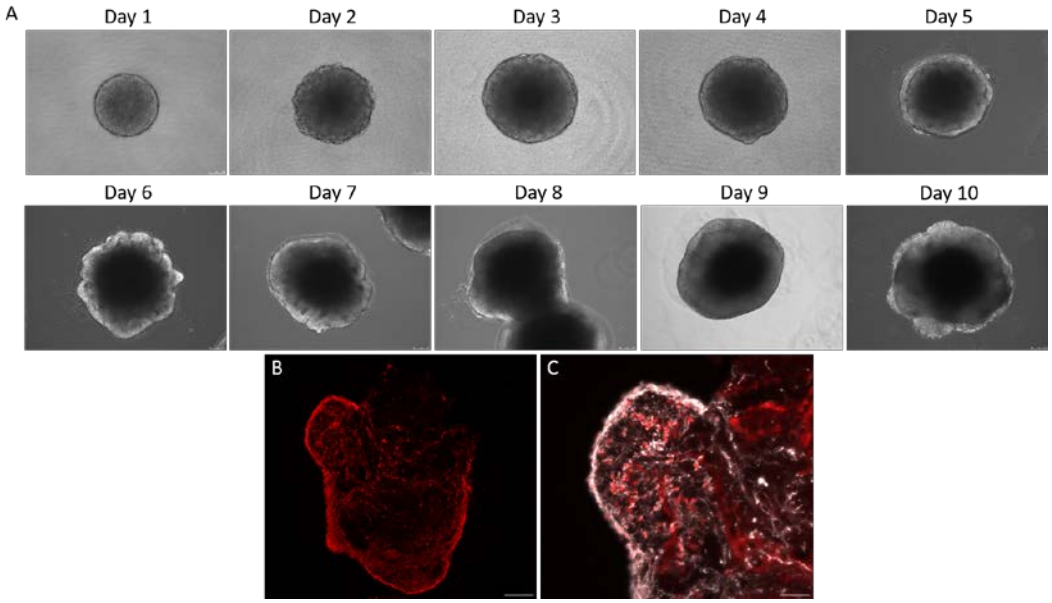

Figure 1. Typical morphologies of embryoid bodies (EBs) at the different stages of differentiation. (A) Different differentiation stages of EBs from day 0 to day 10. R1 mESCs were differentiated using the method described above and around each EB, a clear neuroepithelial layer develops from Day 3. From Day 5 this layer becomes more conspicuous, with optic vesicle and optic cup-like structures becoming apparent from day 6-8. Scale bar: 100µm, (B) A 10 day EB labeled for Beta III tubulin (red). Scale bar: 100 µm and (C) an amplification of an optic cup–like structure in the EB. Note that stem cell derived-RGCs are labelled with Beta III tubulin (white) and Brn3a (red) specific RGC markers. Scale bar: 50µm.

Dissociated EBs were co-cultured on a monolayer of adult mouse Müller cells for 3 days in 3 different media: NBA/B27 with and without 10% FBS (more favorable to neurons), and DMEM +`10% FBS (more favorable to glia). When stem cell derived RGCs were grown on Müller cells, their survival was enhanced in each of the media used relative to the control conditions in the absence of Müller cells: 778.22 ± 45.66% with NBA/B27 + 10% FBS medium, 987.61 ± 118.64 % with NBA/B27 medium, and 816.02 ± 43.43% with DMEM + 10% FBS medium (Figure 2).

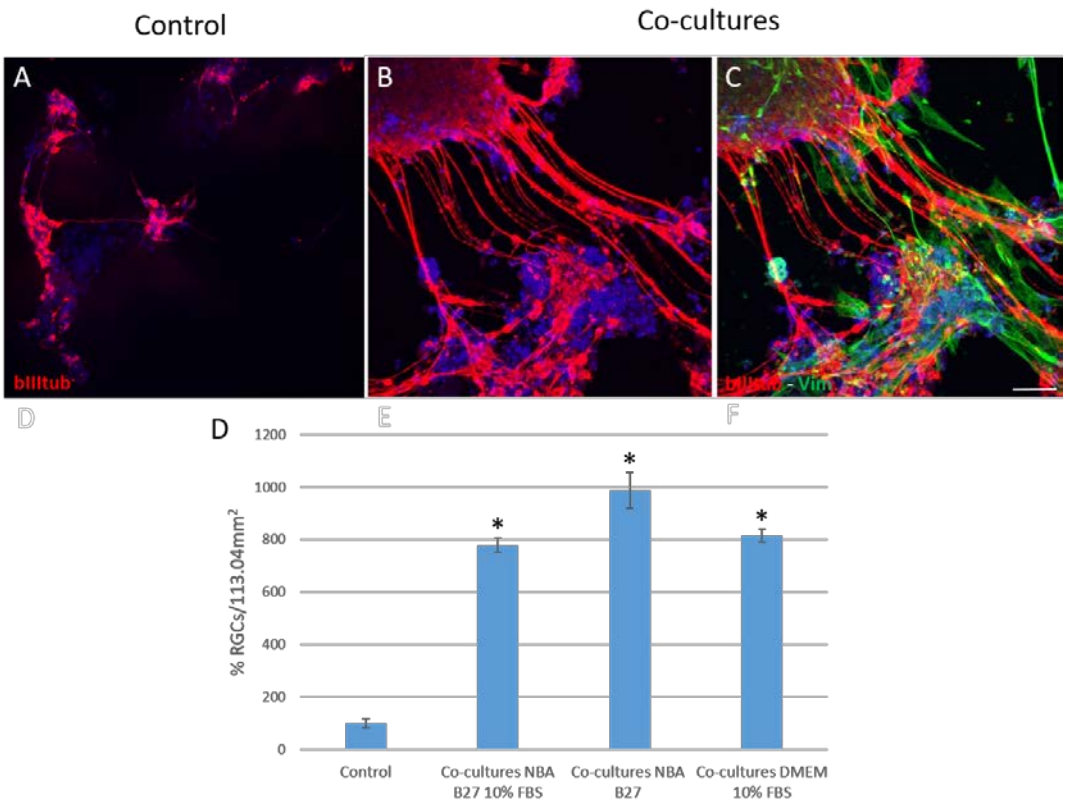

Figure 2. Effect of Müller cells on stem cell derived RGC survival. Images of stem cell derived RGCs in (A) control conditions or (B) and (C) when co-cultured with adult Muller cells. The cells were labeled with antibodies against β-III-tubulin (red) for RGCs, Vimentin (green) for Müller cells and nuclei stained with DAPI (blue). (D) The percentage of RGCs in control conditions or in the presence of Muller cells, cultured in NBA/B27 + 10% FBS, NBA/B27 and DMEM + 10% FBS: *p-value < 0.05 relative to the controls. Scale bar: 50 µm.

To analyze if the factors secreted by adult Müller cells have the same effect on the survival of stem cell derived-RGCs as in the co-cultures, dissociated EBs were cultured with adult mouse Müller cells CM. When RGCs cultured in NBA/B27 (control) were compared to those maintained in NBA/B27 medium: Müller cell CM (1:1), there were more RGCs when the cells were maintained in the presence of Müller cell CM. Exposing the cultured stem cell derived-RGCs to the CM enhanced the survival of RGCs from (100 ± 29.97%) in the controls to 177.76 ± 38.48% when cultured with Müller cell CM (Figure 3).

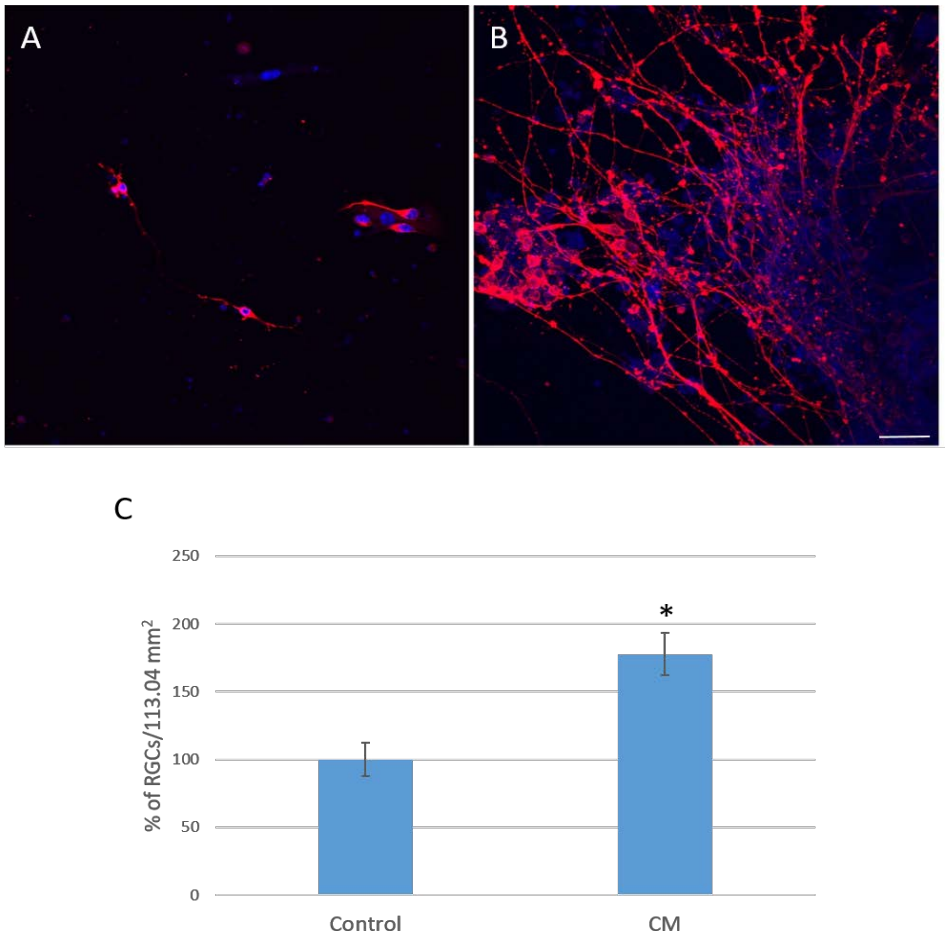

Figure 3. Effect of Muller cell conditioned medium (CM) on the survival of stem cell derived-RGCs *in vitro.* Images of stem cell derived RGCs maintained in (A) control conditions or (B) cultured with adult Müller cell CM. The cells were labeled with antibodies against β-III-tubulin (red) and nuclei stained with DAPI (blue). (C) The percentage of RGCs in control conditions or in the presence of CM: *p-value < 0.05. Scale bar: 50µm.

The effect of the Muller cell CM on the survival of stem cell derived-RGCs was also assessed in non-dissociated EBs by analyzing the expression of Brn3a and Math5 by qPCR. Brn3a is a protein expressed in most RGCs, while Math5 is a transcription factor that is transiently expressed during early retinal histogenesis and that is necessary for RGC development. Both, Brn3a and Math5 expression is enhanced when EBs were cultured with adult Müller cell CM (Figure 4).

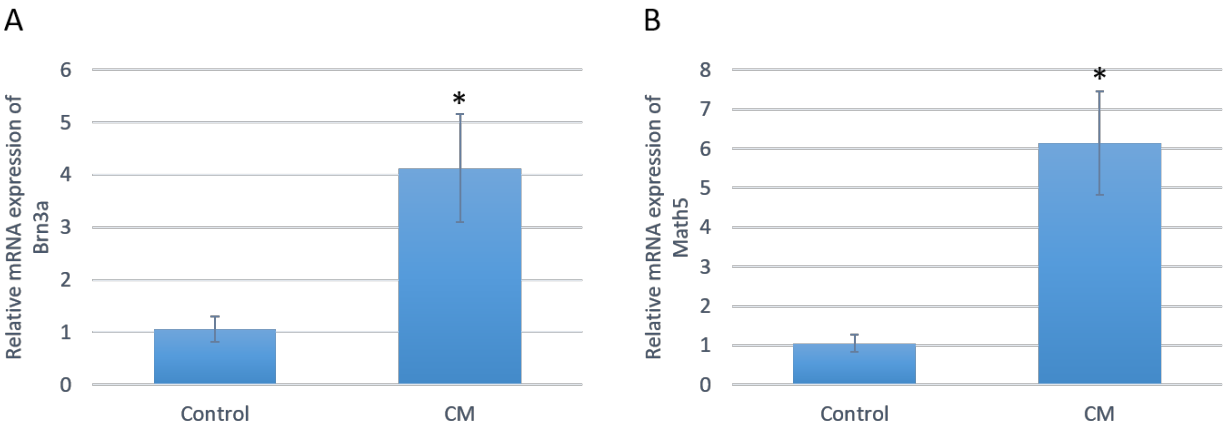

Figure 4. Brn3a and Math5 Expression in EBs cultured with adult Müller cell conditioned media (CM). Relative Brn3a (A) and Math5 (B) expression in EBs maintained in Muller cell CM: *p-value < 0.05.

Discussion

Retinal Müller glia can enhance the survival and activity of neurons like RGCs, the neurons affected in diseases like glaucoma, diabetes and retinal ischemia. Müller glia release neurotrophic factors that support RGC (von Toerne et al., 2014; Ruzafa et al., 2018) and photoreceptor (Balse et al., 2005) survival, and they are known to synthesize neurotrophic factors like BDNF (Vecino et al., 1999), CTNF (Rhee and Yang, 2010), basic fibroblast growth factor (bFGF: Bringmann et al., 2009), pigment epithelium derived growth factor (PEDGF: Zhou et al., 2009), or glial-derived neurotrophic factor (GDNF: Hauck et al., 2006). Given the strong neuroprotective effect of Müller glia on neurons, we developed a strategy to using adult Müller cells to enhance the survival of stem cell-derived RGCs during retinal differentiation.

Retinal differentiation from pluripotent stem cells is based on their commitment to neural lineages in a stepwise manner. At early time points, these pluripotent cells down-regulate many markers of their undifferentiated state and they form a polarized neuroepithelium that expresses many markers of a developing neural plate/tube. Subsequently, these cells form early retinal progenitors and they express transcription factors representative of the eye field, before finally differentiating into post-mitotic retinal neurons, including RGCs (Miltner and La Torre, 2019). The number of RGCs

decreases over time in 3D cultures (Aparicio et al., 2017), as might be expected given that newly born RGCs undergo two waves of programmed cell death during normal development as they become dependent on trophic support from their synaptic targets (Isenmann et al., 2003). Such apoptotic events could compromise the use of these cells in transplant therapies, raising interest in our results showing that co-culturing Müller cells and dissociated EBs notably enhances the survival of stem cell derived-RGCs.

Having confirmed the effect of Müller cells on the survival of stem cell derived-RGCs, dissociated EBs cultures were maintained with medium conditioned by Müller cells to assess whether the increase of stem cell derived RGCs survival is due to factors released by these glia. This conditioned medium also significantly increased the survival of stem cell derived-RGCs, indicating that the effect of Müller cells does not seem to be mediated by the substrate alone. However, as the effect of Müller cells in co-culture on the survival of stem cell derived-RGCs is stronger than that observed with the CM alone, there appears to be a synergistic effect of membrane bound and diffusible factors.

The CM enhanced the expression of Brn3a in non-dissociated EBs, a transcription factor expressed exclusively by RGCs (Nadal-Nicolas et al., 2012). Hence, the increase in Brn3a expression indicates that the CM increases the survival of stem cell derived-RGCs in non-dissociated EBs, as well as that of isolated cells. The expression of Math5 was also assessed, a transcription factor that is transiently expressed during early retinal histogenesis and that is necessary for RGC differentiation (Brown et al., 2001; Brzezinski et al., 2012). Our results show that Math5 expression is enhanced in EBs grown in CM, suggesting that the factors released by Müller cells not only enhance the survival of RGCs but also, they increase the progenitor cells that can differentiate into RGCs.

In conclusion, Müller cells release factors that enhance the survival of stem cell derived-RGCs and that also contribute to the differentiation of progenitor cells to RGCs. These findings could improve RGC-derived stem cell-based transplant strategies currently under development.

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ANEXO 4

Neuroprotective effect of Müller cells according to their localization in the retina *in vitro.* *(In preparation)*

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Abstract

Neurodegenerative diseases like Glaucoma are characterized by progressive damage to retinal ganglion cells (RGCs), provoking irreversible blindness. Müller cells are the main glial cells in the retina, and they provide an endogenous source of neurotrophic and neuroprotective factors. Only a subset of Müller cells respond to retinal injury, which suggests that distinct subpopulations of these glia are endowed with different neuroprotective capabilities. Thus, the aim of this study was to analyze the neuroprotection afforded to RGCs by Müller cells *in vitro* depending on their position in the retina. As such, different combinations of pure pig and rat RGC and Müller cell cultures from different of the retinal locations were established, as well as co-cultures of these cells. We also tested the effect of Müller cell conditioned media on RGC survival when it was obtained from Müller cells isolated from the visual streak, center or periphery of the retina. We found that RGC survival was enhanced substantially when they were co cultured with Muller cells from the periphery or simply by exposing these cells to medium conditioned by these peripheral Müller cells. No significant effect on survival was found with cells or condition media from other parts of the retina. In the cultures from the three areas of the retina, there was an increase in the immunocytochemical detection of the Beta-III-Tubulin neural marker and α-SMA dedifferentiation marker in peripheral cells only. In conclusion, peripheral Müller cells enhance the neuroprotection of RGCs *in vitro* by contact and through released factors. Moreover, the increase in the dedifferentiation markers in peripheral Müller cells suggest that this phenotype enhances RGC neuroprotection *in vitro*.

Introduction

Retinal ganglion cells (RGCs) are the neurons that transmit visual information from the eye to the brain and thus, their survival is critical for vision. However, these neurons are very sensitive to insults and damage to RGC axons in the optic nerve may lead to rapid RGC death in acute diseases like ischemic optic neuropathy or optic neuritis, or in chronic diseases like glaucoma. Thus, enhancing RGC viability (neuroprotection) or RGC function (neuroenhancement) is an important goal in basic and translational research (Goldberg and Corredor, 2009).

Müller cells are the principal macroglial cells in the retina and they are radially oriented cells that span the entire thickness of the retina. These cells are responsible for the homeostatic and metabolic support of retinal neurons, making them essential for neuron survival. Reactive Müller cells can maintain extracellular homeostasis during retinal damage, thereby protecting retinal neurons (Bringmann et al., 2009). The neuroprotective effects of Müller cells can be mediated by diverse mechanisms, enhancing physiological functions like glutamate or potassium uptake. Moreover, neuronal function can be further preserved by enhancing the expression of free radical scavengers or neuroprotective factors by Müller cells (Garcia et al., 2002).

Müller glia represent a heterogeneous population in which some of these cell types are more prone to participate in retinal repair than others. Although little is known about the heterogeneity of Müller cells, the Chx10 transcription factor is also only expressed in a subset of Müller glia (Rowan and Cepko, 2004) and 30% of the Müller cells in vitro express class II MHC antigen, suggesting that they might be involved in immune reactions (Roberge et al., 1985). In terms of their distribution in the retina, Müller cell morphology varies in relation to retinal topography (Reichenbach, 1987). The central retina is thicker than the periphery, with a higher density of neurons and Müller cells. Moreover, Müller cells from the central retinal are longer and thinner, and they have a smaller volume but a higher surface to volume ratio than those in the periphery (Reichenbach and Bringmann, 2015). In the chick retina, the region in which proliferating Müller cells are found in response to retinal injury becomes increasingly confined to the periphery (Fischer and Reh, 2003). Moreover, while mammalian Müller cells have lost the capacity to proliferate and regenerate, peripheral Müller cells express proteins characteristic of stem cells after damage, like CD44 (Too et al., 2017) and the neural progenitor marker, Nestin (Suga et al., 2014).

These differences in the expression of stem cell like proteins depending on their location in the retina may indicate that Müller cells fulfil different functions in the retina depending on their location. Thus, the aim of this study was to analyze the neuroprotective influence of Müller cells on RGCs *in vitro* based on their localization in the retina.

Materials and Methods

Animals

All animal experimentation adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. For Müller cell cultures, adult porcine eyes were obtained from a local slaughterhouse and transported to the laboratory in cold CO_2-independent medium (Life Technologies, Carlsbad, CA, USA) containing 0.1% gentamicin (Life Technologies, Carlsbad, CA, USA). For RGC cultures, eyes were obtained from adult female Sprague Dawley rats (200–250 g). Animals were housed on a 12 hour light-dark cycle with ad libitum access to food and water, and they were sacrificed humanely by exposure to CO_2.

Retinal cultures

Adult porcine eyes were dissected within 1 to 2 h of enucleation and co-cultures of RGCs and Müller cells, or pure Müller cell cultures, were prepared. Briefly, the major blood vessels were removed and the retina was washed in CO_2-independent medium. The retinas were dissected out and three different areas of the retina were cut using an 8 mm diameter dissecting trephine (Biomedical Research Instruments, Silver Spring, MD, USA): Visual streak, center and periphery (Figure 1). The retinal tissue was dissociated for 30 min at 37 °C in 0.2% activated papain (Worthington, Lakewood, NJ, USA) with 10% DNAse I (Worthington, Lakewood, NJ, USA). Enzyme activity was stopped by the addition of Müller medium and DNase I, and the tissue was then disaggregated by gentle trituration using pipette tips of decreasing diameter.

Müller cell were cultured in DMEM (Life Technologies) with 10% fetal bovine serum (FBS: Life Technologies, Carlsbad, CA, USA) or co-cultured in Neurobasal A medium (NBA: Life Technologies, Carlsbad, CA, USA) supplemented with 10% FBS and 2%

B27 (Life Technologies). In addition, 1% L-glutamine (2 mM: Life Technologies) and 0.1% gentamicin (50 mg mL−1: Life Technologies) were added to all the media.

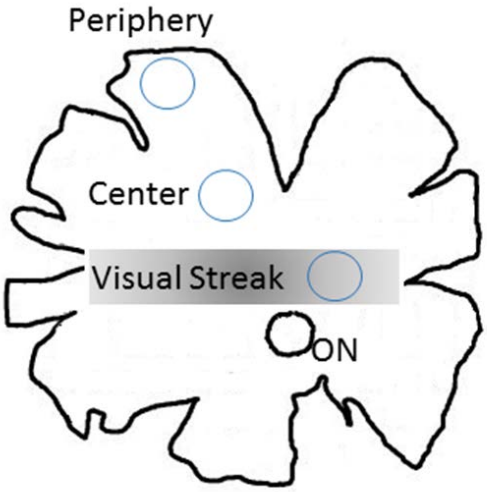

Figure 1. Scheme of the pig retina. The image shows the three different areas of the pig retina selected for culture. The blue circles represent the 8 mm plugs (ON, optic nerve).

Dissociated cells were recovered by centrifugation (1200 rpm, 5 min), resuspended in Müller medium and plated at the same density on poly-L-lysine (100 µg/mL: Sigma-Aldrich, St. Louis, MO, USA) and laminin (10 µg/mL: Sigma-Aldrich, St. Louis, MO, USA) coated 13 mm glass coverslips in 24-well plates. The cells were maintained in a humidified incubator at 37 °C in an atmosphere of 5% CO_2, and the unattached cells were removed after 24 h by changing the entire medium. For maintenance, half the medium was replaced every 3 days.

The conditioned medium (CM) from Müller cell cultures was collected when the cultures had reached confluency (day 7), first washing the wells three times with NBA medium and subsequently, adding fresh NBA medium to each well for 3 h before the medium was changed to eliminate the rest of the FBS and B27. Fresh NBA medium was added and conditioned over 2 days before it was collected, sterilized by passing through a 0.22 µm filter and frozen in aliquots at -20 °C. Finally, the Müller cells were fixed for 10 min with methanol at -20 °C. At least three replicates of each culture were made and the procedure was performed in triplicate.

RGC Cultures

Retinal ganglion cell cultures were prepared as described previously (Vecino et al., 2015a). Briefly, the retinas from pigs and rats were dissected out to obtain a mixed suspension of retinal cells. Pig retinal cells were obtained from the visual streak, and

from the center and periphery of the retina (Figure 1), and they were dissociated enzymatically using the Papain Dissociation Kit (Worthington Biochemical Lakewood, NJ, USA) according to the manufacturer's instructions. Briefly, the retinal tissue was digested for 90 min at 37 °C in 0.2% activated papain with 10% DNAse I and the tissue was then disaggregated by gentle trituration using pipette tips of decreasing diameter. After purification, the survival of pig RGCs from different regions of pig retina was assessed in pure cultures of the cells plated on 13 mm poly-L-lysine (100 μg/mL, Sigma-Aldrich, St. Louis, MO, USA) and laminin (10 μg/mL: Sigma-Aldrich, St. Louis, MO, USA) coated glass coverslips in 24-well plates. The pig and rat cells were then seeded at 10^5 viable cells per well in order to test the activity of the different CM. Rat RGCs were also seeded on confluent Müller cell cultures from the visual streak, and from the center and periphery of the retina. The cultures were maintained in Neurobasal A medium (Life Technologies, Carlsbad, AC, USA) supplemented with 2% B27, 1% L-glutamine (2 mm: Life Technologies, Carlsbad, CA, USA) and 0.1% gentamicin (50 mg/mL: Life Technologies, Carlsbad, CA, USA). Both types of cultures were maintained for 6 days at 37 °C in a humidified atmosphere containing 5% CO_2 and the medium was changed every 3 days. Finally, the RGCs were fixed for 10 min with methanol −20 °C on day 6.

To test the activity of the CM, RGCs were cultured in NBA/B27 medium (control) or NBA/B27 medium:CM (1:1), conditioned by Müller cells that come from the visual streak, or from the center and periphery of the retina. The RGCs were seeded at 10^5 viable cells per well and all the media used contained 1% L-glutamine and 0.1% gentamicin.

At least four replicates were performed for each analyses described, repeating each independent experiment three times.

Immunochemistry

After fixing in methanol and washing with PBS (phosphate buffered saline, pH 7.0), the cells were immunostained. After blocking the binding of non-specific antigens with blocking buffer (3% BSA and 0.1% Triton X-100 in PBS), cells were incubated with antibodies against vimentin as a specific marker of Müller cells (mouse monoclonal antibody diluted 1:10,000, Dako, Glostrup, Denmark), β-III-tubulin as a specific RGC marker (rabbit polyclonal antiserum diluted 1:2,000, Promega, Madison, WI, USA), p75

as a specific marker for Müller cells (rabbit polyclonal antiserum diluted 1:2,000, Abcam, Cambridge, UK), and alpha-smooth muscle actin (αSMA) to evaluate Müller cell dedifferentiation (mouse monoclonal antibody diluted 1:1000, Abcam, Cambridge, UK). After washing again, these antibodies were detected with anti-mouse Alexa Fluor 488, anti-rabbit Alexa Fluor 555, anti-mouse Alexa Fluor 555 and anti-rabbit Alexa Fluor 488 goat secondary antibodies (Life Technologies, Carlsbad, CA, USA), diluted 1:1,000, and the cells were finally counterstained with the nuclear marker DAPI (Life Technologies, Carlsbad, CA, USA), diluted 1:10,000.

Quantification of RGCs and Statistical Analysis

RGCs were analyzed on an epifluorescence microscope (Zeiss, Jena, Germany) coupled to a digital camera (Zeiss Axiocam MRM, Zeiss, Jena, Germany). At least three coverslips were analyzed for each experimental condition to test the activity of the conditioned medium) and from three independent experiments. In pure RGC cultures, cell density was quantified and the cells were classified as: 1) cells with no neurites; 2) cells with a longest neurite <50 μm; 3) cells with the longest neurite between 50 and 200 μm; and 4) cells with neurites longer than 200 μm. The total number of RGCs surviving in each condition was recorded. Müller cells from the co-cultures were also analyzed and were counted in images obtained using a filter specific for only one of the antibody markers. Semi-automatic Zen software (Zeiss, Jena, Germany) was used to count the number of nuclei stained with DAPI, taking into consideration the limits of the axis of the nuclei of Müller cells to achieve more accurate measurements. For that purpose, we used a macro designed to specifically measure the limits of the axes (55-70 μm), which was corrected manually for each image.

The cell density was defined as the mean (cells per 13 mm coverslip, which corresponds to 132.66 mm^2) and the data from the different experimental conditions were compared using an analysis of variance (ANOVA), followed by the Tukey or Games-Howell test depending on the homogeneity of the variances. Differences were considered significant for all tests at $p< 0.05$.

Results

The survival of pig RGCs from the visual streak, or the center and periphery of the retina was analyzed in pure cultures. In cultures from the visual streak the survival of RGCs was significantly higher (173 ± 53.28 RGCs) than that of those from the central (60 ± 11.51 RGCs) or peripheral (86.75 ± 21.15 RGCs) retina (Figure 2).

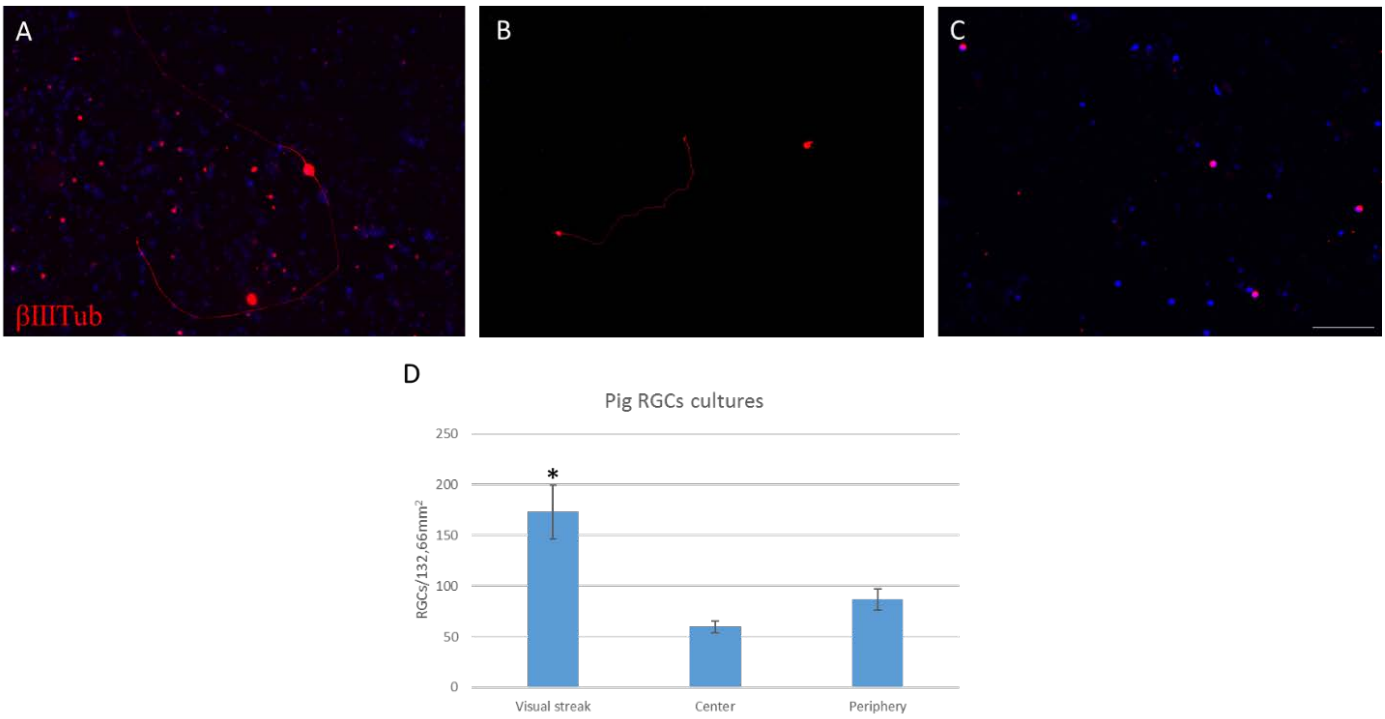

Figure 2. The survival of pig RGCs from the visual streak, central or peripheral retina in pure cultures. Images of pure RGC cultures from (A) the visual streak, (B) the center or the (C) periphery of the retina. The cells were labelled with antibodies against β-III-Tubulin (red) and their nucleus was stained with DAPI (blue). (D) Histogram representing the number of RGCs in the cultures from each retinal region: *p-value < 0.05.

The effect of Müller cells from the visual streak, center or periphery of the pig retina on the survival of RGCs was also analyzed in co-cultures. The survival of both the peripheral Müller cells and RGCs (61,875.66 ± 21,189.82 and 2,908 ± 630.09 cells/132.66 mm^2, respectively) was significantly higher in co-cultures than that of the cells from the central region of the retina (33,232.16 ± 5,975.41 Müller cells and

1,851.85 ± 594.14 RGCs /132.66 mm^2) and the visual streak (40,184.83 ± 9,280.39 Müller cells and 1,131.57 ± 656.12 RGCs /132.66mm^2: Figure 3).

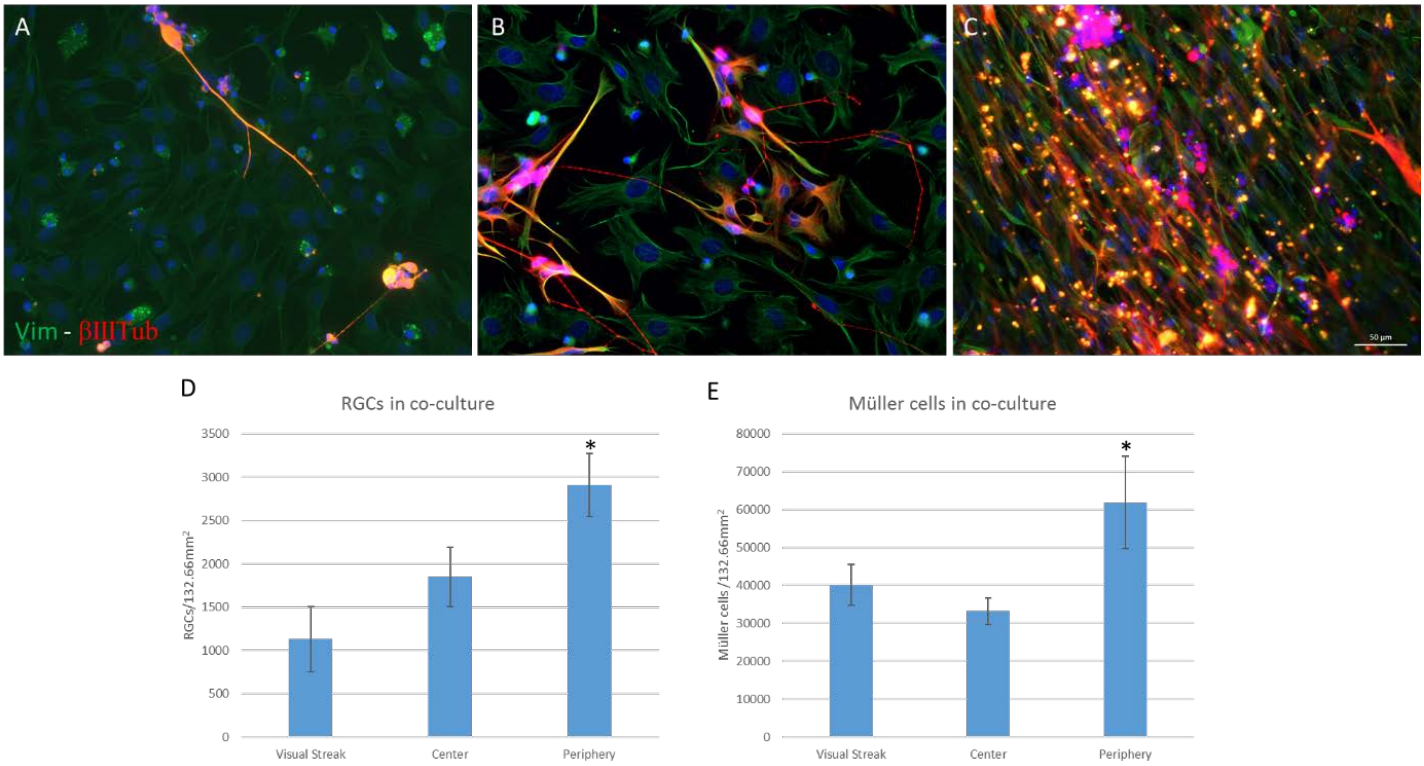

Figure 3. Effect of Müller cells on the survival of RGCs in co-cultures of cells from the visual streak, and the central and peripheral retina. Images of pig Müller cells and RGCs from (A) the visual streak, (B) the center and (C) the periphery of the retina in co-cultures. The cells were labelled with antibodies against β-III-Tubulin (red) and vimentin (green), and the nuclei are stained with DAPI (blue). (D) Number of RGCs in co-cultures from the visual streak, center and periphery of the retina. (E) Number of Müller cells in the co-cultures from the visual streak, center and periphery of the retina: *p-value < 0.05.

In order to confirm the stronger neuroprotective effect of Müller cells from the periphery of the retina, pure rat RGCs were seeded on a monolayer of Müller cells from the visual streak, center and periphery of the retina at 7 days *in vitro* (DIV). The survival of the rat RGCs increased significantly (1,879.75 ± 719.89 RGCs /132.66 mm^2) when they were seeded on Müller cells from periphery of the retina rather than on

Müller cells from the central retina (1,491.87 ± 521.10 RGCs /132.66 mm^2) or the visual streak (987.33 ± 335.68 RGCs /132.66 mm^2) of the retina after 6 days in co-culture (Figure 4).

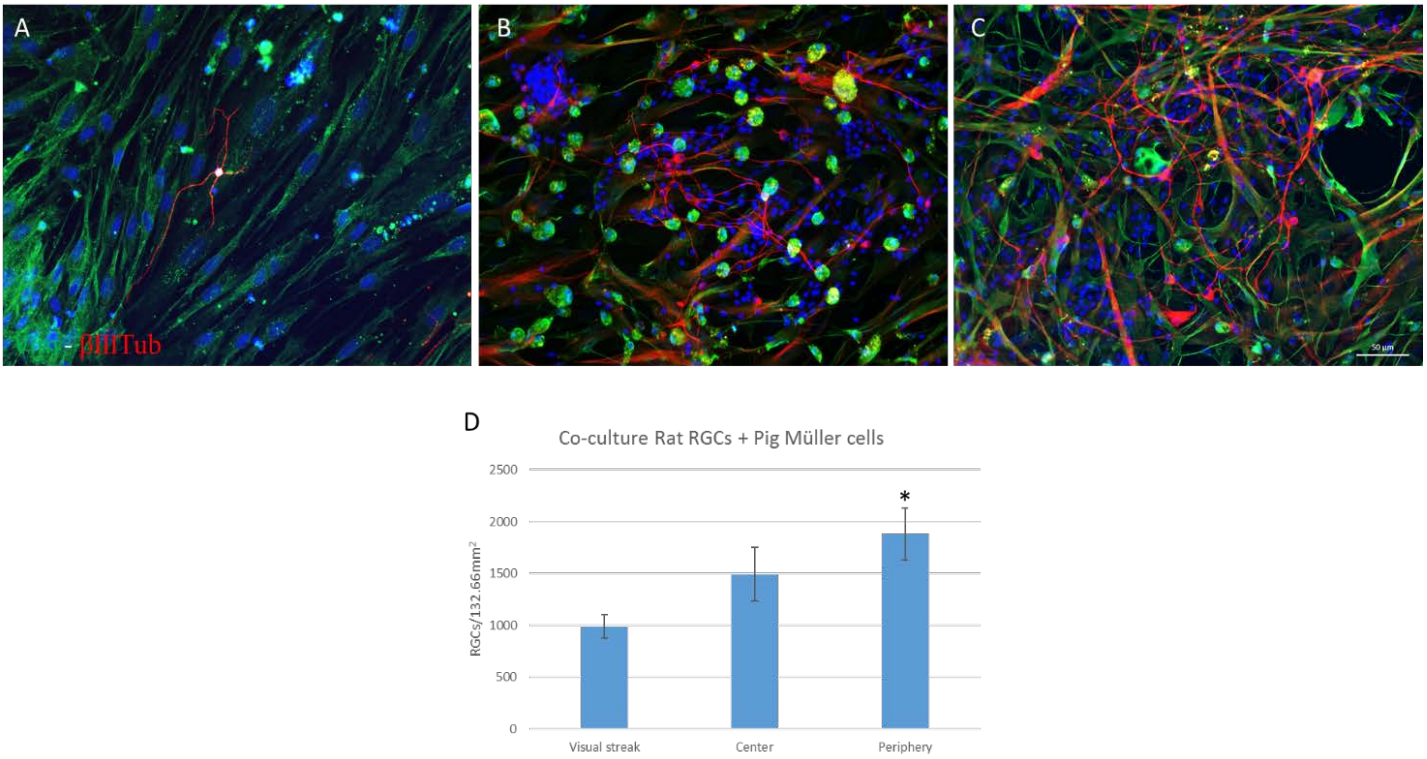

Figure 4. The survival of rat RGCs seeded on confluent pig Müller cells from the visual streak, center and periphery of the retina. Images of rat RGCs seeded on pig Müller cells that came from (A) the visual streak, (B) center, or (C) periphery of the retina. The cells were labelled with antibodies against β-III-Tubulin (red) and vimentin (green), and nuclear stained with DAPI (blue). (D) Number of rat RGCs on pig Müller cell cultures from the visual streak, center or periphery of the retina: *p-value < 0.05.

To analyze whether factors secreted by peripheral Müller cells into the CM may have the same effect on RGC survival as monolayers of Müller cells, rat RGCs were cultured in medium conditioned by Müller cells from the visual streak, and the central and peripheral retina. The RGCs were cultured in NBA/B27 (control) or in NBA/B27 medium:CM (1:1) from the three types of Müller cell cultures, and more RGCs survived (249.33 ± 82.97 RGCs /132.66 mm^2) when the cells were maintained in medium conditioned by peripheral Müller cells than in the CM from control cells (70.66 ± 3.05

RGCs /132.66 mm^2), or in that from Müller cells from the visual streak (113.66 ± 43.29 RGCs /132.66 mm^2) or central retina (94.33 ± 37.89 RGCs/132.66 mm^2: Figure 5D).

The numbers of each type of RGC were calculated for each culture condition to determine whether the different CM affected RGC neurite length (Figure 5E). When RGCs were maintained in CM secreted by peripheral Müller cells, there were more RGCs with short (<50 µm, 146.66 ± 54.60 RGCs /132.66 mm^2) and medium (50-200 µm, 40 ± 7.81 RGCs /132.66 mm^2) neurites than when RGCs were maintained in medium conditioned by Müller cells from the visual streak (61.33 ± 22.59 RGCs with short neurites /132.66 mm^2 and 12 ± 5.19 RGCs with medium neurites /132.66 mm^2) or from the central retina (51 ± 30.80 RGCs with short neurites /132.66 mm^2 and 11 ± 6.08 RGCs with medium neurites /132.66 mm^2).

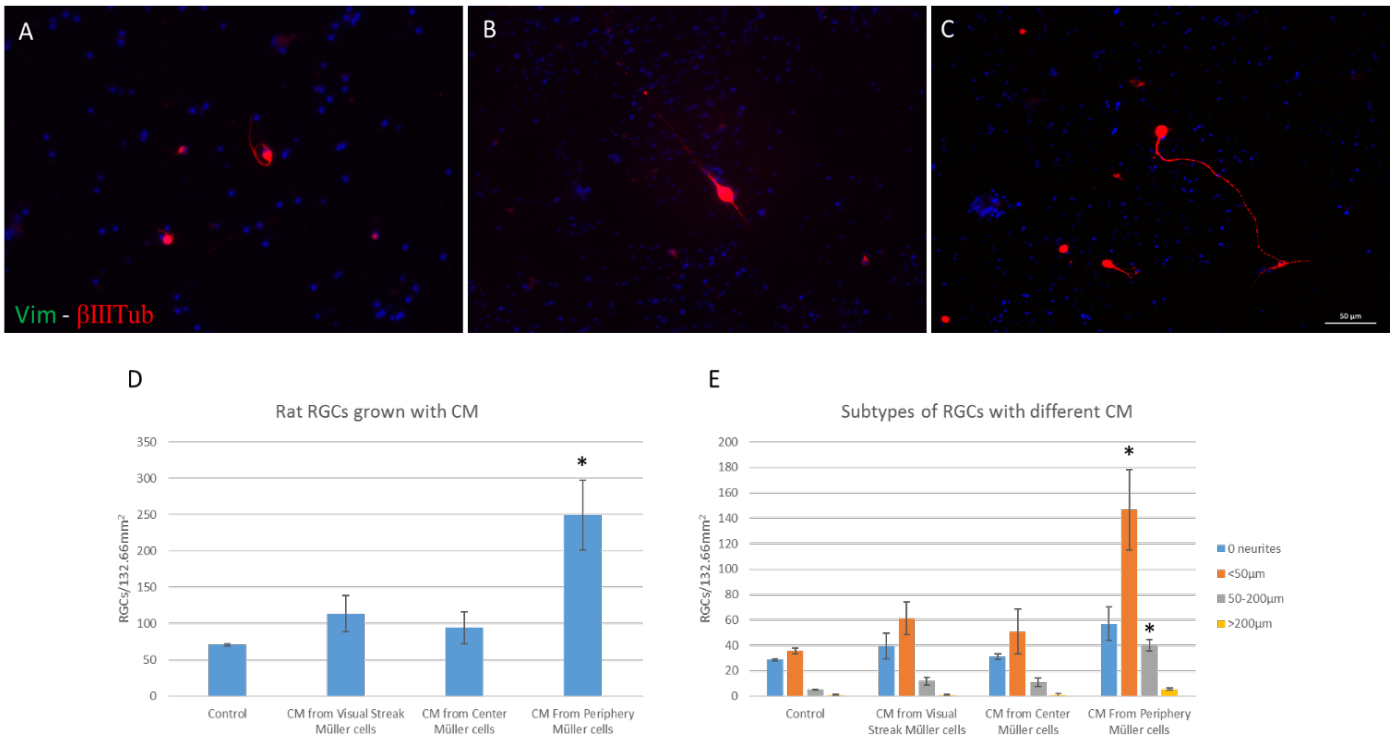

Figure 5. Survival and neuritogenesis of rat RGCs when maintained in CM secreted by Müller cells from the visual streak, central and peripheral retina. Images of rat RGCs maintained with Conditioned medium (CM) secreted by Müller cells that came from the (A) visual streak, or the (B) central or (C) peripheral retina. The cells were labelled with antibodies against β-III-Tubulin (red), vimentin (green) and the nuclei were stained with DAPI (blue). (D) Number of rat RGCs maintained with CM

secreted by Müller cell cultures from the visual streak, the center or the periphery of the retina. (E) To analyze neuritogenesis, the RGCs were classified as RGCs without neurites (blue), with the longest neurite <50 µm (orange), with the longest neurite between 50 and 200 µm (grey), and with neurites longer than 200 µm (yellow). The number of RGCs in each category is shown for control cells and RGCs maintained in the presence of the three types of CM: *p-value < 0.05.

To determine if Müller cells that come from the visual streak, the center or the periphery of the retina respond differently in culture, we analyzed the expression of p75 and vimentin (Müller cell specific markers), and that of β-III-Tubulin (neural marker) and α-SMA (dedifferentiation marker) in pure Müller cell cultures. Although the Müller cell specific markers p75 and Vimentin are expressed strongly in the three types of cultures, β-III-Tubulin and α-SMA were expressed more by Müller cells that come from the peripheral retina than by Müller cells that come from the visual streak or the center of the retina, indicating that peripheral Müller cells are more dedifferentiated (Figure 6).

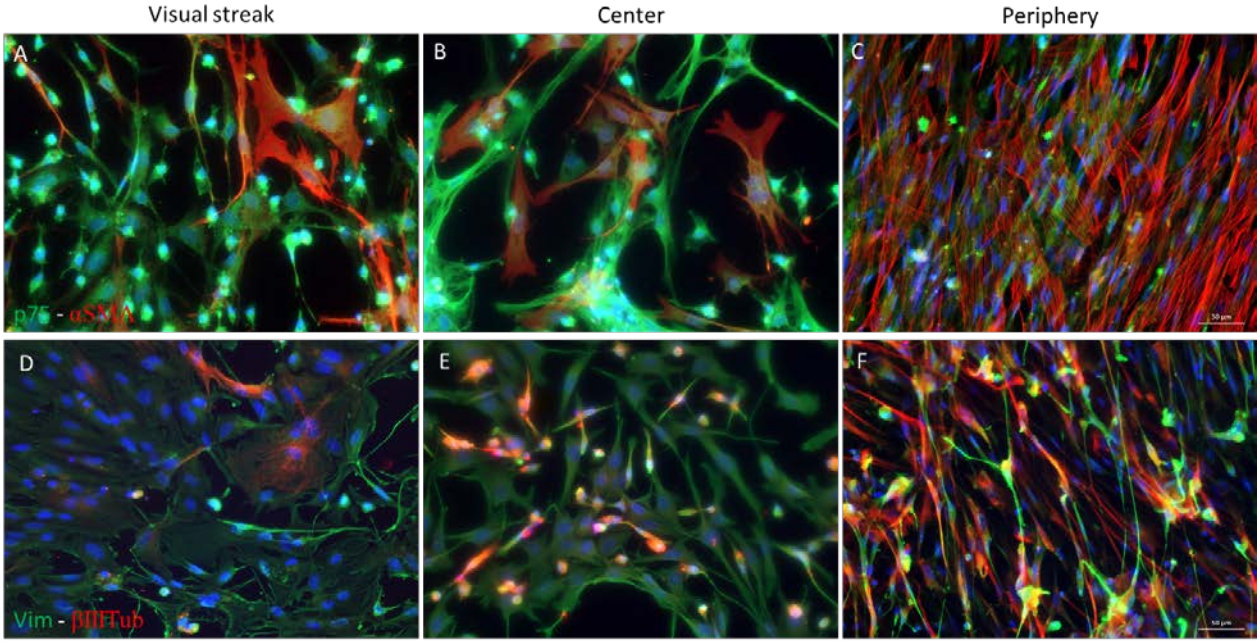

Figure 6: Expression of p75, vimentin, β-III-Tubulin and α-SMA in pure cultures of Müller cells isolated from the visual streak, the center or the periphery of the retina. Images of cultured Müller cells isolated from (A,D) the visual streak, (B,E) or the center and (C,F) periphery of the retina. The cells were labelled with antibodies against (A,B,C) α-SMA (red) and p75 (green), (D,E,F) β-III-Tubulin (red) and vimentin

(green), and the nuclei stained with DAPI (blue). Note the increase in the expression of α-SMA and β-III-Tubulin in peripheral Müller cells.

Discussion

Protecting neurons when their survival is compromised is a good strategy to confront nervous system lesions and neurological disorders (Hayashi and Takagi, 2015). To better understand the fundamental role of glial cells in retinal pathologies, the heterogeneity of these populations must be defined and understood, which could even open novel therapeutic avenues. Here, we have analyzed the neuroprotective effect of Müller cells according to their localization in the retina.

When pig RGCs were cultured alone, we found more RGCs in cultures of tissue isolated from the visual streak of the retina. In general, the distribution of cells is not homogeneous across the retina and it varies with the eccentricity of this organ. Porcine RGCs are concentrated in the center of the retina, forming a visual streak, whereas the density of these cells is lower in the peripheral retina, which is even more evident in larger animals like pigs (Drager and Olsen, 1981; Garca et al., 2005; Salinas-Navarro et al., 2009a; Salinas-Navarro et al., 2009b). Moreover, in pig glaucoma models we found that peripheral RGCs were more sensitive to death (Ruiz-Ederra et al., 2005) than in rats (Urcola et al., 2006), where no significant increase in cell death was found in the periphery relative to the center. However, it is possible that in larger retinas the area considered to be peripheral is more easily dissected for comparison.

By contrast, more RGCs survived when they were co-cultured with Müller cells from the peripheral retina. Hence, Müller cells from the periphery of the retina appear to exert a stronger neuroprotective effect on RGCs than Müller cells from the visual streak of the retina, even though the same number of retinal cells were seeded and there are proportionally fewer RGCs in the center of the retina. In the last decade, the neuroprotective effects of Müller cells have been studied (Garcia et al., 2002; Ruzafa et al., 2018; Ruzafa and Vecino, 2015) and functional heterogeneity was demonstrated in the Müller cell population (Reichenbach and Bringmann, 2015; Vecino et al., 2015b). Here we found that the neuroprotective effect of Müller cells depends on their location within the retina, with Müller cells located in the peripheral retina more neuroprotective that those located at the center. Moreover, markers of dedifferentiation were more

widely expressed in the cultured Müller cells isolated from the peripheral retina. This is consistent with the belief that the periphery of the retina is more undifferentiated, as particularly evident in fish (Vecino et al., 1998) and more residual in humans (Martinez-Navarrete et al., 2008).

The more neuroprotective effect of pig peripheral Müller cells was not only effective in co-cultures with pig RGCs but also when rat RGCs were seeded on pig peripheral Müller cells, confirming that the factors secreted are common to different species. Besides, while Müller cells carry neuroprotective factors that act through contact, they also secrete such factors (Ruzafa et al., 2018). Thus, the effect of CM produced by Müller cells from the three areas of the retina was also assessed in rat RGCs cultures. Here, we found more RGC neuroprotection with medium conditioned by peripheral Müller cells, again showing that the neuroprotective capacity of peripheral Müller cells is higher than that of Müller cells from the central areas of the retina.

Müller cells *in vitro* can rapidly change their protein expression and adopt a fibroblast-like phenotype (Fischer et al., 2004). Indeed, *in vivo* studies found that peripheral Müller cells could express dedifferentiation markers (Suga et al., 2014; Too et al., 2017). Having confirmed the higher neuroprotective capacity of peripheral Müller cells, and to relate differences in dedifferentiation capacity to the neuroprotective effect, we analyzed the expression of specific markers of Müller cells like p75 (Xu et al., 2009) and vimentin (Reichenbach and Bringmann, 2015), and the expression of a neural marker β-III-Tubulin (Jiang et al., 2015) and α-SMA, considered the most reliable marker of a fibroblastic phenotype (Darby et al., 1990). Our results found that on the same day of culture, the Müller cells from each of the three regions of the retina express the specific Müller cell markers p75 and vimentin but notably, peripheral Müller cells express more β-III-Tubulin and α-SMA, suggesting that they are in a more dedifferentiated state. Both β-III-Tubulin and α-SMA are proteins that can be expressed by Müller cells after damage. For example, β-III-Tubulin is overexpressed by Müller cells following retinal detachment (Lewis et al., 1995) and α-SMA expression is dramatically increased in retinas from humans with diabetic retinopathy (Zhou et al., 2017). These changes are remarkably similar to the phenotypic changes described following massive gliosis and they seem to be related to the changes in Müller cell protein expression in vitro, more accused in peripheral Müller cells. Previous studies with dedifferentiated cells like mesenchymal stem cells showed that they can protect

RGCs by releasing neurotrophic and neuroprotective factors (Cui et al., 2017; Osborne et al., 2018; Pan et al., 2019). Accordingly, it might appear that the more dedifferentiated the state of the peripheral Müller cells, the more neurotrophic and neuroprotective factors they might release, enhancing RGCs survival.

In conclusion, peripheral Müller cells enhance the neuroprotection of RGCs *in vitro* by contact and through factors they release. The increase in dedifferentiation markers expressed by peripheral Müller cells suggest that this phenotype enhances the neuroprotection in RGCs. Better understanding the different secreted factors released by subpopulations of Müller cells could identify potential therapeutic targets to enhance retinal neuroregeneration.

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ANEXO 5

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Dexamethasone Protects Retinal Ganglion Cells but not Müller glia against Hyperglycemia *in vitro.* *PloS One*. 2018. 13(11):e0207913

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Abstract

Diabetic retinopathy (DR) is a common complication of diabetes, for which hyperglycemia is a major etiological factor. It is known that retinal glia (Müller cells) and retinal ganglion cells (RGCs) are affected by diabetes, and there is evidence that DR is associated with neural degeneration. Dexamethasone is a glucocorticoid used to treat many inflammatory and autoimmune conditions, including several eye diseases like DR. Thus, our goal was to study the effect of dexamethasone on the survival of RGCs and Müller glial cells isolated from rat retinas and maintained *in vitro* under hyperglycemic conditions. The behavior of primary RGC cell cultures, and of mixed RGC and Müller cell co-cultures, was studied in hyperglycemic conditions (30 mM glucose), both in the presence and absence of Dexamethasone (1 μM). RGC and Müller cell survival was evaluated, and the conditioned media of these cultures was collected to

quantify the inflammatory cytokines secreted by these cells using a multiplex assay. The role of IL-1β, IL-6 and TNFα in RGC death was also evaluated by adding these cytokines to the co-cultures. RGC survival decreased significantly when these cells were grown in high glucose conditions, reaching 54% survival when they were grown alone and only 33% when co-cultured with Müller glia. The analysis of the cytokines in the conditioned media revealed an increase in IL-1β, IL-6 and TNFα under hyperglycemic conditions, which reverted to the basal concentration in co-cultures maintained in the presence of dexamethasone. Finally, when these cytokines were added to co-cultures they appeared to have a direct effect on RGC survival. Hence, these cytokines could be implicated in the death of RGCs when glucose concentrations increase and dexamethasone might protect RGCs from the cell death induced in these conditions.

Introduction

Diabetes is a metabolic disease characterized by high glucose concentrations in the blood. One of the most common complications of this disease is diabetic retinopathy (DR), the leading cause of blindness in the population of working-age in developed countries (Fong et al., 2003). In the symptomatic phase of DR, key clinical alterations to the vascular system occur that are relevant to the diagnosis of the disease. Indeed, for many years DR has been considered a microvascular disease, characterized by increased vascular permeability due to the breakdown of the blood-retinal barrier (BRB) (Madeira et al., 2015). Although vascular changes are a classic hallmark of this disorder, several observations suggest that microangiopathy is only one aspect of a more widespread retinal dysfunction.

The concept that neurons as well as capillaries are affected by diabetes is not new. In the early 1960s, DR was associated with the degeneration of retinal ganglion cells (RGCs) (Bloodworth, 1962; Wolter, 1961) and indeed, apoptosis of rat retinal neurons is enhanced after chemically induced diabetes (Barber et al., 1998). In fact, diabetes-induced changes in retinal neurons and glia may precede the onset of clinically evident vascular injury. Several metabolic impairments have been implicated in the neurodegeneration associated with DR: oxidative stress, characterized by the presence of advanced glycated end products (AGEs) and nitric oxide (NO); excitotoxicity and excess glutamate receptor stimulation that provokes the uncontrolled influx of calcium into neurons; and inflammation, involving the release of chemical mediators and leukostasis (Matteucci et al., 2015).

Müller cells are the principal glia in the retina and they fulfill quite dynamic roles. Müller cells extend throughout the thickness of the retina, providing structural stability and maintaining close contact with the majority of retinal neurons (Lorenzi and Gerhardinger, 2001; Newman and Reichenbach, 1996). They also provide neurons with trophic factors and help to maintain retinal homeostasis, potentially promoting cell survival and repair (Bringmann et al., 2006; Reichenbach and Bringmann, 2013). Although the physiology of these cells was previously thought to be rather simple, studies over the past 2 decades have revealed that Müller cells express a diversity of ion channels and transporters, that they release a range of cytokines and survival factors, and that they express receptors for numerous neurotransmitters and growth factors (Puro, 2002b; Vecino et al., 2016). In fact, it has been shown that under hyperglycemic

conditions, Müller glial cells contribute to the development and progression of diabetes by enhancing caspase-1/IL-1β signaling and mitochondrial stress (Mohr et al., 2002; Vincent and Mohr, 2007). In addition, Müller cells markedly up regulate their expression of glial fibrillary acidic protein (GFAP) early in the course of DR (Rungger-Brandle et al., 2000), a non-specific response to the pathophysiological conditions (Eddleston and Mucke, 1993).

Dexamethasone (DEX) is a synthetic corticosteroid that displays anti-inflammatory and immunosuppressive activity. It was first used for an eye-related disease in 1974, when intravitreal (IVT) injection was employed to treat experimentally induced *Pseudomonas* endophthalmitis in rabbits (Graham and Peyman, 1974). Nowadays, clinical treatment of eye-related conditions with DEX usually involves administration of slow-release intravitreal implants. These are mostly used to treat macular edema (ME) and diabetic ME (DME), producing favorable results on visual acuity (VA) (Busik et al., 2008; Lozano Lopez et al., 2015; Yego et al., 2009), as well as in diabetic patients (Boyer et al., 2014; Callanan et al., 2013; Khurana et al., 2015). Furthermore, a recent long-term study into the use of DEX implants showed that it has the potential to not only delay DR progression but also, it may reduce DR severity over 24 months (Silva et al., 2009). Nevertheless, the mechanisms underlying the effects of this glucocorticoid are not entirely clear. However, IVT DEX injection is known to regulate immune responses and to diminish vascular damage by decreasing cell permeability in rat models of streptozotocin-induced diabetes (Tamura et al., 2005; Wang et al., 2008), possibly acting through the TNFα pathway (Aveleira et al., 2010).

In vitro studies have helped to characterize the retinal damage associated with diabetes. Cell culture models represent simplified systems to assess the effects of different toxic factors in the diabetic milieu, such as excess glucose, glutamate and AGEs. We previously showed that primary cultures of adult rat Müller glia and RGCs express the same trophic factors and receptors as they do *in vivo* (Garcia et al., 2002). Moreover, Müller glia cells contact RGCs in co-culture and the factors secreted by Müller glia into the medium, can protect pig RGCs from death (Garcia and Vecino, 2003; Ruzafa and Vecino, 2015). Studies on endothelial and pericyte cell cultures have helped highlight the mechanisms underlying the cell damage in diabetes, yet studies on retina neuroglial cultures have been scarce (Matteucci et al., 2014). Thus, here we set out to study the effect of high levels of glucose, on RGC survival. In order to identify the role of Müller

glia and their relationship to RGCs, we compared pure RGC cultures with RGC-Müller glia co-cultures in different glucose concentrations *in vitro*. Moreover, to better understand the benefits that DEX produces in DR, we also examined its effect on purified rat RGCs, as well as on RGCs co-cultured with Müller glia maintained under hyperglycemic conditions. The role of inflammation in this process was also assessed by measuring the cytokines that accumulate in the medium of these cultures, thereby identifying cytokines that might protect RGCs from the toxic effects of hyperglycemia.

Materials and Methods

Retinal Cell Cultures

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. The experimental protocol met European (2010/63/UE) and Spanish (RD53/2013) regulations for the protection of experimental animals, and it was approved by the Ethical Committee for Animal Welfare of the University of Basque country. RGCs and Müller glia were isolated from adult female Sprague-Dawley rats, humanely sacrificed by exposure to CO_2, establishing two types of culture: (1) Pure RGC cultures; and (2) co-culture of RGCs and Müller glial cells. The retinas were dissected and dissociated enzymatically with papain (Worthington Papain Dissociation kit, Worthington Biochemical Lakewood, NJ, USA) for 90 (RGCs) or 30 min (co-cultures), according to the manufacturer's instructions. The dissociated cells were recovered by centrifugation and the RGC cultures were prepared as described previously (Ruzafa and Vecino, 2015). Briefly, the dissociated cells were passed through an ovomucoid inhibitor-albumin gradient, where more RGCs than Müller cells pass due to their larger size (this step was excluded when preparing the co-cultures). While this gradient does not purify RGCs to homogeneity, there is only minimal contamination of other cells (data not shown). After purification, the cells were seeded on 13 mm diameter poly-L-lysine (10 g/ml: Sigma–Aldrich, St. Louis, MO, USA) and laminin (10 g/ml: Sigma–Aldrich, St. Louis, MO, USA) coated coverslips in 24-well plates. The RGCs were seeded at 10^5 viable cells per well and the co-culture of RGCs and Müller glia were established at $6x10^6$ viable cells per well (as determined by trypan blue test).

Different media were used for each culture type, containing 1% L-glutamine (2 mM: Life Technologies, Carlsbad, CA, USA) and 0.1% gentamicin (50 mg/ml: Life Technologies, Carlsbad, CA, USA): Neurobasal-A medium supplemented with 2% B27 (Life Technologies, Carlsbad, CA, USA) for RGC cultures; and B27 supplemented Neurobasal-A medium with 10% FBS (Fetal Bovine Serum: Life Technologies, Carlsbad, CA, USA) for RGC and Müller mixed cell cultures. In addition, each culture type was maintained under distinct conditions: i) Control (untreated); ii) 1 μM DEX (Sigma Aldrich, Saint Louise, MO, USA); iii) 10 mM Glucose; iv) 30 mM Glucose; and v) 30 mM Glucose + 1μM DEX. The different conditions were employed from the start of the culture and the medium was changed every 48 hours. At least 4 replicates of each culture type were performed in triplicate. In addition, a further control involved maintaining the cells in the presence of 30 mM mannitol to demonstrate that the osmolarity of the glucose solution did not affect the results. Exposure to mannitol induced no significant changes (results not shown).

Immunocytochemistry

After 6 days in culture, the cells were fixed in cold methanol and washed with PBS (phosphate buffered saline, pH 7.0). After blocking non-specific antigens with blocking buffer (3% BSA and 0.1% Triton X-100 in PBS), the cells were incubated with the following primary antibodies at a dilution of 1:2,000: a mouse anti-βIII-tubulin antibody (Promega Madison, WI, USA) as a specific RGC marker and a rabbit anti-Vimentin antiserum (Abcam, Cambridge, England) as a specific marker of Müller cells. After washing the cells, antibody binding was detected with an anti-mouse Alexa Fluor 488 and an anti-rabbit Alexa Fluor 555 (Life Technologies, Carlsbad, AC, USA) secondary antibodies, diluted 1:1,000. In addition, the cell nuclei were labeled with DAPI (Life Technologies, Carlsbad, AC, USA) at a dilution of 1:10,000.

Cell Quantification

The same number of cells were seeded per plate for each condition and in each experiment, and the cultures were analyzed on an epifluorescence microscope (Zeiss, Jena, Germany) coupled to a digital camera (Zeiss Axiocam MRM, Zeiss, Jena, Germany). The total number of RGCs and Müller cells per coverslip was quantified using the specific markers indicated. The Müller cell counts were normalized to the number of cells grown in the control conditions, considered as 100% survival. The RGCs and Müller cells in co-cultures were counted in images obtained with a filter

specific for only one of the antibody markers. However, since there may be an overlap with vimentin stained cells, the semi-automatic Zen software (Zeiss, Jena, Germany) was used to count the number of nuclei stained with DAPI, taking into consideration the limits of the axis of the nuclei of Müller cells to achieve more accurate measurements. For that purpose, we used a specific macro designed to specifically measure the limits of the axes (50-200 µm), which was corrected manually for each image. At least 4 complete coverslips and 3 independent experiments were analyzed for each experimental condition.

Multiplex cytokine assays

After 6 days in culture, conditioned medium (supernatant) was collected for each experimental condition, filtered and the cytokines present were quantified with a Q-Plex multiplex enzyme-linked immunosorbent assay (Q-Plex Rat Cytokine – Inflammation (9-Plex): Quansys Bioscience, Logan, UT, USA). The presence of 9 cytokines in 150 µl of each sample was assessed in a 98-well Q-Plex plate according to the manufacturer´s instructions: IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IFNγ and TNFα. The standards were measured in duplicate and the cytokine concentrations were calculated using a standard curve. Four replicates were assessed for each sample and arithmetic averages were calculated. The data were normalized per 10,000 cells.

Cell cultures analysis adding IL-1β, IL-6 and TNFα

The direct effects of the IL-1β, IL-6 and TNFα cytokines were assessed in RGC-Müller cells co-cultures. We used the combination of these cytokines because they were found to be overexpressed in the supernatant of the cultures treated with 30 mM glucose. Six different conditions were analyzed: (1) Control (Neurobasal-A medium supplemented with 2% B27 and 10% FBS); (2) 1 µM DEX; (3) a mix of the three cytokines (IL-1β, IL-6 and TNFα: Sigma-Aldrich, St. Louis, MO, USA) at 10 ng/ml, as suggested by the supplier in the data sheet; (4) the cytokine mix at the concentrations in the conditioned medium identified by the multiplex cytokine assay: 16.75 pg/ml for IL-1β, 435 pg/ml for IL-6 and 32.1 pg/ml for TNFα; and (5) and (6) the mixtures of cytokines at both concentrations, respectively plus 1 µM DEX. The cytokines were added to the culture from the fourth (when the culture was stabilized) to the sixth day of culture (a 48 hour treatment), and after the sixth day the culture was fixed, immunostained and the cells were quantified as indicated above.

Statistical analysis

Statistical analyses were carried out using the IBM SPSS Statistical software (v. 21.0), calculating the mean and standard error for each condition. The data from the different experimental conditions were compared using an analysis of variance (ANOVA), followed by the Tukey or Games-Howell test depending on the homogeneity of the variances. Differences were considered significant for all tests at $p< 0.05$.

Results

RGC Cultures

To analyze how hyperglycemia affects RGC survival, the RGCs in primary cultures were counted in the control conditions and following the different experimental manipulations (Figure 1). When considered relative to the controls (considered as 100% survival: Table 1), maintaining RGCs in the presence of 1 μM DEX or 10 mM glucose had no significant effect on cell survival ($p>0.05$). By contrast, RGC survival diminished significantly relative to the controls in the presence of 30 mM glucose ($54.64 \pm 6.74\%$, $p<0.05$). Interestingly, this impairment in RGC survival in the presence of 30 mM glucose was partially recovered by adding 1 μM DEX to the cell cultures ($79.53 \pm 12.24\%$ cell survival), a survival rate that was not significantly different to that recorded in the controls. However, when RGC survival in the presence of 30 mM glucose was compared with that in the presence of 30 mM glucose and DEX, there was no clear difference in survival ($p=0.477$, Figure 1). Hence, DEX appeared to at least partially ameliorate the effect of high glucose on RGC survival in culture.

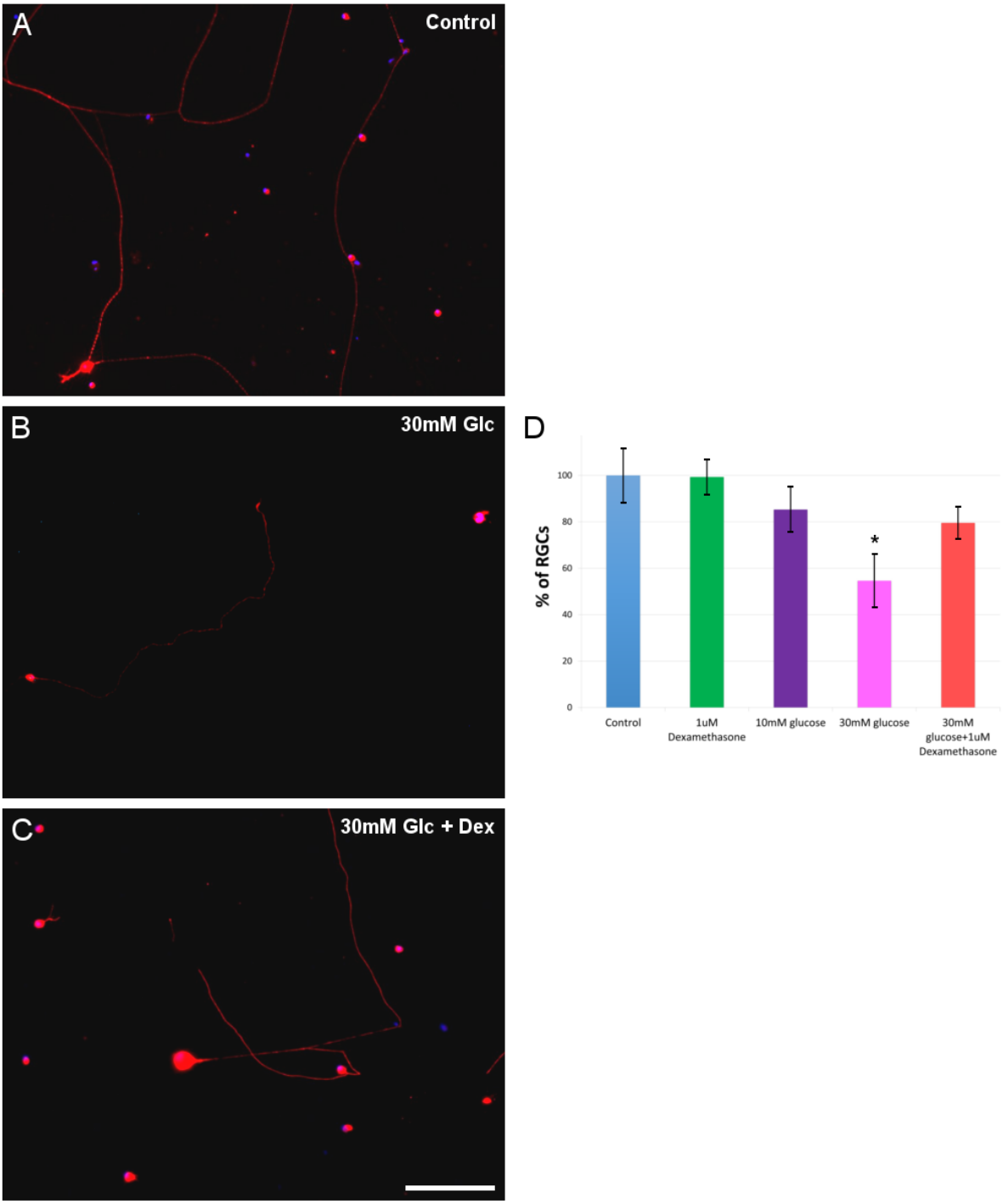

Figure 1: RGC cultures. Images of RGC cultures in control conditions (A), in the presence of 30 mM glucose (B) or 30 mM glucose plus 1µM dexamethasone (C). The RGCs were labeled with an antibody against Beta III-Tubulin (red) and with DAPI for nuclear counterstaining. (D) Percentage of RGCs surviving in the different conditions. A loss of RGC survival is evident in the presence of 30 mM glucose, while the presence

of 1 μM dexamethasone rescued the RGCs from death, reverting survival to the basal values. The data are presented as the mean + SEM. Scale bar = 100 μm, *p<0.05.

Table 1. Survival of RGCs in pure culture of RGCs.

EXPERIMENTAL CONDITION	% of RGCs	n	p value compared to the control
Control	100 ± 11.46	12	-
1 μM Dexamethasone	99.29 ± 7.54	12	1.000
10 mM Glucose	85.30 ± 11.51	12	0.846
30 mM Glucose	54.64 ± 6.74	10	**0.032**
30 mM Glucose + 1 μM Dexamethasone	79.53 ± 12.24	12	0.618

The data are expressed as the proportion of surviving cells (mean + SEM), evaluated by ANOVA followed by the Tukey or Games-Howell test depending on the homogeneity of variances.

Co-cultures

Given the supportive and neuroprotective properties of Müller cells, we assessed the effects of glucose and DEX on the survival of RGCs when they were co-cultured with Müller cells. Taking the control cultures as 100%, 67.35 ± 9.41% ($p<0.05$) of the RGCs survived 6 days in co-culture in the presence of 10 mM glucose, and this figure dropped to 33.10 ± 4.03 ($p<0.05$) in the presence of 30 mM glucose (Table 2). However, this significant decrease in cell survival was rescued by adding 1 μM DEX to the cultures, in the presence of which the survival of RGCs was no different to that in the control cultures. Indeed, RGC survival in the presence of 30 mM glucose improved significantly when DEX was added to the co-cultures ($p=0.001$: Figure 2).

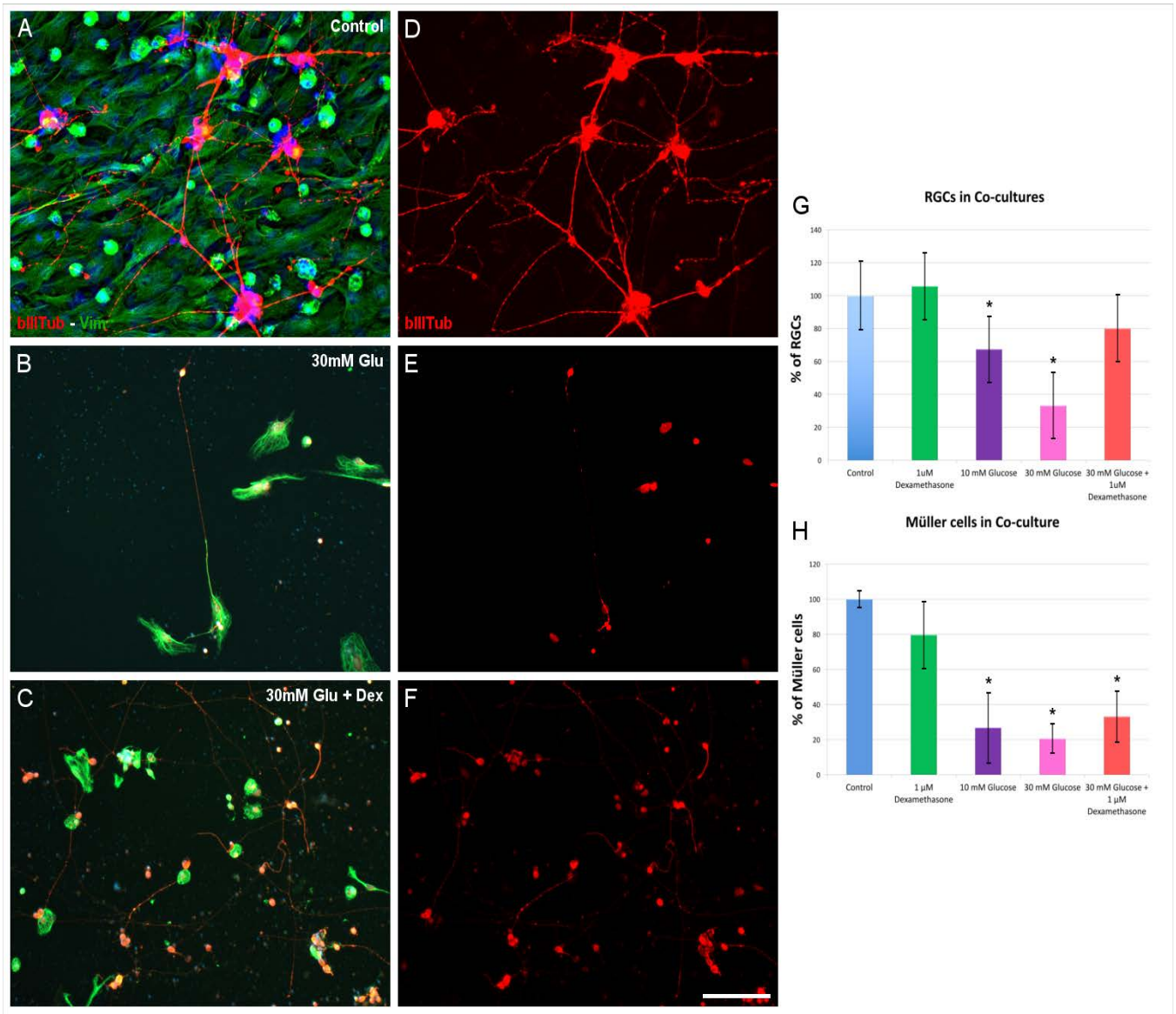

Figure 2: Co-cultures of RGCs and Müller cells. RGCs were labeled with an antibody against Beta III-Tubulin (red) and Müller cells with a Vimentin antibody (green). Note the increased survival of RGCs in the presence of dexamethasone when co-cultures are maintained in the presence of 30 mM Glucose (compare B and E to C and F). (G) RGC survival in the different conditions. RGC survival decreases on exposure to 10 mM (67.35 ± 9.41%, *p<0.05) or 30 mM glucose (33.10 ± 4.03%, *p<0.05), while the presence of 1 µM Dexamethasone enhances the survival of RGCs, reverting it to control levels. (H) The proportion of Müller cells was significantly affected by exposure to 30 mM glucose (19.52 ± 4.84%,*p<0.05), yet this decrease in Müller cell survival was not reversed in the presence of 1 µM dexamethasone. The data represent the mean ± SEM. Scale bar = 100 µm, *p<0.05.

Table 2. Survival of RGCs in co-culture

EXPERIMENTAL CONDITION	% of RGCs	n	p value compared to control
Control	100 ± 7.37	12	-
1 µM Dexamethasone	105.65 ± 8.22	10	0.986
10mM Glucose	67.35 ± 9.41	10	**0.040**
30 mM Glucose	33.10 ± 4.03	11	**0.000**
30 mM Glucose + 1 µM Dexamethasone	80.04 ± 9.66	11	0.366

The data are expressed as the percentage of surviving cells (mean ± SEM), evaluated using ANOVA followed by the Tukey or Games-Howell test depending on the homogeneity of variances.

Indeed, the survival of Müller cells decreased significantly in the co-cultures in the presence of both 10 mM (26.15 ± 8.35%, $p<0.05$) and 30 mM glucose (19.52 ± 4.84% relative to the controls, $p<0.05$: Table 3). However, dexamethasone (1 µM) had no significant effect on the survival of Müller cells in high glucose media (36.82 ± 8.01%), with no differences in cell number relative to the co-cultures maintained in the presence of 30 mM glucose alone ($p= 0.384$) and with significantly fewer Müller cells than in the control conditions ($p<0.05$: Figure 2).

Table 3. Survival of Müller cells in co-culture

EXPERIMENTAL CONDITION	% of Müller cells	n	p value compared to control
Control	100 ± 4.78	10	-
1 µM Dexamethasone	78.84 ± 10.58	9	0.408
10mM Glucose	26.15 ± 8.35	8	**0.000**
30 mM Glucose	19.52 ± 4.84	10	**0.000**
30 mM Glucose + 1 µM Dexamethasone	36.82 ± 8.01	10	**0.000**

The data are expressed as the relative cell survival (mean + SEM), analyzed by ANOVA followed by the Tukey or Games-Howell test depending on the homogeneity of variances.

Cytokine assay

The amounts of the inflammatory cytokines in the conditioned media obtained from the two types of cultures were analyzed for each experimental condition, quantifying the following cytokines: IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IFNγ, and TNFα (Table 4, see Table 4 in S1 File for raw data). With pure RGC cultures, no reliable data were obtained due to the small number of cells in this type of culture (approx. 100 cells per coverslip in the control conditions).

Table 4. Concentration of cytokines in the different conditioned media obtained from RGC and Müller cell co-cultures (data normalized per 10,000 cells).

Cytokine	Control	1 μM Dexamethasone	10 mM Glucose	30 mM Glucose	30 mM Glucose + 1 μM Dexamethasone
IL-1α	40.55±17.36	6.31±6.31	0±0	18.77±11.06	50.34±24.32
IL-1β	0±0	0±0	0±0	61.89±8.23	0±0
IL-2	0±0	0±0	0±0	45.74±37.57	45.86±34.16
IL-4	13.85±0.49	3.44±2.89	3.77±2.57	2.99±1.88	2.56±2.56
IL-6	589.73±31.49	178.92±45.77	764.61±76.95	1209.22±250.89	510.17±96.18
IL-10	5.89±0.18	4.23±0.45	5.89±0.71	11.45±0.97	9.30±0.53
IL-12	15.78±9.26	13.09±7.80	16.40±9.91	14.38±14.17	30.08±9.66
IFNγ	69.39±24.18	97.35±5.61	146.95±13.34	15.15±15.15	146.85±52.15
TNFα	65.60±10.75	18.83±6.66	62.55±4.01	118.61±7.78	59.07±13.08

The data are expressed as pg/ml (mean + SEM).

In the co-cultures, the presence of 30 mM glucose provoked a significant increase in three of the nine cytokines evaluated, with the concentration of IL-6 increasing 172% (2.72 fold, 1609.22 ± 250.89 pg/ml, $p<0.05$) relative to the control condition (589.73 ±

31.49 pg/ml), that of TNFα increasing 81.5% (1.80 fold, 118.61 ± 7.78 pg/ml, $p<0.05$) relative to the control (65.60 ± 10.75 pg/ml), and while IL-1β was undetectable in the control conditions, its concentration increased to 61.89 ± 8.23 pg/ml ($p<0.05$) in these high glucose conditions. When DEX was added to the cultures maintained in the presence of 30 mM glucose, the concentration of these three cytokines (IL-6, TNFα and IL-1β) reverted to the control values (Figure 3).

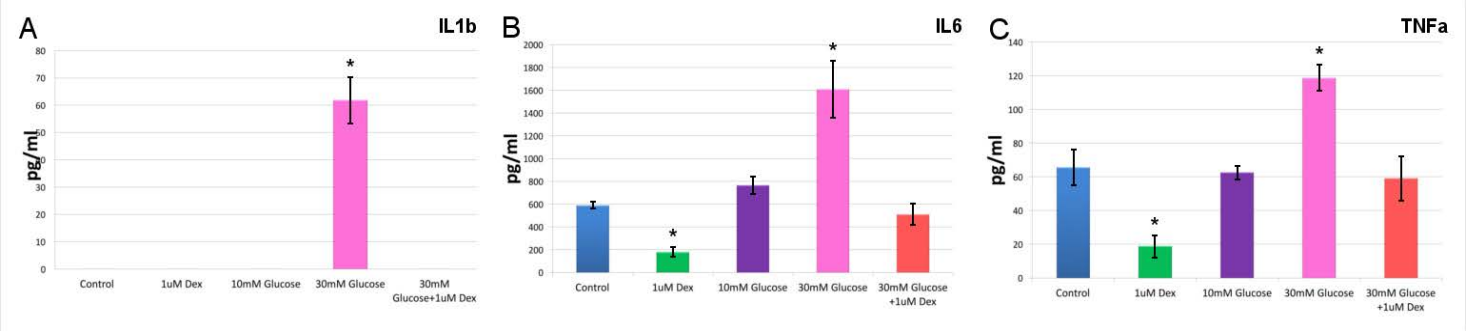

Figure 3: IL-1β, IL-6 and TNFα levels in the co-cultures. The changes in the three most significant cytokines of the 9 analyzed are shown: the IL-1β (A), IL-6 (B) and TNFα (C) in the conditioned medium of the RGC and Müller cell co-cultures under different conditions. The amounts of these cytokines increases in the presence of 30 mM glucose, yet they revert to the control levels when dexamethasone (1 µm) was added (*$p<0.05$).

IL-2 was also undetectable in control conditions, yet the concentration increased to 45.74 ± 37.57 pg/ml in the presence of high glucose. Similarly, the small quantities of IL-10 that were detected in control cultures (5.89 ± 0.18 pg/ml) also increased significantly in the presence of high glucose (11.55 ± 0.97 pg/ml, $p<0.05$). Importantly, DEX did not alter the accumulation of these two cytokines in the presence of 30 mM glucose. No significant changes were evident in the accumulation of the other cytokines analyzed (IL-1α, IL-4, IL-12 and IFNγ) between any of the different experimental conditions relative to the controls (Table 4).

Effect of IL-1β, IL-6 and TNFα on co-cultures

The effect of IL-1β, IL-6 and TNFα on co-cultures was analyzed to confirm the role of these cytokines in RGC death. In addition, the effect of DEX in combination with these three cytokines was also assessed (Figure 4).

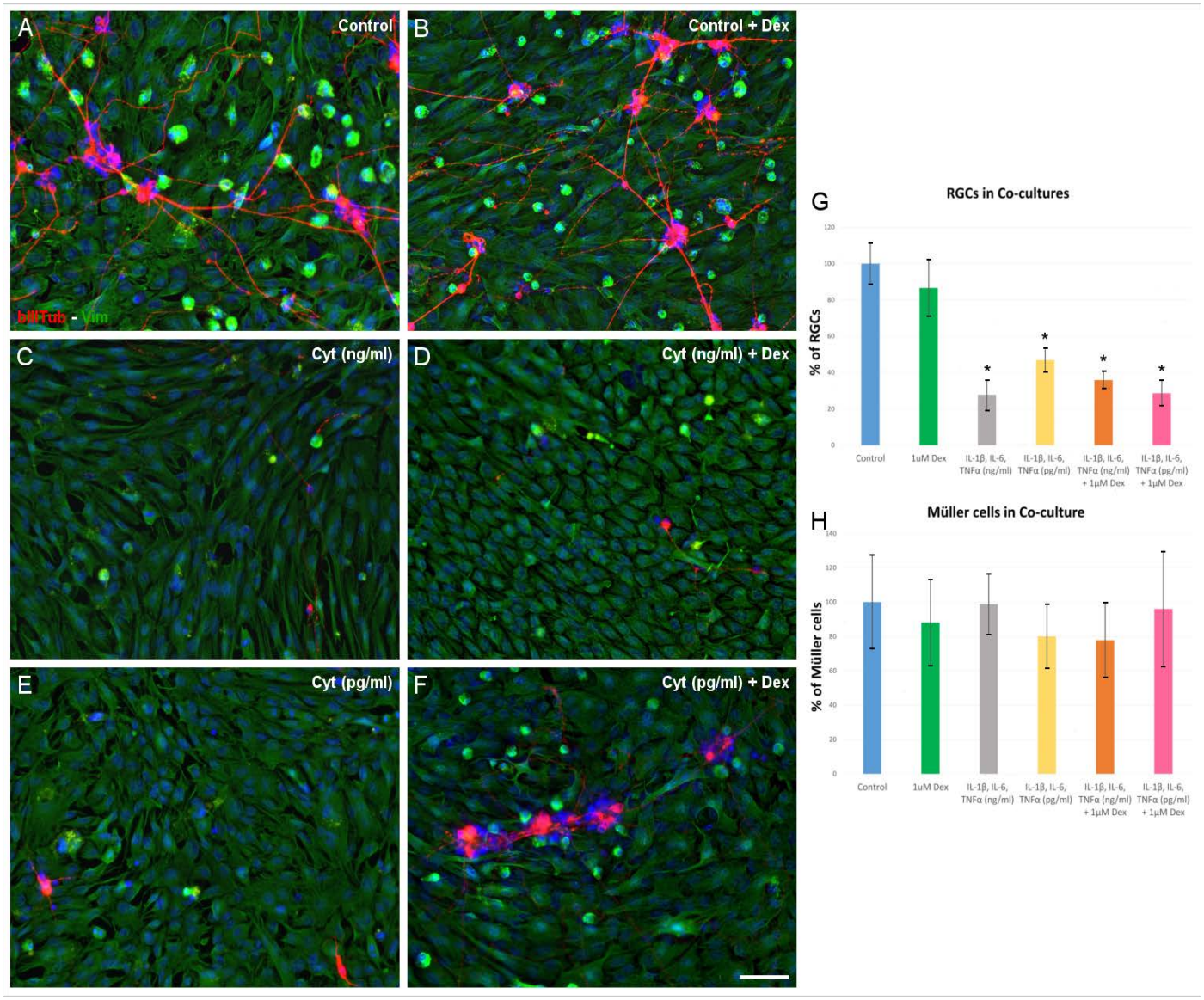

Figure 4: **Effect of IL-1β, IL-6 and TNFα on the co-cultures.**

The RGCs were labeled with an antibody against Beta III-Tubulin (red) and the Müller cells with an antibody against Vimentin (green). Note the decreased RGC survival in the presence of the cytokines at both concentrations (C, E) and in combination with dexamethasone (D, F). (G) The percentage of RGCs surviving in the different conditions. (H) There were no changes in the proportion of Müller cells under each of

the experimental conditions. The data represent the mean ± SEM: *p<0.05. Scale bar = 100 μm.

With the control cultures as a reference for 100% survival (Table 5), 27.78 ± 5.96% (p<0.05) of the RGCs survive after 6 days in culture in the presence of the three cytokines (10 ng/ml) for 48 hours. In the presence of lower concentrations of IL-1β, IL-6 and TNFα (pg/ml) for the same period, 46.89 ± 2.88% of RGCs survive (p<0.05). However, the presence of DEX at either concentration of the cytokines failed to rescue RGCs from death, and the survival rates of RGCs in the presence of DEX were 35.91 ± 5.81% and 28.62 ± 5.13% for the ng/ml and pg/ml concentrations of cytokines, respectively. These rates were significantly lower than in the control conditions (p<0.05) but they were not significantly different to when these cultures were maintained in the presence of these cytokines alone (p= 0.993 and p= 0.811 for the ng/ml and pg/ml cytokine concentrations, respectively).

Table 5. Survival of RGCs in co-culture in presence of cytokines.

EXPERIMENTAL CONDITION	% of RGCs	n	p value compared to control
Control	100 ± 11.56	3	-
1μM Dex	86.62 ± 18.22	5	0.947
IL-1β, IL-6, TNFα (ng/ml)	27.78 ± 5.96	4	**0.003**
IL-1β, IL-6, TNFα (ng/ml) + 1μM Dex	35.91 ± 5.81	4	**0.009**
IL-1β, IL-6, TNFα (pg/ml)	46.89 ± 2.88	4	**0.036**
IL-1β, IL-6, TNFα (pg/ml) + 1μM Dex	28.62 ± 5.13	4	**0.003**

The data are expressed as the percentage of surviving cells (mean ± SEM), assessed by ANOVA followed by the Tukey or Games-Howell test depending on the homogeneity of variances.

Müller cell survival in the co-cultures was not affected by the presence of either concentration of the cytokines, with 98.88 ± 17.68% Müller cell survival in the presence of ng/ml cytokine concentrations and 80.11 ± 18.49% with the pg/ml concentrations of

cytokines, showing no differences relative to the controls (Table 6). Similarly, DEX (1 μM) had no significant effect on the survival of Müller cells at either cytokine concentration: 77.84 ± 21.83% Müller cell survival in the presence of ng/ml cytokines; and 96.02 ± 33.59% in pg/ml concentrations of the cytokines (p = 0.972 by ANOVA, meaning that there are no significant differences between the groups).

Table 6. Survival of Müller cells in co-culture in presence of cytokines

EXPERIMENTAL CONDITION	% of Müller cells	n
Control	100 ± 27.63	4
1μM Dex	88.08 ± 25.06	3
IL-1β, IL-6, TNFα (ng/ml)	98.88 ± 17.68	4
IL-1β, IL-6, TNFα (ng/ml) + 1μM Dex	77.84 ± 21.83	3
IL-1β, IL-6, TNFα (pg/ml)	80.11 ± 18.49	4
IL-1β, IL-6, TNFα (pg/ml) + 1μM Dex	96.02 ± 33.59	3

The data represent the percentage of surviving Müller cells (mean + SEM).

Discussion

Hyperglycemia is considered a major factor in the etiology of DR. Like the brain, the retina primarily utilizes glucose to synthesize ATP and retinal neurons preferentially metabolize glucose rather than lactate *in vitro* (Wood et al., 2005). While RGCs obtain some glucose directly, they also obtain glucose through astrocytes (Bajetto et al., 2002) or Müller glial cells (Gougeon et al., 2013).

As shown here, retinal neurons are particularly susceptible to long-term hyperglycemia. In our experimental set-up, a high concentration of glucose (30 mM) induces RGC death in culture, an effect that is partially reversed by DEX, raising the possibility that RGCs themselves could secrete pro-inflammatory agents that act as autocrine effectors and that possibly induce cell death. We analyzed the secretion of pro-inflammatory cytokines by RGCs, yet the test used was not sufficiently sensitive given the small number of pure RGCs that can be cultured. Neurons are typically examined as targets for cytokine signaling, with various cytokine receptors expressed on neural membranes

(Bajetto et al., 2002; Gougeon et al., 2013). However, neurons and specifically RGCs, are also a source of releasable cytokines including: Interleukin 3 (IL-3), TNF-α, CXCL9, VEGF, L-selectin, IL-4, GM-CSF, IL-10, IL-1Rα, MIP and CCL20 (Freidin et al., 1992; Lim et al., 2016; Yamamoto et al., 2011). Indeed, high levels of glucose activate the overexpression of pro-inflammatory cytokines (IL-1β and IL-18) by RGCs (Shanab et al., 2012), and the expression of TLR-2 and TLR-4 by RGCs, enhancing the secretion of pro-inflammatory factors (Du et al., 2003; Tang and Kern, 2011; Zhao et al., 2016). Although DEX partially rescues RGCs exposed high glucose and we believe that inflammatory pathways could be implicated in this effect, it is possible that other mechanisms are also at play. Many factors could be involved in the death of RGCs associated with hyperglycemia and indeed, such a high-glucose environment could enhance oxidative stress and mitochondrial dysfunction, thereby accelerating the apoptosis of RGCs via caspase-3 activation (Cao et al., 2014; Du et al., 2003; Shanab et al., 2012). It is noteworthy that because of the high-energy demand of RGCs, they have large numbers of mitochondria (Carelli et al., 2004; Osborne and del Olmo-Aguado, 2013), and consequently, it is not surprising that they are particularly vulnerable to any mitochondrial dysfunction in DR. In addition, the expression of the high mobility group box 1 (HMGB-1), a chromatin protein that can promote angiogenesis and induce inflammation, may also be implicated in hyperglycemic RGC death (Zhao et al., 2015).

RGCs are more adversely affected by high glucose concentrations (30 mM) when they are co-cultured with Müller cells than when growing in pure culture. Furthermore, at 10 mM glucose RGCs are significantly affected when they are co-cultured with Müller cells, with the survival of the latter also affected by high glucose conditions. Müller cell death following exposure to high glucose conditions (25–30 mM glucose) has been described elsewhere (Kusner et al., 2004a; Walker et al., 2012), and it has potentially been related to AKT inhibition (Xi et al., 2005). Indeed, when rat retinal Müller glia were exposed to 25 mM glucose for 72 h culture, Akt was inactivated and apoptosis induced. Moreover, hyperglycemia stimulates GAPDH accumulation in the nucleus of retinal Müller cells, in association with the apoptosis of these cells. (Jayaguru and Mohr, 2011; Kusner et al., 2004a). It is possible that Müller cell death could also affect RGCs, accentuating the deleterious effect of glucose on RGCs. Moreover, Müller cell gliosis could damage RGCs under conditions of stress. Gliosis of Müller cells can have both cytoprotective and cytotoxic effects on retinal neurons depending on the extent of

glial activation (Lorenzi and Gerhardinger, 2001). It is already known that diabetes induces abnormalities in retinal Müller cells, including increased GFAP expression, reduced glutamine synthetase and decreased glutamate transporter activity (Li and Puro, 2002). It is well established that Müller cells become activated in DR (Gerhardinger et al., 2005; Kusner et al., 2004b; Puro, 2002a) but nevertheless, the contribution of Müller cells to DR remains unclear (Kusner et al., 2004a).

RGC death caused by exposure to high glucose might be provoked by the up-regulation of secreted pro-inflammatory factors (Zhao et al., 2015; Zhao et al., 2016) and several studies confirm the relevance of Müller glia in the provision of inflammatory factors after activation, such as cytokines. Müller glia are the main source of many cytokines and other inflammatory factors in the gliotic retina *in vitro* (Eastlake et al., 2016), which may serve to protect Müller cells from diabetic insult, and consequently, retinal neurons. The damage produce by these factors in DR might be only a secondary effect (Coughlin et al., 2017). In DR, Müller cells exhibit a reactive phenotype characterized by the activation of inflammatory-related genes (Lorenzi and Gerhardinger, 2001). The proinflammatory response of Müller glia has been associated to the activation of the receptor for advanced glycation end-products (RAGE) and its ligand S100B, which stimulate cytokine production through MAPK signaling pathways (Zong et al., 2010). The accumulation of these inflammatory mediators is thought to enhance early neuronal cell death in the retina (Eastlake et al., 2016), which would explain the contribution of Müller cells to the decrease in RGC survival in our co-cultures.

Inflammation is considered to play a critical role in the progression of diabetic complications, including DR (Yamamoto et al., 2011). Glucocorticoids like DEX are powerful anti-inflammatory compounds. They are steroid hormones that cross the cell membrane and interact with intracellular glucocorticoid receptors (GCRs). The distribution of GCRs in the retina is highly conserved and as they are almost exclusively restricted to Müller glia, and GCRs might enhance survival indirectly through these glial cells (Gallina et al., 2015; Gallina et al., 2014). The data obtained here show that when RGCs are exposed to high glucose, more RGCs die when they are in contact with Müller glia than when they grow alone. However, DEX rescues these cells in both situations, which indicates it has a direct effect on RGCs. Glucocorticoids like DEX can induce endotoxin tolerance, acting as negative regulators of TLR4 signaling, and

efficiently downregulating the production of pro-inflammatory cytokines (Osborne and del Olmo-Aguado, 2013).

The anti-inflammatory properties of DEX may rescue RGCs from death and thus, our results suggest that inflammation plays a critical role in the RGC death provoked by high glucose concentrations *in vitro*. To determine the effect of DEX on RGC survival in such high glucose conditions and its critical influence on inflammation in DR, the implication of cytokines in the inflammatory response caused by hyperglycemia was analyzed in conditioned medium. The levels of IL-1β, IL-6 and TNFα increase significantly in co-cultures maintained in the presence of high glucose, while DEX reverts the concentration of these cytokines to basal values. Hence, these cytokines may be involved in RGC death, since RGC survival decreases when the concentration of these cytokines augments.

When we cultured RGCs and Müller cells with the three cytokines and in the presence of DEX, IL-1β, IL-6 and TNFα appear to be directly involved in RGC death. However, DEX could not rescue RGCs maintained in the presence of the cytokines suggesting it has no effect once they are secreted into the medium. Thus, DEX might be implicated in dampening the release or expression of these cytokines by Müller glia and RGCs. Glucocorticoids like DEX, can exert multiple anti-inflammatory effects, even directly interacting with the transcriptional machinery through the TLRs expressed by Müller cells (Zarbin, 2016) and RGCs (Shanab et al., 2012). TLR signaling pathways may be important targets for DEX (Barber et al., 2001) and thus, DEX could activate the TLR signaling that inhibits the inflammatory response.

IL-1β, IL-6 and TNFα are pro-inflammatory cytokines, and IL-6 is a potential mediator of intraocular inflammation (Lim et al., 2016), inducing numerous physiological and immune responses (Gerhardinger et al., 2005). Müller glia cells release several inflammatory factors and cytokines (Eastlake et al., 2016), and some cytokines are even known to stimulate the production of other cytokines by Müller glia (Yoshida et al., 2001), including IL-6 (Yoshida et al., 2001), IL-1β (Kusner et al., 2004b) and TNFα (Puro, 2002a) in response to different stressors. Moreover, Müller cells are a major source of retinal IL-1β (Busik et al., 2008; Lei et al., 2011; Yego et al., 2009) and they also secrete TNFα, facilitating the apoptotic death of RGCs in response to damage (Tezel and Wax, 2000). Together, these findings suggests that in hyperglycemic conditions, Müller cells could enhance RGC death in co-cultures by releasing these

cytokines, while DEX can reverse this effect by recovering basal cytokine levels. High glucose conditions also increase the concentrations of IL-2 and IL-10, yet DEX does not decrease the production of these cytokines. Increased IL-2 has been seen in experimental models of DR (Tezel and Wax, 2000), while IL-10 is an anti-inflammatory cytokine (de Waal Malefyt et al., 1991), which may be related to protection against the development of late diabetic complications (Hassan et al., 2017). Indeed, DEX induces the release of anti-inflammatory cytokines like IL-10 less than that of pro-inflammatory cytokines (Qiu et al., 2016).

The death of Müller cells in hyperglycemic conditions may be related to other mechanisms not assessed here. This would be consistent with the failure of DEX to rescue these cells and their insensitivity to exogenous cytokine addition. Such effects may be associated with perturbations to essential functions, such as neurotransmitter recycling, the control of glutamate toxicity, a redistribution of ions or the regulation of metabolic pathways, among others (Bringmann et al., 2006; Coughlin et al., 2017). For example, the expression of Kir4.1 (an inwardly rectifying potassium channel) is altered by TNF-α in the Müller cells of diabetic patients (Hassan et al., 2017). Other cytokines not considered here could also alter adaptor molecules like Act 1, thereby provoking damage (Qiu et al., 2016). Also, in a context of diabetes-induced retinal gliosis, long non-coding RNAs affect Müller cell viability and proliferation (Liu et al., 2016). Indeed, oxidative stress suppresses glutamine synthetase activity in diabetic conditions, mediated by the thioredoxin-interacting protein, TXNIP (Zhou et al., 2016). Additionally, Glutaredoxin, an enzyme that catalyzes the deglutationation of key targets like IKK (a kinase that activates NF-κβ receptors), appears to be associated with elevated IL-6 secretion in vitro (Shelton et al., 2009).

Our findings may have therapeutic implications. DME is the main cause of vision loss in patients with DR (Liu et al., 2016) and the most common first line therapy for DME is IVT injection of anti-VEGF agents (Shelton et al., 2009; Zhou et al., 2016). Another therapeutic option for DME is the injection of Ozurdex, an DEX slow release IVT implant that administers this drug over 4 to 6 months. Thus, DEX may also protect RGCs in the context of DR when used in a slow release formula.

In summary (Figure 5), we conclude that high concentrations of glucose (30 mM) decrease the survival of RGCs when cultured alone or with Müller glia. This effect could be at least partially due to the secretion of cytokines, in particular L-1β, IL-6 and TNFα, which were elevated in the milieu after glucose treatment. The influence of these cytokines was confirmed when they were added exogenously. Moreover, DEX prevents RGC death in hyperglycemic conditions, at least partially by dampening the release of cytokines.

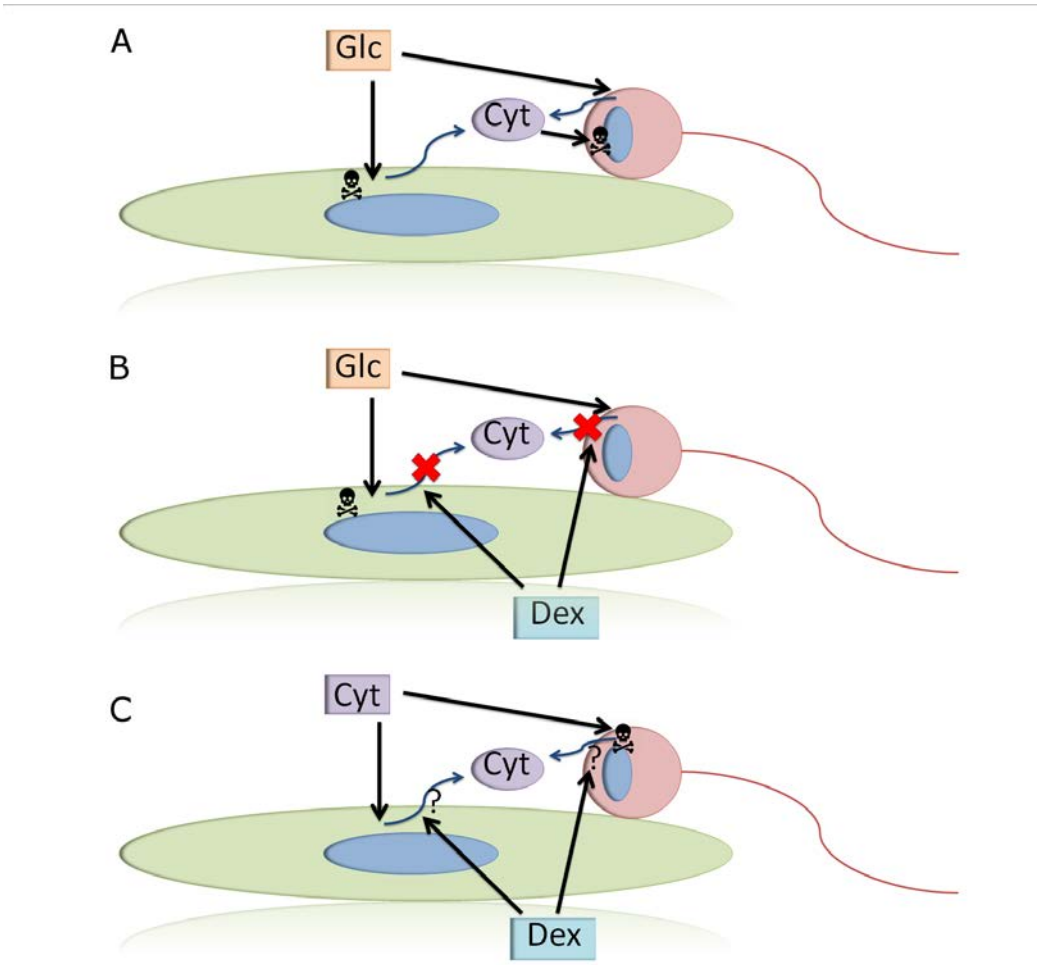

Figure 5: Scheme showing the possible mechanisms active in Müller cell and RGC co-cultures exposed to high glucose, dexamethasone and cytokines. (A) High glucose (30 mM) has a detrimental effect on Müller glia (green) and RGCs (red). Cytokine secretion may be responsible for the effects on cell survival observed. (B) When DEX is added in a high glucose environment, the concentration of cytokines decreases and consequently, RGC survival is enhanced. However, Müller cells were not

rescued by DEX. (C) When cytokines are added to the media, RGCs survival is compromised while that of Müller cells is not affected, either in the presence or absence of DEX. We conclude that DEX could inhibit cytokine release, and that RGCs and Müller cells may exhibit distinct sensitivity to the action of cytokines. More experiments will be necessary to elucidate the precise mechanism(s) that relate high glucose-induced cytokine secretion and retinal cell survival.

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5. Discusión

Las células ganglionares de la retina (RGCs) son responsables de propagar la información visual del ojo al cerebro a lo largo de sus axones, que forman el nervio óptico (Wassle and Boycott, 1991). Estas neuronas son especialmente vulnerables y se ven afectadas en muchas patologías oculares dando lugar a la pérdida de visión. Poseen muchas particularidades respecto a otras neuronas de la retina, presentan una elevada demanda energética, debido a la que sus axones no están mielinizados hasta la lámina cribosa y son también altamente susceptibles a la privación de oxígeno (Della Santina and Ou, 2017). Debido al aumento de la población de personas de edad avanzada en muchos países, el impacto socioeconómico de la discapacidad visual y la ceguera como resultado de tales enfermedades, en los últimos años, se ha incrementado su investigación tanto para conocer más profundamente las patologías en las que se ven involucradas como las vías de neuroprotección.

Las células de Müller, la glía más importante de la retina, son las responsables del soporte homeostático y metabólico de las neuronas de la retina (Reichenbach and Bringmann, 2013). Tienen un papel crucial tanto en la retina sana como enferma y una de sus funciones más importantes y en las que está basada la presente tesis doctoral es la neuroprotección. Por lo que el aumento del conocimiento de esta función de las células de Müller y las respuestas a los estímulos en la retina normal y enferma tendrá un gran impacto para el desarrollo de nuevos enfoques terapéuticos para las enfermedades de la retina.

Para poder llevar a cabo estudios, es de vital importancia el desarrollo de modelos *in vitro* que nos ayuden a entender las enfermedades, el papel de los diferentes tipos celulares en ellas y la búsqueda de nuevos agentes terapéuticos.

En el **ANEXO 1** describimos un protocolo optimizado y detallado para el aislamiento y cultivo de células de Müller adultas. Hasta la fecha, los métodos descritos en la literatura, se centran en el aislamiento de las células de Müller de animales neonatales o muy jóvenes. Sin embargo, la mayoría de enfermedades neurodegenerativas la sufren individuos adultos siendo una herramienta más útil poder estudiar estas enfermedades a partir de tejido adulto. Para encontrar el mejor método para cultivar células de Müller, probamos sistemáticamente diferentes condiciones basadas en los artículos previos en los que se cultivaban este tipo de células: las enzimas para la digestión del tejido, los sustratos y el medio de cultivo.

Para determinar el mejor método para cultivar células de Müller adultas, primero evaluamos dos enzimas diferentes que se han usado comúnmente en protocolos previos para digerir el tejido: la papaína y la tripsina. Se obtuvieron más células Müller cuando las retinas se digirieron con papaína y estos cultivos alcanzaron la confluencia a los 7 días *in vitro* (DIV). Los cultivos digeridos con tripsina tardaron más en alcanzar la confluencia, probablemente debido al menor número de células que sobrevivieron a la digestión. Por lo tanto, llegamos a la conclusión de que la papaína es una enzima más suave para la digestión de las retinas que la tripsina, lo que concuerda con los estudios en los que la tripsina podría ser más tóxica para las neuronas que la papaína (Tabata et al., 2000).

Con respecto al sustrato, cultivamos las células en cubreobjetos de vidrio sin tratar, o en cubreobjetos tratados con poli-L-lisina solo o en combinación con laminina. Como se esperaba, la supervivencia y proliferación de las células de Müller fue mejor cuando se cultivaron en poli-L-lisina + laminina. De hecho, la poli-L-lisina mejora la adherencia celular debido a la interacción entre el polímero cargado positivamente y las células o proteínas cargadas negativamente (Mazia et al., 1975). Estos datos fueron consistentes con los estudios previos del grupo que muestran que la poli-L-lisina y la laminina es el mejor sustrato para las células de la retina, incluidas las células de Müller (Garcia et al., 2002; Ruzafa and Vecino, 2015; Vecino et al., 2015). La laminina ejerce una variedad de actividades biológicas, que no solo median la unión celular, sino que también influyen en la proliferación, diferenciación y motilidad de las células (Paulsson, 1992). De hecho, la membrana limitante interna sobre la que se estructura y polariza las células de Müller, está constituida entre otros componentes por laminina-1 (Mehes et al., 2002; Vecino and Kwok, 2016).

Un paso crucial en el cultivo celular es la selección del medio de cultivo apropiado. De hecho, estudios previos del grupo demostraron que el secretoma de las células de Müller primarias en cultivo está influenciado por las condiciones de cultivo (Ruzafa et al., 2018). Un medio de cultivo típico está compuesto por un complemento de aminoácidos, vitaminas, sales inorgánicas, glucosa y suero como fuente de factores de crecimiento, hormonas y factores de unión. Aquí seleccionamos 3 medios de cultivo diferentes basados en los más utilizados en protocolos anteriores: DMEM + 10% FBS; DMEM-F12; y NBA B27 + 10% FBS. DMEM-F12 es un medio extremadamente rico y complejo que fue diseñado para contener nutrientes, factores de crecimiento y hormonas

en lugar de un suplemento de suero (Mather et al., 1981). Sin embargo, el número de células de Müller que crecieron en DMEM F12 a 7 DIV fue significativamente más bajo que cuando las células se cultivaron en DMEM + FBS al 10%. Esto puede ser debido a que la proliferación de células de Müller es estimulada por numerosos factores de crecimiento y citoquinas derivadas del suero sanguíneo (Ikeda and Puro, 1994), lo que podría explicar las diferencias observadas cuando se utilizó DMEM-F12 sin suero como medio. En el tercer medio probado, NBA B27 + FBS al 10%, no se obtuvieron cultivos puros debido al crecimiento de neuronas en el cultivo. De hecho, el medio Neurobasal-A está diseñado para cumplir con los requisitos específicos de las células neuronales en cultivo. Por lo tanto, el medio DMEM + 10% FBS es la mejor opción para cultivar células de Müller, ya que esta combinación limita la aparición de neuronas en el cultivo al tiempo que fomenta la proliferación de células de Müller.

Para confirmar que las células de Müller conservan sus características después de 7 días in vitro, analizamos la expresión de conocidos marcadores de células de Müller en cultivos de cerdo, rata y ratón: GFAP (Lewis et al., 1988), glutamina sintetasa (Mack et al., 1998), p75NTR (Garcia et al., 2002; Schatteman et al., 1988) y Vimentina (Davidson et al., 1990). Confirmamos la expresión de estos marcadores moleculares en nuestros cultivos de células de Müller de mamíferos adultos, validando nuestro protocolo. Además, la pureza de los cultivos de células de cerdo de Müller en adultos se confirmó mediante un análisis inmunocitoquímico por citometría de flujo ya que un 96% de las células fueron positivas para p75NTR.

Asimismo, en este análisis se detectaron dos subpoblaciones de células de Müller que se distinguían por sus propiedades físicas (tamaño y granulosidad de la membrana). Aunque posiblemente esta heterogeneidad se deba a que las células que son de pequeño tamaño acaben de pasar por el proceso de mitosis, que son aproximadamente un 10%, que además coincide con el porcentaje de células que se encuentran en fase S en el análisis del ciclo celular. La existencia de heterogeneidad de las células gliales ha sido descrita pero sin profusión (Luna et al., 2010; Vecino et al., 2016). De hecho, estas células tienen una expresión heterogénea de diferentes proteínas por ejemplo GFAP (Bringmann et al., 2006). Por lo tanto, no es sorprendente encontrar diferentes subpoblaciones de células de Müller que se distinguen por sus propiedades físicas.

También cabe resaltar la reproducibilidad del método en diferentes tipos de animales en este caso cerdo, rata y ratón. Las células de Müller de los 3 animales se grabaron en un

microscopio adaptado con tecnología “time-lapse” que consiste en sacar una imagen cada cierto periodo de tiempo (10 minutos). Después se analizó el vídeo que forman esas imágenes teniendo en cuenta: el tiempo entre divisiones, el tiempo que tardan en dividirse y el número de células de Müller que se dividen en cada campo y no hubo diferencias significativas entre los cultivos de cerdo, rata y ratón. Además, en el análisis del ciclo celular, un 11.1% de células estaban en fase S y 7.7% en fase G2 / M. Estos resultados, son consistentes con los resultados del “time-lapse”, en los que 10.17% de las células de Müller se dividieron por campo, en un plazo de 8 horas (que es el tiempo mínimo en el que una misma célula de Müller vuelve a dividirse). Además, estos valores corresponden al el perfil típico de un cultivo primario (Pozarowski and Darzynkiewicz, 2004).

Por lo tanto, el método descrito es fiable y fácilmente reproducible para obtener y cultivar células de Müller de retinas adultas y en comparación con otros protocolos publicados, es más rápido y se obtiene un mayor número de células.

El desarrollo de este método, ayudará a avanzar más fácilmente con la investigación de las enfermedades por ejemplo probando los efectos de los medicamentos, comprendiendo las patologías, analizando la capacidad neuroprotectora de estas células o también para el desarrollo de nuevas metodologías para el estudio de moléculas de complejo análisis como por ejemplo los lípidos.

Los avances más recientes en el estudio de los lípidos consideran que son moléculas con importante relevancia tanto en el estudio de la función de la retina normal y patológica, como para el diagnóstico de las enfermedades de la retina. Aun se sabe muy poco acerca de los perfiles lipídicos en la retina fisiológica y las diferencias de los lípidos entre las células que componen la retina. Debido a esto, se propuso identificar los lípidos de cada tipo celular en secciones retinas de cerdo en comparación con los cultivos celulares primarios de RGCs y células de Müller, utilizando MALDI-IMS.

En el **ANEXO 2 se** estudian de forma comparada el perfil lipídico en dos tipos celulares concretos de la retina neuronas (RGCs) y células de la glía (Müller) *in vivo* e *in vitro* Además, se generaron microarrays de células y membranas celulares completas (tanto de RGCs como de células de Müller), lo que permitió realizar múltiples estudios lipídicos en estas muestras y comparar los resultados obtenidos con los lípidos obtenidos de las secciones de la retina.

Los lípidos de las membranas de la retina se han estudiado ampliamente y se ha obtenido mucha información sobre las diferentes clases de lípidos y sobre sus composiciones de ácidos grasos (Fliesler and Anderson, 1983), por ejemplo, se sabe que la bioquímica de los lípidos en la retina humana es bastante singular, sobre todo por la acumulación específica de DHA (22: 6) que está esterificada en diferentes clases de fosfolípidos y especies moleculares dentro de la retina. Sin embargo, todavía no se habían identificado lípidos que pudieran estar más representados en uno u otro tipo celular. Estudios previos, demostraron que ciertos lípidos se distribuyen específicamente en diferentes capas de la retina (Anderson et al., 2014; Zemski Berry et al., 2014), pero ninguno de ellos puede ser atribuido a ningún tipo celular en concreto.

La importancia del conocimiento de los lípidos entre las diferentes células de la retina se debe a que pueden proporcionar información única sobre el estado homeostático de un organismo y el desarrollo de estados patológicos. El protocolo describe una metodología en la que después de un exitoso análisis de correlación en el que los resultados de los experimentos *in vitro* se extrapolaron fácilmente a los resultados *in vivo*, se encontró un grupo de lípidos en los que cuatro picos estaban más significativamente representados en las RGCs y dos que eran más prevalentes en las células de Müller, tanto en secciones y como en los microarrays de los cultivos celulares.

Los lípidos encontrados pertenecen a las familias fosfatidilcolina, fosfatidiletanolamina y fonfatidilinositoles, las familias más abundantes de fosfolípidos en la retina.

Por lo tanto, gracias a la metodología descrita y al uso de cultivos celulares de forma complementaria a las secciones de retina, se puede definir la distribución espacial de varias especies de lípidos, incluidas las PE, las PC y las IP, que representan el primer estudio comparativo entre ensayos *in vivo* e *in vitro* Estudios previos del grupo en 2003 demostraron que los cultivos de neuronas y glía de la retina de cerdo adulto mantenían las características moleculares que poseen in vivo cuando se cultivan in vitro (Garcia et al., 2003). Dado que hay lípidos que son más característicos de las células de Müller o de las RGCs, que, además, pertenecen a algunas de las familias de lípidos más relevantes, sugiere que podrían cumplir una función en diferentes actividades celulares. Por lo que, esta tecnología, podría usarse para comparar la retina sana con la patológica para identificar cambios lipídicos relacionados con distintas enfermedades. Estudios en los que se aplicara esta técnica, proporcionarían más información sobre las

implicaciones de los lípidos en las enfermedades de la retina, identificando nuevas dianas terapéuticas para prevenir la progresión de las enfermedades de la retina.

Los cultivos celulares también nos proporcionan una gran herramienta para estudiar la capacidad de las células de Müller para neuroproteger a las RGCs. Por ello el efecto neuroprotector de las células de Müller fue analizado en las RGCs diferenciadas de células madre.

Las células de Müller liberan factores neurotróficos que apoyan la supervivencia de RGC (Garcia et al., 2002; Ruzafa et al., 2018) y, además, se sabe que su secretoma también contiene moléculas neurotróficas, que también promueven la supervivencia de las RGCs (von Toerne et al., 2014) y de los fotorreceptores (Balse et al., 2005).

En el **ANEXO 3**, probamos el efecto que pudieran tener las células de Müller sobre células progenitoras neuronales y su capacidad para ayudar a la diferenciación en RGCs. Estos estudios los realizamos en ratones durante mi estancia de tres meses en la Universidad de Davis.

En los últimos años, las terapias de trasplantes celulares para el tratamiento de enfermedades neurodegenerativas han crecido mucho y una de las últimas propuestas es el uso de RGCs diferenciadas de células madre para este fin. Sin embargo, se ha demostrado que el número de RGCs obtenido de células pluripontentes disminuye con el tiempo en cultivo (Aparicio et al., 2017). Esto podría impedir el progreso de estas terapias. Nuestros resultados, mostraron que en co-cultivo de las células de Müller adultas y RGCs diferenciadas de células madre obtenidas de la disociación de los cuerpos embriónicos (EBs), las neuronas aumentan notablemente su supervivencia.

Esto se confirmó con el uso del medio condicionado de las células de Müller adultas, se mantuvieron cultivos de los cuerpos embriónicos disociados donde se encontraban las RGCs, con el medio acondicionado de los cultivos de células de Müller para evaluar si el aumento de la supervivencia de las RGCs derivadas de células madre se debe a factores liberados de estas. Nuestros resultados mostraron que con el uso del medio condicionado en los cultivos de RGCs diferenciadas de células madre, también aumenta significativamente su supervivencia. Por lo que el efecto de las células de Müller no parece estar mediado solo por servir de sustrato, sino también, por sus factores secretados. Sin embargo, el efecto de las células de Müller en co-cultivo en la

supervivencia de las RGCs, es incluso más fuerte que solo con el medio condicionado, lo que sugiere posibles efectos sinérgicos del sustrato y factores secretados.

El efecto del medio condicionado también se analizó en los EBs que no se habían disociado. Los resultados obtenidos demostraron el aumento de la expresión de Brn3a. En la retina, el factor de transcripción de Brn3a se expresa exclusivamente en las RGCs (Nadal-Nicolas et al., 2009). En consecuencia, el aumento de la expresión de Brn3a supone que el medio condicionado de las células de Müller adultas también aumenta la supervivencia de las RGCs diferenciadas de células madre en EBs no disociadas. También se evaluó la expresión de Math5, factor de transcripción que se expresa de forma transitoria durante la histogénesis de la retina temprana y es necesario para el desarrollo de las RGCs (Brown et al., 2001; Brzezinski et al., 2012). Nuestros resultados mostraron que en las EBs que crecen con el medio condicionado aumentan la expresión de Math5, lo que sugiere que los factores liberados por las células de Müller no solo aumentan la supervivencia de los RGC, sino que también aumentan las células progenitoras que podrían diferenciarse a RGCs.

Por lo tanto, las células de Müller liberan factores que aumentan la supervivencia de las RGCs derivadas de células madre y, además, también pueden contribuir a determinar la diferenciación de las células progenitoras a RGCs. El uso de los cultivos celulares de células de Müller adultas y su medio condicionado, podrían mejorar las estrategias basadas en terapias de trasplante de RGCs diferenciadas de células madre que se están desarrollando actualmente.

Tal y como se ha comprobado, el uso de los cultivos de células de Müller como estrategia para la protección de las neuronas cuando su supervivencia se ve comprometida, puede ser una buena vía de actuación en las lesiones del sistema nervioso y los trastornos neurológicos (Hayashi and Takagi, 2015). Sin embargo, no todas las células de Müller responden de la misma forma frente a un daño, por lo que definir y comprender la heterogeneidad glial es un objetivo importante, ya que permitiría comprender mejor el papel fundamental de estas células durante las patologías de la retina e incluso optimizar su capacidad neuroprotectora y abrir nuevas vías terapéuticas. Visto que las células de Müller de la periferia de la retina expresan proteínas características de células progenitoras (Suga et al., 2014; Too et al., 2017) y que, en animales no mamíferos como el pollo, la regeneración de la retina se produce gracias a las células de Müller más periféricas .

En el **ANEXO 4,** estudiamos la heterogenicidad de las células de Müller atendiendo a su localización en la retina centro-periferia. Puesto que existen estudios previos (anteriormente mencionados) que indican el distinto posible estado de diferenciación entre la retina central y periférica. Y teniendo en cuenta que en vertebrados inferiores en la escala filogenética, como son los peces, es precisamente en la periferia de la retina donde se inicia la diferenciación de la retina (Vecino, 1998) hemos analizado el efecto neuroprotector de las células de Müller según su localización en la retina.

Primero se comprobó la supervivencia de las RGCs en cultivos de cerdo adulto según su localización en la retina, en estas condiciones, encontramos más RGCs en cultivos que provienen de la banda visual de la retina. Esto concuerda con la disposición de las RGCs en la retina, que no es homogénea y varía con la excentricidad, siendo la densidad de RGCs menor en la retina periférica (Drager and Olsen, 1981; Garca et al., 2005; Salinas-Navarro et al., 2009a; Salinas-Navarro et al., 2009b). Sin embargo, cuando las RGCs se co-cultivan con células de Müller, encontramos un mayor número de RGCs cuando las células (RGCs y células de Müller) provienen de la retina periférica aun habiendo menos RGCs en esta área de la retina. Este hecho sugiere que las células de Müller de la periferia de la retina tienen un efecto neuroprotector mayor en las RGCs que las células de Müller de la banda visual o las del centro de la retina. Este efecto neuroprotector de las células de Müller periféricas no solo fue efectivo en los co-cultivos con RGCs de cerdo, sino también cuando RGCs de rata se sembraron en células de Müller periféricas de cerdo, confirmando los resultados. Además, aunque ya se sabía que los factores neuroprotectores no solo actúan por contacto con las células de Müller, sino también por los factores secretados (Garcia et al., 2002; Ruzafa et al., 2018), el medio condicionando de las células de Müller de las tres áreas de la retina también se evaluó en cultivos de RGCs de rata, confirmando la misma tendencia, el medio condicionado de las células de Müller periféricas también aumentó la supervivencia y la regeneración de las neuritas de las RGCs. Estos resultados también sugieren que la capacidad neuroprotectora de las células de Müller periféricas es mayor que la capacidad de las células de Müller en las áreas más centrales de la retina y esta capacidad también implica la participación de factores secretados.

Estudios *in vivo*, encontraron que las células de Müller periféricas podrían expresar marcadores de desdiferenciación (Suga et al., 2014; Too et al., 2017) por lo que una vez que confirmamos la mayor capacidad de neuroprotección de las células de Müller

periféricas, analizamos la expresión tanto de marcadores específicos de células de Müller como p75NTR (Vecino et al., 1998b; Xu et al., 2009) y vimentina (Reichenbach and Bringmann, 2015), como también también un marcador neural β-III-Tubulina (Jiang et al., 2015) y α-SMA, que se considera el marcador típico de células que presentan un fenotipo fibroblástico (Darby et al., 1990), para comprobar si dependiendo de la localización de la retina de la que provienen había diferencias en relación con la capacidad de desdiferenciación y el efecto neuroprotector. Nuestros resultados encontraron que en el mismo día de cultivo todas las células de Müller de la banda visual, centro y periferia de la retina expresan los marcadores de células de Müller específicos p75NTR y vimentina, sin embargo, las células de Müller periféricas expresan notablemente más β-III-Tubulina y α-SMA, lo que sugiere que se encuentran en un estado más desdiferenciado. Que células más desdiferenciadas tengan más capacidad neuroprotectora puede explicarse con estudios en los que células madre mesenquimales, que en un grado de desdiferenciación mucho mayor aumentan notablemente la supervivencia de las RGCs bajo diferentes condiciones patológicas mediante la liberación de factores neurotróficos y neuroprotectores (Cui et al., 2017; Osborne et al., 2018; Pan et al., 2019). Esto podría sugerir que en un estado más desdiferenciado de las células de Müller periféricas, secreten más factores más neurotróficos y neuroprotectores. Próximos estudios podrían confirmar esta teoría analizando por proteómica los medios condicionados de las células de Müller de las tres áreas de la retina utilizadas en este estudio. Que además, servirían para identificar posibles dianas terapéuticas para fomentar la neuroprotección y regeneración de las RGCs.

Por último, en el **ANEXO 5**, el efecto neuroprotector de las células de Müller también fue evaluado no solo en condiciones fisiológicas sino también en un modelo *in vitro* de retinopatía diabética (RD). La hiperglucemia, se considera un factor importante en la etiología de la RD. Además, las RGCs, obtienen glucosa directamente y también, la obtienen a través de astrocitos o de las células de Müller (Wood et al., 2005).

Para este estudio se utilizaron cultivos de RGCs puras sometidas a altas concentraciones de glucosa (30 mM) en los que se comprobó que, a esta concentración, se induce la muerte de RGCs en el cultivo.

Cuando las RGCs se co-cultivaron con las células de Müller para analizar su efecto neuroprotector en altas concentraciones de glucosa, el porcentaje de supervivencia de

las RGCs fue aún menor que cuando se cultivaron solas. Bajo estas condiciones las células de Müller también veían afectada su supervivencia. En estudios previos, después de la exposición de las células de Müller a condiciones de alta glucosa (25-30 mM de glucosa) también disminuye su supervivencia de las células de Müller (Kusner et al., 2004; Walker et al., 2012), y se ha relacionado con la inhibición de AKT (Xi et al., 2005). Además, la hiperglicemia estimula la acumulación de GAPDH en el núcleo de las células de Müller, y esto se asocia con el proceso de apoptosis en estas células (Jayaguru and Mohr, 2011; Kusner et al., 2004). Por lo que es posible que la muerte de las células de Müller también pueda afectar a las RGCs, acentuando el efecto perjudicial de la glucosa. Hay que tener en cuenta que otro proceso celular por el que las células de Müller podrían estar afectando más a las RGCs y que además es muy común en la RD es la gliosis. La gliosis puede tener efectos tanto citoprotectores como citotóxicos en las neuronas de la retina, según el grado de activación glial (Lorenzi and Gerhardinger, 2001). Aunque está bien establecido que las células de Müller se activan en la RD (Gerhardinger et al., 2005; Kusner et al., 2004; Puro, 2002), la contribución de las células de Müller a la RD no está clara (Kusner et al., 2004).

Una de las características del proceso de gliosis es el aumento de expresión de factores pro-inflamatorios (Zhao et al., 2015; Zhao et al., 2016) y además las células de Müller son capaces de secretarlos (Eastlake et al., 2016). Esta respuesta pro-inflamatoria de las células de Müller es estimulada por la producción de citoquinas a través de las vías de señalización MAPK (Zong et al., 2010), lo que explicaría la contribución de las células de Müller a la disminución de la supervivencia de las RGCs en co-cultivo.

Para confirmarlo, se analizó la secreción de citoquinas causada por la hiperglucemia en medio condicionado de los cultivos. Los niveles de IL-1β, IL-6 y TNFα aumentaron significativamente en los co-cultivos mantenidos en condiciones altas de glucosa. Por lo tanto, estas citoquinas pueden estar involucradas en la muerte de las RGCs, ya que la supervivencia de las RGCs disminuye cuando aumenta la concentración de estas citoquinas.

Para confirmar que la muerte de las RGCs se debe al aumento de IL-1β, IL-6 y TNFα se co-cultivaron a las RGCs y células de Müller esta vez añadiendo IL-1β, IL-6 y TNFα. La supervivencia de las RGCs se vio afectada, lo que sugiere que están directamente involucradas en la muerte de las RGCs.

Los glucocorticoides como la dexametasona son poderosos compuestos antiinflamatorios utilizados comúnmente en el tratamiento de esta enfermedad. Son hormonas esteroides que atraviesan la membrana celular e interactúan con los receptores de glucocorticoides intracelulares (GCR). La distribución de los GCR en la retina está altamente conservada y están restringidos casi exclusivamente a las células de Müller (Gallina et al., 2014; Gallina et al., 2015). Por lo tanto, para determinar el efecto de la dexametasona en la supervivencia de las RGCs se trataron los cultivos con este glucocorticoide combinándolo con altas concentraciones de glucosa. Los datos obtenidos aquí demuestran que la dexametasona rescata a las RGCs tanto solas como en co-cultivo con las células de Müller. Además, cuando se analizaron las citoquinas en el medio secretado, los cultivos tratados con dexametasona bajaron los niveles de IL-1β, IL-6 y TNFα. Esto es debido a que los glucocorticoides como la dexametasona pueden inducir tolerancia a las endotoxinas, actuando como reguladores negativos de la señalización de TLR4, y regulando eficientemente la producción de citoquinas pro-inflamatorias (Osborne and del Olmo-Aguado, 2013).

Sin embargo, la dexametasona no pudo rescatar las RGCs cultivadas en presencia de las citoquinas añadidas al medio. Esto sugiere que no tiene efecto una vez que las células de Müller ya han secretado las citoquinas al medio. Por lo tanto, la dexametasona podría estar implicado en regular la disminución de la liberación o expresión de estas citoquinas por parte de las células de Müller.

Gracias a este estudio y al uso de los cultivos celulares, hemos podido comprobar otra cara de las células de Müller bajo la que pueden contribuir a la neurodegeneración en la retina cuando están sometidas a un estrés, en este caso, condiciones hiperglicémicas. Además, también se ha confirmado el papel neuroprotector de la dexametasona ayudando a esclarecer una de las funciones de este tratamiento en los pacientes con esta patología.

En conclusión, las células de Müller son las células de la glía más importantes de la retina, con múltiples funciones tanto en la retina sana como la enferma. Su estudio se ha incrementado notablemente en los últimos años y su interacción con las RGCs es clave para la comprensión del funcionamiento de estas células en la retina. Una de las funciones estudiadas en esta tesis doctoral ha sido la capacidad neuroprotectora en diferentes situaciones. Para ello, se han establecido cultivos de glía de Müller y estos cultivos también han permitido crear un método para el estudio de los lípidos que, por

primera vez, nos han permitido diferenciar lípidos entre las células de Müller y las RGCs creando un punto de partida para el estudio de enfermedades desde el punto de vista lipídico.

La capacidad neuroprotectora de las células de Müller también ha sido evaluada sobre las RGCs diferenciadas de células madre, mejorando así futuras terapias de trasplantes celulares para pacientes con enfermedades neurodegenerativas como el glaucoma. Además, debido a la heterogeneidad existente en la población de estas células también se estudió la capacidad de neuroprotección según su localización en la retina descubriendo una mayor capacidad neuroprotectora en las células de Müller que procedían de la periferia de la retina. Futuros estudios podrían descubrir nuevas moléculas o una combinación de ellas para su uso como dianas terapéuticas. Por último, también se ha analizado el papel de las células de Müller en un modelo de retinopatía diabética demostrando cómo bajo un estímulo patológico, en este caso alta concentración de glucosa, las células de Müller también pueden contribuir a la neurodegeneración siendo esta revertida gracias al uso de un glucocorticoide, la dexametasona. Todas las conclusiones obtenidas en esta tesis doctoral, ayudarán a la comprensión de las interacciones entre las células de Müller y las RGCs abriendo nuevas vías de investigación, siempre con la intención de mejorar el pronóstico y los tratamientos actuales de las enfermedades degenerativas de la retina.

6. Conclusiones/Conclusions

Tras la realización de esta Tesis Doctoral, se han llegado a las siguientes conclusiones:

1- Hemos desarrollado un método de cultivo fiable, rápido y sencillo para la obtención de cultivos primarios puros de células de Müller de mamíferos adultos para el estudio de enfermedades de la retina.

2- Se ha descrito una huella lipídica que identifica mediante la técnica MALDI-IMS, las neuronas RGCs y glía de Müller manteniendo dicho perfil tanto in vivo como in vitro. sugiriendo que podrían cumplir una función específica en las diferentes actividades específicas de cada tipo celular.

3- Las células de Müller tienen un efecto neuroprotector sobre las células ganglionares diferenciadas desde células madre. Esta función se ejerce tanto por contacto, como mediante factores secretados. Además, promueven la diferenciación neuronal a partir de los progenitores neuronales.

4- El efecto neuroprotector de las células de Müller es mayor cuando proceden de la periferia que cuando proceden de en otras zonas mas centrales de la retina.

5- Condiciones hiperglicémicas provocan la muerte de un alto porcentaje de RGCs, siendo este porcentaje más acusado cuando se co-cultivan con las células de Müller. El efecto maligno es revertido con el uso de la dexametasona al inhibir la secreción de proteínas pro-inflamatorias por parte de las células de Müller.

Following the completion of this Doctoral Thesis, the following conclusions can be drawn:

1- We have developed a reliable, fast and simple culture method to obtain pure primary cultures of Müller glia from adult mammals in order to study retinal diseases.

2- Using the MALDI-IMS technique, a lipid footprint was described that identifies RGCs and Müller glia, a profile maintained in vivo and in vitro. The difference in the lipids that accumulate in these two cell types are likely to reflect their specific functions and activities.

3 - Müller cells have a neuroprotective effect on stem cell derived RGCs, an activity mediated both through contact and by secreted factors. In addition, these glia promote the neuronal differentiation of neuronal progenitors.

4- The neuroprotective effect of Müller cells is stronger when they come from the periphery than when they come from other more central areas of the retina.

5- Hyperglycemic conditions cause the death of a large number of RGCs and this effect is more pronounced when RGCs are co-cultured with Müller cells. The malignant effect is reversed by dexamethasone which inhibits the secretion of pro-inflammatory factors by Müller cells.

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