RESUMEN

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Rol del Ácido Retinoico en el desarrollo de neuronas de retina

La retina de los vertebrados está compuesta por cinco tipos de neuronas: fotorreceptores (FRs, conos y bastones), bipolares, ganglionares, horizontales y amacrinas, y células no neurales entre las que se destacan las células gliales de Müller. Durante el desarrollo, estas neuronas se originan a partir de células progenitoras que pasan a través de una serie de estados de competencia determinados por factores genéticos, celulares y moleculares, lo que permite la aparición ordenada y secuencial de los distintos tipos celulares (Livesey y Cepko, 2001b).

Entre las diversas moléculas y factores tróficos que influencian el desarrollo de los bastones se encuentran el Ácido Retinoico (AR) y el Ácido Docosahexaenoico (ADH). El AR ejerce una amplia variedad de efectos durante el desarrollo de los vertebrados y la diferenciación celular. Juega un rol crucial en la determinación del patrón antero-posterior del cuerpo, en la espermatogénesis, y en la formación y crecimiento de los miembros y de la piel. Además, es crítico para el desarrollo temprano del ojo y diferenciación de los FRs (Stenkamp y col., 1993; Prabhudesai y col., 2005; Hyatt y col., 1996; Khanna y col., 2006). El AR ejerce sus efectos en las células uniéndose y activando a receptores nucleares que funcionan como factores de transcripción y regulan así la transcripción génica. Por otro lado, en nuestro laboratorio se ha establecido que el ADH promueve la supervivencia y diferenciación de los FRs de retina de rata en cultivo, y que sus efectos anti-apoptóticos ocurren a través de la estimulación de la vía de la ERK/MAPK y de la modulación de la expresión de proteínas anti y pro-apoptóticas.

El **objetivo general** de este trabajo fue **estudiar los efectos del AR en el desarrollo de neuronas amacrinas y FRs de retina *in vitro***. Para ello utilizamos cultivos neuronales de retinas de rata postnatal desarrollados en medio químicamente definido, los cuales fueron suplementados con AR y/o ADH.

Dado que el AR es un factor de diferenciación celular nuestra hipótesis fue que, al igual que otros factores tróficos, esta molécula promovería además la supervivencia de los FRs. Sin embargo, cuando el AR se agregó al día 0 se incrementó el porcentaje de FRs apoptóticos, lo cual se correspondió con una pérdida de funcionalidad mitocondrial. Esta apoptosis pudo ser bloqueada completamente por el tratamiento con un pan-inhibidor de caspasas previo a la suplementación con AR. Estos resultados sugieren que el AR induciría la muerte de los FRs a través de un mecanismo apoptótico que involucra la pérdida de la actividad mitocondrial y activación de caspasas. Como el AR está ubicuamente presente en la retina y es esencial para su desarrollo, la preservación de FRs viables requeriría que su efecto pro-apoptótico fuera contrarrestado por la presencia simultánea de moléculas de supervivencia, como el ADH. Para poner a prueba esta hipótesis agregamos ADH a los cultivos previo al tratamiento con AR. Este agregado previno la muerte de los FRs inducida por el AR, respaldando la hipótesis de que durante el desarrollo se requeriría la presencia de otros factores de supervivencia para prevenir esta muerte. Notablemente, la inducción de apoptosis por AR afectó selectivamente a los FRs, resultando inalteradas las neuronas amacrinas.

Dado que el AR es reconocido por sus efectos promotores de la diferenciación, su efecto inductor de la muerte de los FRs fue un hallazgo inesperado. Esta observación hizo necesario verificar si, en las condiciones experimentales ensayadas, el AR favorecía o no la diferenciación. Comprobamos que el AR promovió marcadamente la diferenciación, en paralelo al aumento en el porcentaje de células apoptóticas. Determinamos, por inmunocitoquímica y Western Blot, que el AR incrementó la cantidad de FRs que expresaron opsina y periferina, proteínas características de FRs maduros, y que desarrollaron procesos apicales, rudimentos de los segmentos externos propios de estas neuronas maduras. Además, el AR aumentó el número de FRs que desarrollaron neuritas y la extensión alcanzada por las mismas.

Cabe destacar que a diferencia de los otros parámetros analizados, la estimulación del desarrollo de neuritas no fue selectiva para los FRs: el tratamiento con AR indujo el crecimiento de neuritas también en las neuronas amacrinas.

Dado que el AR y el ADH tienen efectos similares sobre la diferenciación, y que se unen a receptores que forman heterodímeros (RAR y RXR respectivamente), decidimos estudiar sus posibles efectos aditivos o sinérgicos. El tratamiento simultáneo con ambos factores aumentó la expresión de opsina y periferina a valores semejan la suma de los dos por separado. Estos resultados implican que el AR y ADH contribuyen a la diferenciación de los FRs en forma aditiva, y sugieren que estimularían vías independientes para promover sus efectos.

El hecho de que el AR indujera mayor expresión de proteínas y formación de estructuras de neuronas maduras, nos llevó a proponer que la funcionalidad de los FRs también podría estar estimulada. Sin embargo, observamos que el AR no estimuló la hidrólisis del GMPc, característica indicativa de una cascada de fototransducción activa y por consiguiente de capacidad de respuesta a la luz, ni la capacidad de incorporar neurotransmisores (como glutamato en los FRs y GABA en las neuronas amacrinas) del medio extracelular. Estos resultados indican que, aunque el AR promueve la diferenciación de los FRs y neuronas amacrinas, por sí solo no logra la maduración funcional de estas neuronas en cultivo, sugiriendo que se requeriría la presencia de otros factores.

La observación de que el AR inducía simultáneamente la diferenciación y simultáneamente la apoptosis nos hizo suponer que podría tener efectos distintos sobre distintas sub-poblaciones de FRs o sobre sub-poblaciones celulares en distintos estadios de maduración. Para corroborar esta hipótesis, se suplementaron los cultivos con AR al día 0, cuando la proliferación aún era activa, y al día 2, momento en el cual ya no había progenitores en proliferación. Notablemente, al tratar los cultivos al día 2, el AR estimuló la diferenciación de los FRs, aunque ya no se observó un aumento en la apoptosis. Estos resultados indican que el AR actuaría en forma diferencial según el estadio de desarrollo de los FRs, induciendo la apoptosis en una sub-población de aquellos que aun son progenitores indiferenciados y acelerando la diferenciación en los que ya han abandonado el ciclo celular.

Diversos trabajos han demostrado que el AR influye en la proliferación y la adquisición de un fenotipo particular en progenitores de retina embrionarios. Esto sugirió que el incremento en el número de células diferenciadas inducido por el AR podría ser resultado de un mayor número total de FRs debido a que el AR podría estar modificando la proliferación o redirigiendo el destino celular. Sin embargo, al analizar distintos parámetros relacionados con estos eventos, como la incorporación de BrdU, la expresión de p27, nestina, Crx y HPC-1 (marcadores de FRs y neuronas amacrinas, respectivamente), observamos que el AR no indujo una salida temprana del ciclo ni modificó la determinación de la identidad celular. Esto implica que al menos en las condiciones experimentales descritas, y en ese momento del desarrollo postnatal temprano, el AR no altera la salida del ciclo ni regula la identidad celular de estas neuronas *in vitro*.

Para comprender mejor los mecanismos de acción del AR sobre los FRs, estudiamos la modulación de las vías de señalización intracelular implicadas en sus efectos. Se ha involucrado al AR en la activación de la quinasa p38, relacionada con la regulación de la apoptosis en varios tipos celulares. Cuando investigamos si el AR activaba la vía de p38 en los FRs, el análisis por Western Blot e inmunocitoquímica demostró que el AR promovió rápidamente la activación de esta vía de señalización, y que el bloqueo de dicha activación con un inhibidor específico de p38 evitó la apoptosis de los FRs. Paralelamente, la inhibición de esta vía redujo significativamente, aunque no por completo, la diferenciación de los FRs. Esto sugiere que la vía de señalización de p38 sería la preferencialmente activada por el AR para activar la apoptosis de los FRs y al menos una de las involucradas en inducir su diferenciación.

Trabajos previos han mostrado que en la estimulación de la supervivencia de los FRs promovida por ADH interviene la activación de ERK/MAPK. Por ello, sería posible que el efecto deletéreo del AR implicara una modulación de esta vía. Sin embargo, no observamos cambios en la activación de dicha vía, indicando que no estaría afectada en el proceso de muerte inducido por AR. Por otro lado, teniendo en cuenta que la actividad de p38/MAPK podría ser regulada por interacción con la vía de PI3K/Akt, determinamos si el AR era capaz de modular esta vía en los FRs. El tratamiento con AR

redujo la cantidad de P-Akt, respaldando la hipótesis de que el efecto estimulatorio del AR sobre la vía de p38 involucraría también una inhibición de la actividad de PI3K/Akt.

En conjunto, estos resultados muestran que el AR es requerido para promover la diferenciación de los FRs y que este proceso de diferenciación no está necesariamente ligado a la supervivencia de estas neuronas. Su presencia prematura podría inducir la muerte de los progenitores al inducirlos a diferenciarse cuando aun están demasiado inmaduros, lo que resalta la importancia de la presencia simultánea de factores tróficos para prevenir dicha muerte.

En conclusión, este trabajo remarca la importancia de una adecuada sincronización entre los niveles de diferentes señales moleculares esenciales para el desarrollo de los FRs. El AR podría así ser una de las moléculas cruciales que contribuyen a definir el número final de FRs en la retina.

Las principales conclusiones de esta tesis son:

a) El AR induce la muerte por apoptosis en los progenitores de FRs mientras se encuentran en el ciclo celular, por una vía que involucra la pérdida de funcionalidad mitocondrial y la activación de caspasas.
b) El AR induce la diferenciación de los FRs, estimulando la expresión de opsina, periferina y el crecimiento de neuritas.
c) El AR promueve el crecimiento de las neuritas en las neuronas amacrinas.
d) La inducción de apoptosis por parte del AR es selectiva para los FRs.
e) El AR no altera la proliferación ni modifica el destino de los progenitores.
f) La inducción de de la diferenciación es independiente de que las células estén activas o no en el ciclo celular.
g) Los procesos de apoptosis y diferenciación en los FRs inducidos por el AR dependen de la activación de la vía de p38/MAPK, que a su vez interacciona con la vía de PI3K/Akt.
h) Un factor trófico lipídico, el ADH, protege a los FRs de la muerte inducida por AR.

SUMMARY

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Role of Retinoic Acid in the development of retina neurons

The vertebrate retina has five neuronal types: photoreceptors (PHRs, rods and cones), bipolar, ganglion, horizontal and amacrine neurons, and non neuronal cells including the Müller glial cells. During development, these neurons are originated from progenitor cells that undergo a series of competence states, determined by genetic, cellular and environmental factors, thus allowing the sequential and organized appearance of the different cell types (Livesey y Cepko, 2001b).

Retinoic Acid (RA) and Docosahexaenoic Acid (DHA) are among the different molecules and trophic factors that influence the development of rod PHRs. RA exerts a wide variety of effects during vertebrate development and cell differentiation. It plays a major role in the determination of the antero-posterior body axis, spermatogenesis, the formation and growth of body limbs and skin. Moreover, it is critical for the early development of the eye and PHR differentiation (Stenkamp y col., 1993; Prabhudesai y col., 2005; Hyatt y col., 1996; Khanna y col., 2006). RA binds to and activates nuclear receptors that function as transcription factors, thus regulating gene transcription. On the other hand, in our lab we have established that DHA promotes the survival and differentiation of rat PHRs in culture, and that these anti-apoptotic effects require the activation of the ERK/MAPK signaling pathway and the modulation of anti- and pro-apoptotic protein the expression.

The **general purpose** of this work was to **study the effects of RA on the development of amacrine neurons and PHRs *in vitro***. To that end, we used cultures obtained from postnatal rat retinas, developed in chemically defined media, which were supplemented with RA and/or DHA.

Given that RA is a cell differentiation factor; our hypothesis was that, like other trophic factors, this molecule would also promote PHR survival. However, when RA was added at day 0, the percentage of apoptotic PHRs increased, in parallel with a loss of mitochondrial functionality. This apoptosis was completely blocked by incubating the cultures with a caspase inhibitor before RA addition. These results suggest that RA would induce PHR death through an apoptotic mechanism involving a loss of mitochondrial activity and caspase activation. Since RA is ubiquitously present in the retina and it is essential for development, the preservation of viable PHRs would require its pro-apoptotic effects to be counteracted by the simultaneous presence of survival molecules, such as DHA. To test this hypothesis, we added DHA to the cultures prior RA treatment; this addition prevented RA-induced PHR death, supporting the hypothesis of the necessity of other survival factors to prevent death during development. Noteworthy, RA-induced apoptosis was selective for PHRs, since amacrine neurons were not affected.

Since RA is well known for its differentiation-promoting effects, the fact that it induced apoptosis was rather unexpected. This observation led us to test whether, under these experimental conditions, RA would promote or not PHR differentiation. RA indeed promoted differentiation, in parallel with an increase in the percentage of apoptotic PHRs. We determined, by immunocytochemistry and Western Blot, that RA increased the amount of PHRs that expressed opsin and peripherin, characteristic proteins of mature PHRs and of PHRs that developed apical processes, structures that resemble the initial steps of outer segment formation. Moreover, RA increased the percentage of PHRs that developed neurites and promoted neurite outgrowth. It is worth to note that, unlike other evaluated features, the stimulation of neurite outgrowth was not exclusive for PHRs; RA treatment also induced also neurite outgrowth in amacrine cells.

Since RA and DHA have similar effects on differentiation, and they bind to receptors that form heterodimers (RAR y RXR respectively), we evaluated their possible additive or synergistic effects. The simultaneous treatment with both factors increased opsin and peripherin expression up to a value that resembled the sum of both metabolites alone. These results imply that RA and DHA contribute to PHR

differentiation in an additive fashion, and suggest that they stimulate independent pathways to that end.

The fact that RA induced the expression of proteins and formation of structures of mature neurons, led us to propose that the functionality of these cells could also be stimulated. However, RA neither stimulated cGMP hydrolysis, a characteristic that would indicate an active phototransduction cascade and the ability to respond to light, nor the capacity to take up neurotransmitters (like glutamate in PHRs and GABA in amacrine neurons) from the extracellular medium. These results indicate that, although RA promotes PHR and amacrine cell differentiation, it is not enough of a stimulus to achieve functional maturity of these cells, suggesting that this functionality requires the presence of other factors.

The finding that RA simultaneously induced differentiation and apoptosis led us to propose that it might have distinct effects on different PHR sub-populations or on populations at different developmental stages. To test this hypothesis, cultures were supplemented with RA at day 0, when proliferation is still active, and at day 2, when there are no longer proliferating progenitors. Noteworthy, when added at day 2, RA stimulated PHR differentiation, although no increase in apoptosis was evident. These results indicate that RA would act differentially depending on PHRs developmental stages, inducing apoptosis in a sub-population of undifferentiated progenitors and accelerating the differentiation in those which have already abandoned the cell cycle.

Several studies have shown that RA influences proliferation and in the acquisition of a particular phenotype in embryonic retina progenitors. For that reason, the increase in the number of differentiated cells induced by RA could be due to a higher total number of PHRs, since RA might be redirecting cell fate or modifying proliferation. However, when we analyzed a number of parameters related to these events, such as BrdU incorporation and the expression of p27, nestin, CRX and HPC-1 (markers of PHRs and amacrine cells, respectively), we found RA neither induced cell cycle exit nor modified cell fate. This implies that, at least under the described experimental conditions, and at this particular time of development, RA would not alter the cell cycle exit or regulate cell identity.

To better understand the mechanisms by which RA exerted its effects on PHRs, we studied the modulation of signaling pathways. RA has been involved in the activation of p38/MAPK, which related to the regulation of apoptosis in several cell types. When we evaluated whether RA activated the p38 pathway in PHRs, Western Blot and immunocytochemical analyses showed that it induced a rapid activation of this pathway, and the blockade of such activation with a specific inhibitor prevented PHR apoptosis. Moreover, the inhibition of this pathway led to a significant, though not complete, reduction of PHR differentiation. This suggests that the p38/MAPK would be the preferred signaling pathway activated by RA to induce apoptosis in PHRs, and at least one of the involved in the induction of their differentiation.

Previous work has shown that DHA-stimulated survival in PHRs requires the activation of the ERK/MAPK pathway. Hence, the deleterious effect of RA might involve the modulation of this pathway. However, we found no changes in the activation of this pathway, indicating that it would not be related to RA-induced PHR death. On the other hand, given that p38/MAPK activity has been shown to be regulated by interaction with the PI3K/Akt pathway, we determined whether RA was capable of modulating this pathway in PHRs. Treatment with RA reduced the amount of P-Akt, supporting the hypothesis that the stimulatory effect of RA on the p38 pathway would involve the inhibition of PI3K/Akt activity.

As a whole, these results show that RA is required for the induction of PHR differentiation, and that this process is not necessarily linked to the survival of these neurons. The premature presence of RA could elicit progenitor death as it might induce them to differentiate at a stage when they are still too immature, highlighting the need of the simultaneous presence of trophic factors to prevent this death.

In summary, this work underscores the relevance of an adequate synchronization between the levels of different molecular cues essential for PHR development. RA might thus be one of the crucial molecules that contribute to define the final number of PHRs in the retina.

The main conclusions of this thesis are:

a) RA induces PHR progenitor apoptosis while they are active in the cell cycle, through a mechanism that involves the loss of mitochondrial activity and caspase activation.
b) RA induces PHR differentiation, stimulating opsin and peripherin expression, and neurite outgrowth.
c) RA promotes neurite outgrowth in amacrine neurons.
d) RA-induced apoptosis is selective for PHRs.
e) RA does not alter progenitor proliferation or the acquisition of cell fate.
f) The induction of differentiation occurs regardless of the cells being active in the cell cycle or not.
g) RA-induced differentiation and apoptosis processes in PHRs depend on the activation of p38/MAPK, which also interacts with PI3K/Akt.
h) A lipid trophic factor, DHA, protects PHRs from RA-induced apoptosis.

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PUBLICACIONES REALIZADAS DURANTE EL DESARROLLO DE LA TESIS

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