

UC Berkeley

UC Berkeley Electronic Theses and Dissertations

Title

Biosynthesis of all-trans-Retinoic Acid and Regulation of Retinoids Homeostasis in Primary Hippocampal Astrocytes and Neurons

Permalink

<https://escholarship.org/uc/item/3cj8886g>

Author

Wang, Chao

Publication Date

2010

Peer reviewed|Thesis/dissertation

**Biosynthesis of all-*trans*-Retinoic Acid and Regulation of Retinoids Homeostasis in
Primary Hippocampal Astrocytes and Neurons**

By

Chao Wang

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Molecular and Biochemical Nutrition

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Joseph L. Napoli, Chair

Professor Barry Shane

Professor Daniela Kaufer

Fall 2010

**Biosynthesis of all-*trans*-Retinoic Acid and Regulation of Retinoids Homeostasis in
Primary Hippocampal Astrocytes and Neurons**

Copyright 2010

By

Chao Wang

Abstract

Biosynthesis of all-*trans*-Retinoic Acid and Regulation of Retinoids Homeostasis in Primary Hippocampal Astrocytes and Neurons

by

Chao Wang

Doctor of Philosophy in Molecular and Biochemical Nutrition

University of California, Berkeley

Professor Joseph L. Napoli, Chair

All-*trans*-retinoic acid stimulates neurogenesis, dendritic growth of hippocampal neurons and higher cognitive functions, such as spatial learning and memory formation. Although astrocyte-derived atRA has been considered a key factor in neurogenesis, little direct evidence identifies hippocampus cell types and the enzymes that biosynthesize atRA. Nor has any factor been reported to regulate atRA biosynthesis in adult CNS. Here we show that primary rat astrocytes, but not neurons, biosynthesize atRA using multiple retinol dehydrogenases (Rdh) of the short-chain dehydrogenase/reductase (SDR) gene family and retinaldehyde dehydrogenases (Raldh). Astrocytes secrete atRA into their medium; neurons sequester atRA. The first step, conversion of retinol into retinal, is rate-limiting and the second step, conversion of retinal to atRA, is much more active and usually affected in response to different stimuli. Both neurons and astrocytes can synthesize retinyl esters and reduce retinal into retinol. siRNA knockdown indicates that Rdh10, Rdh2 (mRdh1), and Raldh 1, 2 and 3 contribute to atRA production. Knockdown of the Rdh Dhrs9 increased atRA synthesis ~40% by increasing Raldh1 expression. Immunocytochemistry revealed cytosolic and nuclear expression of Raldh1 and cytosol and perinuclear expression of Raldh2. atRA autoregulated its concentrations by inducing retinyl ester synthesis *via* lecithin:retinol acyltransferase and stimulating its catabolism *via* inducing Cyp26B1. Raldh1, Raldh2, Rdh2, Rdh10 and Dhrs9 increase their expression as the elongation of *in vitro* culture time. Rdh1^{-/-} and Crbp1^{-/-} mice astrocytes showed similar changes of retinoids metabolism except RE formation and partially overlap genes compensation. Though shown a broad and strong expression pattern in pure cultured astrocytes, Raldh1 expression dramatically dropped in astrocytes mixed cultured with neurons or in hippocampus *in vivo*. In contrast, Raldh1 is widely expressed in cultured neurons, with special intense signals on axons. CA1 neurons and mossy fibers enriched Raldh1 expression pattern was confirmed as a postnatal development process by immunohistochemistry. As a proinflammatory cytokine, TNF α oppositely down regulate Raldh1 expression *via* JNK and MAPK pathway and up regulate Raldh3 expression partially through P38 pathway, which resulted in different overall effect on atRA

biosynthesis in young and old astrocytes. These data show that adult hippocampus astrocytes rely on multiple Rdh and Raldh to provide a paracrine source of atRA to neurons, and atRA regulates its own biosynthesis in astrocytes directing flux of retinol. Besides redundancy, different Rdh and Raldh may have unique function according to cell types and/or subcellular locations. Cross talk between first and second step dehydrogenation indicate a novel regulation mechanism that control the atRA homeostasis in astrocytes.

Table of Contents

Abstract	1
Table of Contents	i
List of Figures and Tables	iii
Acknowledgement	v

Chapter I Literature Review

Part I General Review of Retinoids Metabolism	1
Vitamin A and retinoic acid	1
Structure and retinoid property	2
Retinoid uptake and processing	2
Cellular retinoid metabolism	5
Cellular uptake and processing retinol	5
ADHs	6
SDRs	7
Retinal dehydrogenases	9
Cellular retinoic acid binding proteins	10
Retinoic acid degradation--cytochrome P450 enzymes	10
RA receptors	11
RA target genes-possible autoregulation pathway	12
Part II Retinoic Acid Signaling and Function in Adult CNS	13
Overview	13
RA signaling components in adult CNS	13
Physiological function of RA in CNS- learning and synaptic plasticity	14
RA and song learning	14
Physiological function of RA signaling in adult hippocampus	14
Pathological association of RA signaling on CNS diseases	16
RA source in adult hippocampus	18

Part III Regulation of RA biosynthesis by hormones, cytokines or endotoxins	19
Estrogen and RA signaling	19
LPS, PGE2, TNF α and RA signaling	20
References	22

Chapter II Multiple Retinol and Retinal Dehydrogenases Catalyze

All-*trans*-Retinoic Acid Biosynthesis in Astrocytes

Introduction	34
Materials and Methods	36
Results.....	39
Discussion.....	46
Figures and Legends	50
References	69

Chapter III Localization *in vitro* and *in vivo* of RALDH1 Expression

Introduction	74
Materials and Methods	76
Results.....	78
Discussion.....	81
Figures and Legends	85
References	92

Chapter IV TNF α Regulates all-*trans*-RA Biosynthesis in Astrocytes through Oppositely Changing RALDH1 and RALDH3 Expression

Introduction	94
Materials and Methods	96
Results.....	97
Discussion.....	100
Figures and Legends	103
References	108

Chapter V Summary and Future Directions

Part I Retinoids metabolism in cultured astrocytes and neurons	110
Part II Enzymes that participate in the retinoids homeostasis in CNS	113
Part III Future directions.....	115
Part IV Conclusion.....	116
Figures and Legends	118

List of Figures and Tables

Figure II-1 Time course of atRA and RE biosynthesis from retinol by primary astrocytes 50

Figure II-2 Effects of substrate concentrations on retinoid metabolism in primary astrocytes 51

Figure II-3 Retinoid metabolism catalyzed by primary hippocampus neurons 52

Figure II-4 Co-cultured with neurons changed the atRA distribution proportion in medium and cells 53

Figure II-5 Neurons sequester atRA secreted by astrocytes. 54

Figure II-6 Knockdown reveals Raldh that contribute to atRA biosynthesis 56

Figure II-7 Confocal images of Raldh1 and 2 expression 58

Figure II-8 Multiple Rdh contribute to atRA biosynthesis in astrocytes 60

Figure II-9 Dhhr9 functions as a retinol dehydrogenase in astrocytes 61

Figure II-10 atRA induces Cyp26B1 and Lrat mRNA in astrocytes 63

Figure II-11 Effect of culture duration on retinoid metabolism by primary astrocytes 65

Figure II-12 Compensation effect of atRA biosynthesis in Rdh1^{-/-} and CrbpI^{-/-} astrocytes 66

Figure II-13 Retinol homeostasis in astrocytes and neurons 67

Table II-1 Rate constants for retinol metabolism 68

Figure III-1 RALDH1 expression in primary cultured neuronal cells 85

Figure III-2 RALDH1 expression in adult rat hippocampus 86

Figure III-3 RALDH1 expression in astrocytes and oligodendrocytes in hippocampus 87

Figure III-4	RALDH1 is expressed in dendrites of CA1 but not CA3 neurons	88
Figure III-5	RADLH1 is co-localized with axons present in CA3 region	89
Figure III-6	RALDH1 is expressed on mossy fibers	90
Figure III-7	Developmental changes of RALDH1 expression in adult rat hippocampus	91
Figure IV-1	atRA production from retinol in 1 month old astrocytes remains stable upon different stimuli.	103
Figure IV-2	TNF α affect retinal dehydrogenation in astrocytes through oppositely changing RALDH1 and RALDH3 expression	104
Figure IV-3	TNF α induces similar change of RALDH1 and RALDH3 expression but different atRA production in 2weeks and 3months old astrocytes	106
Figure IV-4	TNF α affect RALDH1 expression via JNK and MEK pathway; RALDH3 expression partially via P38 pathway	107
Figure V-1	Retinoids homeostasis model in adult hippocampus	118

Acknowledgement

It's my pleasure to acknowledge the contributions of many people to this work. I have been blessed with generous help and support from them, to whom I would like to express my deepest gratitude.

First and foremost, I would like to give my sincere gratitude to my PI, Dr. Joseph L. Napoli, for giving me the opportunity to study this important and meaningful project, for his keen wisdom, solid support and generous guidance during my past 6 years Ph.D. research. I truly appreciate Dr. Barry Shane for serving on all my committees and for his thoughtful concerns and suggestions on my projects. I am very grateful to have Dr. Daniela Kaufer on my dissertation committee. I thank Dr. Kaufer for informative suggestions on Raldh1 expression project and generous sharing of specific antibodies. I very much appreciate Dr. Nancy Amy, Dr. Leonard F. Bjeldances, Dr. Robert O. Ryan and Dr. John Ngai for serving on my qualify exam committee and providing important guidance on this projects.

I would like to give my special thank my lab member Dr. Maureen Kane for her altruistic help on measurement of retinoids and collaboration on the ethanol projects. The training and guidance I received from Dr. Kane was the footstone for my whole project. Her accurate and timely data analysis, patience to answer my questions and quickly response to my requirements insure the progress of my project. I also want to thank Dr. Na Chen for her patient guidance on primary cell culture and basic experimental technologies; Dr. Charles Krois for assistance on animal handling and Dr. Kristin Obrochta for help on immunohistochemistry. I also greatly appreciate all other colleges, Sunny Wang, Nan Li, Alexandra Folias, Weiya Jiang, Min Zhang, Peirong Hu, James Chithalen, Wentao Hao, Hua Tran, for sharing their knowledge and expertise, giving suggestions and enjoying the fun of research.

I want to give special thanks to Dr. Xiao Shu and Dr. Aaron Li for being such wonderful friends, not only giving me generous help on my research but also sharing all the joy and despair, failure and success during my six years of life in Berkeley. I am deeply grateful for the help and friendships from Roger Wong, Nan Wang, Su Yan, Yuyang Tian, Maryam Ahmadian, Brie Fuqua, Seung-Min Lee and many others.

Last but not least, I would like express my deepest gratitude to my parents. Their unconditional love and support have been my powerful and secure backing all the times in my life.

Chapter I

Literature Review

Part I General Review of Retinoid Metabolism

Vitamin A and retinoic acid

Vitamin A, a fat-soluble vitamin, is essential for most forms of life, especially in chordates. The vitamin has numerous functions information and maintenance of many body tissues, for example, skin, bone, vasculature, and eye as well as the immune system (Blomhoff and Blomhoff, 2006; Evans and Kaye, 1999; Kim, 2008). Vitamin A is indispensable for normal vision, maintenance of epithelial cells, immune competence, reproduction and embryo development and growth, and the maintenance and regulation of both embryonic and adult central nervous system (Theodosiou *et al.*, 2010). Vitamin A is converted into an activated metabolite, all-*trans*-retinoic acid (atRA), through which it performs its multiple effects on embryogenesis, cell proliferation, differentiation and apoptosis (Petkovich, 2001). atRA is a rapid diffusing signaling molecule and exerts its functions by controlling gene expression through activation of specific nuclear receptors (Blomhoff and Blomhoff, 2006).

In humans, lower vitamin A intake than needed, impairs vision, featured by xerophthalmia and even blindness in severe situations (Bendich and Langseth, 1989). Deficiency of vitamin A (VAD) also increases susceptibility to infectious diseases, because of impaired immune resistance (Kim, 2008). VAD is a serious problem in developing countries. The WHO estimates that between 140 and 250 million preschool children are at the risk of subclinical vitamin A deficiency, 3 million are clinically vitamin A deficient and 1 million childhood deaths are associated with vitamin A deficiency annually (Organization, 1995).

In contrast, vitamin A could function as a double-edged sword if uptake is excessive. Excess dietary vitamin A, sometimes even marginally above the RDI (recommended dietary intake) can result in damage to liver, skin, internal organs, the musculoskeletal system, the central nervous system and embryo development (Blomhoff and Blomhoff, 2006; Collins and Mao, 1999). Hypervitaminosis A causes embryonic malformations, reduced bone mineral density, and increased hip fractures (Melhus *et al.*, 1998; Rothman *et al.*, 1995). Hypervitaminosis A results from large intake of dietary vitamin A, from animal liver for example, or from drugs containing retinoids (synthetic or naturally occurring compounds with vitamin A activity).

The function of vitamin A in the visual cycle was first revealed decades ago. 11-*cis*-Retinal serves as the chromophore of visual pigment (Wald, 1968). Early research also found that either hyper or hypo-vitaminosis A affect differentiation of epithelial cells and atRA is a powerful signaling molecules in chordates (Wolbach and Howe, 1925). The

mechanism of atRA action was not resolved until retinoic acid receptors (RAR) were discovered in 1987, which demonstrated that atRA affects gene expression through nuclear transcription factors (Giguere *et al.*, 1987). However, this is not the only mechanism through which vitamin A exerts its biological activity. Additional mechanisms of vitamin A actions have been identified, as detailed below.

Structure and retinoid property

Vitamin A is the term that designates any compound possessing the biological activity supported by retinol (after conversion into metabolites such as atRA). Some of synthetic compounds, even though they don't have a retinol-like structure, have greater activity in some assays of vitamin A function. So Sporn and Roberts proposed that 'a retinoid should be defined as a substance that can elicit specific biologic responses by binding to and activating a specific receptor or set of receptors'. Today, most researchers accept a combination of definitions that the class of retinoids consists of RA analogs (with or without biologic activity) and several compounds not structural close to RA that can exert the biological activity of vitamin A.

All-*trans*-retinol is the parent retinoid compound containing a primary alcohol that can be oxidized to other active retinoids. Retinyl palmitate is the predominant retinoid in most animal tissues. However, other forms of fatty acid esters, such as retinyl oleate and retinyl stearate also exist in natural materials. The all-*trans* configuration is the major naturally occurring isomer, but *cis* configurations are also present in nature; for example, 11-*cis*-retinal is present in the retina of eye, 13-*cis*-retinoic acid is present in many tissues. All retinoids, including retinol and atRA, are highly unstable in the presence of oxygen and light. Exposure to oxidants or light will lead to degradation or isomerization. This feature requires that any reagents or biological samples that containing retinoids must be stored and handled under dim illumination; for example, yellow light causes the least decay of retinoids (Gundersen and Blomhoff, 2001). In addition, glassware is required when dealing with retinoids because plastic equipment can easily absorb retinoids, which might result in shift of experiment results. Due to the instability of retinoids, well-designed controls are especially required for each experiment.

All-*trans*-retinol is a fat soluble vitamin. There is increased water solubility from retinol, retinal to atRA (60, 110, 210 nM) which means atRA can diffuse efficiently in both aqueous phases, such as plasma and hydrophobic phases, such as membranes (Blomhoff and Blomhoff, 2006). Most retinoids are bound with specific binding-proteins during transportation between hydrophilic plasma or extra- and intracellular fluids. Binding proteins also function in retinoid metabolism, such as the oxidation of retinol or degradation of atRA (Napoli, 1997).

Retinoid uptake and processing

International units (IUs) are used for nutritional recommendations of vitamin A. The term 'retinol equivalents' (RE) is used to convert all kinds of performed retinol and provitamin A carotenoids into a single unit. No animal species have ability to synthesize

vitamin A. All dietary vitamin A is from plant and microorganisms or from the tissues of other animals that have gotten vitamin A in their diets. Carotenoids serve as provitamin A compounds in plants and are cleaved to form retinoids in animals. Carotenoids are the pigments responsible for the yellow, orange, red, or purple colors of many vegetables, fruits and flowers. β -Carotene represents the most efficient precursor of vitamin A. One β -carotene molecule can be cleaved into two retinal molecules in the small intestine and further reduced into retinol. Alternatively, animals can obtain retinol by eating animal tissues that have already converted carotenoids into retinoids. RE (retinyl esters), particularly palmitate, represent the most abundant storage form, with liver serving as the main, but not the only tissue for storage. The primary function of retinol and RE is serving as the precursors of biologically active retinoids. This process occurs in both liver and non-hepatic tissues. The most established pathway of retinol metabolism includes esterification of retinol into RE in liver or other tissues, reversible conversion of retinol into retinal and then irreversible conversion of retinal into atRA. There are six isoforms of biologically-active retinol derivatives, including all-*trans*, 9-*cis*, 13-*cis*, 9,13-*cis*, 11-*cis* and 11,13-di-*cis*, with all-*trans* being the predominant physiological form. Some, but not all endogenous retinoids with direct biological activity, include: atRA, 11-*cis*-retinaldehyde, 3,4-didehydro-RA and 9-*cis*-RA.

In general, carotenoids are absorbed by enterocytes in the small intestine through passive diffusion. After uptake, β -carotene is cleaved centrally to produce two molecules of all-*trans*-retinaldehyde by the enzyme, β -carotenecyclooxygenase-I (BCOI). Dietary RE is converted into retinol in the intestinal lumen before its uptake by triglyceride lipase. Retinol is absorbed by enterocytes by a carrier-mediated process.

Retinol, either from β -carotene or RE, is bound to cellular retinol binding protein, type II (CRBP II) for esterification in enterocytes. CRBP II is expressed primarily in the absorptive cells in the intestine. CRBP II knock-out mice have reduced retinol uptake, but when they are kept on a vitamin A-enriched diet, they develop and reproduce normally, indicating that CRBP II functions in retinol absorption, especially during low amounts of dietary vitamin A—the normal situation (E *et al.*, 2002). It is generally believed that the role of CRBP II is to solubilize retinol, protect retinol from degradation and more importantly, deliver retinol to the esterification enzyme lecithin: retinol acetyltransferase (LRAT) for conversion into RE. LRAT is widely expressed in many tissues and highly expressed in the intestine, liver and the retinal-pigmented epithelial cells in eye (Herr and Ong, 1992). It is the major enzyme that converts all-*trans*-retinol into RE. LRAT knock-out mice develop normally with slight changes in the retina. Only trace levels of RE are found in the liver, kidney and lung; indicating that this enzyme is important for RE formation. However, there is an increase of RE storage level in adipose tissue in LRAT^{-/-} mice and during vitamin A deficiency, which are mobilized from adipose tissue (O'Byrne *et al.*, 2005). In LRAT^{-/-} mice, retinol is absorbed as free retinol into cells, but RE can be still found in circulation. This kind of retinol esterification is performed *via* an acyl-CoA-dependent enzymatic process, which is catalyzed by acyl-CoA: retinol

acetyltransferase (ARAT) (Batten *et al.*, 2004).

RE in enterocytes are secreted either into lymph as chylomicrons or into the portal circulation as unesterified retinol. Chylomicrons consist of aggregates of triacylglycerol and phospholipids packed together with carotenoids, RE, small amounts of retinol, cholesterol esters and apolipoproteins. Chylomicrons in general circulation are reduced to chylomicron remnants that contain high amounts of RE, which are either transported to target tissues such as bone marrow, spleen, adipose tissue, skeletal muscle and kidney, or cleared by the parenchymal cells of the liver.

In hepatocytes, RE is hydrolyzed. The released retinol binds with retinol-binding protein (RBP), which is highly expressed in the hepatocyte endoplasmic reticulum (ER). Retinol-RBP translocates from the ER to the Golgi complex, followed by secretion into the plasma (Kanai *et al.*, 1968). Besides secretion into the plasma, the majority of unesterified retinol is transferred to stellate cells in the liver, where free retinol will be re-esterified into RE and stored. In mammals, 50%-80% of total retinol is present in hepatic stellate cells (Senoo, 2004). The stellate cells' ability of extensively storing or mobilizing RE provides an adequate supply of vitamin A and a mechanism of ensuring steady blood retinol level. Through RE storage and mobilization, the plasma retinol concentration is maintained at a steady state of about 2 μ M in spite of fluctuation of daily vitamin A intakes. CRBPI and LRAT are highly expressed in hepatic stellate cells which are responsible for RE formation. Mice null in CRBPI or LRAT had impaired stellate cell RE storage (Batten *et al.*, 2004; Ghyselinck *et al.*, 1999). Similar to CRBP II, one of the functions of CRBPI is to deliver retinol to LRAT for esterification. CRBPs belong to the greater family of fatty acid binding-proteins. CRBPI knock-out mice appear normal, but have low storage of hepatic RE (Ghyselinck *et al.*, 1999). Lipid droplets in the liver are smaller and less abundant in CRBPI null mice. The plasma retinol concentration remained the same as wild type mice, which suggests that CRBPI doesn't participate in the release of RBP-retinol.

Besides hepatic stellate cells which can store around 80% of the total retinol and retinyl esters (RE) in vertebrates, some extra hepatic tissues, for examples, cells in the intestine, lung and kidney may accumulate RE in lipid droplets (Nagy *et al.*, 1997). This might result from these organs which have a high demand for retinol. For example, RE storage in retinal pigment epithelial cells is a prerequisite for normal visual function. atRA biosynthesis is also highly active in those tissues, which indicates that storage of RE could provide a quick, localized source of precursors when the demand for RA changes. CRBPs (I, II and III) plus LRAT and ARAT are considered to be important for the esterification of retinol in these tissues using similar mechanism as in liver. However, different tissues might utilize different combination of those binding proteins and enzymes to esterify retinol.

RBP has been proved to be important but not indispensable for retinol transportation, as long as the animal has a diet copious in vitamin A. Mice with RBP^{-/-} have been generated and show several phenotypes. Surprisingly, RBP^{-/-} mice are viable and fertile

with impaired function of retina only when mice was fed with a diet low in vitamin A. Adult RBP^{-/-} mice fed with copious vitamin A diet are phenotypically normal (Quadro *et al.*, 1999; Quadro *et al.*, 2003). Because RBP is the only known carrier for retinol in circulation, the retinol level in RBP^{-/-} mice is ten times lower than that in wild-type littermates, while there is a significant increase of stored RE in liver. These data indicate that RBP is required for the retinol mobilization and storage. These data also indicate that RBP is not essential, but enhances the efficiency of uptake of retinol. The mechanism of this RBP independent retinol transportation has not been well characterized. It might be due to the simple passive diffusion of those unbound retinol in plasma.

When the RBP-retinol complex is transported in the circulation, it associates with transthyretin (TTR) at 1:1 ratio, which reduces the glomerular filtration of retinol by kidney. Mice null for TTR are viable and healthy without any development defects, as long as they are fed diets copious in vitamin A. In Ttr^{-/-} mice, the plasma level of retinol is extremely low (almost zero) compared with wild-type littermates. However, retinol and RE levels in liver, testes, kidney, spleen and eye in Ttr^{-/-} mice are normal, indicating that TTR is not involved in retinol uptake and storage in liver, and in retinoids delivery to these target tissues (Gottesman *et al.*, 2001).

Besides retinol and RE, many other retinoids, including all-*trans*-RA, 13-*cis*-RA, 13-*cis*-4-oxo-RA, all-*trans*-4-oxo-RA and all-*trans*-retinyl β -glucuronide are transported in plasma. With exception of all-*trans*-retinyl β -glucuronide, the other retinoids are transported in plasma bound to albumin at concentration about 5-10 nM (Wyss and Bucheli, 1997). The level of those retinoids fluctuates according to intake of vitamin A. Two to four fold increases in these retinoids will occur after large doses of vitamin A (Hartmann *et al.*, 2005). Besides retinoids, carotenoids also can be transported in plasma bound with lipoproteins.

Cellular retinoid metabolism

In some retinoid signaling, the active retinoids are synthesized in target cells. The source of these active retinoids is RBP-bound retinol, which is taken up by the cells. Besides that, cellular uptake of lipoproteins, such as chylomicrons containing retinol, RE and carotenoids or the RE stored in the target cells or neighboring cells can contribute to the biosynthesis of active retinoids. In some tissues, which have low demand for active retinoids, circulation retinoids serve as a source of atRA.

Among all those active retinoids, atRA is the major active cellular retinoid metabolite and has been widely studied and well characterized. The biosynthesis of atRA is a two-step oxidation process. The rate limiting step of atRA biosynthesis is oxidation of retinol to retinal. This two-step oxidation is catalyzed by specific enzymes and tightly regulated. The major function of atRA is to act as a ligand of transcription factors.

Cellular uptake and processing of retinol

Holo-RBP delivers retinol to target cells. There is a specific cell surface receptor for

RBP present in target cells. This kind of receptor was first reported on retinal pigment epithelium and intestinal epithelial cells in 1970s. Since then, accumulative data support the presence of this receptor in many other tissues and cell types, such as placenta, choroid plexus, testis and macrophages. This receptor was finally identified in 2007 as stimulated by retinoic acid gene 6 (STRA6) (Kawaguchi *et al.*, 2007). STRA6 is a widely expressed multi-transmembrane protein. It mediates cellular retinol uptake and the mutation of STRA6 cause a broad spectrum of malformations including anophthalmia, congenital heart defects, and diaphragmatic hernia (Blaner, 2007, Pasutto *et al.*, 2007). STRA6 is broadly expressed in the murine embryo, but in the adult its expression becomes more restricted. STRA6 mRNA is also up regulated in mammary gland tumors and human colorectal tumors.

In target tissues, free retinol will associate with CRBP to form holo-CRBP for further processing. CRBP encapsulates retinol and isolates this fat soluble molecule from the aqueous environment by burying its hydroxyl function deep into the protein to stabilize retinol and facilitate further processing of retinol, such as deliver retinol to enzymes for oxidation or for esterification (Gottesman *et al.*, 2001). In rat liver, the concentration of retinol is about 5 μM and CRBP concentration is about 7 μM . This would result in the 'free' retinol (non-CRBP-associated retinol) is about 0.25 nM, which is about 20,000 fold lower than the holo-CRBP concentration. So the holo-CRBP is believed to be the most potential substrate for atRA biosynthesis (Napoli, 1999).

ADHs

In some *in vitro* studies, both hepatic and extrahepatic alcohol dehydrogenases (ADH) can catalyze oxidation of retinol into retinaldehyde. There are four classes of ADHs, ADH1-ADH4. All vertebrate groups from bony fish to the human contain ADH1 and ADH3. ADH4 is present in both rodent and human (Galter *et al.*, 2003).

ADH1^{-/-} mice exhibit a normal phenotype when maintained on a vitamin A sufficient diet. ADH3 or ADH4 knock-out mice show some developmental defects only when fed with VAD diet (Molotkov *et al.*, 2002b; Pares *et al.*, 2008). However, critical evidence don't support the notion that ADHs function as the enzymes to catalyze retinol dehydrogenation *in vivo*. Although ADH can oxidize retinol *in vitro*, even for ADH4, which shows the highest retinol preference among all ADHs, it is still more than 10-fold lower activity compared to ethanol as substrate. In addition, ADHs can't utilize holo-CRBP as substrate for retinol oxidation, which is, as mentioned before, the predominating form of retinol in cells. In cultured cells, expression of ADH didn't catalyze retinol metabolism; reduced ADH expression didn't associate with a phenotype of insufficient retinol activation (Pares *et al.*, 2008). Careful inspection suggests that the expression loci of ADH do not correlate with the putative sites of atRA biosynthesis during embryogenesis, except for the widely expressed ADH3, which was not characterized kinetically for retinol dehydrogenase activity. In ADH1, ADH3 and ADH4 knockout reports, no phenotypic or metabolic alterations are reported, which are consistent with failing of retinol activation or genes changes that can compensate this

vitamin A activation interruption. No endogenous atRA level has been reported in those knockout mice. Besides that, ADH1 was only proved to convert high dose of retinol (50 mg/kg) into atRA (Molotkov *et al.*, 2002a). This dose of retinol provides ~100-fold more than the recommended daily intake for a mouse and drive serum atRA about 2000-fold over the steady-state value. It is believed that this high dose of retinol overwhelm homeostatic mechanisms which can concentrate low level retinol *in vivo* to control the atRA signaling sensitivity. ADH1 might be forced to participate into retinol metabolism in this high concentration of retinol.

No genetic, metabolic or function-related data demonstrate that ADH can metabolize physiological level of retinol *in vivo* or in cultured cells, which turn our focus on SDR/RDHs, the *in vivo* retinol dehydrogenases.

SDRs

Besides ADHs which are cytosolic enzymes, retinoid-metabolizing activity can also be found in the membrane fractions of many cells. The first two kinds of those membrane-bound enzymes were rat retinol dehydrogenase 1-RoDH1 (now rename as RDH2) purified from rat liver and RDH5 purified from bovine retinal pigment epithelium (Chai *et al.*, 1995; Suzuki *et al.*, 1993). RDH2 can recognize holo-CRBPI as substrate whereas RDH5 can oxidize 11-*cis*-retinol to 11-*cis*-retinaldehyde indicating that it is important for vision cycle and recognizes CRABP as substrate. The sequence identity of these two enzymes is about 50% suggesting common evolutionary origins. After these two short chain dehydrogenase/reductases (SDR) were cloned, numerous retinoid-active SDRs were identified. To date, at least 17 different SDRs are identified in humans, rats and mice, such as RDH1, RDH5, RDH11, RDH10, DHRS9, CRAD1-3 (Pares *et al.*, 2008).

Different from ADHs, microsome, which is the major location of SDRs, produce retinaldehyde from retinol bound to CRBPI, the physiological form of intracellular retinol. The microsomal rate of retinaldehyde production from holo-CRBPI exceeds the cytosolic rate by 10-50 folds in different tissues, and microsomes harbor 80-90% of retinaldehyde generating capacity (enzyme units) from holo-CRBPI. Two kinds of enzymes crosslink with holo-CRBPI: RDH and LRAT. Pyridine nucleotide cofactors are required for this crosslinking and apo-CRBPI cannot crosslink with RDH or LRAT (Napoli, 1999). Those data promote the effort to clone rat RDH2 (mouse ortholog RDH1) and RDH5 and following work has identified some SDRs with retinoid metabolizing activity *in vitro*. Three of those SDRs have all-*trans*-retinol dehydrogenase potentials, including RDH1 (rat RDH2), RDH10 and DHRS9. Some of them have been discovered several times or in multiple tissues, accounting for several different names.

There are at least 7 genes with high homology to RDH1 which cluster closely in mouse chromosome 10D3. However, RDH1 is the only one with high activity for all-*trans*-retinol. The others either lack the activity or have high activity with *cis*-retinoids. Two enzymes in rat, originally named Rodh2 and Rodh1/3, are homologs of mouse RDH1. Rat RDH2 and RDH2_rat have only 1 amino acid difference and might have

different promoters.

Rat RDH2/RDH2_{rat} (mouse RDH1), which exhibit widespread expression starting as early as E7.5, serve as primary candidates for retinol oxidation enzymes because they have the highest V_m/K_m value of all SDR with all-*trans*-retinol. The expression pattern of RDH2 also correlates with atRA activity regions. RDH1 mRNA expresses throughout the embryo, especially enriched in the neural plate at E7.5 and in the neural tube, gut, neural crest, and Rathke's pouch at E10.5. The mRNA of RDH1 is also expressed in the developing eye, ventral regions, cartilage, liver and lung, but less intense in other tissues. This expression pattern suggests that RDH1 participates in the precise atRA generation during embryogenesis. However, RDH1 knockout mice didn't show any growth defects on any level vitamin A diet, from marginal (0.6 IU to >30 IU) (Zhang *et al.*, 2007). H&E staining revealed no obvious differences in morphologies of multiple tissues in RDH1-null mice compared to wild type mice. Further investigation showed that RDH1 inactivation lead to decrease liver Cyp26A1 mRNA and protein, a major atRA catabolic enzyme, to spare retinoids in liver, indicating that RDH1 participate in the coordinate modulation of atRA homeostasis.

Dhrs9 recognizes both CRBPI-bound and free retinol as substrate, and is strongly expressed in epidermis in the atRA-dependent strata (Soref *et al.*, 2001). Dhrs9 is associated with intestinal development in zebra fish, and atRA biosynthesis in colon; especially, Dhrs9 expression in colon cancer is impaired (Nadauld *et al.*, 2004). During estrus, Dhrs9 co-expressed with CRBPI and CRABPII in uterine lining epithelium. Dhrs9 expression is regulated by estrogen *in vivo* (Li *et al.*, 2004). However, Dhrs9 knockout mouse is currently not available to precisely understand the contribution of this enzyme to atRA biosynthesis.

RDH10 is associated with the development of renal progenitor cells and with preimplantation mouse development (Romand *et al.*, 2008; Wu *et al.*, 2004). PPAR γ can induce atRA biosynthesis in dendritic cells through inducing RDH10. Recently, Sandell *et al.* and Ashique *et al.* have stochastically produced mutants of RDH10 by ENU mutagenesis, which generated T251C and A196V, respectively (Sandell *et al.*, 2007; Siegenthaler *et al.*, 2009). Surprisingly, the mutant died at e13 and e17, respectively, due to the cortex development defects which make RDH10 the only known RDH that is indispensable for embryogenesis under normal vitamin A supply. RDH10 is currently the only known NADP⁺-dependent retinol dehydrogenase and highly conserved among different species, with 98.6% protein sequence identity between human and mouse (Wu *et al.*, 2002).

A retinaldehyde reductase (RRD), also known as human 2,4-dienoyl-CoA reductase, was described in peroxisomes of liver (Lei *et al.*, 2003).

In summary, many SDRs that can oxidize retinol into retinaldehyde have been identified, but their tissue specific contributions to physiological retinol oxidation and atRA biosynthesis remain to be clarified. Like ADHs, SDRs are also widely distributed in metazoans and this SDR dependent retinol processing can be an evolutionary conserved

process.

Retinal dehydrogenases

The second step oxidation of atRA biosynthesis, following the reversible oxidation of retinol into retinaldehyde by SDR, is an irreversible process that converts retinaldehyde into atRA. This reaction is catalyzed by retinaldehyde dehydrogenases (RALDHs). There are generally three RALDHs of the ALDH1A class in vertebrates, which are named as ALDH1A1 or RALDH1, ALDH1A2 or RALDH2, and ALDH1A3 or RALDH3. Another RALDH of the ALDH8 class called RALDH4 is mainly present in mouse liver (Theodosiou *et al.*, 2010).

Retinaldehyde dehydrogenase 1 is highly expressed in the dorsal retina of embryos and in several adult epithelial tissues. The proposed function of RALDH1 is involved in dorsoventral patterning of the eye and axonal path finding of retinal ganglion cells (Li *et al.*, 2000). However, only some minor effects were observed in the dorsal retina and the axonal projection of *Raldh1*^{-/-} mice, suggesting that this enzyme is not essential for RA biosynthesis in most tissues (Duester *et al.*, 2003). Overexpression of RALDH1 in *Xenopus* embryos results in premature RA synthesis, which confirms that RALDH1 perform a retinal oxidation function *in vivo* (Duester *et al.*, 2003). RALDH1 is also reported to perform some function beyond as a retinal dehydrogenase, for example, a thyroid binding protein (Yamauchi *et al.*, 1999). This data indicate that expression of *Raldh1* is not always associated with retinal dehydrogenation.

RALDH2 is widely expressed in many embryonic and adult tissues. Overexpression of RALDH2 in *Xenopus* embryos also results in high levels of atRA indicating the *in vivo* RA synthesis function of RALDH2 (Haselbeck *et al.*, 1999). RALDH2 can be first detected at E7.5 during mouse embryogenesis, the same time RA can be detected. In mouse embryos, RALDH2 is mainly present in mesenchymal tissues, such as lung bud mesoderm, proximal limb bud, trunk mesoderm and heart until midgestation (Duester *et al.*, 2003). *Raldh2*^{-/-} mice is embryonic lethal at E8.75 due to the failure development of the heart. Besides that, *Raldh2* null mice embryos have shortening of the anteroposterior axis and defects on limb buds formation due to the absence of atRA, which is caused by the reduced Hox expression. RALDH2 knockout mice also exhibit development defects on hindbrain and neural crest. External administration of atRA can rescue the RALDH2^{-/-} phenotypes to a considerable extent (Niederreither *et al.*, 1999; Niederreither *et al.*, 2002a; Niederreither *et al.*, 2001; Niederreither *et al.*, 2002b). All these data lead to the conclusion that RALDH2 is essential and indispensable for development. However, the importance of RALDH2 in adult atRA biosynthesis hasn't been fully investigated.

RALDH3 is mainly expressed in mouse and chicken retina, lens, and olfactory pit as well as in the ureteric buds and surface ectoderm adjacent to the developing forebrain (Duester *et al.*, 2003). *In vitro* research proves that RALDH3 can oxidize retinal to atRA. RALDH3 null mice died within 10 hr after birth because of the defects in nasal and ocular development which cause respiratory distress (Dupe *et al.*, 2003). This defect is similar with the phenotypes observed in VAD mice or retinoid receptors null mice and

can be reversed by a simple maternal treatment with atRA.

RALDH4 is highly expressed in mouse liver and kidney and prefers 9-*cis*-retinaldehyde rather than all-*trans*-retinaldehyde as substrate, indicating that this enzyme might play an important role in 9-*cis*-RA biosynthesis (Lin *et al.*, 2003).

In summary, different organs and cells utilize different RALDHs to synthesize RA in different life stage.

Cellular retinoic acid binding proteins

Two kinds of cellular RA binding proteins (CRABPI and CRABPII) bind newly synthesized RA for either gene activation or RA degradation. CRABPs play an important role in RA transportation, either entering into the nucleus to activate transcription (autocrine) or delivered to nearby target cells (paracrine) (Napoli, 1996). CRABPI binds all-*trans*-RA with higher affinity than CRABPII and both of them bind 9-*cis*-RA with lower affinity than all-*trans*-RA. CRABPII is suggested to function as a RA signaling facilitator which can quickly translocate to the nucleus upon binding with RA, where the complex associates directly with retinoid receptors and facilitates the RA-receptor interaction (Delva *et al.*, 1999). In contrast, CRABPI is believed to transfer RA to the RA catabolism enzymes like cytochrome P450s and assist the RA degradation process (Noy, 2000). Overexpression of CRABPI in F9 stem cell lines result in an increased rate of RA degradation and lower sensitivity to RA compared to untransfected cells. Interestingly, CRABPI and CRABPII double null mice are normal, as long as they are fed a diet copious in vitamin A, and don't show any altered sensitivity to teratology after retinoid administration (Lampron *et al.*, 1995).

Retinoic acid degradation--cytochrome P450 enzymes

The atRA concentration is precisely controlled by the balance between atRA biosynthesis and atRA degradation. Catabolism of atRA into more oxidized metabolites, such as 4-hydroxy-RA, 18-hydroxy-RA and 4-oxo-RA, is catalyzed mainly by the enzymes that belong to Cyp26 family. Three Cyp26 enzymes, CYP26A1, CYP26B1 and CYP26C1, have been identified in both humans and rodents that function as the major RA degradation enzymes (Thatcher and Isoherranen, 2009). Some other CYPs also have been implicated in catabolizing atRA *in vitro*. However, whether these *in vitro* data can be applied *in vivo* remained unclear (Theodosiou *et al.*, 2010).

The first cytochrome P450 enzyme for RA catabolism CYP26A1 was cloned by White *et al.* in zebrafish and shortly thereafter the human, mouse and rat homolog was cloned (White *et al.*, 1996). When transfected into COS cells, it can metabolize all-*trans*-RA into more polar metabolites, such as 4-oxo-RA, 18-hydroxy-RA. CYP26A1 is highly expressed in the liver, duodenum, colon, placenta and some region of the brain. An RARE is found in the proximal upstream promoter region of CYP26A1 and the transcripts are inducible by RA, indicating that CYP26A1 can sense the concentration of RA and regulate the oxidation metabolism of RA accordingly. CYP26A1 null mice die

during mid to late gestation and display a prominent defect of spina bifida (Abu-Abed *et al.*, 2001). Generally speaking, CYP26A1 knockout mice show similar morphogenetic defects as RA teratogenicity.

CYP26B1 was identified shortly after CYP26A1 and can also metabolize all-*trans*-RA into polar metabolites including 4-oxo-RA, 4-hydroxy-RA and 18-hydroxy-RA. CYP26B1 is mainly expressed in CNS, such as cerebellum and pons of the brain, whereas CYP26A1 is expressed in low intensity in the brain. They have similar catabolic activity, indicating that CYP26B1 might be the major catabolic enzyme that regulates atRA in adult CNS (White *et al.*, 2000). Besides brain, CYP26B1 mRNA is also abundantly expressed in the placenta, ovary, testes and intestine, but not detectable in liver. Overall, CYP26B1 expression is more widespread than CYP26A1. CYP26B1^{-/-} mice is also embryonic lethal as is CYP26A1^{-/-}, but they show distinct development defects. CYP26B1 null mice die shortly after birth, which is attributed to respiratory failure and show greater defects in limb bud (Yashiro *et al.*, 2004).

Most recently, Taimi *et al.* cloned the third enzyme CYP26C1 which can also convert all-*trans*-RA to polar metabolites similar to CYP26A1 and B1 in transfected cells (Taimi *et al.*, 2004). However, CYP26C1 show higher activity with 9-*cis*-RA compared with CYP26A1 and B1. CYP26C1 is mainly expressed during embryonic development. CYP26C1^{-/-} mice are viable and didn't show any defects in embryonic development, as long as fed a diet copious in vitamin A.

Generally speaking, the non-overlapping expression pattern of three CYP26s indicates individual roles for each CYP enzymes in the regulation of RA concentration both in embryo development and in adulthood.

RA receptors

The effects of atRA are mediated through its binding to specific retinoid receptors, which belong to the family of nuclear receptors (NR). There are three RARs (RAR α , RAR β and RAR γ). Both all-*trans*-RA and 9-*cis*-RA bind to RAR. RXRs (RXR α , RXR β and RXR γ) show only 9-*cis*-RA binding activity (Chambon, 1996). RXR, originally identified as an orphan receptor, serves as a heterodimeric partner to RAR, and many other nuclear receptors, involved in regulating intermediary metabolism, such as thyroid hormone receptor (TR), vitamin D receptor (VDR) or peroxisome proliferator-activated receptor (PPAR) (Chambon, 1996). Transcriptional activation by RAR is determined by the binding of RAR/RXR heterodimer to a specific DNA binding sequence called RAR elements (RARE) (or RXR elements-RXRE) located within the promoters of target genes. The binding recruits co-activators and co-repressors. The net effect may be either gene repression, the release of gene repression, gene activation or gene transrepression (Wei, 2003). Knockout mice of three RARs showed obvious redundancy for each single RAR in RA signaling. Mice null of RAR α display some phenotypes of vitamin A deficiency such as decreased viability, growth deficiency and male sterility (Mark *et al.*, 2009). RAR β ^{-/-} mice display a selective loss of striosomal compartmentalization in the rostral

striatum and locomotor defects, which are correlated with dopamine signaling and suggest that RAR β might play an important role in brain function (Krezel *et al.*, 1998; Liao *et al.*, 2008). RAR γ is abundantly expressed in skin. Deletion of this gene shows defects associated with vitamin A deficiency (Lohnes *et al.*, 1993). The single RAR knockout mice indicate some function redundancy among RARs. In contrast, the double knockout mice exhibit more dramatic growth defects, which increase the mortality of embryos or new pups (Lohnes *et al.*, 1994; Mendelsohn *et al.*, 1994). The RXR α and RXR β , but not RXR γ knockouts showed much lower viability than RARs null mice, indicating that RXR is essential for retinoid signaling *in vivo* and each RXR has its own function (Theodosiou *et al.*, 2010).

Besides RAR and RXR, other nuclear receptors are also believed to participate in retinoid signaling, and somehow compete with RAR/RXR heterodimer to regulate gene expression. For example, atRA was previously reported to be a ligand for the PPAR β/δ orphan receptor, which can induce differentiation and shows anti-apoptotic activities through regulation of the PDK-1/Akt survival pathway. FABP5 is believed to act as the binding protein that delivers RA to PPAR β/δ (Shaw *et al.*, 2003; Tan *et al.*, 2004). So one model is the relative ratio of FABP5 and CRABP determine which transcription factors are selectively activated and result in the regulation of target genes (Schug *et al.*, 2007).

RA target genes--possible autoregulation pathway

RA target genes, some of them are involved in RA homeostasis have been discovered, including RAR β , CRBP, CRABP, CYP26, RALDH, laminin B1, Hox genes (Napoli, 1999). Some genes are found to be direct target of the classical RAR/RXR-dependent RARE pathway, however, there are much more other genes have been proved as the regulatory targets of RA but this regulatory effect is indirect through intermediate transcription factors or non-classical association of receptors with other proteins (Theodosiou *et al.*, 2010).

Among those direct target genes, CRBP, LRAT, RALDH and CYP26 are the genes that can be autoregulated by RA itself and then feedback regulate RA metabolism. In the presence of RA, cells will increase CRBP and LRAT expression to convert more retinol to RE, which will decrease the rates of atRA synthesis. In some examples, atRA can decrease RALDH1 expression and increase CYP26 expression, which will result in the similar effect as regulation of CRBP and LRAT—catabolize extra atRA and decrease atRA synthesis (Napoli, 1999). In summary, atRA itself can regulate cellular retinol uptake by inducing CRBP, enhances retinol esterification by inducing LRAT and limits its own concentration by inducing CYP26.

Besides RAR/RXR dependent gene transcription signaling, a non-genomic signaling of RA has been described recently. One example is the RA-dependent regulation of a type of homeostatic synaptic plasticity in hippocampal neurons (Chen and Napoli, 2008). Chen *et al.* showed that unliganded RAR α serve as an RNA-binding protein associated with mRNAs, such as glutamate receptor 1 (GluR1) to suppress translation. RA binding to

RAR α releases RAR α from the complex and relieves translational repression.

Part II Retinoic Acid Signaling and Function in Adult CNS

Overview

One of the first functions of atRA identified in the embryonic or early postnatal CNS development, include patterning of anteroposterior axis of neural tube, neuronal cell differentiation, and neurite outgrowth (Maden, 2002). The development of embryonic hindbrain, retina, inner ear, olfactory system and spinal cord also is regulated by atRA (Maden, 2002, 2006; Romand *et al.*, 2006). Recently, cumulative evidence indicates the importance of RA signaling in the adult CNS (Drager, 2006; Lane and Bailey, 2005). The components of RA signaling, such as retinal dehydrogenase and binding proteins, are present in the adult CNS, although the distribution pattern differs from that observed in the embryo CNS. The presence of retinoid receptors in the adult CNS also indicates that RA signaling might be important in specific areas of the adult brain, such as the hippocampus, cortex, olfactory bulb and hypothalamus. Many neural genes are regulated by atRA, directly or indirectly (Mey and McCaffery, 2004). RARE have been identified in some of those genes. Disruption of signaling pathways indicates that RA signaling participates in cognitive function of the brain, especially learning and memory formation in the hippocampus. RA signaling is also implicated in the pathology of some neurological diseases, such as Alzheimer's disease, schizophrenia, Huntington's disease and Parkinson's disease.

RA signaling components in adult CNS

All-*trans*-RA biosynthesis is present in adult rabbit, mouse and rat brain. A comparable or even relative higher rate of atRA biosynthesis than the rate in animal liver was demonstrated in the cerebrum, cerebellum and meninges (Dev *et al.*, 1993; Wagner *et al.*, 2002; Werner and Deluca, 2002).

RA signaling components have been discovered in the adult nervous system, including synthetic and catabolic enzymes, binding proteins, retinoids receptors and RARE located in many neuronal genes (Mey and McCaffery, 2004). RALDHs were found in the pia mater, meninges, basal ganglia, hippocampus and auditory afferents in the adult brain. In addition, RALDH1 was found throughout the blood vessels in the brain and RALDH2 was highly present in the meninges and perivascular cells of the olfactory bulb (McCaffery and Drager, 1994; Thompson Haskell *et al.*, 2002; Wagner *et al.*, 2002). The expression pattern of CRBPs and CRABPs in adult CNS is quite different from that in embryos. In adult brain, CRBPI is the major CRBP and the distribution pattern parallels that of the RALDHs with high expression in the meninges, hippocampus and the olfactory bulb (Zetterstrom *et al.*, 1999). CRABPI is mainly expressed in the hippocampus and olfactory bulb, whereas CRABPII is restricted to cholinergic neurons in the basal forebrain and nucleus accumbens and the pia mater (Zetterstrom *et al.*, 1999). The expression of those CRABPs does not entirely match RALDH expression. However, the presence of RALDHs alone doesn't necessarily indicate the presence of atRA

synthesis. So the function of CRABPs in adult brain remains unclear and need to be further investigated. For retinoids receptors, RAR α and RAR β were detected at high levels in adult CNS with low expression of RAR γ . RAR α protein is generally widely distributed with particularly high expression in hippocampus and cortex. In contrast, RAR β and RXR γ localizes in restricted areas, such as the caudate/putamen and nucleus accumbens (Krezel *et al.*, 1999). Besides retinoid receptors, RARE have been identified in many neuronal genes, indicating that RA signaling is physiological active and important in adult brain. Interestingly, RARE can bind not only RAR/RXR heterodimers, but also some other transcription factor such as COUP-TF receptors which might indicate a competitive regulation of gene expression (Pfahl *et al.*, 1994).

Large numbers of neuronal specific genes are regulated by retinoids (Lane and Bailey, 2005). Those genes regulate different subgroups of neuronal events, including transporters, metabolic enzymes, G-protein coupled receptors, ionotropic receptors, Ion transport protein, cytoskeletal proteins and intracellular signaling molecules. Individual neurotransmitter systems may be regulated by retinoids at different levels. Retinoids can up regulate expression of glutamic acid decarboxylase (the enzyme involved in GABA synthesis), the GABA transporter, and GABA receptor γ 2 subunit. Among all those genes, some, for example, oxytocin and neurogranin, have been confirmed as directly interacting with RA *via* RAREs; some of them have been proved to be directly regulated by atRA, but RARE haven't been proved present in their promoter regions. Many other genes are indirect targets of retinoids—the regulation of gene expression might be a secondary effect of retinoids (Lane and Bailey, 2005). However, all these data indicate that retinoids are important for regulation of adult neuronal function.

Physiological function of RA in CNS- learning and synaptic plasticity

RA and song learning: Continued neurogenesis has been discovered in the high vocal center of song birds, a region that can integrate auditory and motor activity and important for male song birds to acquire their specie specific songs (Alvarez-Buylla and Kirn, 1997; Denisenko-Nehrbass *et al.*, 2000). RA signaling is believed to have an important function in this process. Gene screen reveals that a mouse RALDH2 homolog is present in HVC, which can utilize retinaldehyde to synthesize RA. This RA synthesis ability is present in both young and adult song birds. But only the inhibition of RA synthesis in young song birds by RALDH inhibitors can influence song production. These data indicate that RA signaling is necessary at least for synaptic plasticity during song production acquisition.

Physiological function of RA signaling in adult hippocampus: Hippocampus is a seahorse-shaped structure in the limbic lobe, a region of phylogenetically and architectonically primitive cortex (McCaffery *et al.*, 2006). The major function of hippocampus is associated with the generation of intermediate, episodic, declarative and spatial learning and memory and to consolidate these memories to permanent form. Neural plasticity, the ability of the adult brain to actively remodel the pattern of neuronal

pathways, includes many neuronal events such as modulation of synaptic strength in the form of long-term potentiation (LTP) and long-term depression (LTD), turnover of synapses, neuritic reorganization and neurogenesis (Drager, 2006). RA signaling alters many of these neural events from transcriptional or translational level to impact the synaptic plasticity and finally affect the cognitive function of hippocampus (McCaffery *et al.*, 2006).

Components of RA signaling pathways are present in the adult hippocampus, including RALDH2, which is restricted to the meninges, and CRBPI and CRABPI mRNA. In addition, RAR α and RXR α,β,γ mRNA was detected in the hippocampus and RAR α is expressed at a relative high level compared with other receptors (Zetterstrom *et al.*, 1999; Zetterstrom *et al.*, 1994). Although these components were discovered in adult hippocampus, the concentration of RA in hippocampus is uncertain due to methods limitations. In our lab, we use LC/MS/MS to accurately measure atRA in discrete areas of adult brain, including hippocampus. We found a relative high concentration of atRA in the adult mice hippocampus, which is consistent with the hypothesis that hippocampus is a RA signaling activating region in the adult brain (Kane *et al.*, 2008).

RA signaling has been implicated in changes of LTP and LTD in adult hippocampus. In RAR β -null or RAR β -RXR γ double knockout mice, LTP and LTD is impaired, and LTP is totally abolished in CA1 region. Interestingly, only LTD is impaired in RXR γ knockout mice, whereas LTP is normal, which indicates that RAR β is necessary for both LTP and LTD, but RXR γ is only required for LTD (Chiang *et al.*, 1998). This kind of impairment of LTP and LTD might be a development deficit due to the lack of RA signaling receptor during embryonic development. However, electron microscopy reveals that no ultrastructural abnormalities were observed in these mutant mice. Presynaptic neurotransmissions are also normal in the mutants. This evidence indicates that the RAR β and/or RXR γ dependent RA signaling function during adulthood affect the synaptic plasticity in hippocampus. However, RAR β is not detected in adult hippocampus, so this effect must be through some indirect regulation of RAR β dependent signaling pathway, such as neurogranin and neuromodulin which can regulate calcium availability and are regulated by atRA (Chiang *et al.*, 1998). Though RAR α is the most abundant retinoid receptor in the hippocampus, RAR α null mice are early postnatal lethal. The importance of RA signaling in synaptic plasticity is also supported by vitamin-A deficient mice and aged mice (Etchamendy *et al.*, 2003; Etchamendy *et al.*, 2001; Misner *et al.*, 2001). 12 weeks of VAD mice showed significantly reduced LTP and LTD, whereas LTD was totally lost after 15 weeks. Both phenomena were improved after feeding a vitamin A-sufficient diet. Similarly, in aged mice, the reduced mRNA level of RAR β , RXR β and/or γ concurred with a diminished level of LTP and LTD which can be reversed by administration RA and be exacerbated by application of RAR antagonist.

LTP and LTD are generally considered to be physiologically associated with spatial learning and memory formation. Retinoid receptors null mice, VAD mice/rats and aged mice have all been used to study the effects of RA signaling on learning and memory

formation (Mey and McCaffery, 2004). Consistent with the effect on LTP and LTD, all these animal models show correlative impairment of learning and memory formation. RAR β or RAR β /RXR γ null mice, but not RXR γ null mice showed impaired learning and memory formation in the Morris Water Maze test (Chiang *et al.*, 1998). Rats fed with a VAD diet for 12 weeks made significantly more errors than controls in the radial maze spatial learning tasks, an effect that could be reversed by replenishing with a vitamin A-sufficient diet for about 2 weeks (Misner *et al.*, 2001). However, it is more difficult to induce similar effects in mice. In one study, 28 weeks of VAD diet was needed for mice to achieve obvious symptoms of vitamin A deficiency. Similar deficits in spatial learning ability as observed in 12 weeks VAD rats were not observed until 39 weeks of feeding mice the VAD diet, and this effect was not be reversed by dosing 150 μ g/kg atRA for 10 days, which indicate that it is more difficult to induce a vitamin A deficient status in mice and once it is induced, or has gone too far, the deleterious effect might be permanent (Etchamendy *et al.*, 2003). Aged mice provide another model to study the effects of RA signaling on learning and memory. Old mice (21-23months) displayed a significant impaired relational memory formation compared with young mice (4-5months), which is consistent with reduced expression of RAR genes. This effect could be reversed by administration of atRA for 10 days. Co-administration of an RAR antagonist blocked this reversal (Etchamendy *et al.*, 2001). Interestingly, another retinoid, 13-*cis*-RA also induces the deficits in the performance of radial arm maze task, which is attributed to decreased hippocampus neurogenesis (Crandall *et al.*, 2004).

Taking all these data together, there is a clear link between RA signaling and synaptic plasticity, as well as learning and memory formation. Absence of vitamin A or reduced RA signaling by altering RAR, leads to reduce expression of neurogranin and other important neuronal genes, which affects hippocampal synaptic plasticity and correlates with impaired spatial learning. RA signaling may directly modify dendritic trees. Chen *et al* have shown that atRA can quickly induce spine formation, which is associated with new synapse formation, in cultured hippocampal neurons *via* a RAR α -dependent translational modification.

Pathological association of RA signaling on CNS diseases

In the corpus striatum, many RA signaling components were found, including CRABPI, CRABPII and CRBPI. RAR β is the major RA receptor in the striatum and all three RXRs were also present. Besides that, a high level of RALDH1 is present in dopaminergic neurons of the substantia nigra (Krezel *et al.*, 1998; Zetterstrom *et al.*, 1999; Zetterstrom *et al.*, 1994). Two dopamine receptors, dopamine D2 receptor, which has an RARE, (the knockout mice show a parkinsonian-like phenotype), and the D1 receptor rely on RA regulation. The survivals of nigrostriatal dopaminergic neurons, whose lesions are associated with Parkinson's disease, are also regulated by RA (Krezel *et al.*, 1998).

RA signaling is also associated with motoneuron disease. In vitamin A depleted rats, astrocytosis and a significant loss of motoneurons was found in the spinal cord ,which

indicates that RA signaling is not only important for embryonic neuron differentiation, but also required for adult neuron survival (Maden, 2002). Interestingly, a small population of spontaneous amyotrophic lateral sclerosis (ALS) patients showed significant decrease of RAR α and RALDH2 expression, which may contribute to neuron loss in ALS (Maden and Hind, 2003).

13-*cis*-RA, which is the major component of Accutane (an oral treatment for severe acne), may cause depression after chronic use. Recently, evidence pointed to hippocampus as the major functional point for 13-*cis*-RA's deleterious effect in adult brain (Crandall *et al.*, 2004). A clinical dose (1 mg/kg/d) of 13-*cis*-RA administration significantly suppresses hippocampal neurogenesis and severely disrupts hippocampus dependent learning ability.

Indirect evidence indicates a link between RA signaling and schizophrenia. The chromosomal location of loci of some RA signaling component genes are suggested to link to schizophrenia. Abnormal RA signaling during development may increase the susceptibility of schizophrenia (Goodman, 1998). Reelin, a secreted protein that may promote synaptogenesis in GABAergic cells and is significantly down regulated in schizophrenia, is controlled by RA through methylation of its promoters (Chen *et al.*, 2002).

The fact that RA can guide nervous system development raises the possibility that RA can also act as a signal for nerve regeneration. Indirect evidence come from retinoids receptors and binding proteins expression after nerve injury and explant cultures of dorsal root ganglia, retina and spinal cord. A supportive effect of RA on axon growth and neuronal survival is suggested due to increased secretion of some cytokines, such as IL-1, IL-6 and TGF β or transcriptional activation of the neurotrophin receptor genes, such as Trk and GF-R α 1 through RAR α . RA can also induce neuron and glia cells differentiation. In cell culture experiments with embryonic tissues, RA increased axonal outgrowth from the spinal cord, cerebellum and sympathetic ganglia. RA can also promote regeneration of axons from differentiated nerve cells. These effects are usually synergistic with nerve growth factor (NGF), BDNF and NT-3. RAR β 2, but not other RARs, seems to be a prerequisite for RA induced axonal growth, at least in spinal cord neurons. In mammalian PNS, where axonal regeneration is available, the entire RA signaling components are present, including CRBPI, CRABPI and II, enzymes for RA synthesis and CYP26A1, which indicate the *in vitro* results discovered before might be physiologically important for axon regeneration *in vivo* (For review, see Mey and McCaffery, 2004).

RA signaling has been implicated in the pathology of Alzheimer's disease (AD), a disease characterized by the progressive memory impairment and deteriorating cognitive ability. Formation of amyloid plaques, presence of neurofibrillary tangles and neuronal loss is clinical phenotype of AD. A number of AD related genes are regulated by RA signaling *in vitro*, including amyloid precursor protein, presenilins, and choline acetyltransferase. Genetic analysis also reveals the association of chromosomes 10q23 and 12q13, the loci which include RBP4 and RAR γ , with Alzheimer's disease. Recently,

the effects of RA signaling on amyloid plaque formation have been directly tested and atRA might prevent the formation of amyloid plaque. atRA signaling has also been demonstrated to affect the cholinergic neurotransmission which might also contribute to the pathology of AD (For review, see Goodman, 2006; Lane and Bailey, 2005).

RA source in adult hippocampus

The relative high concentration of atRA detected in adult brain, especially in hippocampus, as well as the evidence showing that RA signaling contributes to cognitive function, raises questions about the sources of atRA. As mentioned before, the circulating concentration of atRA is relatively low and may not support high demand for atRA in the adult brain. Given that in other atRA signaling active organs, such as kidney and lung, atRA is mainly from localized biosynthesis, as well as the presence of RA biosynthesis components in adult CNS, our hypothesis is that a similar mechanism of atRA biosynthesis is also present in adult brain, especially in RA signaling active regions such as the hippocampus. Werner *et al.* and her colleagues showed that only ³H labeled retinol but not ³H labeled atRA could be taken in by adult CNS, which provides direct evidence to exclude the possibility that atRA in adult brain is from circulation, but supports the hypothesis that localized biosynthesis of atRA might be present, because the substrate can cross the blood-brain barrier (Werner and Deluca, 2002).

The primary goal of my project was to identify the cell types in the adult hippocampus responsible for atRA biosynthesis, and to identify the enzymes active in atRA biosynthesis in the hippocampus. Two major cell types are present in the adult hippocampus, pyramidal neurons and glia cells. Pyramidal neurons are the acknowledged functional unit for all cognitive roles in hippocampus, for example LTP and LTD. RA signaling response elements, such as RAR has been detected in pyramidal neurons. Pyramidal neurons can also respond to exogenous atRA and significantly increase the spine numbers and spine lengths within 30 min treatment (Chen and Napoli, 2008). atRA biosynthesis enzymes, such as RALDH1, are also found in pyramidal neurons. Pyramidal neurons are candidates for atRA biosynthesis.

Glia cells are the other major cell type present in hippocampus. Generally speaking, there are more glia cells than neurons in adult CNS. The ratio of glia cells: neurons is 10:1 in human and about 3:1 in rodents. The major function of glia cells is to provide structural, nutritional and functional support to neurons (Farina *et al.*, 2007). There are different subtypes of glia cells. Astrocytes are the most abundant subtype that has been well studied. Originally, astrocytes are only thought to provide metabolic support to pyramidal neurons in adult hippocampus. However, more and more evidence has shown that astrocytes are also implicated in the dynamic regulation of neurogenesis, synaptic network formation, neuron electrical activity and specific neurological disease (Nedergaard *et al.*, 2003). To regulate and optimize the environment within which neurons function, astrocytes maintain a tight control of ion concentration and pH homeostasis, store glycogen and export lactate to neurons to provide metabolic support, metabolize neurotransmitters including glutamate, and secrete neurotrophic factors to

support neurons differentiation and synaptogenesis. In addition, astrocytes are also involved in the induction and maintenance of blood-brain barrier, and participate in tissue repair and nerve regeneration after CNS injury. Astrocytes can also regulate the immune response in the CNS by secreting cytokines, such as TNF α , IL-1 β (For review, see Farina *et al.*, 2007; Markiewicz and Lukomska, 2006; Mori *et al.*, 2005; Nedergaard *et al.*, 2003; Ransom *et al.*, 2003).

Astrocytes are good candidates for RA biosynthesis. Astrocytes induce neurogenesis from all kinds of neuronal stem cells, such as adult hippocampal stem cells, neonatal and adult SVZ stem cells, NE-4C neuroectodermal cells and mouse ES cells in contacted or non-contacted co-culture system (Nakayama *et al.*, 2003; Song *et al.*, 2002; Wuarin *et al.*, 1990). Glial condition medium also has similar effects, indicating that astrocytes can secrete intrinsic factors to induce neurogenesis. As atRA has long been recognized as an autocrine that induces neuronal stem cell differentiation, the intrinsic factor could be atRA. atRA showed a trophic effect on spinal neuron survival in the absence of astrocytes. Retinol only has similar effects in the presence of astrocytes (Wuarin *et al.*, 1990). Metabolic labeling of retinol suggests that in the presence of astrocytes, retinol could be oxidized into atRA. atRA biosynthesis components are also detected in some reports. For example, Smith *et al.* found that RALDH1 is co-localized with GFAP⁺ cells in olfactory tissue (Asson-Batres and Smith, 2006). All these data indicate that astrocytes might be a source for RA biosynthesis in adult hippocampus.

Astrocytes also occupy a strategic position, interposed between blood vessels and neurons. This position facilitates astrocytes to intake precursors from circulation, process them and finally provide the metabolites to neurons (Paemeleire, 2002). Astrocytes also have some cytoarchitectural structures to perform this function. For example, astrocytes project their processes toward blood vessels so called endfeet, which almost cover the whole surface of blood vessels. On the other hand, astrocytes also project processes that surround synapses of neurons. One example of how astrocytes utilize this cytoarchitectural structure to support neurons' function is so called neuron-glia metabolic coupling and plasticity. A glutamate-dependent astrocytes uptake and processing glucose results in the release of lactate from astrocytes, which can be transported to neurons to support the activity-dependent fuelling of the neuronal energy demands associated with synaptic regulation (Magistretti, 2006).

Part III Regulation of RA biosynthesis by hormones, cytokines or endotoxins

We're trying to find out whether atRA biosynthesis can be regulated by some other factors besides atRA itself. Those factors include the inflammatory cytokine TNF α , the endotoxin lipopolysaccharide (LPS), prostaglandin E2 (PGE2), and the female sex hormone estrogen.

Estrogen and RA signaling

It is believed that in the female reproductive system, there is interplay between RA signaling and the ovarian hormone estrogen (Bucco *et al.*, 1996; Li *et al.*, 2004; Li and

Ong, 2003; Prins *et al.*, 2002; Rexer and Ong, 2002). Vitamin A levels fluctuate in serum and ovary, as do retinoid binding protein expression in the uterus during the human menstrual cycle and the rat estrous cycle (Bucco *et al.*, 1996). In estrogen treatment of prepubertal rats uterus, increased RA synthesis is coincident with a down regulation of RBP and CRBP mRNA and protein expression as well as an up regulation of CRABP II mRNA and protein (Bucco *et al.*, 1996). Further investigation proved that CRABP II is directly induced by estrogen, but not atRA, in rat uterus (Li and Ong, 2003). In ovariectomized rats, estrogen administration markedly increased uteri mRNA level of Drhs9 and RALDH 2, which indicated that RA biosynthesis in rat uterus is directly controlled by estrogen (Li *et al.*, 2004). A similar effect was also found in adult rat prostate where RAR α , RAR β and atRA were markedly elevated in response to neonatal estrogenic exposure (Prins *et al.*, 2002). Thus, estrogen may be a regulator of RA signaling.

For more than a decade, it has been known that estrogen influences synaptic plasticity in hippocampus. Ovariectomy of female rats resulted in decreased dendritic spine density and disruption of LTD on CA1 pyramidal neurons in the hippocampus, while systemic application of estradiol to these rats caused an increase in the number of dendritic spines and magnitude of LTD in this region (Gould *et al.*, 1990). Along this line, the fluctuation of spine density was observed during the estrus cycle in female rats. Chen *et al.* also demonstrated that atRA treatment induces dendritic filopodia growth and leads to spine formation. Considering that estrogen is a key regulator of RA biosynthesis in the reproductive system, it is possible that estrogen can also regulate RA biosynthesis in the hippocampus and further affect synaptic plasticity.

LPS, PGE2, TNF α and RA signaling

LPS is the major component of the outer membrane of Gram-negative bacteria, acting as a prototypical endotoxin and inducing a strong response from normal animal immune systems by promoting the secretion of pro-inflammatory cytokines in many cell types, especially in macrophages. atRA works synergistically with LPS to increase prostanoid concentrations in rat, indicating that there might be cross talk between atRA and LPS's signaling pathway (Devaux *et al.*, 2001).

The pro-inflammatory cytokines induced by LPS include PGE2 and TNF α . PGE2 stimulates osteoblasts to release factors which stimulate bone resorption by osteoclasts. PGE2 is also an important inflammatory cytokine that can induce immune responses such as fever. atRA induces PGE2 synthesis in human neuroblastoma cells (Alique *et al.*, 2007).

TNF α is a pleiotropic cytokine implicated in the pathogenesis of neurological and neurodegenerative disorders (Sriram and O'Callaghan, 2007). TNF α is a double-edge sword for the normal function of adult brain. Under normal physiological conditions, TNF α performs many different functions, including immune defense, cellular homeostasis and protection against neurological insults. However, TNF α is also elicited

after some brain injury such as ischemia, infection such as HIV and in some neurodegeneration such as Alzheimer's disease. The presence of TNF α in these conditions is believed to be harmful and to delay recovery from injury or exacerbate the disease (Sriram and O'Callaghan, 2007). Activation of different signal pathways by TNF α , such as NF- κ B, ERK1/2, P38 and JNK under different regulation might explain how TNF α has such multifarious functions in the immune response in CNS (Wajant *et al.*, 2003). Astrocytes and microglia are the two major cell types in adult brain that can synthesize and secrete this cytokine. atRA is associated with the ability of astrocytes and microglia to produce TNF α (Dheen *et al.*, 2005). However, whether TNF α could affect atRA production is unknown.

References

- Abu-Abed, S., P. Dolle, *et al.* (2001). "The retinoic acid-metabolizing enzyme, CYP26A1, is essential for normal hindbrain patterning, vertebral identity, and development of posterior structures." Genes Dev **15**(2): 226-240.
- Akita, J., M. Takahashi, *et al.* (2002). "Neuronal differentiation of adult rat hippocampus-derived neural stem cells transplanted into embryonic rat explanted retinas with retinoic acid pretreatment." Brain Res **954**(2): 286-293.
- Alique, M., J. F. Herrero, *et al.* (2007). "All-trans retinoic acid induces COX-2 and prostaglandin E2 synthesis in SH-SY5Y human neuroblastoma cells: involvement of retinoic acid receptors and extracellular-regulated kinase 1/2." J Neuroinflammation **4**: 1.
- Alliot, F., N. Delhaye-Bouchaud, *et al.* (1988). "Role of astroglial cell clones in the survival and differentiation of cerebellar embryonic neurons." Brain Res Dev Brain Res **44**(2): 247-257.
- Alvarez-Buylla, A. and J. R. Kirn (1997). "Birth, migration, incorporation, and death of vocal control neurons in adult songbirds." J Neurobiol **33**(5): 585-601.
- Aoto, J., C. I. Nam, *et al.* (2008). "Synaptic signaling by all-trans retinoic acid in homeostatic synaptic plasticity." Neuron **60**(2): 308-320.
- Asson-Batres, M. A. and W. B. Smith (2006). "Localization of retinaldehyde dehydrogenases and retinoid binding proteins to sustentacular cells, glia, Bowman's gland cells, and stroma: potential sites of retinoic acid synthesis in the postnatal rat olfactory organ." J Comp Neurol **496**(2): 149-171.
- Banker, G. A. (1980). "Trophic interactions between astroglial cells and hippocampal neurons in culture." Science **209**(4458): 809-810.
- Batten, M. L., Y. Imanishi, *et al.* (2004). "Lecithin-retinol acyltransferase is essential for accumulation of all-trans-retinyl esters in the eye and in the liver." J Biol Chem **279**(11): 10422-10432.
- Bendich, A. and L. Langseth (1989). "Safety of vitamin A." Am J Clin Nutr **49**(2): 358-371.
- Blaner, W. S. (2007). "STRA6, a cell-surface receptor for retinol-binding protein: the plot thickens." Cell Metab **5**(3): 164-166.
- Blomhoff, R. and H. K. Blomhoff (2006). "Overview of retinoid metabolism and function." J Neurobiol **66**(7): 606-630.
- Brodeur, H., I. Gagnon, *et al.* (2003). "Cloning of monkey RALDH1 and characterization of retinoid metabolism in monkey kidney proximal tubule cells." J Lipid Res **44**(2): 303-313.
- Bucco, R. A., W. L. Zheng, *et al.* (1996). "Regulation and localization of cellular retinol-binding protein, retinol-binding protein, cellular retinoic acid-binding protein (CRABP), and CRABP II in the uterus of the pseudopregnant rat." Endocrinology **137**(7): 3111-3122.

- Chai, X., M. H. Boerman, *et al.* (1995). "Cloning of a cDNA for liver microsomal retinol dehydrogenase. A tissue-specific, short-chain alcohol dehydrogenase." J Biol Chem **270**(8): 3900-3904.
- Chambon, P. (1996). "A decade of molecular biology of retinoic acid receptors." FASEB J **10**(9): 940-954.
- Chandrasekaran, V., Y. Zhai, *et al.* (2000). "Retinoic acid regulates the morphological development of sympathetic neurons." J Neurobiol **42**(4): 383-393.
- Chen, N. and J. L. Napoli (2008). "All-trans-retinoic acid stimulates translation and induces spine formation in hippocampal neurons through a membrane-associated RARalpha." FASEB J **22**(1): 236-245.
- Chen, N., B. Onisko, *et al.* (2008). "The nuclear transcription factor RARalpha associates with neuronal RNA granules and suppresses translation." J Biol Chem **283**(30): 20841-20847.
- Chen, Y., R. P. Sharma, *et al.* (2002). "On the epigenetic regulation of the human reelin promoter." Nucleic Acids Res **30**(13): 2930-2939.
- Chetyrkin, S. V., O. V. Belyaeva, *et al.* (2001). "Characterization of a novel type of human microsomal 3alpha -hydroxysteroid dehydrogenase: unique tissue distribution and catalytic properties." J Biol Chem **276**(25): 22278-22286.
- Chiang, M. Y., D. Misner, *et al.* (1998). "An essential role for retinoid receptors RARbeta and RXRgamma in long-term potentiation and depression." Neuron **21**(6): 1353-1361.
- Cocco, S., G. Diaz, *et al.* (2002). "Vitamin A deficiency produces spatial learning and memory impairment in rats." Neuroscience **115**(2): 475-482.
- Collins, M. D. and G. E. Mao (1999). "Teratology of retinoids." Annu Rev Pharmacol Toxicol **39**: 399-430.
- Connor, M. J. and N. Sidell (1997). "Retinoic acid synthesis in normal and Alzheimer diseased brain and human neural cells." Mol Chem Neuropathol **30**(3): 239-252.
- Cooper, D. L., N. R. Isola, *et al.* (1993). "Members of the ALDH gene family are lens and corneal crystallins." Adv Exp Med Biol **328**: 169-179.
- Cowherd, R. M., R. E. Lyle, *et al.* (1999). "Molecular regulation of adipocyte differentiation." Semin Cell Dev Biol **10**(1): 3-10.
- Crandall, J., Y. Sakai, *et al.* (2004). "13-cis-retinoic acid suppresses hippocampal cell division and hippocampal-dependent learning in mice." Proc Natl Acad Sci U S A **101**(14): 5111-5116.
- Delva, L., J. N. Bastie, *et al.* (1999). "Physical and functional interactions between cellular retinoic acid binding protein II and the retinoic acid-dependent nuclear complex." Mol Cell Biol **19**(10): 7158-7167.
- Denisenko-Nehrbass, N. I., E. Jarvis, *et al.* (2000). "Site-specific retinoic acid production in the brain of adult songbirds." Neuron **27**(2): 359-370.
- Dev, S., A. J. Adler, *et al.* (1993). "Adult rabbit brain synthesizes retinoic acid." Brain Res **632**(1-2): 325-328.

- Devaux, Y., C. Seguin, *et al.* (2001). "Retinoic acid and lipopolysaccharide act synergistically to increase prostanoid concentrations in rats in vivo." J Nutr **131**(10): 2628-2635.
- Dheen, S. T., Y. Jun, *et al.* (2005). "Retinoic acid inhibits expression of TNF-alpha and iNOS in activated rat microglia." Glia **50**(1): 21-31.
- Douville, J., R. Beaulieu, *et al.* (2009). "ALDH1 as a functional marker of cancer stem and progenitor cells." Stem Cells Dev **18**(1): 17-25.
- Drager, U. C. (2006). "Retinoic acid signaling in the functioning brain." Sci STKE **2006**(324): pe10.
- Duester, G. (2000). "Families of retinoid dehydrogenases regulating vitamin A function: production of visual pigment and retinoic acid." Eur J Biochem **267**(14): 4315-4324.
- Duester, G. (2001). "Genetic dissection of retinoid dehydrogenases." Chem Biol Interact **130-132**(1-3): 469-480.
- Duester, G., F. A. Mic, *et al.* (2003). "Cytosolic retinoid dehydrogenases govern ubiquitous metabolism of retinol to retinaldehyde followed by tissue-specific metabolism to retinoic acid." Chem Biol Interact **143-144**: 201-210.
- Dupe, V., N. Matt, *et al.* (2003). "A newborn lethal defect due to inactivation of retinaldehyde dehydrogenase type 3 is prevented by maternal retinoic acid treatment." Proc Natl Acad Sci U S A **100**(24): 14036-14041.
- E, X., L. Zhang, *et al.* (2002). "Increased neonatal mortality in mice lacking cellular retinol-binding protein II." J Biol Chem **277**(39): 36617-36623.
- Edwards, R. B., A. J. Adler, *et al.* (1992). "Synthesis of retinoic acid from retinol by cultured rabbit Muller cells." Exp Eye Res **54**(4): 481-490.
- Etchamendy, N., V. Enderlin, *et al.* (2003). "Vitamin A deficiency and relational memory deficit in adult mice: relationships with changes in brain retinoid signalling." Behav Brain Res **145**(1-2): 37-49.
- Etchamendy, N., V. Enderlin, *et al.* (2001). "Alleviation of a selective age-related relational memory deficit in mice by pharmacologically induced normalization of brain retinoid signaling." J Neurosci **21**(16): 6423-6429.
- Evans, T. R. and S. B. Kaye (1999). "Retinoids: present role and future potential." Br J Cancer **80**(1-2): 1-8.
- Farina, C., F. Aloisi, *et al.* (2007). "Astrocytes are active players in cerebral innate immunity." Trends Immunol **28**(3): 138-145.
- Galter, D., A. Carmine, *et al.* (2003). "Distribution of class I, III and IV alcohol dehydrogenase mRNAs in the adult rat, mouse and human brain." Eur J Biochem **270**(6): 1316-1326.
- Ghyselinck, N. B. *et al.* (1999). "Cellular retinol-binding protein I is essential for vitamin A homeostasis." EMBO J **18**(18): 4903-4914.
- Giguere, V., E. S. Ong, *et al.* (1987). "Identification of a receptor for the morphogen retinoic acid." Nature **330**(6149): 624-629.

- Ginestier, C., M. H. Hur, *et al.* (2007). "ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome." Cell Stem Cell **1**(5): 555-567.
- Goodman, A. B. (1998). "Three independent lines of evidence suggest retinoids as causal to schizophrenia." Proc Natl Acad Sci U S A **95**(13): 7240-7244.
- Goodman, A. B. (2006). "Retinoid receptors, transporters, and metabolizers as therapeutic targets in late onset *alzheimer* disease." J Cell Physiol **209**(3): 598-603.
- Gottesman, M. E., L. Quadro, *et al.* (2001). "Studies of vitamin A metabolism in mouse model systems." Bioessays **23**(5): 409-419.
- Gould, E., C. S. Woolley, *et al.* (1990). "Gonadal steroids regulate dendritic spine density in hippocampal pyramidal cells in adulthood." J Neurosci **10**(4): 1286-1291.
- Grun, F., Y. Hirose, *et al.* (2000). "Aldehyde dehydrogenase 6, a cytosolic retinaldehyde dehydrogenase prominently expressed in sensory neuroepithelia during development." J Biol Chem **275**(52): 41210-41218.
- Gundersen, T. E. and R. Blomhoff (2001). "Qualitative and quantitative liquid chromatographic determination of natural retinoids in biological samples." J Chromatogr A **935**(1-2): 13-43.
- Halilagic, A., V. Ribes, *et al.* (2007). "Retinoids control anterior and dorsal properties in the developing forebrain." Dev Biol **303**(1): 362-375.
- Hartmann, S., O. Brors, *et al.* (2005). "Exposure to retinyl esters, retinol, and retinoic acids in non-pregnant women following increasing single and repeated oral doses of vitamin A." Ann Nutr Metab **49**(3): 155-164.
- Haselbeck, R. J., I. Hoffmann, *et al.* (1999). "Distinct functions for Aldh1 and Raldh2 in the control of ligand production for embryonic retinoid signaling pathways." Dev Genet **25**(4): 353-364.
- Herr, F. M. and D. E. Ong (1992). "Differential interaction of lecithin-retinol acyltransferase with cellular retinol binding proteins." Biochemistry **31**(29): 6748-6755.
- Jacobs, S., D. C. Lie, *et al.* (2006). "Retinoic acid is required early during adult neurogenesis in the dentate gyrus." Proc Natl Acad Sci U S A **103**(10): 3902-3907.
- Jette, C., P. W. Peterson, *et al.* (2004). "The tumor suppressor adenomatous polyposis coli and caudal related homeodomain protein regulate expression of retinol dehydrogenase L." J Biol Chem **279**(33): 34397-34405.
- Jiang, F., Q. Qiu, *et al.* (2009). "Aldehyde dehydrogenase 1 is a tumor stem cell-associated marker in lung cancer." Mol Cancer Res **7**(3): 330-338.
- Jones-Villeneuve, E. M., M. A. Rudnicki, *et al.* (1983). "Retinoic acid-induced neural differentiation of embryonal carcinoma cells." Mol Cell Biol **3**(12): 2271-2279.
- Jurukovski, V., N. G. Markova, *et al.* (1999). "Cloning and characterization of retinol dehydrogenase transcripts expressed in human epidermal keratinocytes." Mol

- Genet Metab **67**(1): 62-73.
- Kanai, M., A. Raz, *et al.* (1968). "Retinol-binding protein: the transport protein for vitamin A in human plasma." J Clin Invest **47**(9): 2025-2044.
- Kane, M. A., N. Chen, *et al.* (2005). "Quantification of endogenous retinoic acid in limited biological samples by LC/MS/MS." Biochem J **388**(Pt 1): 363-369.
- Kane, M. A., A. E. Foliass, *et al.* (2008). "Quantitative profiling of endogenous retinoic acid in vivo and in vitro by tandem mass spectrometry." Anal Chem **80**(5): 1702-1708.
- Kane, M. A., A. E. Foliass, *et al.* (2010). "Ethanol elevates physiological all-trans-retinoic acid levels in select loci through altering retinoid metabolism in multiple loci: a potential mechanism of ethanol toxicity." FASEB J **24**(3): 823-832.
- Kawaguchi, R., J. Yu, *et al.* (2007). "A membrane receptor for retinol binding protein mediates cellular uptake of vitamin A." Science **315**(5813): 820-825.
- Kim, C. H. (2008). "Roles of retinoic acid in induction of immunity and immune tolerance." Endocr Metab Immune Disord Drug Targets **8**(4): 289-294.
- Klann, R. C. and A. C. Marchok (1982). "Effects of retinoic acid on cell proliferation and cell differentiation in a rat tracheal epithelial cell line." Cell Tissue Kinet **15**(5): 473-482.
- Kornyei, Z., E. Gocza, *et al.* (2007). "Astroglia-derived retinoic acid is a key factor in glia-induced neurogenesis." FASEB J **21**(10): 2496-2509.
- Krezel, W., N. Ghyselinck, *et al.* (1998). "Impaired locomotion and dopamine signaling in retinoid receptor mutant mice." Science **279**(5352): 863-867.
- Krezel, W., P. Kastner, *et al.* (1999). "Differential expression of retinoid receptors in the adult mouse central nervous system." Neuroscience **89**(4): 1291-1300.
- Labrecque, J., P. V. Bhat, *et al.* (1993). "Purification and partial characterization of a rat kidney aldehyde dehydrogenase that oxidizes retinal to retinoic acid." Biochem Cell Biol **71**(1-2): 85-89.
- Laird, M. D., J. R. Vender, *et al.* (2008). "Opposing roles for reactive astrocytes following traumatic brain injury." Neurosignals **16**(2-3): 154-164.
- Lampron, C., C. Rochette-Egly, *et al.* (1995). "Mice deficient in cellular retinoic acid binding protein II (CRABPII) or in both CRABPI and CRABPII are essentially normal." Development **121**(2): 539-548.
- Lane, M. A. and S. J. Bailey (2005). "Role of retinoid signalling in the adult brain." Prog Neurobiol **75**(4): 275-293.
- Lei, Z., W. Chen, *et al.* (2003). "Reduction of all-trans-retinal in the mouse liver peroxisome fraction by the short-chain dehydrogenase/reductase RRD: induction by the PPAR alpha ligand clofibrate." Biochemistry **42**(14): 4190-4196.
- Li, H., E. Wagner, *et al.* (2000). "A retinoic acid synthesizing enzyme in ventral retina and telencephalon of the embryonic mouse." Mech Dev **95**(1-2): 283-289.
- Li, X. H., B. Kakkad, *et al.* (2004). "Estrogen directly induces expression of retinoic acid biosynthetic enzymes, compartmentalized between the epithelium and underlying

- stromal cells in rat uterus." Endocrinology **145**(10): 4756-4762.
- Li, X. H. and D. E. Ong (2003). "Cellular retinoic acid-binding protein II gene expression is directly induced by estrogen, but not retinoic acid, in rat uterus." J Biol Chem **278**(37): 35819-35825.
- Liao, W. L., H. C. Tsai, *et al.* (2008). "Modular patterning of structure and function of the striatum by retinoid receptor signaling." Proc Natl Acad Sci U S A **105**(18): 6765-6770.
- Lin, M., M. Zhang, *et al.* (2003). "Mouse retinal dehydrogenase 4 (RALDH4), molecular cloning, cellular expression, and activity in 9-cis-retinoic acid biosynthesis in intact cells." J Biol Chem **278**(11): 9856-9861.
- Lohnes, D., P. Kastner, *et al.* (1993). "Function of retinoic acid receptor gamma in the mouse." Cell **73**(4): 643-658.
- Lohnes, D., M. Mark, *et al.* (1994). "Function of the retinoic acid receptors (RARs) during development (I). Craniofacial and skeletal abnormalities in RAR double mutants." Development **120**(10): 2723-2748.
- Maden, M. (2002). "Retinoid signalling in the development of the central nervous system." Nat Rev Neurosci **3**(11): 843-853.
- Maden, M. (2006). "Retinoids and spinal cord development." J Neurobiol **66**(7): 726-738.
- Maden, M. (2007). "Retinoic acid in the development, regeneration and maintenance of the nervous system." Nat Rev Neurosci **8**(10): 755-765.
- Maden, M. and M. Hind (2003). "Retinoic acid, a regeneration-inducing molecule." Dev Dyn **226**(2): 237-244.
- Magistretti, P. J. (2006). "Neuron-glia metabolic coupling and plasticity." J Exp Biol **209**(Pt 12): 2304-2311.
- Mark, M., N. B. Ghyselinck, *et al.* (2009). "Function of retinoic acid receptors during embryonic development." Nucl Recept Signal **7**: e002.
- McCaffery, P. and U. C. Drager (1994). "High levels of a retinoic acid-generating dehydrogenase in the meso-telencephalic dopamine system." Proc Natl Acad Sci U S A **91**(16): 7772-7776.
- McCaffery, P., O. Koul, *et al.* (2004). "Ethanol increases retinoic acid production in cerebellar astrocytes and in cerebellum." Brain Res Dev Brain Res **153**(2): 233-241.
- McCaffery, P., J. Zhang, *et al.* (2006). "Retinoic acid signaling and function in the adult hippocampus." J Neurobiol **66**(7): 780-791.
- Melhus, H., K. Michaelsson, *et al.* (1998). "Excessive dietary intake of vitamin A is associated with reduced bone mineral density and increased risk for hip fracture." Ann Intern Med **129**(10): 770-778.
- Mendelsohn, C., D. Lohnes, *et al.* (1994). "Function of the retinoic acid receptors (RARs) during development (II). Multiple abnormalities at various stages of organogenesis in RAR double mutants." Development **120**(10): 2749-2771.
- Mey, J. and S. Hammelmann (2000). "OLN-93 oligodendrocytes synthesize

- all-trans-retinoic acid in vitro." Cell Tissue Res **302**(1): 49-58.
- Mey, J. and P. McCaffery (2004). "Retinoic acid signaling in the nervous system of adult vertebrates." Neuroscientist **10**(5): 409-421.
- Misner, D. L., S. Jacobs, *et al.* (2001). "Vitamin A deprivation results in reversible loss of hippocampal long-term synaptic plasticity." Proc Natl Acad Sci U S A **98**(20): 11714-11719.
- Molotkov, A., L. Deltour, *et al.* (2002). "Distinct retinoid metabolic functions for alcohol dehydrogenase genes Adh1 and Adh4 in protection against vitamin A toxicity or deficiency revealed in double null mutant mice." J Biol Chem **277**(16): 13804-13811.
- Molotkov, A., X. Fan, *et al.* (2002). "Stimulation of retinoic acid production and growth by ubiquitously expressed alcohol dehydrogenase Adh3." Proc Natl Acad Sci U S A **99**(8): 5337-5342.
- Molotkov, A., X. Fan, *et al.* (2002). "Excessive vitamin A toxicity in mice genetically deficient in either alcohol dehydrogenase Adh1 or Adh3." Eur J Biochem **269**(10): 2607-2612.
- Mori, T., A. Buffo, *et al.* (2005). "The novel roles of glial cells revisited: the contribution of radial glia and astrocytes to neurogenesis." Curr Top Dev Biol **69**: 67-99.
- Moro, J. R., M. Iwata, *et al.* (2008). "Vitamin effects on the immune system: vitamins A and D take centre stage." Nat Rev Immunol **8**(9): 685-698.
- Nadauld, L. D., I. T. Sandoval, *et al.* (2004). "Adenomatous polyposis coli control of retinoic acid biosynthesis is critical for zebrafish intestinal development and differentiation." J Biol Chem **279**(49): 51581-51589.
- Nagy, N. E., K. B. Holven, *et al.* (1997). "Storage of vitamin A in extrahepatic stellate cells in normal rats." J Lipid Res **38**(4): 645-658.
- Nakayama, T., T. Momoki-Soga, *et al.* (2003). "Astrocyte-derived factors instruct differentiation of embryonic stem cells into neurons." Neurosci Res **46**(2): 241-249.
- Napoli, J. L. (1996). "Retinoic acid biosynthesis and metabolism." FASEB J **10**(9): 993-1001.
- Napoli, J. L. (1999). "Interactions of retinoid binding proteins and enzymes in retinoid metabolism." Biochim Biophys Acta **1440**(2-3): 139-162.
- Napoli, J. L. (2010). Vitamin A (Retinoids). Encyclopedia of Biological Chemistry, Elsevier Ltd: in press.
- Nedergaard, M., B. Ransom, *et al.* (2003). "New roles for astrocytes: redefining the functional architecture of the brain." Trends Neurosci **26**(10): 523-530.
- Niederreither, K., V. Subbarayan, *et al.* (1999). "Embryonic retinoic acid synthesis is essential for early mouse post-implantation development." Nat Genet **21**(4): 444-448.
- Niederreither, K., J. Vermot, *et al.* (2002). "Retinaldehyde dehydrogenase 2 (RALDH2)-independent patterns of retinoic acid synthesis in the mouse embryo." Proc Natl

- Acad Sci U S A **99**(25): 16111-16116.
- Niederreither, K., J. Vermot, *et al.* (2001). "Embryonic retinoic acid synthesis is essential for heart morphogenesis in the mouse." Development **128**(7): 1019-1031.
- Niederreither, K., J. Vermot, *et al.* (2002). "Embryonic retinoic acid synthesis is required for forelimb growth and anteroposterior patterning in the mouse." Development **129**(15): 3563-3574.
- Noy, N. (2000). "Retinoid-binding proteins: mediators of retinoid action." Biochem J **348 Pt 3**: 481-495.
- O'Byrne, S. M., N. Wongsiriroj, *et al.* (2005). "Retinoid absorption and storage is impaired in mice lacking lecithin:retinol acyltransferase (LRAT)." J Biol Chem **280**(42): 35647-35657.
- Ong, D. E., P. N. MacDonald, *et al.* (1988). "Esterification of retinol in rat liver. Possible participation by cellular retinol-binding protein and cellular retinol-binding protein II." J Biol Chem **263**(12): 5789-5796.
- Organization, W. H. (1995). "Global prevalence of vitamin A deficiency." Geneva.
- Paemeleire, K. (2002). "The cellular basis of neurovascular metabolic coupling." Acta Neurol Belg **102**(4): 153-157.
- Pares, X., J. Farres, *et al.* (2008). "Medium- and short-chain dehydrogenase/reductase gene and protein families : Medium-chain and short-chain dehydrogenases/reductases in retinoid metabolism." Cell Mol Life Sci **65**(24): 3936-3949.
- Pasutto, F., H. Sticht, *et al.* (2007). "Mutations in STRA6 cause a broad spectrum of malformations including anophthalmia, congenital heart defects, diaphragmatic hernia, alveolar capillary dysplasia, lung hypoplasia, and mental retardation." Am J Hum Genet **80**(3): 550-560.
- Penniston, K. L. and S. A. Tanumihardjo (2006). "The acute and chronic toxic effects of vitamin A." Am J Clin Nutr **83**(2): 191-201.
- Pereira, F., E. Rosenmann, *et al.* (1991). "The 56 kDa androgen binding protein is an aldehyde dehydrogenase." Biochem Biophys Res Commun **175**(3): 831-838.
- Petkovich, P. M. (2001). "Retinoic acid metabolism." J Am Acad Dermatol **45**(5): S136-142.
- Pfahl, M., R. Apfel, *et al.* (1994). "Nuclear retinoid receptors and their mechanism of action." Vitam Horm **49**: 327-382.
- Posch, K. C., R. D. Burns, *et al.* (1992). "Biosynthesis of all-trans-retinoic acid from retinal. Recognition of retinal bound to cellular retinol binding protein (type I) as substrate by a purified cytosolic dehydrogenase." J Biol Chem **267**(27): 19676-19682.
- Prins, G. S., W. Y. Chang, *et al.* (2002). "Retinoic acid receptors and retinoids are up-regulated in the developing and adult rat prostate by neonatal estrogen exposure." Endocrinology **143**(9): 3628-3640.
- Quadro, L., W. S. Blaner, *et al.* (1999). "Impaired retinal function and vitamin A

- availability in mice lacking retinol-binding protein." EMBO J **18**(17): 4633-4644.
- Quadro, L., L. Hamberger, *et al.* (2003). "Understanding the physiological role of retinol-binding protein in vitamin A metabolism using transgenic and knockout mouse models." Mol Aspects Med **24**(6): 421-430.
- Ransom, B., T. Behar, *et al.* (2003). "New roles for astrocytes (stars at last)." Trends Neurosci **26**(10): 520-522.
- Rexer, B. N. and D. E. Ong (2002). "A novel short-chain alcohol dehydrogenase from rats with retinol dehydrogenase activity, cyclically expressed in uterine epithelium." Biol Reprod **67**(5): 1555-1564.
- Rochette-Egly, C. and P. Germain (2009). "Dynamic and combinatorial control of gene expression by nuclear retinoic acid receptors (RARs)." Nucl Recept Signal **7**: e005.
- Rodnight, R., C. A. Goncalves, *et al.* (1997). "Control of the phosphorylation of the astrocyte marker glial fibrillary acidic protein (GFAP) in the immature rat hippocampus by glutamate and calcium ions: possible key factor in astrocytic plasticity." Braz J Med Biol Res **30**(3): 325-338.
- Romand, R., P. Dolle, *et al.* (2006). "Retinoid signaling in inner ear development." J Neurobiol **66**(7): 687-704.
- Romand, R., T. Kondo, *et al.* (2008). "Dynamic expression of the retinoic acid-synthesizing enzyme retinol dehydrogenase 10 (rdh10) in the developing mouse brain and sensory organs." J Comp Neurol **508**(6): 879-892.
- Ross, A. C. (2003). "Retinoid production and catabolism: role of diet in regulating retinol esterification and retinoic Acid oxidation." J Nutr **133**(1): 291S-296S.
- Ross, A. C. and R. Zolfaghari (2004). "Regulation of hepatic retinol metabolism: perspectives from studies on vitamin A status." J Nutr **134**(1): 269S-275S.
- Rothman, K. J., L. L. Moore, *et al.* (1995). "Teratogenicity of high vitamin A intake." N Engl J Med **333**(21): 1369-1373.
- Sandell, L. L., B. W. Sanderson, *et al.* (2007). "RDH10 is essential for synthesis of embryonic retinoic acid and is required for limb, craniofacial, and organ development." Genes Dev **21**(9): 1113-1124.
- Schug, T. T., D. C. Berry, *et al.* (2007). "Opposing effects of retinoic acid on cell growth result from alternate activation of two different nuclear receptors." Cell **129**(4): 723-733.
- Senoo, H. (2004). "Structure and function of hepatic stellate cells." Med Electron Microsc **37**(1): 3-15.
- Shapiro, M. L. and H. Eichenbaum (1999). "Hippocampus as a memory map: synaptic plasticity and memory encoding by hippocampal neurons." Hippocampus **9**(4): 365-384.
- Shaw, N., M. Elholm, *et al.* (2003). "Retinoic acid is a high affinity selective ligand for the peroxisome proliferator-activated receptor beta/delta." J Biol Chem **278**(43): 41589-41592.

- Shearer, K. D., T. H. Goodman, *et al.* (2010). "Photoperiodic regulation of retinoic acid signaling in the hypothalamus." J Neurochem **112**(1): 246-257.
- Shudo, K., H. Fukasawa, *et al.* (2009). "Towards retinoid therapy for Alzheimer's disease." Curr Alzheimer Res **6**(3): 302-311.
- Siegenthaler, J. A., A. M. Ashique, *et al.* (2009). "Retinoic acid from the meninges regulates cortical neuron generation." Cell **139**(3): 597-609.
- Silman, I. and J. L. Sussman (2005). "Acetylcholinesterase: 'classical' and 'non-classical' functions and pharmacology." Curr Opin Pharmacol **5**(3): 293-302.
- Song, H., C. F. Stevens, *et al.* (2002). "Astroglia induce neurogenesis from adult neural stem cells." Nature **417**(6884): 39-44.
- Soref, C. M., Y. P. Di, *et al.* (2001). "Characterization of a novel airway epithelial cell-specific short chain alcohol dehydrogenase/reductase gene whose expression is up-regulated by retinoids and is involved in the metabolism of retinol." J Biol Chem **276**(26): 24194-24202.
- Sriram, K. and J. P. O'Callaghan (2007). "Divergent roles for tumor necrosis factor-alpha in the brain." J Neuroimmune Pharmacol **2**(2): 140-153.
- Steven W. Levison, J. d. V., and James E. Goldman (2005). "Astrocyte development." Developmental Neurobiology, 4th ed.
- Suzuki, Y., S. Ishiguro, *et al.* (1993). "Identification and immunohistochemistry of retinol dehydrogenase from bovine retinal pigment epithelium." Biochim Biophys Acta **1163**(2): 201-208.
- Taimi, M., C. Helvig, *et al.* (2004). "A novel human cytochrome P450, CYP26C1, involved in metabolism of 9-cis and all-trans isomers of retinoic acid." J Biol Chem **279**(1): 77-85.
- Takahashi, J., T. D. Palmer, *et al.* (1999). "Retinoic acid and neurotrophins collaborate to regulate neurogenesis in adult-derived neural stem cell cultures." J Neurobiol **38**(1): 65-81.
- Tan, N. S., L. Michalik, *et al.* (2004). "Peroxisome proliferator-activated receptor-beta as a target for wound healing drugs." Expert Opin Ther Targets **8**(1): 39-48.
- Thatcher, J. E. and N. Isoherranen (2009). "The role of CYP26 enzymes in retinoic acid clearance." Expert Opin Drug Metab Toxicol **5**(8): 875-886.
- Theodosiou, M., V. Laudet, *et al.* (2010). "From carrot to clinic: an overview of the retinoic acid signaling pathway." Cell Mol Life Sci **67**(9): 1423-1445.
- Thompson Haskell, G., T. M. Maynard, *et al.* (2002). "Retinoic acid signaling at sites of plasticity in the mature central nervous system." J Comp Neurol **452**(3): 228-241.
- Toresson, H., A. Mata de Urquiza, *et al.* (1999). "Retinoids are produced by glia in the lateral ganglionic eminence and regulate striatal neuron differentiation." Development **126**(6): 1317-1326.
- Unsicker, K., H. Reichert-Preibsch, *et al.* (1987). "Astroglial and fibroblast growth factors have neurotrophic functions for cultured peripheral and central nervous system neurons." Proc Natl Acad Sci U S A **84**(15): 5459-5463.

- Vaccarino, F. M., D. M. Fagel, *et al.* (2007). "Astroglial cells in development, regeneration, and repair." Neuroscientist **13**(2): 173-185.
- Wagner, E., T. Luo, *et al.* (2002). "Retinoic acid synthesis in the postnatal mouse brain marks distinct developmental stages and functional systems." Cereb Cortex **12**(12): 1244-1253.
- Wajant, H., K. Pfizenmaier, *et al.* (2003). "Tumor necrosis factor signaling." Cell Death Differ **10**(1): 45-65.
- Wald, G. (1968). "The molecular basis of visual excitation." Nature **219**(5156): 800-807.
- Wang, X., P. Penzes, *et al.* (1996). "Cloning of a cDNA encoding an aldehyde dehydrogenase and its expression in Escherichia coli. Recognition of retinal as substrate." J Biol Chem **271**(27): 16288-16293.
- Wei, L. N. (2003). "Retinoid receptors and their coregulators." Annu Rev Pharmacol Toxicol **43**: 47-72.
- Werner, E. A. and H. F. Deluca (2002). "Retinoic acid is detected at relatively high levels in the CNS of adult rats." Am J Physiol Endocrinol Metab **282**(3): E672-678.
- White, J. A., Y. D. Guo, *et al.* (1996). "Identification of the retinoic acid-inducible all-trans-retinoic acid 4-hydroxylase." J Biol Chem **271**(47): 29922-29927.
- White, J. A., H. Ramshaw, *et al.* (2000). "Identification of the human cytochrome P450, P450RAI-2, which is predominantly expressed in the adult cerebellum and is responsible for all-trans-retinoic acid metabolism." Proc Natl Acad Sci U S A **97**(12): 6403-6408.
- Wolbach, S. B. and P. R. Howe (1925). "Tissue Changes Following Deprivation of Fat-Soluble a Vitamin." J Exp Med **42**(6): 753-777.
- Wu, B. X., Y. Chen, *et al.* (2002). "Cloning and characterization of a novel all-trans retinol short-chain dehydrogenase/reductase from the RPE." Invest Ophthalmol Vis Sci **43**(11): 3365-3372.
- Wu, B. X., G. Moiseyev, *et al.* (2004). "Identification of RDH10, an All-trans Retinol Dehydrogenase, in Retinal Muller Cells." Invest Ophthalmol Vis Sci **45**(11): 3857-3862.
- Wuarin, L., N. Sidell, *et al.* (1990). "Retinoids increase perinatal spinal cord neuronal survival and astroglial differentiation." Int J Dev Neurosci **8**(3): 317-326.
- Wyss, R. and F. Bucheli (1997). "Determination of endogenous levels of 13-cis-retinoic acid (isotretinoin), all-trans-retinoic acid (tretinoin) and their 4-oxo metabolites in human and animal plasma by high-performance liquid chromatography with automated column switching and ultraviolet detection." J Chromatogr B Biomed Sci Appl **700**(1-2): 31-47.
- Yamauchi, K. and J. Nakajima (2002). "Effect of coenzymes and thyroid hormones on the dual activities of Xenopus cytosolic thyroid-hormone-binding protein (xCTBP) with aldehyde dehydrogenase activity." Eur J Biochem **269**(8): 2257-2264.
- Yamauchi, K., J. Nakajima, *et al.* (1999). "Xenopus cytosolic thyroid hormone-binding protein (xCTBP) is aldehyde dehydrogenase catalyzing the formation of retinoic

- acid." J Biol Chem **274**(13): 8460-8469.
- Yang, Y., W. Ge, *et al.* (2003). "Contribution of astrocytes to hippocampal long-term potentiation through release of D-serine." Proc Natl Acad Sci U S A **100**(25): 15194-15199.
- Yashiro, K., X. Zhao, *et al.* (2004). "Regulation of retinoic acid distribution is required for proximodistal patterning and outgrowth of the developing mouse limb." Dev Cell **6**(3): 411-422.
- Yost, R. W., E. H. Harrison, *et al.* (1988). "Esterification by rat liver microsomes of retinol bound to cellular retinol-binding protein." J Biol Chem **263**(35): 18693-18701.
- Zetterstrom, R. H., E. Lindqvist, *et al.* (1999). "Role of retinoids in the CNS: differential expression of retinoid binding proteins and receptors and evidence for presence of retinoic acid." Eur J Neurosci **11**(2): 407-416.
- Zetterstrom, R. H., A. Simon, *et al.* (1994). "Localization of cellular retinoid-binding proteins suggests specific roles for retinoids in the adult central nervous system." Neuroscience **62**(3): 899-918.
- Zhang, M., W. Chen, *et al.* (2001). "Molecular characterization of a mouse short chain dehydrogenase/reductase active with all-trans-retinol in intact cells, mRDH1." J Biol Chem **276**(47): 44083-44090.
- Zhang, M., P. Hu, *et al.* (2007). "Altered vitamin A homeostasis and increased size and adiposity in the rdh1-null mouse." FASEB J **21**(11): 2886-2896.
- Zhao, D., P. McCaffery, *et al.* (1996). "Molecular identification of a major retinoic-acid-synthesizing enzyme, a retinaldehyde-specific dehydrogenase." Eur J Biochem **240**(1): 15-22.
- Ziouzenkova, O., G. Orasanu, *et al.* (2007). "Retinaldehyde represses adipogenesis and diet-induced obesity." Nat Med **13**(6): 695-702.

Chapter II

Multiple Retinol and Retinal Dehydrogenases Catalyze

All-trans-Retinoic Acid Biosynthesis in Astrocytes

Introduction

Vitamin A (retinol) metabolism produces the autacoid *all-trans*-retinoic acid (atRA), which regulates multiple processes required for vertebrate reproduction, embryonic development, immunity, growth and systems homeostasis (Maden, 2007; Moro *et al.*, 2008; Napoli, 1999; Penniston and Tanumihardjo, 2006; Ross and Zolfaghari, 2004). atRA regulates proliferation, differentiation and apoptosis of many cell types, including epithelial, preadipocytes, and neuronal stem cells (Cowherd *et al.*, 1999; Klann and Marchok, 1982; Wuarin *et al.*, 1990). Molecular, cellular, and behavioral studies confirm that central nervous system development and function rely on atRA (Cocco *et al.*, 2002; Etchamendy *et al.*, 2003; Misner *et al.*, 2001). atRA functions in the nervous system *via* the nuclear hormone receptors RAR to regulate both transcription and translation (Chen *et al.*, 2008; Mark *et al.*, 2009; Rochette-Egly and Germain, 2009). For example, disrupting atRA signaling by knocking out RAR β severely compromises performance in the Morris water maze test, commonly used to evaluate hippocampus-dependent spatial learning in rodents (Chiang *et al.*, 1998). Impairing atRA signaling impairs long-lasting, activity-dependent changes in synaptic efficacy, including long-term potentiation and long-term depression, viewed as potential cellular learning mechanisms (Shapiro and Eichenbaum, 1999). atRA enhances hippocampus neuron function by stimulating dendritic growth (Chen and Napoli, 2008). atRA also induces neurogenesis of adult neural stem cells in culture and *in vivo*, and neuronal differentiation of embryonic carcinoma cells (Akita *et al.*, 2002; Jacobs *et al.*, 2006; Jones-Villeneuve *et al.*, 1983; Takahashi *et al.*, 1999).

A complex metabolic pathway, consisting of multiple steps and enzymes, controls atRA homeostasis (Napoli, 2010). Depending on cell needs, *all-trans*-retinol undergoes storage as RE, catalyzed primarily by lecithin: retinol acyltransferase (Lrat) (Ong *et al.*, 1988; Yost *et al.*, 1988). Alternatively, dehydrogenation into *all-trans*-retinal, catalyzed by retinol dehydrogenases (Rdh) that belong to the short-chain dehydrogenase/reductase gene family, initiates atRA biosynthesis (Chai *et al.*, 1995; Chetyrkin *et al.*, 2001; Jurukovski *et al.*, 1999; Wu *et al.*, 2002; Zhang *et al.*, 2001). Dehydrogenation of *all-trans*-retinal, catalyzed by retinal dehydrogenases (Raldh), which belong to the Aldh gene family, produces atRA (Grun *et al.*, 2000; Posch *et al.*, 1992; Wang *et al.*, 1996; Zhao *et al.*, 1996). Catabolism by members of the Cyp gene family balances biosynthesis of atRA (Thatcher and Isoherranen, 2009). These steps function collectively to establish

the presence and amount of atRA at specific loci. The two dehydrogenation reactions, and the catabolic reaction are each catalyzed by multiple isozymes. Rdh physiologically active in generating atRA include Rdh2 (rRdh2, mRdh1), Rdh10, and Dhhs9. Knockout or inadequate expression of each Rdh produces a phenotype associated with impaired retinoid function, including enhanced adiposity (mRdh1), defects in head and body development (Rdh10), or enhanced tumorigenesis (Dhhs9) (Jette *et al.*, 2004; Siegenthaler *et al.*, 2009; Zhang *et al.*, 2007). The three Raldh also have been associated with generating atRA physiologically, through modifying adiposity (Raldh1) or supporting embryonic development (Raldh2 and 3) (Dupe *et al.*, 2003; Halilagic *et al.*, 2007; Niederreither *et al.*, 1999; Ziouzenkova *et al.*, 2007).

Astrocytes, the predominant glia cell type in the hippocampus, provide structural, metabolic and functional support to neurons by secreting factors that induce neurogenesis and formation of synaptic networks (Markiewicz and Lukomska, 2006). atRA has been identified as one of the astrocyte-derived factors that instruct neural stem cell differentiation (Kornyei *et al.*, 2007; Nakayama *et al.*, 2003; Song *et al.*, 2002; Wuarin *et al.*, 1990). Consistent with supplying atRA to neurons, evidence has been generated indicating that astrocytes biosynthesize atRA, including astrocytes from the rat spinal cord, glial cells in the lateral ganglion eminence, Müller cells, and various brain areas (Edwards *et al.*, 1992; Kornyei *et al.*, 2007; McCaffery *et al.*, 2004; Toresson *et al.*, 1999; Wuarin *et al.*, 1990). Expression of Raldh1 and Raldh2 has been detected in glial cells and astrocytes (Asson-Batres and Smith, 2006; McCaffery *et al.*, 2004). Expression of Raldh also has been detected in human neural cells and rat superior cervical ganglion neurons; the later also express Rdh. These data suggest that neurons also biosynthesize atRA (Chandrasekaran *et al.*, 2000; Connor and Sidell, 1997). Other research indicates that the cortex meninges and hypothalamus tanycytes secrete atRA to diffuse throughout nearby brain regions (Shearer *et al.*, 2010; Siegenthaler *et al.*, 2009). Given the complexity of the brain, the specialized functions of brain regions, and the complexity of retinoid homeostasis, conceivably atRA generation may occur in a region-specific manner.

The goals of this research were to determine sources of atRA in the hippocampus, the nature of the Rdh and Raldh that contribute to atRA generation, and to provide insight into pathway regulation. We identify astrocytes as the primary, if not sole source, of hippocampus atRA, and show that Rdh2, Dhhs9, Rdh10 and all three Raldh likely contribute to astrocyte atRA biosynthesis. atRA autoregulates its homeostasis in astrocytes by redirecting retinol metabolism to RE biosynthesis and inducing its catabolism. We also report a novel cross talk between Dhhs9 and Raldh1, indicating a new mechanism of regulating atRA production. These data reveal a complex path with multiple functional isoenzymes for atRA biosynthesis in the hippocampus.

Materials and Methods

Cell culture—Primary cultures of astrocytes were prepared as described with some modifications (Yang *et al.*, 2003). Briefly, hippocampi without meninges from 2-day-old Sprague-Dawley rats were dissected and resuspended in 2 ml Hanks' Balanced Salt Solution containing 10 mM HEPES, pH 8.0 and 1,000 units/ml Penicillin-Streptomycin. Hippocampi were dissociated by 0.05% trypsin and trituration through a series of Pasteur pipettes with gradually reduced diameters. Astrocytes were plated in DMEM supplemented with 10% FBS in 175 cm² tissue culture flasks, and incubated in a 37°C incubator with 5% CO₂. The medium was changed after 24 hr. Astrocytes formed a confluent layer 14 days after plating. Flasks were sealed and shaken at 300 rpm for 6 hr to separate oligodendrocytes and microglia cells. Astrocytes were trypsinized and re-cultured in 6-well plates or 22 x 22 mm glass coverslips at a cell density of 2x10⁵ cells/well after an additional two weeks in culture.

To prepare the pure neuronal cultures, as described (Chen and Napoli, 2008), hippocampal neurons were dissected and dissociated from E18 Sprague-Dawley rat hippocampus in the same way as astrocytes, and plated in Neurobasal medium containing 2% B27 supplement (Invitrogen) on plates or glass cover slips pre-coated with poly-D-Lysine(Sigma) at a cell density of 2x10⁵ cells/well in 24-well plates or 1x10⁶ cells/well in 6-well plates. Half the medium was replenished each week. After 1 week culture, 2 μM Ara-C (Sigma) was added to prevent glia proliferation. Co-cultured neurons were prepared in the same manner as pure neuronal cultures except that neurons were plated on a layer of 1 month primary astrocytes rather than directly on poly-D-lysine-coated plates and no Ara C was added.

Purities of cultured astrocytes and neurons were confirmed by immunostaining with the astrocyte marker glial fibrillary acidic protein (GFAP) or neuron dendrite marker microtubule-associated protein 2 (MAP2).

Retinoid metabolism assays—Primary cultured astrocytes, neurons or co-cultured astrocytes and neurons were incubated with the indicated concentrations of substrates all dissolved in DMSO for the indicated times in DMEM containing 3% FBS. The medium was aspirated and saved, cells were lysed on the plate with reporter lysis buffer (Promega). atRA in the media and lysed cells, and RE and retinol in lysed cells were extracted as described (Kane *et al.*, 2008, 2010). atRA was quantified by LC/MS/MS with atmospheric pressure chemical ionization. Retinol and RE were quantified by HPLC (Kane *et al.*, 2005). Retinoids were handled under yellow lights using only glass/stainless steel containers, pipettes, and syringes. Background was assessed from control incubations without cells. Results are averages of triplicates ± S.D, normalized to pmol/million cells/4 hr or to control groups and shown as fold change.

RNA interference—The small interfering RNAs set (siRNA) of Raldh1 (#L-093355), Raldh2 (#L-095884), Raldh3 (#L-098021), Rdh10 (#L-098615), Rdh2 (#L-095187), Dhhrs9 (#L-091764), Cyp26B1 (#L-093105) and non-targeting control siRNA

(#D-001810) were obtained from ON-TARGET *plus* SMART pool of Dharmacon Research (Lafayette, CO). Each siRNA set was designed to target four different regions of the specific gene. Astrocytes were plated in 6-well plates at a density of 2×10^5 /well 1 day before the transfection and transfected with 100 nM siRNA using DharmaFECT siRNA transfection reagent I (Dharmacon) following manufacturer's protocol. 48 to 96 hr post-transfection, the level of target gene knockdown was assayed by RT-PCR or western blot. atRA was assayed 96 hr post-transfection.

RNA extraction, RT-PCR and quantitative RT-PCR analysis—Total RNA was extracted from astrocytes with Trizol reagent (Invitrogen) following the manufacturer's protocol. RNA concentration was determined by absorption at 260 nm; the ratio 260/280 nm was > 1.9 . Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was done using oligo (dT)_{12–18} as primer and superscript II reverse transcriptase (Invitrogen) in a 20- μ L reaction mixture. The cDNA was diluted and amplified with Taq polymerase at following conditions: initial denaturing step at 93 °C for 3 min followed by 30-35 cycles of denaturation (93 °C, 30 s), annealing (60 °C, 30 s), and extension (72 °C, 30 s), then further extension at 72 °C for 10 min. Exon-specific primers were (forward and reverse, respectively):

Raldh1, 5'-GAAGGGGACAAGGCAGATG-3', 5'-CCACACACCCCAATAGGTTC-3';
Raldh2, 5'-TTCTTCATTGAGCCTACCGTGTTC-3', 5'-TCTCTCCCATTTCAGACATCTTG-3';
Raldh3, 5'-CGATAAGCCCGATGTGGAC-3', 5'-CTGTGGATGATAGGAGATGTC-3';
Rdh10, 5'-ACCTGTGATGTGGGGAAGAG-3', 5'-CAAGGTAAGGGCAAACC AAA-3';
Rdh2, 5'-GGACCAGACCAGCTCAGAAG-3', 5'-CAGGCTTTGGAGAAGTC CAG-3';
Dhrs9, 5'-ATGCTGCTTTGGGTGTTGGCCCTC-3', 5'-TCACACAGCTTGGG GATTGGCCAG-3';
Cyp26A1, 5'-TTCTGCAGATGAAGCGCAGG-3', 5'-TTTCGCTGC TTGTGCGAGGA-3';
Cyp26B1, 5'-CAGCTAGTGAGCACGGAGTG-3', 5'-CGGCAGA GAGAAGACATTCTC-3';
Lrat, 5'-AGGAGGCACAGGGAAGAAA-3', 5'-CACAAGCA GAACGGGATG-3';
 β -actin, 5'-GGCATCCTGACCCTGAAGTAC-3', 5'-ACCCTCATAG ATGGGCACAG-3'. PCR products were electrophoresed in 1.5% agarose gels in the presence of ethidium bromide, visualized by ultraviolet fluorescence, and recorded by a digital camera connected to computer (ChemImager 4400, Alpha Innotech). The cDNA of rat β -actin was adopted as an intrinsic standard during RT-PCR.

Pre-designed real-time PCR primers for Raldh1 (Rn00755484_m1), Raldsh2 (Rn00588079_m1), Raldh3 (Rn00596232_m1), Rdh2 (Rn01505848_g1), Rdh10 (Rn00710727_m1), Dhrs9 (Rn00590763_m1) were obtained from Applied Biosystem (Foster City, CA). The 20ul reaction mixture included 10ul TaqMan Universal PCR master-mix, 9 μ l diluted cDNA, 1 μ l 20X TaqMan Gene Expression Assay Mix. Real-time PCR was performed in 7500 real-time PCR system (Applied Biosystems). The thermal cycling program consisted of 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. Reactions were quantified by selecting the amplification cycle when the PCR product was first detected (threshold cycle, Ct). Each reaction was performed in triplicate and the average Ct value was used in analysis. To

account for variability in total RNA input, expression was normalized to β -actin and shown as (target gene/ β -actin) $\times 10^5$.

Western-blot analysis—Astrocytes were lysed with reporter lysis buffer following manufacturer's protocol; protein content was normalized using protein assay kits (Bio-Rad Laboratories). Protein was subjected to SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane *via* standard protocol. The odyssey western blotting system (LI-COR bioscience) was used for quantification. The membrane was incubated in odyssey blocking buffer (1:1 dilution in PBS) for 1 hr and then incubated with rabbit anti-Raldh1 antibody (1:200, abcam) overnight at 4 °C. After incubation with IRDye infrared secondary antibodies (1:10,000 for IRDye800 goat anti-rabbit; 1:15,000 for IRDye680 goat anti-mouse), the fluorescent signal was visualized and density was measured by the Odyssey Infrared Imaging System. Results are fold change compared with controls. Values are at least two independent reactions.

Construction of plasmid and transfection—To obtain a mammalian expression vector, the full length open reading frame of rat Dhars9 (NM_130819) was obtained by PCR amplification from a rat brain cDNA library using forward primer 5'-CGCGAATTCGCCACCATGCTGCTTTGGGTGTTGGCC-3', containing an *EcoRI* site, and reverse primer 5'-CCGGGATCCCACAGCTTGGGGATTGGCCAG-3', containing a *BamHI* site. To obtain a FLAG-tagged Dhars9 (Dhars9-FLAG), the gel-purified PCR product was ligated into pFLAG-CMV-5.1 (Sigma) vector, which appends the FLAG tag to the C terminus of the protein. The sequence of the expression vector was confirmed by sequencing. COS cell or primary cultured astrocytes were transfected with Dhars9-FLAG using Lipofectamine 2000 (Invitrogen) for 24 hr. Expression of FLAG-tagged Dhars9 was detected by immunofluorescent staining with anti-FLAG antibody.

Immunofluorescent staining—Primary cultured neurons and astrocytes or transfected COS cells and astrocytes grown on cover slips were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100 for 5 min and blocked with PBS containing 10% goat serum or chicken serum for 1 hr at room temperature. Cells were incubated with mouse anti-MAP2 (1:500, Millipore), mouse anti-GFAP (1:500, Millipore), mouse monoclonal anti-FLAG M2 (1:500, Sigma), rabbit polyclonal anti-Raldh1 (1:200, Abcam), goat polyclonal anti-Raldh2 (1:10, Santa Cruz) overnight at 4 °C, washed three times for 5-min each with PBST (1 x PBS + 0.25% Tween 20) followed by incubation with goat anti-mouse Alexa 555, goat anti-rabbit Alexa 488, donkey anti-mouse Alexa 555 or chicken anti-goat Alexa 488 secondary antibody (Invitrogen) for 1 hr. After three times washing for 5 min each with PBST, coverslips were mounted on slides by Vectashield mounting medium (with DAPI, Vector Laboratories Inc. Burlingame, CA). Images were captured with a LSM 510 Meta UV/Vis confocal microscope.

Statistical analysis—Statistical significance was assessed with two-tailed, unpaired student's *t*-tests. Data are means \pm S.D.

Results

Retinoids in adult rat hippocampus—We have reported concentrations of atRA in adult mouse hippocampus using a sensitive liquid chromatography-tandem mass spectrometry assay (Kane *et al.*, 2005; Kane *et al.*, 2008). Here we used the method to quantify retinoids in adult rat hippocampus. The atRA concentration was $\sim 3 \pm 0.9$ pmol/g tissue in hippocampus of 2 month old male Sprague-Dawley rats. Retinol and RE concentrations were $\sim 56 \pm 7.7$ and $\sim 539 \pm 62$ pmol/g tissue ($n = 8$).

Hippocampus astrocytes biosynthesize atRA—To test directly whether hippocampus astrocytes metabolize retinol, we prepared primary cultures. Astrocyte purity was assessed by assaying for expression of glial fibrillary acidic protein (GFAP), an astrocyte marker (Rodnight *et al.*, 1997). Immunostaining showed >98% of the cultured cells were GFAP positive (Figure II-1A).

Rates of atRA and RE biosynthesis from retinol by primary astrocytes were determined as a function of incubation time. The rates of total atRA produced, measured in medium and cells, increased over 24 hr (Figure II-1B and C). At each time, astrocytes secreted most atRA into the medium, resulting in a linear increase of atRA in the medium, with the proportion remaining in the cells decreasing with incubation time. This linear increase of atRA in the medium indicates a lack of feedback inhibition of synthesis and secretion. RE increases were linear during the first 4 hr of incubation, but continued to increase over 24 hr (Figure II-1D). Four hr incubation was selected as the incubation time in the following experiments to remain in the linear range of rate vs. time.

Rates of astrocytes converting retinol into atRA and RE depended on the retinol concentration (Figure II-2A and B). Once again, astrocytes secreted the majority of the synthesized atRA into their medium, with the proportion reaching $\sim 96\%$ with retinol concentrations >1 μM . The substrate-rate curve of atRA biosynthesis (total atRA in cells plus medium) had sigmoidal kinetics with an apparent $K_{0.5}$ of ~ 2.1 μM and a Hill coefficient of ~ 1.8 . In contrast, RE synthesis was not saturated with retinol concentrations as high as 8 μM , and no RE was detected in the medium. RE biosynthesis was much faster than atRA biosynthesis, consistent with the much higher RE than atRA concentrations in serum and tissues. The rate of retinal dehydrogenation into atRA was not saturated at substrate concentrations up to 2 μM (Figure II-2C). In contrast, reduction of retinal into retinol had an apparent $K_{0.5}$ value of 0.3 μM (Figure II-2D). The rate of retinal reduction into retinol exceeded the rate of dehydrogenation into atRA, at the lower concentrations of retinal normally found *in vivo* (≤ 100 nM). This rate of reduction may contribute to the sigmoidal kinetics observed during the production of atRA from retinol, rather than reflecting a cooperative process. In other words, at the lower concentrations of retinol the reduction of newly formed retinal would compete with the dehydrogenation of retinal into atRA, reducing the overall rate of atRA biosynthesis. As the retinal concentrations increases, however, the rate of dehydrogenation would exceed the rate of reduction. Based on these data, we selected 2 μM retinol, 1 μM retinal, and 4 hr

incubation to monitor atRA biosynthesis in astrocytes, as reflecting the forward reactions for both steps.

Retinoid metabolism by hippocampus neurons—Next, we tested capacity of neurons to metabolize retinoids. About 95% of the cells in primary cultures of DIV14 neurons expressed the dendritic marker MAP2 (Figure II-3A). Although atRA was detected in a retinol concentration-dependent manner with the LC/MS assay, the amounts were linear with substrate concentration, and the values were close to the limit of quantification of the assay (Figure II-3B). Therefore, they are not as reliable as the data with astrocytes. Neurons produced even less atRA from retinal, and atRA was at the limit of detection (Figure II-3C). These data do not support the conclusion that neurons generate atRA. In contrast, neurons showed a relatively robust rate of retinal reduction into retinol (Figure II-3D). Retinal reduction by neurons was sigmoidal with an apparent $K_{0.5}$ of 0.23 μM and a Hill coefficient of 1.9. This likely reflects true reductase activity in neurons, because the values are well within the capability of the HPLC assay used to quantify retinal, and are too high relative to astrocytes to result solely from astrocyte contamination. Neurons generated RE from retinol in a sigmoidal relationship with an apparent $K_{0.5}$ of 5.9 μM and a Hill coefficient of 1.4 (Figure II-3E). These data indicate that rat hippocampus neurons have fairly robust retinal reductase and retinol acyltransferase activities, but low if any retinol and retinal dehydrogenase activities.

Neurons sequester atRA secreted by astrocytes— Because hippocampus neurons do not seem to generate atRA, or do so at a relatively low rate, secretion of atRA by astrocytes might serve as a source of atRA for neurons. To test this hypothesis, we co-cultured astrocytes and neurons. Neuron precursors were co-cultured on the top of 1 month old astrocytes monolayer at an incremental ratio for 14 days (Figure II-4). If the cultured neurons can uptake RA, there should be less RA accumulated in the co-culture medium and more atRA accumulated in co-culture cells. As expected, when the ratio of neurons increased, the atRA accumulated in the medium is significantly decreased compared with the add up of total atRA accumulated by the same amount of neurons and astrocytes which were cultured separately (Figure II-4A and 4D); in the meantime, a significant increase of atRA accumulation, which was also correlated with the percentage of neurons, was found in co-cultured system compare with the add up of separate atRA accumulation in same amount of astrocytes and neurons(Figure II-4B and 4E). Besides 2 μM retinol we usually used to assay the atRA biosynthesis, we also incubated the cells with a lower concentration of retinol—0.5 μM which is more close to physiological concentration of retinol. There is a similar change of atRA distribution proportion for low concentration of substrate incubation which indicates that atRA transportation between neurons and astrocytes could also happen under physiological condition. Notice that the absolutely increased amount of RA in co-cultured cells (i.e. 0.32 pmol in 1:5 co-culture system with 0.5 μM retinol) is less than the decreased amount of RA in the medium(4.55 pmol in 1:5 co-culture system with 0.5 μM retinol). This might reflect that neurons can not only take up atRA but also utilize and degrade those RA within the incubation time.

Previous experiments show that both neurons and astrocytes have the ability to synthesize RE from retinol and retain RE in the cells. Since there's no RE secretion, we hypothesize that no RE was transported between neurons and astrocytes. In the co-culture system, no matter what's the ratio of neurons, the RE produced in co-cultured cells is equal to the summation of RE synthesized separately by same amount of neurons and astrocytes (Figure II-4C and 4F). This result indicate that the RE formation is independent in neurons and astrocytes and the co-culture doesn't change the individual RE biosynthesis.

However, since the astrocytes were cocultured with neurons for 2 weeks, we can't exclude the possibility that the change of atRA distribution proportion is caused by changing of astrocytes' atRA biosynthesis and secretion ability. We also don't know whether neurons need direct cell-cell contact to sequester atRA secreted by astrocytes. To test those possibilities, primary neurons were cultured on the surfaces of poly-D-lysine coated plates and astrocytes were cultured on glass coverslips (Figure II-5A). A third configuration involved placing the coverslips on the tops of neurons with the astrocytes facing up. The coverslips prevented direct cell-cell contact between neurons and astrocytes, and allowed easy separation of the neurons and astrocytes to quantify atRA and RE after incubation with retinol. The coverslips covered about half the surface area of the neurons, which ensured that at least half of the cultured neurons were exposed directly to the medium.

Individual cultures revealed less atRA in neurons relative to astrocytes, and coculture did not change the amount of atRA in astrocytes, but increased the amount of atRA in neurons ~10-fold, relative to neurons cultured in the absence of astrocytes (Figure II-5B). The amount of atRA in the medium did not change significantly, as expected given the disparity between medium and cell atRA concentrations (Figure II-5C). We also quantified RE and consistent with the intracellular retention of RE, independent culture *vs.* co-culture made no difference in the intracellular concentrations of RE in neurons or astrocytes (Figure II-5D). These results indicate that neurons can sequester atRA secreted by astrocytes without requiring cell-cell contact.

Raldh contribute to atRA production in astrocytes—Raldh catalyze the second dehydrogenation step in the pathway of atRA biosynthesis. Q-PCR revealed that astrocytes express three Raldh (Raldh1, 2 and 3) (Figure II-6A). Expression varied greatly, with ~500-fold higher expression of Raldh1 than Raldh2 or 3. To determine which of these Raldh participate in astrocyte atRA biosynthesis, we used siRNA to sequentially knockdown each mRNA. siRNA transfection reduced Raldh 1 and 3 mRNA ~80%, and Raldh2 mRNA ~60% after 3 days (Figure II-6B) and reduced Raldh1 protein ~90% after 4 days (Figure II-6C). Each Raldh knockdown occurred without affecting β -actin expression (mRNA and protein), verifying the specificities of the siRNAs. Astrocytes transfected with Raldh1 siRNA had a 64% reduction in atRA production from retinol and a 77% reduction from retinal (Figure II-6D and 6E). Raldh2 knockdown also caused significant reduction of atRA production: ~37% from retinol and ~44% reduction from retinal. These results indicate that both Raldh1 and 2 contribute to atRA

biosynthesis in hippocampus astrocytes. Although the knockdown of Raldh3 mRNA was as efficient as Raldh1, it caused only ~24% and ~13% reduction of atRA production from retinol or retinal, respectively. Notably, no complementary up regulation of Raldh2 or 3 mRNA was observed during Raldh1 knock down (Figure II-6F); similar results were observed when Raldh2 or 3 were knocked down (data not shown).

Although Raldh1 and 2 localize to the soluble fraction after differential centrifugation of tissues or after heterologous expression in *E. coli*, each could occur in different intracellular microenvironments (Brodeur *et al.*, 2003; Labrecque *et al.*, 1993; Wang *et al.*, 1996). To test this premise, primary astrocytes were immunostained with Raldh1 or 2 antibodies together with the astrocyte marker GFAP. Both Raldh1 and 2 expressed throughout the cytoplasm (Figure II-7A and 7C). In addition, Raldh1 localized strongly to the nuclei of astrocytes, whereas Raldh2 expressed more strongly in perinuclear areas than throughout the cytoplasm and the nuclei (Figure II-7B and 7D). All GFAP-positive cells expressed Raldh1 and 2, but GFAP-negative cells also expressed both, indicating wider expression in glial cells.

Multiple Rdh contribute to atRA biosynthesis in astrocytes—Primary astrocytes express Rdh10 and Dhhr9 much more robustly than Rdh2 (Figure II-8A). We knocked down each using siRNA to evaluate their relative contributions to atRA production. siRNA transfection eliminated ~50% of Rdh10 and >90% of Rdh2 and Dhhr9 mRNAs without affecting β -actin (Figure II-8B). Knockdown of Rdh10 or Rdh2 caused ~25% and ~20% reduction of atRA production from retinol, respectively (Figure II-8C). To exclude one Rdh complementing for the loss of another by increased expression, we assayed the impact of siRNA knockdown on the non-targeted Rdh by RT-PCR. Knockdown of Rdh10 or Rdh2 did not result in marked up regulation of the non-targeted paralog (Figure II-8D and 8E). Knockdown of Dhhr9, surprisingly, caused ~40% increase of atRA production, indicating its participation in retinoid metabolism in astrocytes—a result seeming inconsistent with its purported function as a retinol dehydrogenase (Figure II-8C).

Dhhr9 functions as a retinol dehydrogenase—To confirm its function, Dhhr9 was expressed in COS cells and over expressed in primary astrocytes. We confirmed expression of FLAG-tagged Dhhr9 by immunostaining with an anti-FLAG antibody, and tested the cells for ability to convert retinol into atRA (Figure II-9A). Expressing Dhhr9 in both COS cells and primary astrocytes increased atRA production from retinol, confirming Dhhr9 function as a dehydrogenase. Next, we tested whether Dhhr9 impacted the rate of atRA catabolism. Knockdown of Dhhr9 in astrocytes did not change the atRA degradation rate (Figure II-9B). Consistent with this result, the mRNA of Cyp26A1 and 26B1, two of the main enzymes that catalyze atRA catabolism, did not decrease with knockdown of Dhhr9 (Figure II-9C). Nor did the mRNA of Rdh10 and Rdh2 increase after Dhhr9 knockdown. These data do not support a decrease in atRA catabolism or an increase in expression of the enzymes in the first dehydrogenation step for the increase in atRA production with knockdown of Dhhr9. To test whether Dhhr9 knockdown enhances dehydrogenation or reduces reduction of retinal, atRA and retinol production from retinal

was measured in astrocytes transfected with Dhhrs9 siRNA. Knockdown of Dhhrs9 did not change the rate of retinal reduction into retinol, confirming that Dhhrs9 does not function as a retinal reductase (Figure II-9D). atRA production from retinal, however, increased with time after knockdown up to ~50% relative to the non-targeting siRNA transfected cells, indicating an increase in retinal dehydrogenase activity (Figure II-9E). RT-PCR revealed no increase of Raldh2 and 3 mRNA after Dhhrs9 knockdown (Figure II-9C), but an increase in Raldh1 mRNA (Figure II-9F, upper panel). Real-time PCR confirmed the increase in Raldh1 mRNA with time after Dhhrs9 knockdown (Figure II-9F, left lower panel). An increase in protein accompanied the increase in Raldh1 mRNA (Figure II-9F, right lower panel). To determine whether changes in retinal or atRA contribute to the changes in Raldh1 expression, astrocytes were treated with either retinal or atRA, and Raldh1 mRNA was measured by RT-PCR (Figure II-9G). Neither retinal nor atRA caused changes in Raldh1 mRNA expression.

atRA autoregulates its homeostasis through Cyp26B1 and Lrat—To test the contributions of redirecting retinol metabolism to RE biosynthesis and increasing catabolism on maintaining atRA homeostasis, astrocytes were incubated with 1 μ M atRA from 3 hr to 3 days. atRA induced Lrat and Cyp26B1 mRNA as early as 6 hr after treatment, but did not affect Cyp26A1 mRNA (Figure II-10A). To test whether increases in RE formation and/or decreases in atRA accompany these gene changes, atRA and RE biosynthesis from retinol were measured in astrocytes exposed to graded doses of atRA for 72 hr (Figure II-10B). As low as 0.1 μ M atRA increased RE formation and decreased atRA accumulation, with 0.3 to 0.5 μ M producing maximum effects.

Because atRA induces Cyp26B1 in astrocytes, we tested whether Cyp26B1 serves as the major catalyst of atRA degradation. Knockdown of Cyp26B1 mRNA by siRNA resulted in a 5-fold increase in atRA recovery after incubating astrocytes with retinol (Figure II-10C and 10D). To confirm this insight, the elimination $t_{1/2}$ of atRA was measured (Figure II-10E). atRA was relatively stable in medium without cells with a $t_{1/2}$ of ~139 hr. Astrocytes transfected with the non-targeting construct catabolized half of the atRA in ~9 hr. Cyp26B1 knockdown increased the $t_{1/2}$ of atRA to ~17 hr, confirming a major contribution of Cyp26B1 to atRA catabolism by astrocytes. These data indicate that atRA autoregulates its homeostasis in astrocytes by re-directing retinol use from atRA to RE formation, and by increasing the rate of atRA catabolism.

Rates of retinol metabolism change with time of astrocytes in primary culture—The preceding work was done with primary astrocytes cultured for 1 month. To determine whether length of time in culture alters ability of astrocytes to metabolize retinol, we compared two additional times in culture to the 1 month cultures—2 weeks and 3 months (Figure II-11A). Rates of atRA biosynthesis from retinol increased 4-fold after 1 month compared to 2 weeks, and 19-fold after 3 months relative to 2 weeks in culture. Rates of atRA biosynthesis from retinal increased 7-fold after 1 month compared with 2 weeks and 11-fold after 3 months relative to 2 weeks in culture. RE production, in contrast, decreased ~15% after 1 month of culture relative to 2 weeks, and ~34% after 3 months of

culture relative to 2 weeks. We used Q-PCR to determine gene expression changes that might underlie the changes in metabolic rates (Figure II-11B). Raldh1 mRNA expression underwent the largest changes with time, with a nearly 30-fold increase from 2 weeks to 1 month in culture. By 3 months, Raldh1 expression increased 47-fold relative to 2 weeks. Increased Raldh1 protein also was observed by western blot (data not shown). Raldh2 mRNA increased 1000-fold from 2 weeks to 1 month, and 2.6-fold from 1 month to 3 months, yet remained far less robustly expressed at all times than Raldh1. Raldh3 did not change markedly with time in culture, and at 1 month was similar in expression to Raldh2, as first observed in Figure II-6A. Rdh2 increased only ~30% from 2 weeks to 1 month, and ~70% from 2 weeks to 3 months. Rdh10 mRNA doubled from 2 weeks to 3 months, but did not change from 2 weeks to 1 month. Dhhrs9 increased 57% from 2 weeks to 1 month and >5-fold from 2 weeks to 3 months. Cyp26A1 mRNA expression did not change (data not shown), but Cyp26B1 mRNA increased with time in culture (Figure II-11C). No change was detected of Lrat mRNA at 3 months.

Rdh1 or CrbpI knock out result in similar compensation of atRA biosynthesis and transcriptional change in mice astrocytes--Rdh1 is the mouse homolog of rat Rdh2 and the knockout mice is physiological normal with no significant defects during the development on any level of vitamin A diet. However, Cyp26A1 has been proved to be down regulated in liver to spare retinoids in Rdh1 null mice. CRBPI is the retinol binding protein that facilitate retinol metabolism though the CRBPI null mice is completely normal. To test the contribution of these two genes on retinoids metabolism in astrocytes, 1 month old primary cultured astrocytes from either Rdh1 null mice or CrbpI null mice were incubated with retinol or retinal for 4hr to measure retinoids production (Figure II-12A-12D). The expression of Rdh1 was barely detected in primary astrocytes by RT-PCR (data now shown), however, Rdh1^{-/-} astrocytes showed ~2 fold increase of atRA production from retinol or retinal compared with astrocytes from wild-type mice. No change of RE production from retinol or retinol reduction from retinal was observed in Rdh1^{-/-} astrocytes. These data indicate that Rdh1 knockout result in some over-complementary effect in cultured astrocytes when treated with high concentration substrates. To find out what genes are changed when Rdh1 is knocked out, we tested expressions of Raldh1-3, Rdh10, Dhhrs9 and Cyp26A1 and B1 by RT-PCR (Figure II-12E). A significant up regulation of Raldh2 and Dhhrs9 in Rdh1^{-/-} astrocytes, as well as a dramatic decrease of Cyp26A1, contributed to the increased ability of Rdh1^{-/-} astrocytes to synthesize and accumulate more atRA from retinol and retinal. Interestingly, an increase expression of Cyp26B1 was detected in Rdh1^{-/-} astrocytes, which is consistent with feedback autoregulation of atRA in astrocytes.

A similar fold increase of atRA production from retinol was also detected in CrbpI^{-/-} astrocytes compared with Rdh1^{-/-} astrocytes; whereas when incubated with retinal, ~4 fold increase of atRA biosynthesis was detected (Figure II-12A and 12B). 50% reduction of RE formation in CrbpI^{-/-} astrocytes is consistent with the previous report that CrbpI facilitate the esterification of retinol by delivering substrate to Lrat (Figure II-12C). Same

as *Rdh1*^{-/-} astrocytes, knockout of *CrbpI* doesn't affect the reduction of retinal, which also indicates that the increase of atRA production from retinal must be due to the complementary up regulation of *Raldhs* (Figure II-12D). Among three *Raldhs*, same as *Rdh1*^{-/-} astrocytes, *Raldh2* is the one that is unregulated in *CrbpI*^{-/-} astrocytes (Figure II-12F). No change of *Rdh10* or *Dhrs9* was observed (data now shown). *CrbpI* expression was confirmed by RT-PCR and no *CrbpI* mRNA was detected in null mice (data not shown). We also measured the expression of *CrbpII* mRNA and a robust up regulation of this retinol binding protein which is usually expressed relatively low in CNS was detected, indicating a complementary up regulation of retinol binding proteins in astrocytes. Consistent with atRA level, a similar down regulation of *Cyp26A1* and up regulation of *Cyp26B1* was also observed in the *CrbpI*^{-/-} mice astrocytes, indicating that *Rdh1*^{-/-} mice and *CrbpI*^{-/-} might share a homologous regulation system to combat with loss of one of the key components in retinoids metabolism. All these data demonstrated that *RDH1* and *CRBPI* participate into the retinoids homeostasis in adult astrocytes.

Discussion

Hippocampus neurons respond to atRA *via* RAR α by quickly increasing dendrite outgrowth through a novel mechanism of regulating translation to increase CamKII kinase and GluR1 (Chen and Napoli, 2008; Chen *et al.*, 2008). The source of atRA in the hippocampus had not been established, whether from neurons, astrocytes or more distance sources (*e.g.* meninges). Here we applied a sensitive LC/MS/MS assay to specifically quantify atRA bio-generation in astrocytes and neurons. Our results do not support major atRA biosynthesis by hippocampus neurons, suggesting a paracrine source. Astrocytes seemed a likely source. Astrocytes are abundant in the adult CNS, accounting for nearly half of the total cell content of brain (Laird *et al.*, 2008). Astrocytes occupy a strategic position, interposed between blood vessels and neurons. Astrocyte structures, known as endfeet, contact blood vessel walls, enabling capture of retinol from circulation. Astrocytes also extend processes that contact synapses, and regulate synaptic efficacy through modulating uptake and release of neurotransmitters and neurotrophic factors. This cytoarchitecture provides metabolic support for neurons by absorbing glucose from circulation and exporting lactate for neuron use (Alliot *et al.*, 1988; Banker, 1980; Unsicker *et al.*, 1987). Astrocytes or astrocyte-conditioned medium can induce neural fate differentiation of ES cells or adult neural stem cells. atRA, and retinol in the presence of astrocytes, induce neuronal differentiation, but retinol alone cannot induce differentiation (Wuarin *et al.*, 1990). Thus, our data considered with these previous reports, support the hypothesis that hippocampus astrocytes absorb retinol from blood vessels and export atRA to neurons to regulate synaptic plasticity. Nevertheless, our data do not exclude the possibility that oligodendrocytes and microglia also synthesize atRA.

Our results show that Rdh2 (mRdh1), Rdh10 and Dhhrs9 are all present and active in hippocampus astrocytes. Rdh10 and Dhhrs9 were expressed more intensely than Rdh2, yet knockdown of Rdh2 decreased atRA biosynthesis, verifying a contribution. The result with Dhhrs9 knockdown also verifies a contribution to retinoid homeostasis, even though its knockdown increased atRA biosynthesis by inducing Raldh1. The knockdowns verify contributions for these Rdh, but do not necessarily reflect relative contribution. Compensatory mechanisms may not be obvious, as illustrated by Dhhrs9, or retinol substrate may redirect to an unaffected Rdh. Moreover, these data do not exclude the possibility that additional Rdh participate in retinol dehydrogenation. As for Raldh, astrocytes expressed Raldh1 far more intensely than Raldh2 and 3, but all three catalyzed atRA production in intact cells. These data show that multiple Rdh and Raldh are expressed in and contribute to atRA biosynthesis in astrocytes.

These data stand in contrast to conclusions about sites of atRA biosynthesis in the CNS, and “the essential enzyme” based on localization of one enzyme in one step, as has been attempted for some Raldh, without verifying catalytic activity by quantifying atRA. Such an approach prompts several concerns. Absence of one Rdh or Raldh does not imply absence of the others. On the other hand, the presence of a single enzyme does not define

a path: the enzyme may not have access to its substrate and/or it may not function catalytically. Acetylcholine esterase, *e.g.* may have non-classical functions in which it does not hydrolyze acetylcholine, but prompts neurite outgrowth and promotes adhesion during synapse formation (Silman and Sussman, 2005). Members of the Aldh gene family, including Raldh1, serve as corneal crystallins, which protect the eye from UV damage *via* both catalytic and non-catalytic mechanisms (Cooper *et al.*, 1993). Thus, it is important to determine loci of multiple enzymes and to verify their function to generate realistic models of atRA homeostasis.

Other than redundancy, other purposes may account for multiple catalytically active Rdh and Raldh in the same cell type. Even though all GFAP positive astrocytes express both Raldh1 and 2, and despite the fact that each isolates with the cytosolic fraction upon differential centrifugation, each isolates to distinct subcellular loci when assayed by immunocytochemistry (30, 31). These loci include, but are not restricted to the cytoplasm. The perinuclear locus of Raldh2 and the nuclear locus of Raldh1 imply generation of distinct atRA pools. Distinct isozymes might also provide opportunity for differential regulation. For example, proinflammatory cytokines induce astrocyte Raldh3, but not Raldh1 or 2 (Wang and Napoli, unpublished data). These insights indicate that the generation of atRA in the astrocyte is far from a simple process, and the complexity involves both steps in generating atRA from retinol.

In contrast to differences in atRA biosynthesis, both neurons and astrocytes can synthesize RE from retinol and reduce retinal into retinol. In astrocytes, conversion of retinol into RE serves both to store retinol for future use and regulate the amount of retinol shunted into atRA biosynthesis, whereas neurons may only sequester retinol as RE to prevent atRA biosynthesis. Further insight into the flux of retinol in both neurons and astrocytes can be deduced by comparing kinetic constants (V_m and $K_{0.5}$ values) to the 60 nM retinol concentration in rat hippocampus (Table II-1). A substrate concentration much lower than the apparent $K_{0.5}$ with a relatively high V_m indicates an enzyme poised to efficiently convert substrate into product and react to small increases in substrate concentration with a disproportionate increase in rate. Such is the case for the retinoid metabolizing enzymes in astrocytes and neurons. In astrocytes, the rate of RE biosynthesis exceeds the overall rate of atRA biosynthesis (Figure II-13). The conversion of retinal into atRA also exceeds the overall rate of retinol conversion into atRA by a large margin, indicating retinal biosynthesis provides the rate limiting step of atRA biosynthesis. The lower rate of retinal conversion into retinol *vs.* retinal conversion into atRA confirms the latter as a fast step dependent on the retinal supply (retinol dehydrogenation) and as a non-rate-limiting step. In the presence of atRA, Lrat induction exacerbates this competition in favor of RE formation, while induction of catabolism further decreases atRA concentrations. These studies support a model of atRA in astrocytes controlling its steady-state concentrations by controlling its rate of catabolism and the retinol concentration *via* Lrat, and neurons directing retinol into RE. This model is consistent with previous conclusions that atRA induces Lrat and Cyp26 in liver (Ross,

2003; Ross and Zolfaghari, 2004).

Because Dhhrs9 had been reported as a retinol dehydrogenase, we were surprised to observe that its knockdown in astrocytes resulted in increased atRA production (Chetyrkin *et al.*, 2001; Rexer and Ong, 2002; Soref *et al.*, 2001). We excluded the most obvious possibility that Dhhrs9 functioned as a reductase, and instead noted that Dhhrs9 knockdown causes an increase in Raldh1 expression. This observation confirms function for both Dhhrs9 and Raldh1 in astrocyte atRA biosynthesis, and provides the first evidence of communication between the two steps. The mechanism remains unresolved. Neither atRA nor retinal changes atRA production by feedback inhibition, seemingly excluding either as interfering directly with Raldh1 action. It is tempting to speculate that Dhhrs9 mRNA may regulate Raldh1 expression. Future research will address this possibility. The observation, nevertheless, provides novel insight into regulation of atRA homeostasis.

Neurons and astrocytes increase proliferation and then differentiation during the first month of postnatal CNS development (Steven W. Levison, 2005; Vaccarino *et al.*, 2007). This coincides with the onset of increases in atRA biosynthetic enzymes in primary astrocytes, likely to supply atRA to support rapid postnatal CNS development. The quantitatively largest increase occurred in Raldh1 and seems to account for most, but not all, of the increase in atRA biosynthesis, indicating a primary function for Raldh1 in the adult nervous system.

The availability of Rdh1 null and CrbpI null mice provided another good model to evaluate the contribution of them on retinoids homeostasis in astrocytes. Knockout of Rdh1 or CrbpI both resulted in a similar change of atRA biosynthesis, which confirmed the participation of Rdh1 and CrbpI in atRA biosynthesis in astrocytes. The increased atRA production from both retinol and retinal elucidated that a complementary up regulation of other retinoids homeostasis genes compensate loss of one of the first step dehydrogenation enzymes or the binding proteins. This was confirmed by the gene express pattern examination. However, this complementary effect should not be considered as over compensation because i) the concentration of the substrate we used was much higher than the physiological concentration. Given Raldh2 is an efficient enzyme to synthesize atRA, the overdose of substrate could result in a robust over-synthesized atRA in our *in vitro* model. ii) The continued increase of Raldhs and Sdrs expression in prolonged cultured astrocytes and low Raldh1 expression in mixed culture neurons and astrocytes (results in Chapter III) indicated that the presence of neurons might feedback regulate atRA homeostasis in astrocytes and inhibit atRA synthesis enzyme expression. In our pure *in vitro* astrocytes culture, lack of neurons inhibition might result in over expression of either Raldh2 or Dhhrs9, the genes have been approved to contribute to atRA synthesis in astrocytes. The decrease of RE production in CrbpI null astrocytes confirmed the function of this retinol binding protein in directing retinol esterification. However, increased availability of retinol in CrbpI^{-/-} astrocytes didn't result in increase of atRA production from retinol compared with Rdh1^{-/-} astrocytes, indicating a tight control of atRA homeostasis at this concentration of

substrate in astrocytes. No change of retinal reduction indicated that Rdh1 doesn't function as a retinal reductase and astrocytes don't up regulate atRA synthesis through increasing the availability of retinal by decreasing retinal reduction.

Rdh1^{-/-} and CrbpI^{-/-} astrocytes shared some similar changes of retinoids metabolism genes, including: i) increasing of Raldh2 and decreasing Cyp26A1, which result in net increase of atRA accumulation; ii) increasing of Cyp26B1, which might prevent any over accumulation of atRA. Besides these similar changes, Rdh1 knockout also specifically increased Dhhrs9 expression and CrbpI knockout specifically resulted in robust increased expression of CrbpII, indicating that CrbpII, which is usually expressed in relatively low level in CNS, function as a backup protein for any loss function of CrbpI. Some of these genes changes are consistent with siRNA knockdown conclusions. For example, Dhhrs9 function as one of the first step dehydrogenation enzyme and Cyp26B1 is the major enzyme feedback regulated by excess atRA. Different from rat astrocytes, we detected a relative high expression of Cyp26A1 in mice astrocytes and it seems like Cyp26A1 does participate in the regulation of atRA homeostasis in mice. This might be due to species specific function of Cyp26 enzymes. Both Rdh1 and CrbpI knockout astrocytes, which means an impairment of the first step dehydrogenation, resulted in the increase of the second step dehydrogenation. This phenomenon is consistent with increase of Raldh1 expression when Dhhrs9 is knocked down in rat astrocytes though a different retinal dehydrogenase, Raldh2, is up regulated in mice astrocytes. We don't know whether this difference is gene specific (Rdh1 vs. Dhhrs9) or species specific (mice vs. rat). However, these data indicate that up regulation of retinal dehydrogenation through increasing Raldhs expression to compensate decrease of retinol dehydrogenation might be a common mechanism that astrocytes utilize to regulate atRA homeostasis.

In summary, our data support multiple Rdh and Raldh constituting a complex metabolic system in astrocytes generating atRA to support neurons. This complexity may reflect redundancy, except overlapping but distinct subcellular expression patterns of Raldh1 and Raldh2 suggests more than redundancy, and perhaps generation of distinct atRA pools. Cross talk between the first step and the second step dehydrogenation provides another manner of regulation of atRA biosynthesis. Age related decreases in relational memory and some neurodegenerative diseases have been related to lower atRA levels in the brain (Etchamendy *et al.*, 2001; Shudo *et al.*, 2009). It is probable that impaired expression or regulation of this synthetic and catabolic machinery contribute to the atRA deficit.

Figures and Legends

Figure II-1

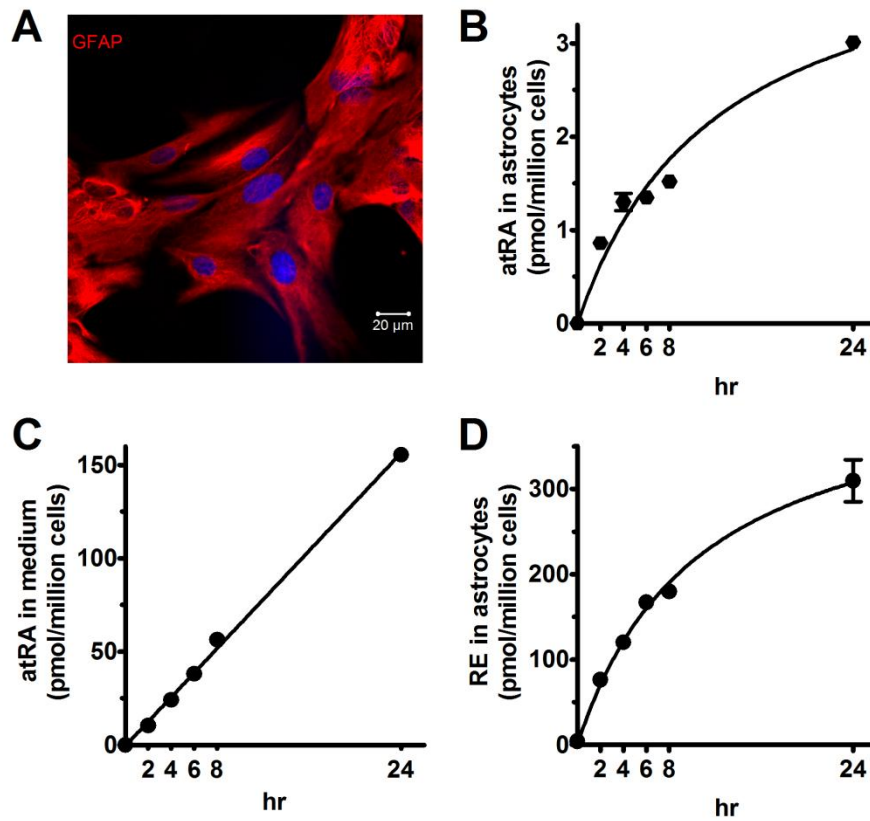


Figure II-1 Time course of atRA and RE biosynthesis from retinol by primary astrocytes. (A) Astrocyte purity was determined by visualization with GFAP. atRA concentrations were quantified in (B) cells and (C) medium, and (D) RE concentrations were quantified in cells after incubation with 2 μM retinol for the times indicated.

Figure II-2

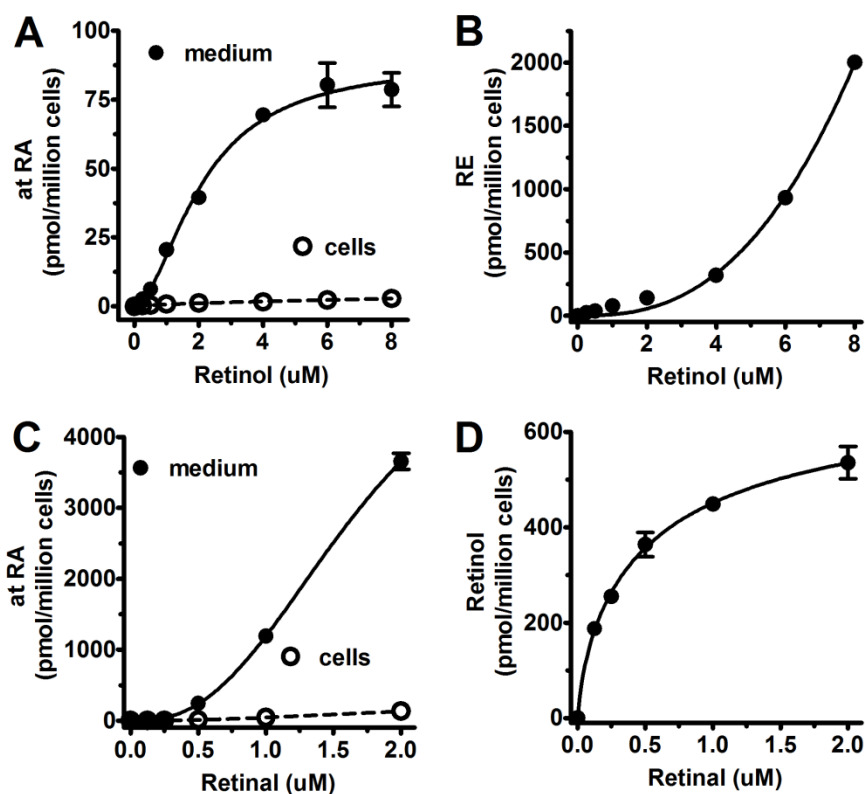


Figure II-2 Effects of substrate concentrations on retinoid metabolism in primary astrocytes. Primary astrocytes were cultured for 1 month and were incubated 4 hr with graded concentrations of retinol or retinal. atRA concentrations in the medium (solid line) and in cells (dashed line) were quantified by LC/MS/MS: (A) retinol; (C) retinal. (B) RE biosynthesis from esterification of retinol and (D) retinol biosynthesis from reduction of retinal in cells.

Figure II-3

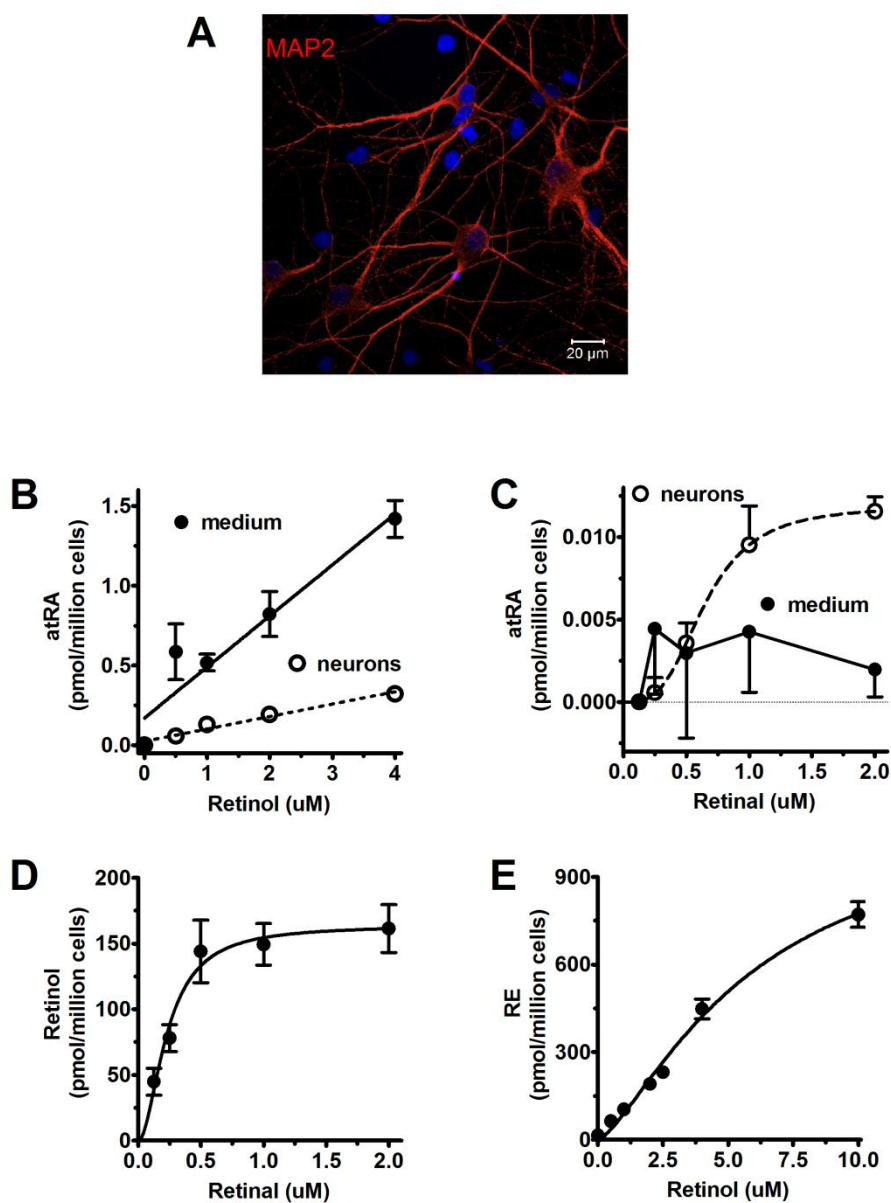


Figure II-3 Retinoid metabolism catalyzed by primary hippocampus neurons. (A) The purity of neurons (DIV14) was determined by MAP2 visualization. Primary cultured neurons were incubated 4 hr with retinol or retinal. atRA accumulation in the medium (solid line) and in cells (dashed line) from (B) retinol or (C) retinal was quantified by LC/MS/MS. (D) Retinol biosynthesis from retinal and (E) RE production from retinol was quantified by HPLC

Figure II-4

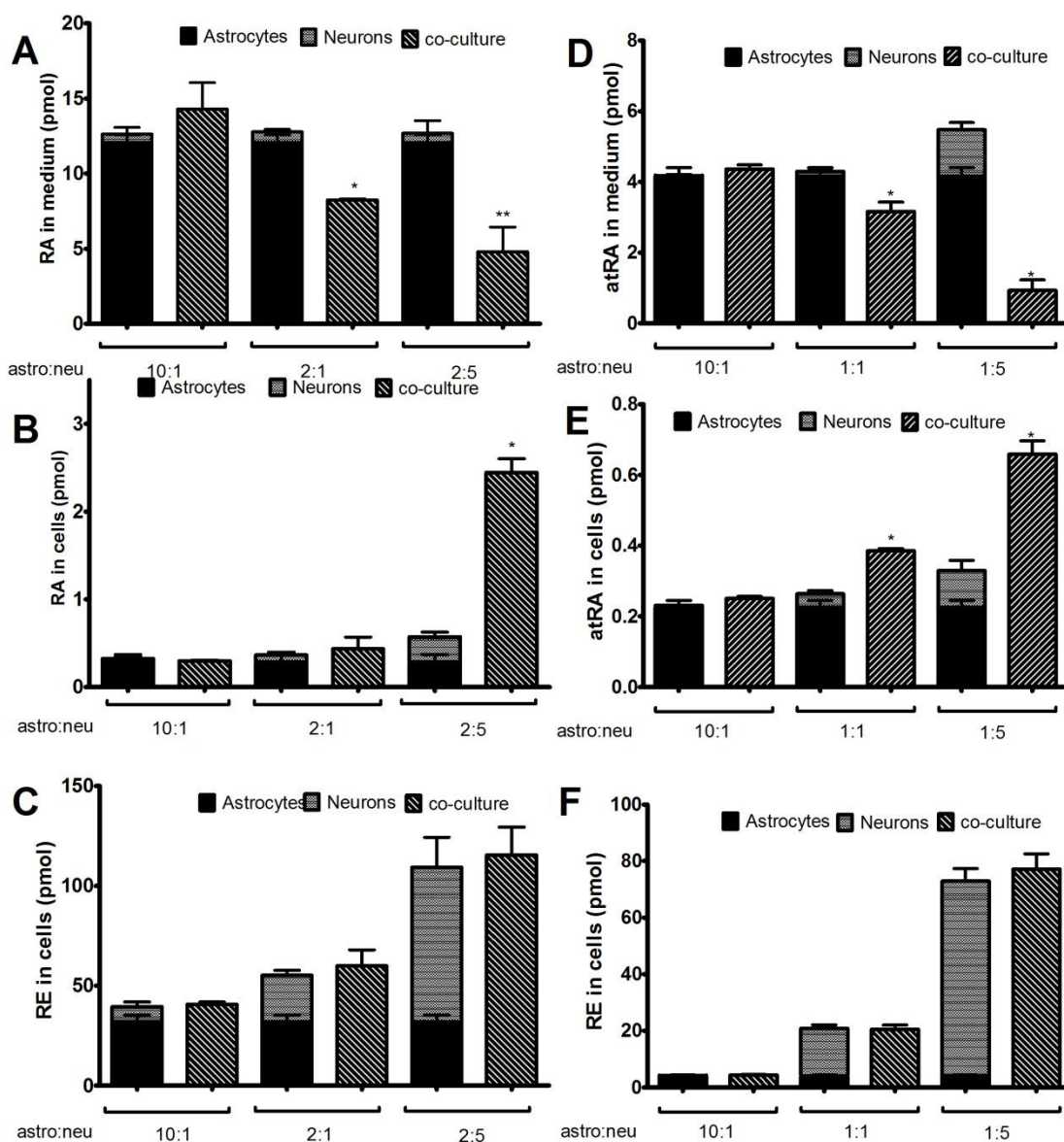


Figure II-4 Co-cultured with neurons changed the atRA distribution proportion in medium and cells. 2×10^5 1 month old primary astrocytes were co-cultured with indicated amount of neurons: the astrocytes:neurons ratio was ranged from 10:1 to 1:5, neuron precursors were counted and placed on the top of confluent monolayer astrocytes. Co-cultured or individually cultured cells were incubated with 2 μM retinol (A-C) or 0.5 μM retinol (D-F) for 4 hours, atRA in medium (A and D) or cells (B and E) were measured by LC/MS/MS, RE (C and F) was measured by HPLC.

Figure II-5

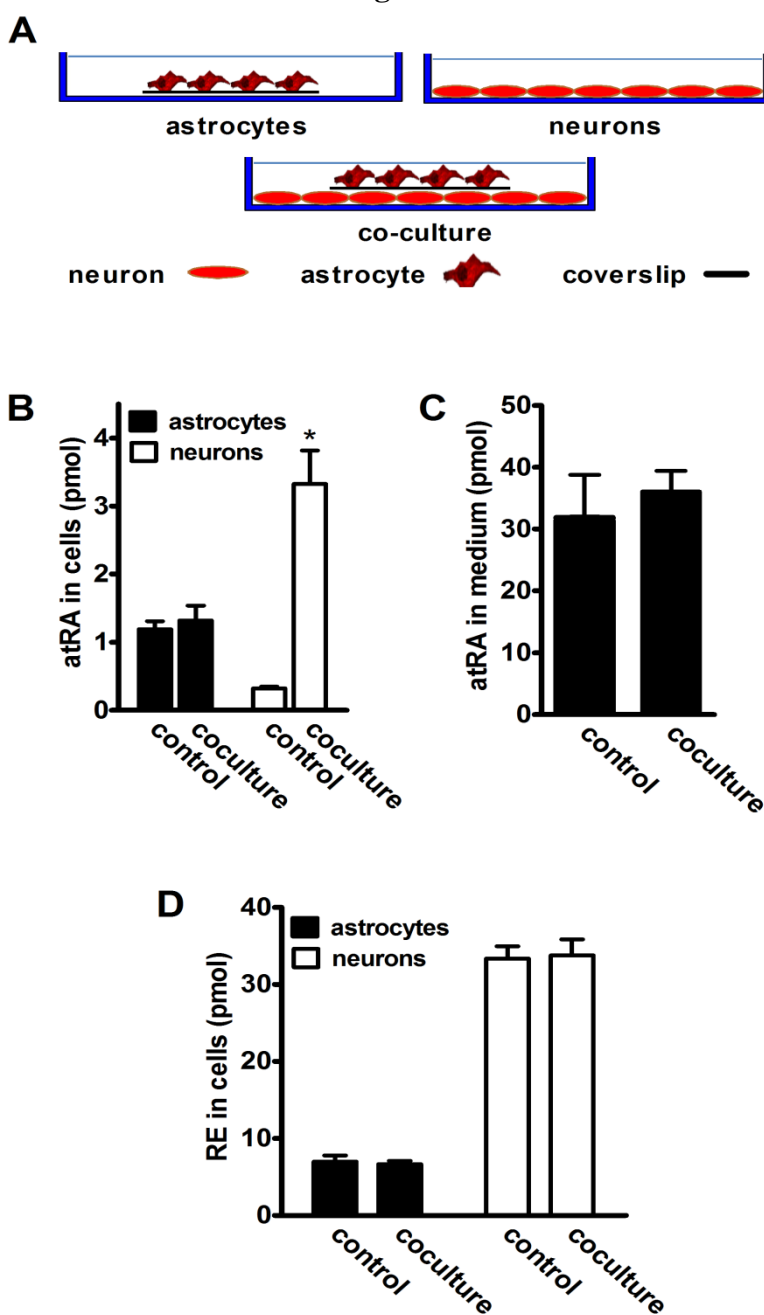


Figure II-5 Neurons sequester atRA secreted by astrocytes. (A) Astrocytes (2×10^5) were cultured on 22 x 22 mm glass coverslips and neurons (6×10^5) were cultured directly in plates. In cultures, coverslips were placed on the top of neurons with astrocytes facing up to avoid direct contact between neurons and astrocytes. Cells were incubated 4 hr with 2 μ M retinol. atRA in the medium was quantified. Neurons and astrocytes in cultures were separated and analyzed individually for atRA and RE in the cells. (B) Intracellular atRA in astrocytes, neurons and cultures of astrocytes and neurons:

* $P=0.0005$, $n = 3$. (C) atRA in the medium of astrocyte cultures or cultures of astrocytes and neurons. (D) Intracellular RE in astrocytes, neurons and cultures of astrocytes and neurons

Figure II-6

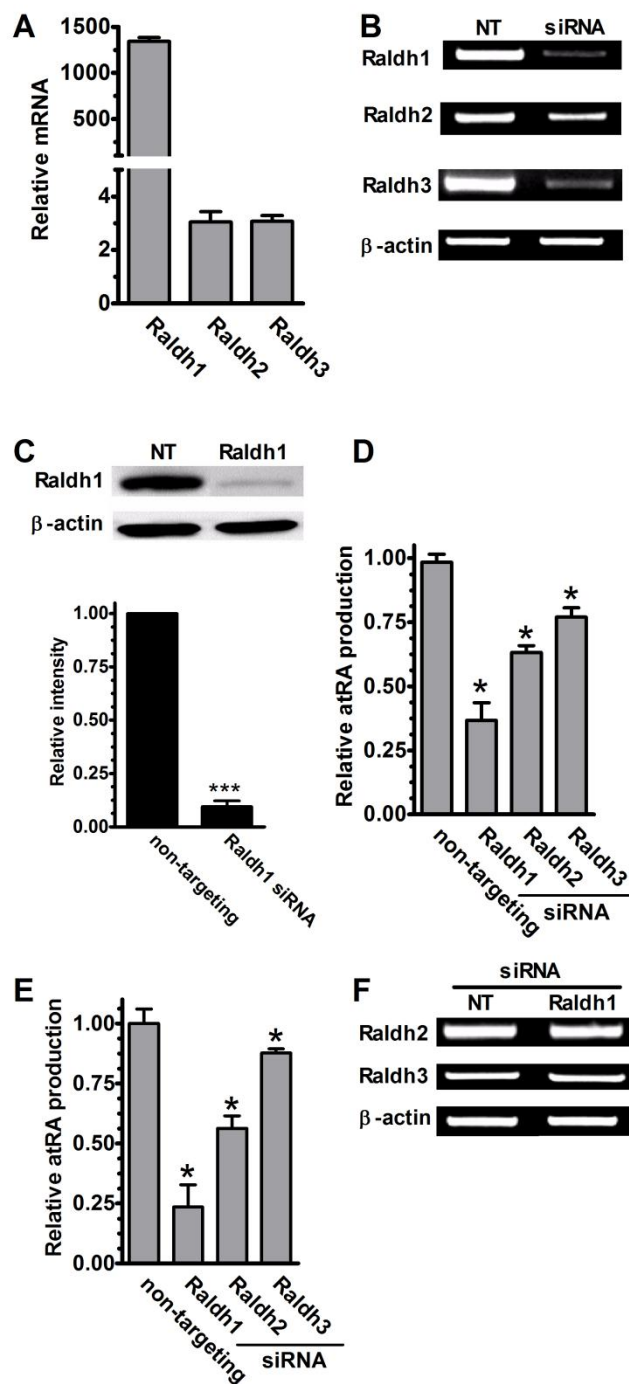


Figure II-6 Knockdown reveals Raldh that contribute to atRA biosynthesis. (A) Q-PCR analysis of Raldh mRNA expression in primary astrocyte cultures normalized to β -actin mRNA. (B) Results of transfecting primary astrocytes with non-targeting siRNA or with siRNA targeted to Raldh1, 2 or 3. mRNA was detected by RT-PCR 72 hr after

transfection. (C) Raldh1 was quantified by western blot 96 hr after transfection: *** $P < 0.001$; (D) atRA biosynthesis from 2 μM retinol after 4 hr incubation. (E) atRA biosynthesis from 1 μM retinal after 4hr incubation. (F) Seventy-two hr after Raldh1 siRNA transfection, Raldh2 and 3 mRNA were measured by RT-PCR. D and E data were normalized to non-targeting siRNA transfected group: * $P < 0.01$; n = 3. Four hr incubations with substrates were done 96 hr after transfections. NT: Non-targeting.

Figure II-7

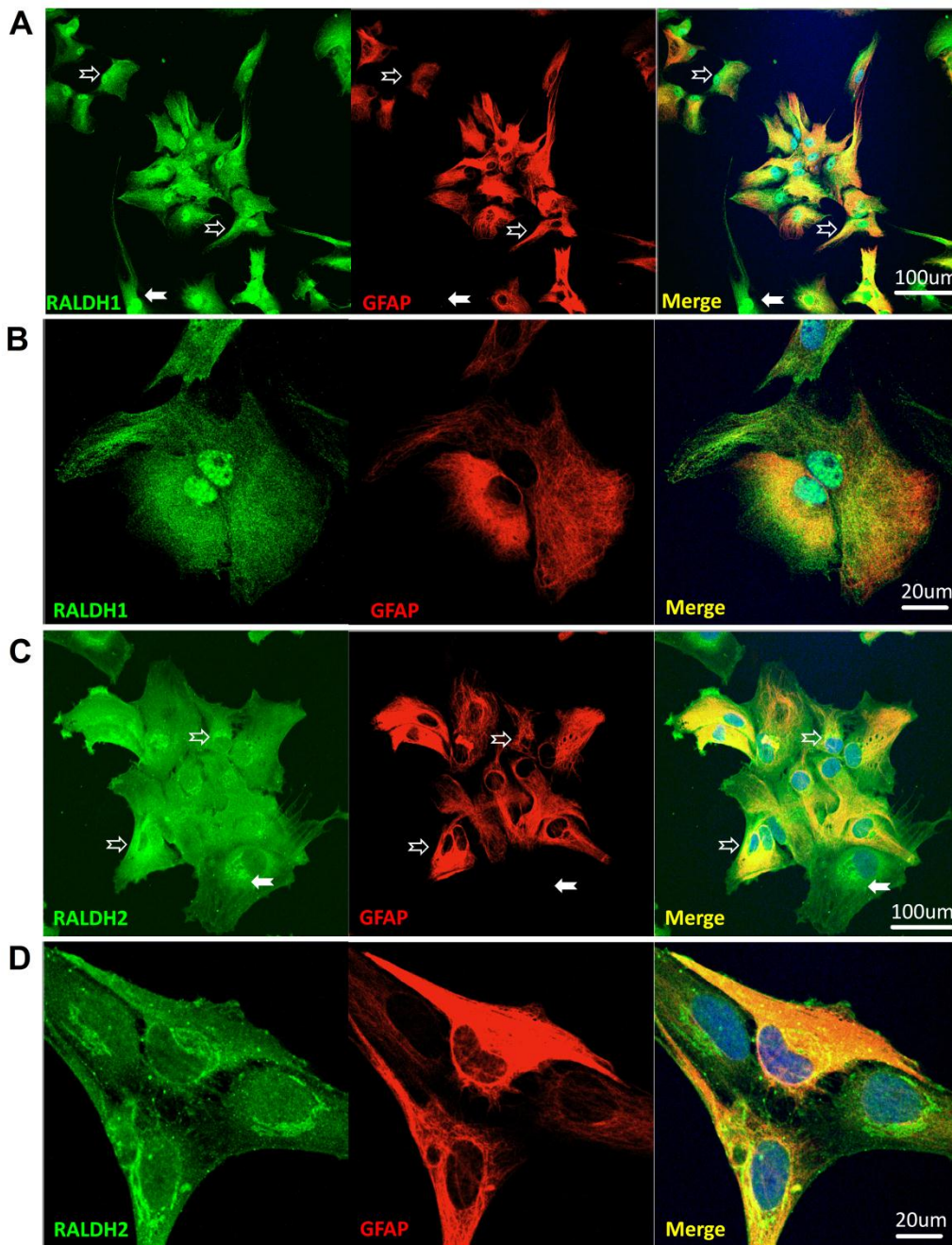


Figure II-7 Confocal images of Raldh1 and 2 expression. (A) Primary cultured astrocytes were fixed and stained for Raldh1 (green), GFAP (red) and DAPI (blue). The three channels of each set were merged (merge). (B) Higher magnification of representative astrocytes with staining as in A. (C) Astrocytes were fixed and stained for Raldh2 (green), GFAP (red) and DAPI (blue) and the three channels were merged (merged). (D) Higher magnification of representative astrocytes with staining as in C. Hollow arrow: astrocytes with either strong or weak GFAP expression. Solid arrow: cells

without GFAP expression which expressed Raldh1 or 2. No signals were detected in negative controls, which consisted of eliminating primary antibodies (data not shown).

Figure II-8

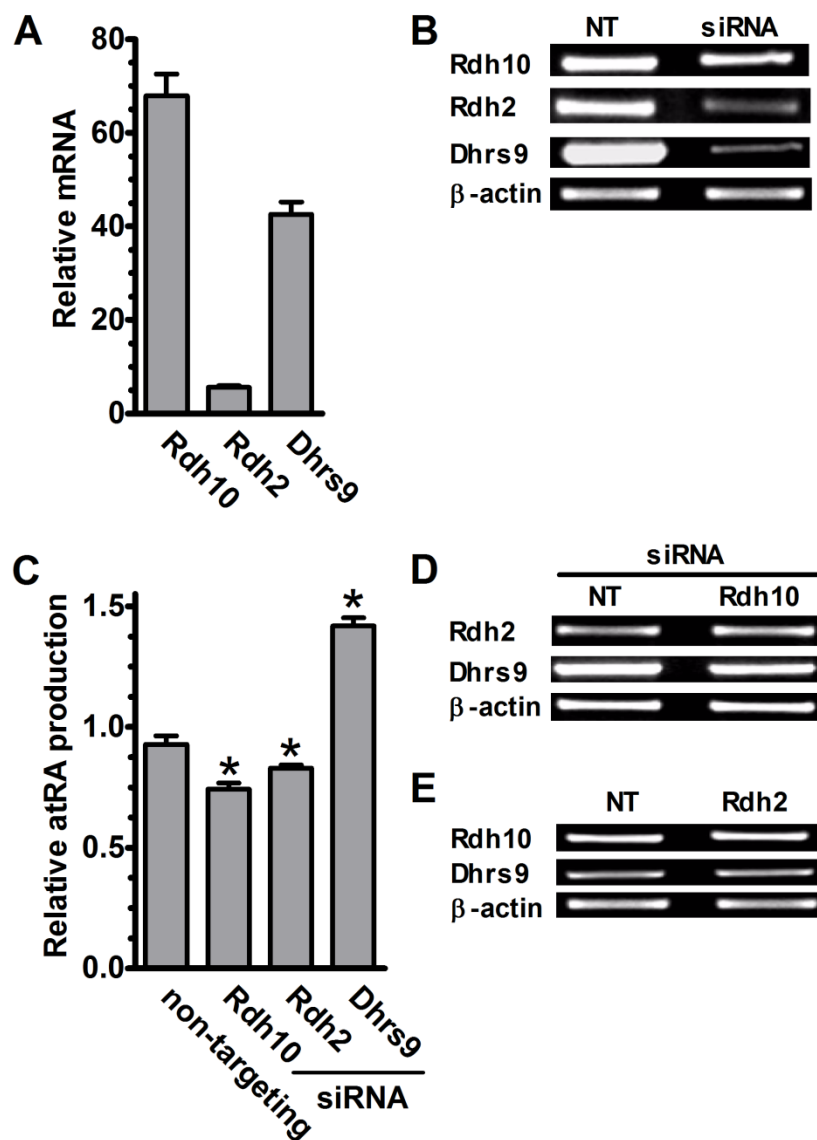


Figure II-8 Multiple Rdhs contribute to atRA biosynthesis in astrocytes. (A) Q-PCR analysis of Rdh10, Rdh2 and Dhhrs9 mRNA expression in astrocytes. (B) Effects of transfecting astrocytes with non-targeting siRNA or siRNA homologous with Rdh10, Rdh2 and Dhhrs9. Rdh mRNA were detected by RT-PCR 72 hr after transfection. (C) Total atRA production from 2 μ M retinol in 4 hr was quantified 96 hr after transfection and was normalized to the non-targeting cultures: * $P < 0.01$, $n = 4$. (D) mRNA expression of Rdh2 and Dhhrs9 was evaluated by RT-PCR 72 hr after Rdh10 siRNA transfection. (E) mRNA expression of Rdh10 and Dhhrs9 was evaluated by RT-PCR 72 hr after Rdh2 siRNA transfection

Figure II-9

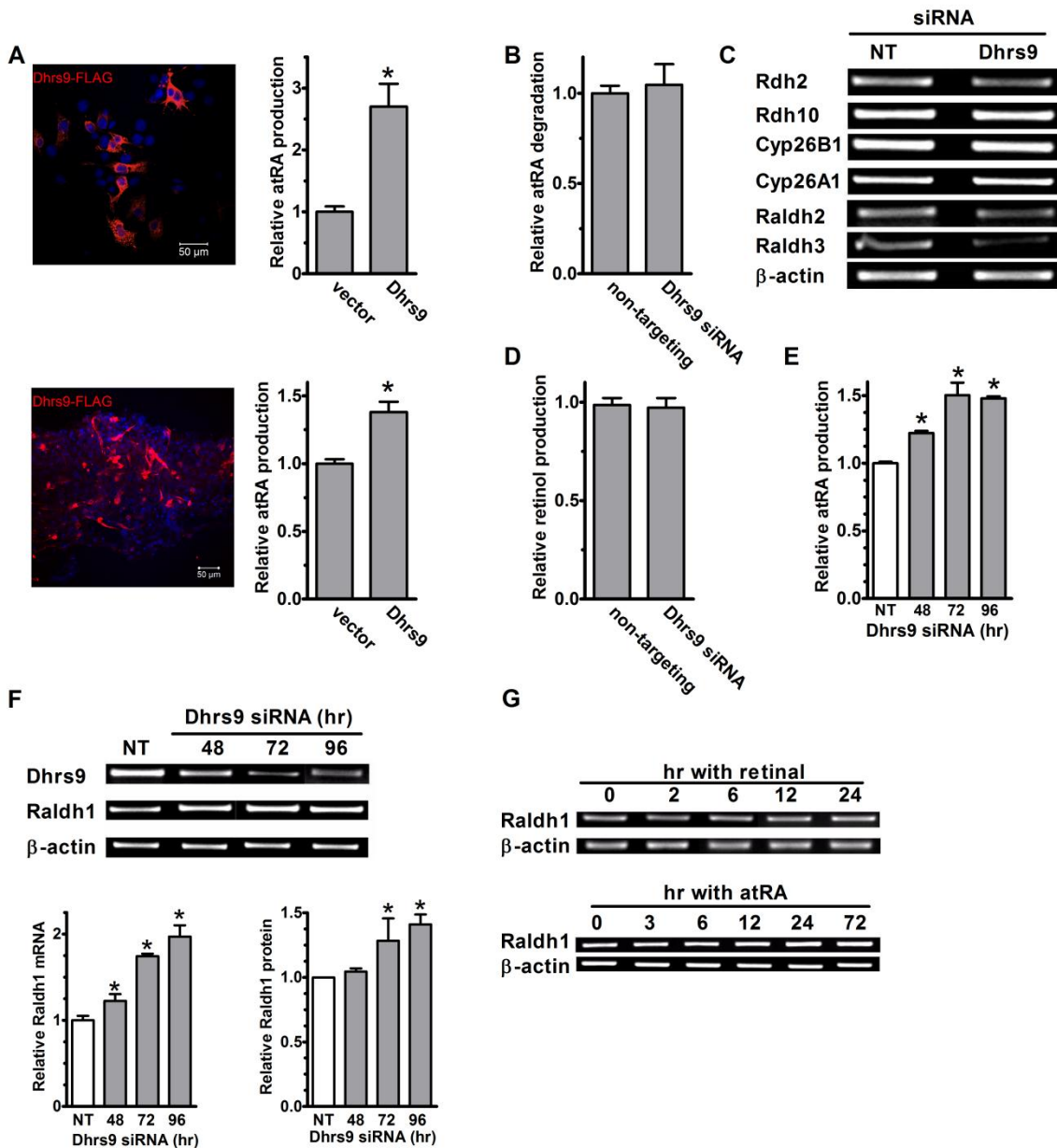


Figure II-9 Dhrs9 functions as a retinol dehydrogenase in astrocytes. (A) COS cells (top) or primary astrocytes (bottom) were transfected with an empty vector or a vector that expressed FLAG-tagged Dhrs9. Anti-FLAG antibody (red) and nuclei (DAPI blue) images in cells transfected with the expression vector. Bar graphs show atRA production from 2 μ M retinol over 4 hr from cells transfected with empty vectors or Dhrs9-expressing vectors: * P <0.005, n = 3. (B) Recovery of atRA was measured in astrocytes transfected with non-targeting or Dhrs9 siRNA and incubated with 4 nM atRA

for 6 hr. (C) mRNA expression was measured by RT-PCR 72 hr after transfection with Dhhrs9 siRNA. (D) Retinol production was measured in astrocytes transfected with non-targeting or Dhhrs9 siRNA, incubated 4 hr with 1 μ M retinal. (E) atRA production from 1 μ M retinal with time after transfection with non-targeting or Dhhrs9 siRNA: $*P < 0.0001$, $n = 3$. (F) Dhhrs9 and Raldh1 mRNAs were detected by RT-PCR (upper panel). Raldh1 mRNA levels were quantified by Q-PCR (left lower panel). Raldh1 protein levels were quantified by western blot (right lower panel) with time after transfection with Dhhrs9 siRNA: $*P < 0.05$, $n = 3$. (G) Astrocytes were incubated with: (upper panel) 1 μ M retinal or (lower panel) 1 μ M atRA. Radlh1 mRNA was measured by RT-PCR.

Figure II-10

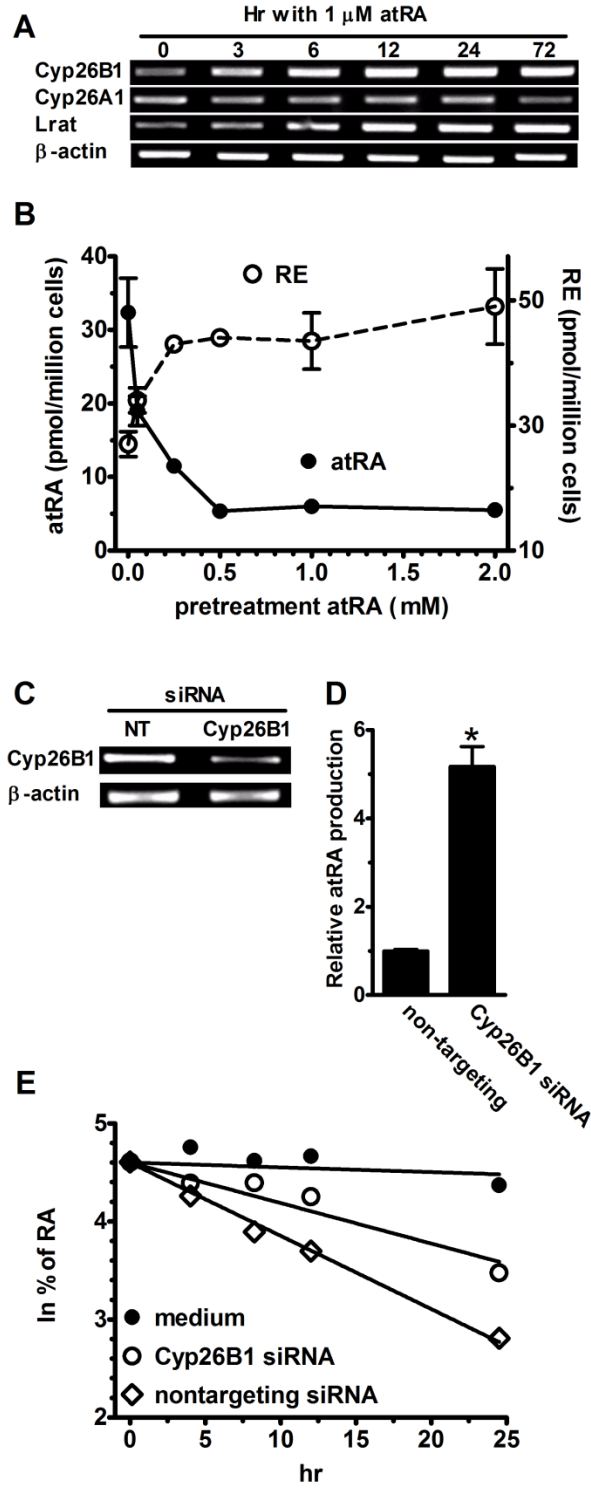


Figure II-10 atRA induces Cyp26B1 and Lrat mRNA in astrocytes. (A) RT-PCR of Cyp26A1, Cyp26B1 and Lrat mRNAs after incubating primary astrocytes with 1 μ M atRA. (B) Astrocytes were pre-incubated with graded concentrations of atRA for 72 hr. atRA was removed, cells were incubated 4 hr with 2 μ M retinol, atRA (solid line) and RE (dashed line) production was quantified. (C) Astrocytes were transfected with non-targeting or Cyp26B1 siRNA for 72 hr and Cyp26B1 mRNA was measured by RT-PCR. (D) Astrocytes were transfected 96 hr with Cyp26B1 siRNA and then incubated 4 hr with 2 μ M retinol. The amount of atRA produced was normalized to the amount detected in cells transfected with non-targeting siRNA: $*P < 0.0001$, $n = 3$. (E) Astrocytes were transfected with non-targeting or Cyp26B1 siRNA (96 hr) and then incubated with 1 nM atRA. The natural log of the percent atRA remaining was plotted vs. incubation time: filled circles, medium without cells; open circles, cells transfected with Cyp26B1 siRNA; open diamonds, cells transfected with non-targeting siRNA.

Figure II-11

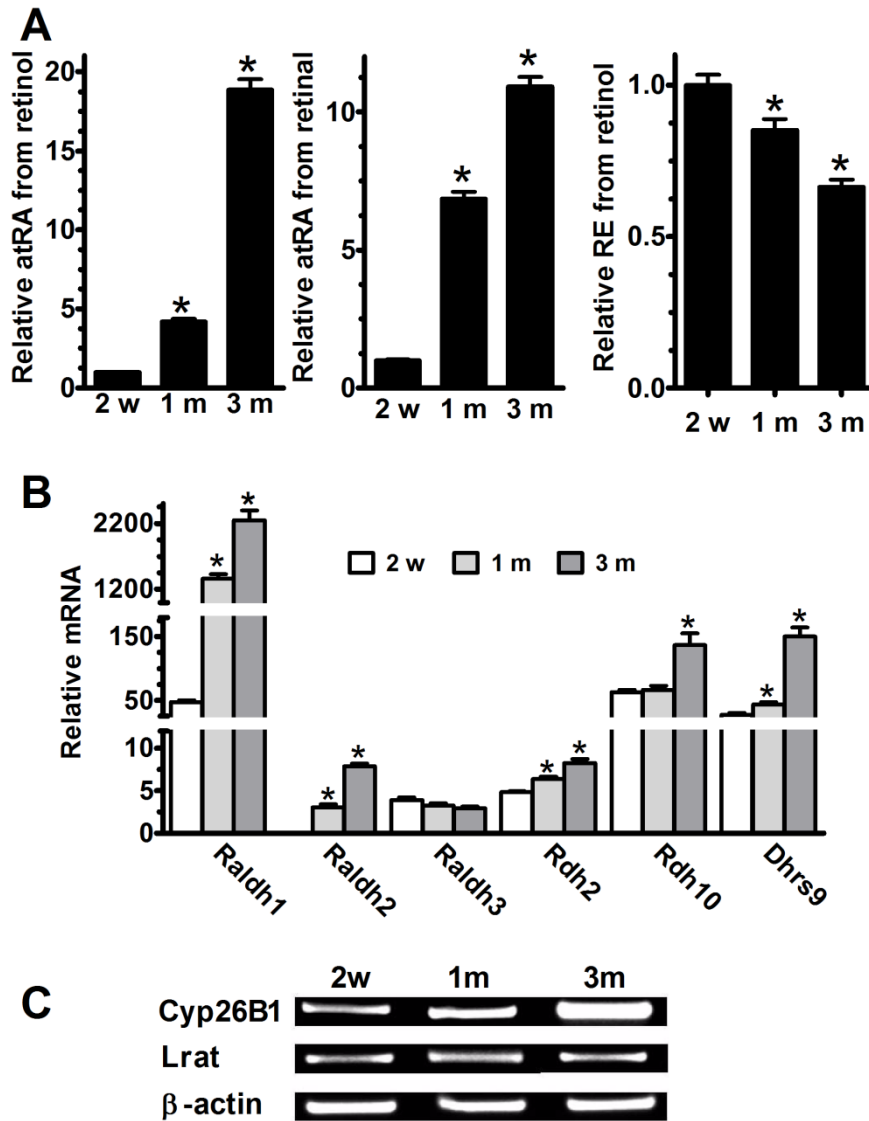


Figure II-11 Effect of culture duration on retinoid metabolism by primary astrocytes. (A) atRA and RE production was quantified from primary astrocytes incubated for 4 hr with 2 μ M retinol or 1 μ M retinal as a function of days in culture: * P <0.001 relative to 2 weeks in culture. (B) mRNA expression levels of Raldh1, 2 and 3, Rdh2 and 10, and Dhrs9 in primary astrocytes with days in culture were quantified by Q-PCR: * P <0.001 relative to 2 weeks in culture. (C) Cyp26B1 and Lrat mRNA expression in astrocytes with days in culture were detected by RT-PCR.

Figure II-12

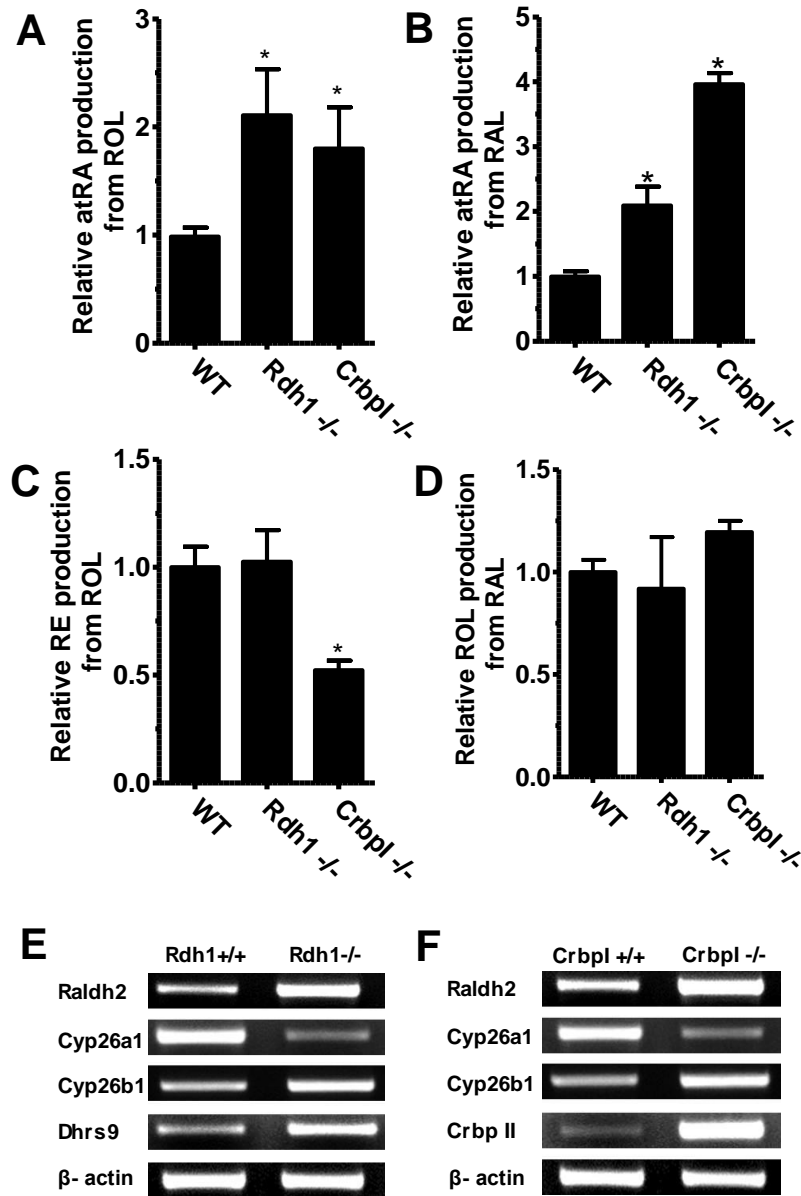


Figure II-12 Compensation effect of atRA biosynthesis in Rdh1^{-/-} and CrbpI^{-/-} astrocytes. Primary astrocytes from wild-type (WT) and Rdh1 knockout (Rdh1^{-/-}) or CrbpI (CrbpI^{-/-}) knockout mice cultured for 1 month were incubated 4 hr with 2 μM retinol or 1 μM retinal, atRA production from retinol (A) or retinal (B), RE production from retinol (C) and retinol production from retinal (D) were quantified and normalized to wild-type group. * P<0.0001, n=3. (E) Expressions of various mRNAs in wild-type or Rdh1^{-/-} mice astrocytes were measured by RT-PCR. (F) Expressions of various mRNAs in wild-type or CrbpI^{-/-} mice astrocytes were measured by RT-PCR.

Figure II-13

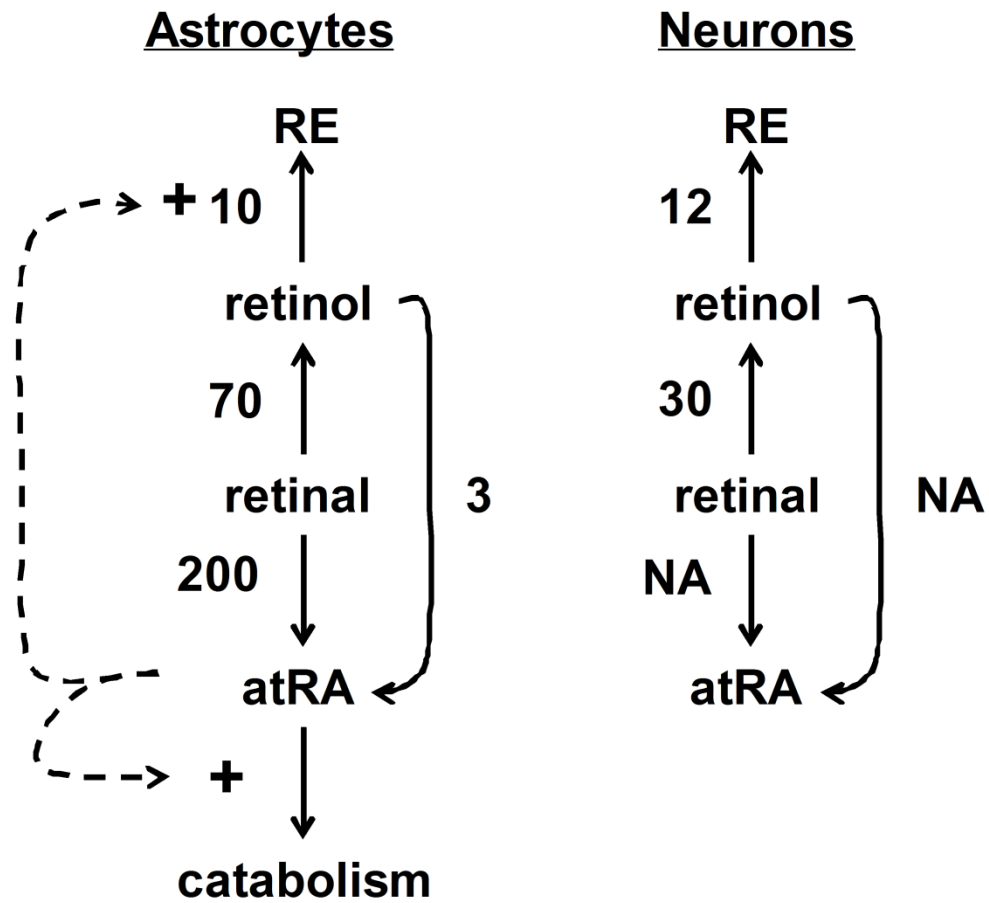


Figure II-13 Retinol homeostasis in astrocytes and neurons. The numbers next to each step designate the rate of the step at 60 nM substrate relative to the other steps, calculated from the data of Table 1. The dotted lines represent atRA control of Lrat and Cyp26B1 expression. NA, not applicable.

Table II-1

Rate constants for retinol metabolism.

Reaction	Astrocytes (V_m , $K_{0.5}$, rate)	Neurons (V_m , $K_{0.5}$, rate)
retinol through atRA	90, 2, 3	not applicable
retinal into atRA	6200, 2, 200	not applicable
retinal into retinol	740, 0.5, 70	160, 0.2, 30
retinol into RE	very high, 10	1200, 6, 12

Units are: V_m , pmol/million cells/4 hr; $K_{0.5}$, μM ; rate, pmol/million cells/4 hr at 60 nM substrate.

References

- Akita, J., M. Takahashi, *et al.* (2002). "Neuronal differentiation of adult rat hippocampus-derived neural stem cells transplanted into embryonic rat explanted retinas with retinoic acid pretreatment." Brain Res **954**(2): 286-293.
- Alliot, F., N. Delhaye-Bouchaud, *et al.* (1988). "Role of astroglial cell clones in the survival and differentiation of cerebellar embryonic neurons." Brain Res Dev Brain Res **44**(2): 247-257.
- Asson-Batres, M. A. and W. B. Smith (2006). "Localization of retinaldehyde dehydrogenases and retinoid binding proteins to sustentacular cells, glia, Bowman's gland cells, and stroma: potential sites of retinoic acid synthesis in the postnatal rat olfactory organ." J Comp Neurol **496**(2): 149-171.
- Banker, G. A. (1980). "Trophic interactions between astroglial cells and hippocampal neurons in culture." Science **209**(4458): 809-810.
- Brodeur, H., I. Gagnon, *et al.* (2003). "Cloning of monkey RALDH1 and characterization of retinoid metabolism in monkey kidney proximal tubule cells." J Lipid Res **44**(2): 303-313.
- Chai, X., M. H. Boerman, *et al.* (1995). "Cloning of a cDNA for liver microsomal retinol dehydrogenase. A tissue-specific, short-chain alcohol dehydrogenase." J Biol Chem **270**(8): 3900-3904.
- Chandrasekaran, V., Y. Zhai, *et al.* (2000). "Retinoic acid regulates the morphological development of sympathetic neurons." J Neurobiol **42**(4): 383-393.
- Chen, N. and J. L. Napoli (2008). "All-trans-retinoic acid stimulates translation and induces spine formation in hippocampal neurons through a membrane-associated RARalpha." FASEB J **22**(1): 236-245.
- Chen, N., B. Onisko, *et al.* (2008). "The nuclear transcription factor RARalpha associates with neuronal RNA granules and suppresses translation." J Biol Chem **283**(30): 20841-20847.
- Chetyrkin, S. V., O. V. Belyaeva, *et al.* (2001). "Characterization of a novel type of human microsomal 3alpha -hydroxysteroid dehydrogenase: unique tissue distribution and catalytic properties." J Biol Chem **276**(25): 22278-22286.
- Chiang, M. Y., D. Misner, *et al.* (1998). "An essential role for retinoid receptors RARbeta and RXRgamma in long-term potentiation and depression." Neuron **21**(6): 1353-1361.
- Cocco, S., G. Diaz, *et al.* (2002). "Vitamin A deficiency produces spatial learning and memory impairment in rats." Neuroscience **115**(2): 475-482.
- Connor, M. J. and N. Sidell (1997). "Retinoic acid synthesis in normal and Alzheimer diseased brain and human neural cells." Mol Chem Neuropathol **30**(3): 239-252.
- Cooper, D. L., N. R. Isola, *et al.* (1993). "Members of the ALDH gene family are lens and corneal crystallins." Adv Exp Med Biol **328**: 169-179.
- Cowherd, R. M., R. E. Lyle, *et al.* (1999). "Molecular regulation of adipocyte

- differentiation." Semin Cell Dev Biol **10**(1): 3-10.
- Dupe, V., N. Matt, *et al.* (2003). "A newborn lethal defect due to inactivation of retinaldehyde dehydrogenase type 3 is prevented by maternal retinoic acid treatment." Proc Natl Acad Sci U S A **100**(24): 14036-14041.
- Edwards, R. B., A. J. Adler, *et al.* (1992). "Synthesis of retinoic acid from retinol by cultured rabbit Muller cells." Exp Eye Res **54**(4): 481-490.
- Etchamendy, N., V. Enderlin, *et al.* (2003). "Vitamin A deficiency and relational memory deficit in adult mice: relationships with changes in brain retinoid signalling." Behav Brain Res **145**(1-2): 37-49.
- Etchamendy, N., V. Enderlin, *et al.* (2001). "Alleviation of a selective age-related relational memory deficit in mice by pharmacologically induced normalization of brain retinoid signaling." J Neurosci **21**(16): 6423-6429.
- Grun, F., Y. Hirose, *et al.* (2000). "Aldehyde dehydrogenase 6, a cytosolic retinaldehyde dehydrogenase prominently expressed in sensory neuroepithelia during development." J Biol Chem **275**(52): 41210-41218.
- Halilagic, A., V. Ribes, *et al.* (2007). "Retinoids control anterior and dorsal properties in the developing forebrain." Dev Biol **303**(1): 362-375.
- Jacobs, S., D. C. Lie, *et al.* (2006). "Retinoic acid is required early during adult neurogenesis in the dentate gyrus." Proc Natl Acad Sci U S A **103**(10): 3902-3907.
- Jette, C., P. W. Peterson, *et al.* (2004). "The tumor suppressor adenomatous polyposis coli and caudal related homeodomain protein regulate expression of retinol dehydrogenase L." J Biol Chem **279**(33): 34397-34405.
- Jones-Villeneuve, E. M., M. A. Rudnicki, *et al.* (1983). "Retinoic acid-induced neural differentiation of embryonal carcinoma cells." Mol Cell Biol **3**(12): 2271-2279.
- Jurukovski, V., N. G. Markova, *et al.* (1999). "Cloning and characterization of retinol dehydrogenase transcripts expressed in human epidermal keratinocytes." Mol Genet Metab **67**(1): 62-73.
- Kane, M. A., N. Chen, *et al.* (2005). "Quantification of endogenous retinoic acid in limited biological samples by LC/MS/MS." Biochem J **388**(Pt 1): 363-369.
- Kane, M. A., A. E. Folias, *et al.* (2008). "Quantitative profiling of endogenous retinoic acid in vivo and in vitro by tandem mass spectrometry." Anal Chem **80**(5): 1702-1708.
- Kane, M. A., A. E. Folias, *et al.* (2010). "Ethanol elevates physiological all-trans-retinoic acid levels in select loci through altering retinoid metabolism in multiple loci: a potential mechanism of ethanol toxicity." FASEB J **24**(3): 823-832.
- Klann, R. C. and A. C. Marchok (1982). "Effects of retinoic acid on cell proliferation and cell differentiation in a rat tracheal epithelial cell line." Cell Tissue Kinet **15**(5): 473-482.
- Korneyi, Z., E. Gocza, *et al.* (2007). "Astroglia-derived retinoic acid is a key factor in glia-induced neurogenesis." FASEB J **21**(10): 2496-2509.

- Labrecque, J., P. V. Bhat, *et al.* (1993). "Purification and partial characterization of a rat kidney aldehyde dehydrogenase that oxidizes retinal to retinoic acid." Biochem Cell Biol **71**(1-2): 85-89.
- Laird, M. D., J. R. Vender, *et al.* (2008). "Opposing roles for reactive astrocytes following traumatic brain injury." Neurosignals **16**(2-3): 154-164.
- Maden, M. (2007). "Retinoic acid in the development, regeneration and maintenance of the nervous system." Nat Rev Neurosci **8**(10): 755-765.
- Mark, M., N. B. Ghyselinck, *et al.* (2009). "Function of retinoic acid receptors during embryonic development." Nucl Recept Signal **7**: e002.
- Markiewicz, I. and B. Lukomska (2006). "The role of astrocytes in the physiology and pathology of the central nervous system." Acta Neurobiol Exp (Wars) **66**(4): 343-358.
- McCaffery, P., O. Koul, *et al.* (2004). "Ethanol increases retinoic acid production in cerebellar astrocytes and in cerebellum." Brain Res Dev Brain Res **153**(2): 233-241.
- Misner, D. L., S. Jacobs, *et al.* (2001). "Vitamin A deprivation results in reversible loss of hippocampal long-term synaptic plasticity." Proc Natl Acad Sci U S A **98**(20): 11714-11719.
- Moro, J. R., M. Iwata, *et al.* (2008). "Vitamin effects on the immune system: vitamins A and D take centre stage." Nat Rev Immunol **8**(9): 685-698.
- Nakayama, T., T. Momoki-Soga, *et al.* (2003). "Astrocyte-derived factors instruct differentiation of embryonic stem cells into neurons." Neurosci Res **46**(2): 241-249.
- Napoli, J. L. (1999). "Interactions of retinoid binding proteins and enzymes in retinoid metabolism." Biochim Biophys Acta **1440**(2-3): 139-162.
- Napoli, J. L. (2010). Vitamin A (Retinoids). Encyclopedia of Biological Chemistry, Elsevier Ltd: in press.
- Niederreither, K., V. Subbarayan, *et al.* (1999). "Embryonic retinoic acid synthesis is essential for early mouse post-implantation development." Nat Genet **21**(4): 444-448.
- Ong, D. E., P. N. MacDonald, *et al.* (1988). "Esterification of retinol in rat liver. Possible participation by cellular retinol-binding protein and cellular retinol-binding protein II." J Biol Chem **263**(12): 5789-5796.
- Penniston, K. L. and S. A. Tanumihardjo (2006). "The acute and chronic toxic effects of vitamin A." Am J Clin Nutr **83**(2): 191-201.
- Posch, K. C., R. D. Burns, *et al.* (1992). "Biosynthesis of all-trans-retinoic acid from retinal. Recognition of retinal bound to cellular retinol binding protein (type I) as substrate by a purified cytosolic dehydrogenase." J Biol Chem **267**(27): 19676-19682.
- Rexer, B. N. and D. E. Ong (2002). "A novel short-chain alcohol dehydrogenase from rats with retinol dehydrogenase activity, cyclically expressed in uterine

- epithelium." Biol Reprod **67**(5): 1555-1564.
- Rochette-Egly, C. and P. Germain (2009). "Dynamic and combinatorial control of gene expression by nuclear retinoic acid receptors (RARs)." Nucl Recept Signal **7**: e005.
- Rodnight, R., C. A. Goncalves, *et al.* (1997). "Control of the phosphorylation of the astrocyte marker glial fibrillary acidic protein (GFAP) in the immature rat hippocampus by glutamate and calcium ions: possible key factor in astrocytic plasticity." Braz J Med Biol Res **30**(3): 325-338.
- Ross, A. C. (2003). "Retinoid production and catabolism: role of diet in regulating retinol esterification and retinoic Acid oxidation." J Nutr **133**(1): 291S-296S.
- Ross, A. C. and R. Zolfaghari (2004). "Regulation of hepatic retinol metabolism: perspectives from studies on vitamin A status." J Nutr **134**(1): 269S-275S.
- Shapiro, M. L. and H. Eichenbaum (1999). "Hippocampus as a memory map: synaptic plasticity and memory encoding by hippocampal neurons." Hippocampus **9**(4): 365-384.
- Shearer, K. D., T. H. Goodman, *et al.* (2010). "Photoperiodic regulation of retinoic acid signaling in the hypothalamus." J Neurochem **112**(1): 246-257.
- Shudo, K., H. Fukasawa, *et al.* (2009). "Towards retinoid therapy for Alzheimer's disease." Curr Alzheimer Res **6**(3): 302-311.
- Siegenthaler, J. A., A. M. Ashique, *et al.* (2009). "Retinoic acid from the meninges regulates cortical neuron generation." Cell **139**(3): 597-609.
- Silman, I. and J. L. Sussman (2005). "Acetylcholinesterase: 'classical' and 'non-classical' functions and pharmacology." Curr Opin Pharmacol **5**(3): 293-302.
- Song, H., C. F. Stevens, *et al.* (2002). "Astroglia induce neurogenesis from adult neural stem cells." Nature **417**(6884): 39-44.
- Soref, C. M., Y. P. Di, *et al.* (2001). "Characterization of a novel airway epithelial cell-specific short chain alcohol dehydrogenase/reductase gene whose expression is up-regulated by retinoids and is involved in the metabolism of retinol." J Biol Chem **276**(26): 24194-24202.
- Steven W. Levison, J. d. V., and James E. Goldman (2005). "Astrocyte development." Developmental Neurobiology, 4th ed.
- Takahashi, J., T. D. Palmer, *et al.* (1999). "Retinoic acid and neurotrophins collaborate to regulate neurogenesis in adult-derived neural stem cell cultures." J Neurobiol **38**(1): 65-81.
- Thatcher, J. E. and N. Isoherranen (2009). "The role of CYP26 enzymes in retinoic acid clearance." Expert Opin Drug Metab Toxicol **5**(8): 875-886.
- Toresson, H., A. Mata de Urquiza, *et al.* (1999). "Retinoids are produced by glia in the lateral ganglionic eminence and regulate striatal neuron differentiation." Development **126**(6): 1317-1326.
- Unsicker, K., H. Reichert-Preibsch, *et al.* (1987). "Astroglial and fibroblast growth factors have neurotrophic functions for cultured peripheral and central nervous

- system neurons." Proc Natl Acad Sci U S A **84**(15): 5459-5463.
- Vaccarino, F. M., D. M. Fagel, *et al.* (2007). "Astroglial cells in development, regeneration, and repair." Neuroscientist **13**(2): 173-185.
- Wang, X., P. Penzes, *et al.* (1996). "Cloning of a cDNA encoding an aldehyde dehydrogenase and its expression in Escherichia coli. Recognition of retinal as substrate." J Biol Chem **271**(27): 16288-16293.
- Wu, B. X., Y. Chen, *et al.* (2002). "Cloning and characterization of a novel all-trans retinol short-chain dehydrogenase/reductase from the RPE." Invest Ophthalmol Vis Sci **43**(11): 3365-3372.
- Wuarin, L., N. Sidell, *et al.* (1990). "Retinoids increase perinatal spinal cord neuronal survival and astroglial differentiation." Int J Dev Neurosci **8**(3): 317-326.
- Yang, Y., W. Ge, *et al.* (2003). "Contribution of astrocytes to hippocampal long-term potentiation through release of D-serine." Proc Natl Acad Sci U S A **100**(25): 15194-15199.
- Yost, R. W., E. H. Harrison, *et al.* (1988). "Esterification by rat liver microsomes of retinol bound to cellular retinol-binding protein." J Biol Chem **263**(35): 18693-18701.
- Zhang, M., W. Chen, *et al.* (2001). "Molecular characterization of a mouse short chain dehydrogenase/reductase active with all-trans-retinol in intact cells, mRDH1." J Biol Chem **276**(47): 44083-44090.
- Zhang, M., P. Hu, *et al.* (2007). "Altered vitamin A homeostasis and increased size and adiposity in the rdh1-null mouse." FASEB J **21**(11): 2886-2896.
- Zhao, D., P. McCaffery, *et al.* (1996). "Molecular identification of a major retinoic-acid-synthesizing enzyme, a retinaldehyde-specific dehydrogenase." Eur J Biochem **240**(1): 15-22.
- Ziouzenkova, O., G. Orasanu, *et al.* (2007). "Retinaldehyde represses adipogenesis and diet-induced obesity." Nat Med **13**(6): 695-702.

Footnotes

The abbreviations used are: atRA, all-*trans*-retinoic acid; DIV, days *in vitro*; CNS, central nervous system; Cyp26A1, Cytochrome P450 26A1; Cyp26B1, Cytochrome P450 26B1; Dhhr9, dehydrogenase/reductase SDR family member 9; RAR, retinoic acid receptor; GFAP, glial fibrillary acidic protein; HPLC, High-performance liquid chromatography; LC/MS/MS, liquid chromatography/mass spectrometry; Lrat, lecithin:retinol acyltransferase; MAP2, microtubule-associated protein 2; Raldh, retinaldehyde dehydrogenase; RE, retinyl ester; Rdh, retinol dehydrogenase; SDR, short-chain dehydrogenase/reductase; siRNA, small interfering RNA.

Chapter III

Localization *in vitro* and *in vivo* of RALDH1 Expression

Introduction

We have demonstrated that RALDH1 is a key component of atRA biosynthesis in adult astrocytes, because knockdown of RALDH1 reduced atRA biosynthesis in astrocytes. Astrocytes also up regulate RALDH1 expression to compensate for impairment of atRA biosynthesis due to the loss of *Dhrs9*, indicating that RALDH1 is at least one of the enzymes that the adult nervous system uses to control atRA homeostasis. An *in vitro* culture time dependent increase of RALDH1 expression, which correlates with increased atRA production in astrocytes also supports the conclusion that RALDH1 is a major enzyme that contributes to atRA biosynthesis in astrocytes. Besides what we found, the presence of RALDH1 has been reported in adult hippocampal neurons (Aoto *et al.*, 2008) and GFAP positive tissue in the adult olfactory bulb (Asson-Batres and Smith, 2006).

Immunostaining of RALDH1 in pure cultured astrocytes, showed wide expression in cultured glia cells, not only in GFAP⁺ cells, but also in glia cells that do not express GFAP. RALDH1 is expressed in both cytoplasm and nucleus of astrocytes, an uncommon location that has not been reported before, to the best of our knowledge. As we know, RALDH2 is a critical enzyme that provides atRA for embryo development, but in cultured mature astrocytes, RALDH1 had the highest copy numbers of mRNA. We hypothesize that there is a time-dependent increased expression of RALDH1 in hippocampus, and this increment might correlate with the appearance and development of astrocytes in adult hippocampus.

The anatomic structure of adult hippocampus is quite unique. Neurons in the hippocampus are centralized in three areas, granule cells in the dentate gyrus, and pyramidal neurons in CA1 and CA3 regions (Gross, 1993). The primary afferent to the hippocampus is the perforant pathway (Witter *et al.*, 2000). This pathway arises from the entorhinal cortex and projects to the dentate gyrus. Granule cells in the dentate gyrus project to mossy cells in the hilus of the dentate gyrus and the CA3 region of Ammon's horn. The CA3 neurons in turn project to the CA1 by way of the Shaffer collateral. The CA1 regions projects to the deep layers of the entorhinal cortex, thereby completing a circuit. The axons that project from the dentate gyrus to the CA3 region are called mossy fibers (Kobayashi, 2010). The pathway was so named by Ramon Cajal because the axons display varicosities all along their lengths, giving them a 'mossy' appearance. These mossy fibers form multiple synapses with the elaborate dendritic spines of CA3 pyramidal cells in the stratum lucidum. A single mossy fiber projection may make as many as 37 contacts with a single pyramidal cell. In rodents, the size of mossy fiber

projections show large interindividual variation, which show strong correlations with spatial learning (McBain, 2008).

As shown in chapter II, no atRA production was found in cultured neurons, even incubated with retinal. However, RALDH1 has been reported to be expressed in cultured neurons (Aoto *et al.*, 2008). Besides that, the *in vivo* RALDH1 location has never been reported. Through immunostaining and immunohistochemistry, for the first time, we elucidated the specific localization of RALDH1 in cultured astrocytes, neurons and in adult hippocampus. Through co-immunostaining with other cell specific markers, we demonstrated that RALDH1 expresses throughout the cultured neurons with particularly high expression in axons; RALDH1 is also highly expressed in adult CA1 pyramidal neurons and mossy fibers in the adult hippocampus. In contrast, RALDH1 is only highly expressed in small portions of astrocytes, when in mixed culture with neurons, but has low expression levels in the other astrocytes. Our data indicate that RALDH1 may not function as a retinal dehydrogenase in neurons and its expression in astrocytes might be regulated by neurons.

Materials and Methods

Cell culture--Mono neuronal precursors were obtained the same way as pure neurons from the E18 Sprague-Dawley rat. To obtain mixed cultured glia-neurons, the precursors were plated on poly-D-Lysine coated glass coverslips with Neurobasal medium containing 2% B27 and 10% FBS at a density of 1×10^5 cells/well in 24-well plates. Half of the total medium was replenished every week. Mixed culture cells were fixed and immunostained with RALDH1 antibody and cell marker after 2 weeks incubation in a 37°C incubator with 5% CO₂.

Diluted pure neurons were cultured to obtain a clearly individual neuron image. E18 rat mono neuronal precursors were plated at the density of 5×10^3 cells/well in 24-well plate on glass coverslips pre-coated with poly-D-lysine. Pure neurons were cultured as described in Chapter II Materials and Methods.

RALDH1 construction of plasmid and transfection--To obtain a mammalian expression vector, the full length open reading frame of mouse RALDH1 (NM_013467) was obtained by PCR amplification from mouse brain cDNA library using the forward primer 5'-ATGGATCCATGTCTTCGCCTGCACAAC-3', containing a *Bam*HI site, and the reverse primer 5'-AAGAATTCTTAGGAGTTCTTCTGAGATATCTTC-3', containing a *Eco*RI site. The gel-purified PCR product was cloned into pcDNA3.1⁺ vector. The proper sequence of the expression vector was confirmed by sequencing. COS cells were transfected with pcDNA3.1(+)-RALDH1 vector using Lipofectamine 2000 (Invitrogen). After 24h transfection, cells were fixed for immunostaining of RALDH1.

Preparation of hippocampal slices—2 weeks to 1 month old Sprague-Dawley rats were anesthetized with CO₂ and decapitated. Brains were quickly excised and trimmed, leaving a rectangular block of tissue containing the dorsal hippocampus. The tissue blocks were immersed into liquid nitrogen slowly for 1 min until the tissue was completely frozen. Frozen tissue blocks were embedded in O.C.T. compound (Tissue-Tek) and glued to the stage of a cryostat-microtome. Six μ M frozen hippocampal slices were sectioned coronally and placed on the surfaces of glass slides. The slides were immersed into 4% paraformaldehyde for half an hour before immunostaining procedures.

Immunocytochemistry and Immunohistochemistry--Immunocytochemistry was performed in the same way as described in Chapter II materials and methods. Monoclonal Antibody against pan-axonal neurofilament marker SMI-312 (1:500) was used to identify axons in cultured neurons. For immunohistochemistry, fixed hippocampus slices were washed three time for 5-min each in PBST (1xPBS+0.25% Tween 20), permeabilized with 0.2% Triton X-100 for 15 min, and blocked with PBS containing 10% goat serum or donkey serum overnight, depending on the origin of secondary antibody. Hippocampal slices were then incubated with anti-NeuN (1:500, Millipore), rabbit polyclonal anti-RALDH1 (1:200 abcam), mouse anti MBP (1:200), rabbit anti MAP2 (1:1000), mouse anti Tau (1:500), or goat polyclonal anti Synaptoporin (1:10) overnight at 4 °C, washed three times

for 5-min each with PBST, followed by incubation with goat or donkey anti-mouse Alexa 555, goat or donkey anti-rabbit Alexa 488, chicken anti-goat Alexa 488 secondary antibody (Invitrogen) or rat cy3 red for 1 hr. After washing three times for 5 min each with PBST, coverslips were mounted on slides with Vectashield mounting medium (with DAPI, Vector Laboratories Inc. Burlingame, CA). Images were captured by a LSM 510 Meta UV/Vis confocal microscope.

Results

RALDH1 was highly expressed in pure neurons, but not astrocytes mixed cultured with neurons—To test the specificity of the RALDH1 antibody, COS cells transfected with rat-RALDH1-pcDNA3.1 expression vector or empty vector were immunostained with the antibody (Figure III-1A). A strong cytoplasmic and weak nucleus expression pattern of RALDH1 was found in transfected cells group but not in control cells.

In previous research, we found that RALDH1 was widely expressed in cultures of pure astrocyte, not only in GFAP positive cells, but also in cells that did not express GFAP. In pure glia cell cultures, astrocytes lost their *in vivo* stellate-like shape (Figure II-1A) and the connection with pyramidal neurons during the differentiation. To address the question whether the presence of neurons would affect RALDH1 expression, we mixed cultured neurons and astrocytes. Immunostaining of GFAP showed that astrocytes in mixed cultured neural cells maintained their star-like shape (Figure III-1B). RALDH1 was unevenly expressed in these mixed cultured astrocytes (Figure III-1B, top panel). Most of the astrocytes showed fairly low levels of RALDH1 expression (Figure III-1B, middle panel), but a few (about 5%) showed strong expression of RALDH1 (Figure III-1B, bottom panel). Surprisingly, we also found that RALDH1 was present in GFAP negative cells in mixed cultures, especially highly present in some process-like structures. To test whether those cells and processes with strong RALDH1 expression were neurons, individual pyramidal neurons were immunostained with RALDH1 antibody together with neuron markers (Figure III-1C). The neuronal markers included: NeuN—neuron nucleus and cytoplasm marker, MAP2—neuron dendrite marker and SMI 312—neuron axon marker. RALDH1 co-localized with all these cell markers, indicating that RALDH1 was expressed throughout intact neurons, including soma, dendrites and axons. Compared with other parts of neurons, the strongest signal of RALDH1 was detected on axons (Figure III-1B, top panel).

RALDH1 is highly expressed in the CA1 and CA3 regions of adult rat hippocampus—To elucidate the *in vivo* expression pattern of RALDH1 in adult rat hippocampus, 2 month old adult male rat hippocampus slice were immunostained with RALDH1 and NeuN antibody (Figure III-2A). Strong expression of RALDH1 was found in the CA1 neuron cell body region. A closer examination of the CA1 region showed that RALDH1 was expressed in CA1 soma of pyramidal neurons—colocalizing with the NeuN marker and processes in the stratum radiatum layer (Figure III-2B, left panel). This expression pattern correlated with the *in vitro* distribution of RALDH1 in pyramidal neurons. Besides the strong RALDH1 expression in CA1 region, an even more intense RALDH1 signal was detected in an area starting from the hilus of the dentate gyrus and extending along with CA3 region. However, higher magnification showed that the strong RALDH1 signals in CA3 region excluded the CA3 neuron soma (Figure III-2B, right panel), indicating that CA3 neurons don't express RALDH1 in their cell bodies.

RALDH1 is not expressed in astrocytes and oligodendrocytes in adult hippocampus— We

next tested whether the structures highly expressing RALDH1 in the CA3 region belonged to glia cells. Hippocampus slices double stained with RALDH1 and GFAP clearly showed that i) a few astrocytes partially expressed RALDH1, which is consistent with the *in vitro* mixed culture conclusions; ii) the intense RALDH1 signal in the CA3 region (for example, two selected rectangle areas) didn't co-localize with the GFAP marker, which excluded the possibility that this structure belonged to astrocyte or its processes (Figure III-3A). In summary, this *in vivo* RALDH1 expression pattern in astrocytes didn't match what we observed in pure cultured astrocytes but matched more closely with the mixed cultured results, indicating that the *in vivo* micro-environment will affect RALDH1 expression. Myelin basic protein (MBP) is a protein important in the process of myelination of nerves and is a major constituent of the myelin sheath of oligodendrocytes (Montague *et al.*, 2006). In mixed culture neural cells, RALDH1 is weakly expressed in MBP positive oligodendrocytes (Figure III-3B, upper panel). However, RALDH1 is not detected *in vivo* in MBP positive cells in the CA3 region (Figure III-3B, lower panel). All these data suggest that the structure that expressed RALDH1 was highly expressed in CA3 region was not belonged to glia cells in adult hippocampus.

RALDH1 is localized in dendrites of CA1 neurons but not dendrites in the CA3 region—In cultured neurons, we found RALDH1 expressed in dendrites of pyramidal neurons. We next tested whether RALDH1 was also expressed in dendrites of pyramidal neurons *in vivo*. The co-localization of RALDH1 with MAP2 in the CA1 region indicated that RALDH1 was expressed in the dendrites of CA1 neurons, consistent with *in vitro* cultured neurons results (Figure III-4, left panel). However, high RALDH1 expression structures in the CA3 region were not co-localized with MAP2, indicating that dendrites in CA3 region do not express RALDH1 (Figure III-4, right panel). Given that RALDH1 is expressed in CA1 neuron soma, but not in CA3 neuron soma, primary cultured neurons seem to mimic the RALDH1 expression pattern of CA1 neurons but not neurons in CA3 region.

RALDH1 is highly expressed in axons in CA3 region and co-localized with mossy fibers—Tau proteins stabilize microtubules present in dendrites and are primarily in distal parts of axons (Kempf *et al.*, 1996). Co-immunostaining of both RALDH1 and Tau further confirmed expression of RALDH1 in axons of cultured neurons (Figure III-5A). Immunostaining of Tau proteins in the adult hippocampus exhibited a similar expression pattern of RALDH1 in CA3 region (Figure III-5B). Higher magnification of the RALDH1 expression structure in CA3 region showed perfect co-localization with Tau signals, which indicated that the high RALDH1 expression structure in CA3 region are the distal axons bundles (Figure III-5C).

Mossy fibers are the varicosities axons that projected in CA3 region from granule cells in dentate gyrus and show similar structure as the Tau staining pattern. We then tested whether those axon bundles that highly expressed RALDH1 in this area are mossy fibers. Three selected areas in CA3 region all exhibited co-localization of RALDH1 and

synaptoporin, a mossy fiber specific marker (Figure III-6) (Sun *et al.*, 2006). However, these two proteins do not 100% overlap with each other. This result further confirmed the mossy fibers-specific enriched RALDH1 expression in CA3 region. But RALDH1 might have a different subcellular compartmentation with synaptoporin in mossy fibers.

Postnatal development of RALDH1 in adult hippocampus—Finally, we tested the time-scale of RALDH1 expression in adult hippocampus to see whether its expression, especially in mossy fibers, is a transitional or developmental change. RALDH1 was barely detected in the hippocampus of P2 rats (Figure III-7). Weak expression of RALDH1 was detected in one week old rat hippocampus, mainly in the CA3 region. The expression level of RALDH1 gradually increased in the following weeks and reached a steady level after 3 weeks of postnatal development (Figure III-7, left panel). A close examination clearly showed the incremental expression of RALDH1 in CA3 region (Figure III-7, right panel). In the same time, RALDH1 also began to appear in CA1 regions, including neurons soma and dendrites (data not shown). All these data indicate that RALDH1 expression changes with postnatal development.

Discussion

Distinct expression levels and distribution patterns of RALDH1 *in vitro* and *in vivo* in astrocytes

RALDH1 is a major enzyme responsible for atRA biosynthesis in astrocytes, as shown by knockdown experiments in primary cultures. The distribution pattern and expression level of RALDH1 in astrocytes serves as an indicator of atRA biosynthesis. However, one of the disadvantages of using the primary cell culture model is the different developmental environment for these cells. The media used to culture primary astrocytes and pyramidal neurons only contains basic necessary nutrients and growth factors that maintain cell survival and stimulate proliferation. However, more and more evidence indicates that the development of complex nervous systems requires many integrated signals that function in a spatial and temporal specific manner (Chen and Tonegawa, 1997). For example, formation of synaptic plasticity relies on soluble factors secreted by astrocytes and contact with oligodendrocytes (Markiewicz and Lukomska, 2006). Absence of these essential factors during the *ex vivo* culture of neural cells may cause those cells to undergo unconventional changes. Our pure cultured astrocytes lost the star-like processes morphology. In the mixed culture system, astrocytes showed morphology close to the *in vivo* morphology, which may be due to the presence of pyramidal neurons, which partially compensate for loss of micro-environmental factors required for astrocyte development.

RALDH1 showed an expression pattern in pure astrocyte cultures of particularly high expression level in the nucleus. However, RALDH1 is only highly expressed in a small portion of astrocytes in the mixed culture system, leaving most of the astrocytes with a relative low level of RALDH1 expression. This uneven expression pattern is even more obvious in hippocampus slices, with only a few astrocytes showing low level of RALDH1, whereas the others don't show detectable level of RALDH1. The differences in RALDH1 expression might indicate that astrocytes have ability to change atRA biosynthesis by changing the level of RALDH1 during different circumstances. The presence of mature neurons might have a negative effect on RALDH1 expression in nearby astrocytes. In pure cultured astrocytes, lacking this inhibitory effect from neurons, a culture-time dependent increase of RALDH1 expression was seen (Chapter II). The presence of neurons in mixed culture systems or *in vivo* restricts RALDH1 expression to a relative low and stable level perhaps to regulate the biosynthesis of atRA. This is necessary for maintaining the normal function of neurons, because astrocytes have ability to efficiently synthesize and secrete atRA into the environment and atRA can stimulate spine growth of nearby neurons. The high level of RALDH1 might result in excessive atRA secretion, which may damage synaptic plasticity or affect neurogenesis. The inhibitory mechanism, either due to soluble factors secreted by neurons, or neuron-astrocyte contact, need to be further investigated.

Although most astrocytes showed relative low levels of RALDH1 expression, a small

portion of mixed cultured astrocytes exhibited strong RALDH1 expression all throughout the cell body; immunohistochemistry of hippocampus slices also revealed scattered astrocytes, which had detectable RALDH1 expression, indicating that those astrocytes reserve or regain the high atRA biosynthesis ability. The signals and regulatory mechanisms that control RALDH1 expression in this small number of astrocytes are unclear. Figuring out the age of these astrocytes or the developmental stage of the neurons contacted with these astrocytes may provide a possible explanation, because either the young astrocytes or the developmental neurons might need more atRA for differentiation or formation of synaptic plasticity.

Function of RALDH1 in neurons

Our previous results didn't support the conclusion that primary cultured neurons can synthesize atRA. Even incubated with retinal, no obvious atRA production can be detected in cells or in medium. However, a relatively strong signal of RALDH1 was detected in cultured neurons, which challenges this conclusion.

The high level of RALDH1 was detected all throughout cultured neurons, from soma to dendrites, with particularly strong signal in axons. Similar results have been reported by other groups (Aoto *et al.*, 2008). There are several possibilities that could explain the conflict between high expression of RALDH1 and barely detectable atRA after retinal incubation in pyramidal neurons.

First, if RALDH1 in neurons functions as a retinal dehydrogenase, the low production of atRA could be due to the low accessibility of RALDH1 to substrate or a fast and efficient degradation of synthesized atRA. However, the latter situation is less likely because in the astrocytes-neurons co-culture experiment, we detected significantly accumulation of atRA in neurons. To exclude this possibility, CYP26 enzymes inhibitors could be used to block atRA degradation. Retinol or retinal are small hydrophobic molecules and can easily diffuse across the cell membrane to reach target enzymes in cell culture models. This property is observed in many cells types such as astrocytes, COS cells and CHO cells. However, neurons are a specific cell type in CNS and tightly control the uptake of substances from the environment. Whether the pyramidal neurons in hippocampus have a specific mechanism to load retinol or retinal, or this loading needs specific assistance from other glia cells, for example, astrocytes, needs to be further investigated. Moreover, the presence of one 'atRA synthesis essential enzyme' is not sufficient for atRA production. Other components for atRA biosynthesis need to be detected to evaluate the presence of atRA biosynthesis in neurons.

Secondly, besides function as a retinal dehydrogenase, RALDH1 was also reported as a binding protein. Pereira *et al.* reported the 56 kD androgen binding protein in genital skin fibroblasts is an aldehyde dehydrogenase (Pereira *et al.*, 1991). A cytosolic thyroid-hormone-binding protein (xCTBP), predominantly responsible for the major binding activity of T3 in the cytosol of *Xenopus* liver, is identical to aldehyde dehydrogenase class 1 (ALDH1) (Yamauchi *et al.*, 1999). Whether RALDH1 in neurons also function as a hormone binding protein is unknown and this possibility should be

considered as a potential function in neurons.

It is also possible that RALDH1 shows both aldehyde dehydrogenase activity and hormone binding activity. There might be other factors in neurons, for example, NAD⁺ or NADPH, that could modulate balance between enzyme activity and binding activity (Yamauchi and Nakajima, 2002).

In vivo RALDH1 expression in adult hippocampus

In vivo RALDH1 expression in CA1 neurons is highly consistent with the *in vitro* culture neurons, with particular high expression in neuron soma and dendrites. The high expression of RALDH1 in axons *in vitro* is barely seen *in vivo* because axons are too thin to be clearly observed in the hippocampus slice. Neurons in the cortex also show similar RALDH1 expression pattern with particular strong expression on dendrites (data not shown). These data indicate that this wide RALDH1 expression pattern is generally present in neurons *in vivo*. However, not all the neurons in hippocampus showed homogeneous RALDH1 expression pattern.

Oligodendrocytes are the major cell type responsible for myelination of axons in the CNS (Bradl and Lassmann, 2010). Relative low expression of RALDH1 in oligodendrocytes *in vitro* and *in vivo* suggests that atRA signaling might not be active in this cell type in adult hippocampus. However, we could not exclude the presence of other types of RADLH in oligodendrocytes. Actually, atRA biosynthesis was observed in the OLN-93 cell line, a permanent cell line derived from spontaneously transformed cells in rat brain glial cultures and resembling primary oligodendrocytes in their antigenic properties (Mey and Hammelmann, 2000).

Granule cells are the major neurons in the dentate gyrus and also undergo adult neurogenesis. A very weak RALDH1 signal was detected in these cells' bodies. However, the processes near the granule cell layer, with axons like structures, showed strong RALDH1 expression pattern. This area belongs to the subgranular zone, one of two areas in which neurogenesis persisted in the adult hippocampus. The presence of RALDH1 in this region might be associated with active atRA signaling that contributes to survival and differentiation of newly formed neurons.

CA3 neuron is the important participant of the perforant pathway, receiving input from mossy fibers and exporting signals to CA1 neurons. However, RALDH1 expression in CA3 neurons is totally different from CA1 region. No RALDH1 signals were detected in CA3 neurons. However, the strongest RALDH1 signals were detected in the mossy fibers which encompass the CA3 neurons bodies and dendrites. The perfect co-localization of RADLH1 with Tau proteins is consistent with *in vitro* observation of high RALDH1 signal on axons. In adult, synaptopodin is found exclusively in the mossy fiber terminals present in the hilar region of the dentate gyrus and the regio inferior of the cornu ammonis (Sun *et al.*, 2006). Co-localization of RALDH1 with synaptopodin further confirmed its unique mossy fiber enriched expression pattern. The expression of RALDH1 on mossy fiber is a typical postnatal developmental change, with obvious RALDH1 expression from 1-month-old rat. The function of this highly expressed

RALDH1 in mossy fiber is unknown. If it functions as a retinal dehydrogenase, it might indicate a highly active atRA signaling present in mossy fibers, which may contribute to the dynamic synaptic plasticity changes in this area.

Besides function as retinal dehydrogenase and binding proteins, RALDH1 is also reported as stem cell marker, especially tumor stem cell (Douville *et al.*, 2009; Ginestier *et al.*, 2007; Jiang *et al.*, 2009). So the presence of RALDH1 in adult rat hippocampus might have a broad potential functions rather than atRA biosynthesis. The cell type and subcellular location specific expression pattern of RALDH1 further imply the possible complex functions of this protein. Glia specific or neurons specific knock out RALDH1 would be a good model to study the exact function of RALDH1 in different area of adult CNS.

Figures and Legends
Figure III-1

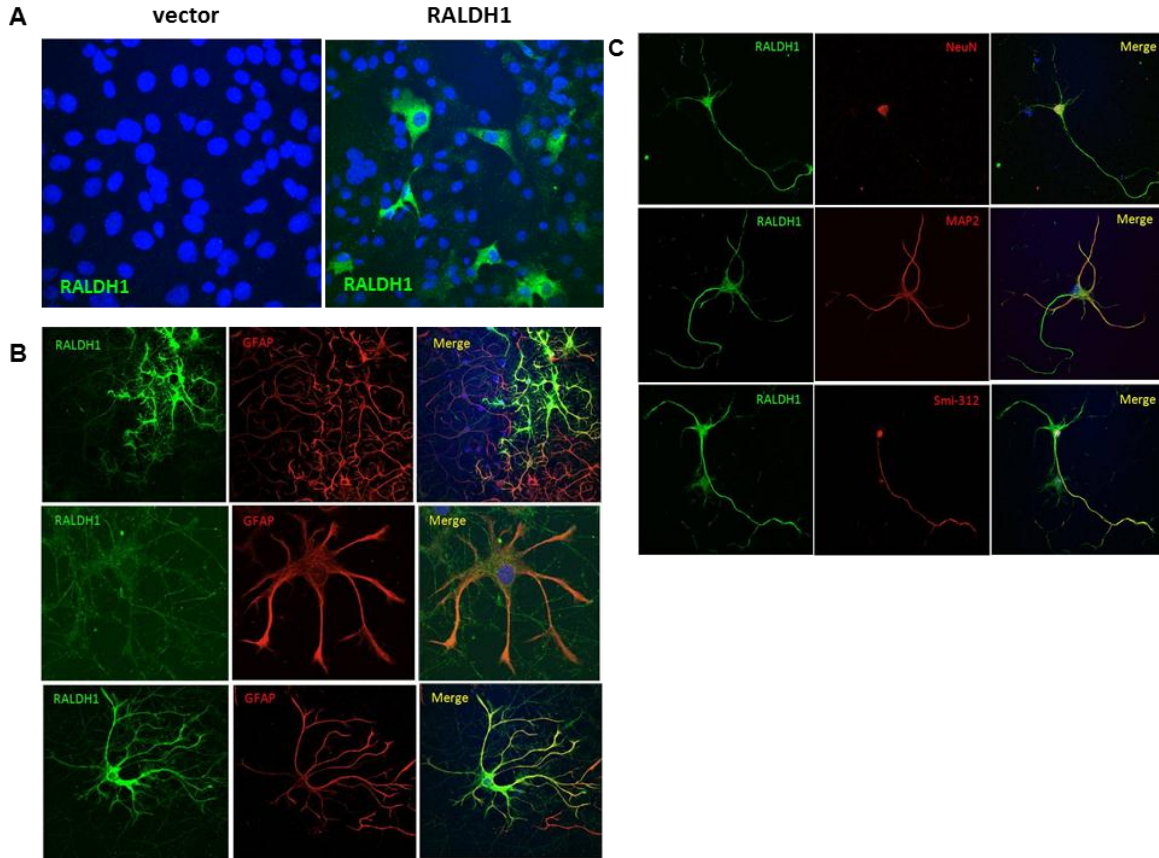


Figure III-1 RALDH1 expression in primary cultured neuronal cells. (A) COS cell transfected with empty vector or RALDH1-pcDNA3.1+ construct were fixed and stained with RALDH1 (green) and DAPI (blue). (B) Mixed cultured neurons-astrocytes were fixed and stained with RALDH1 (green), GFAP (red) and DAPI (blue). The three channels of each set were merged (merged). (C) Cultured neurons were fixed and stained with RALDH1 (green), NeuN, MAP2, SMI-312 (red) and DAPI (blue). The three channels of each set were merged (merged).

Figure III-2

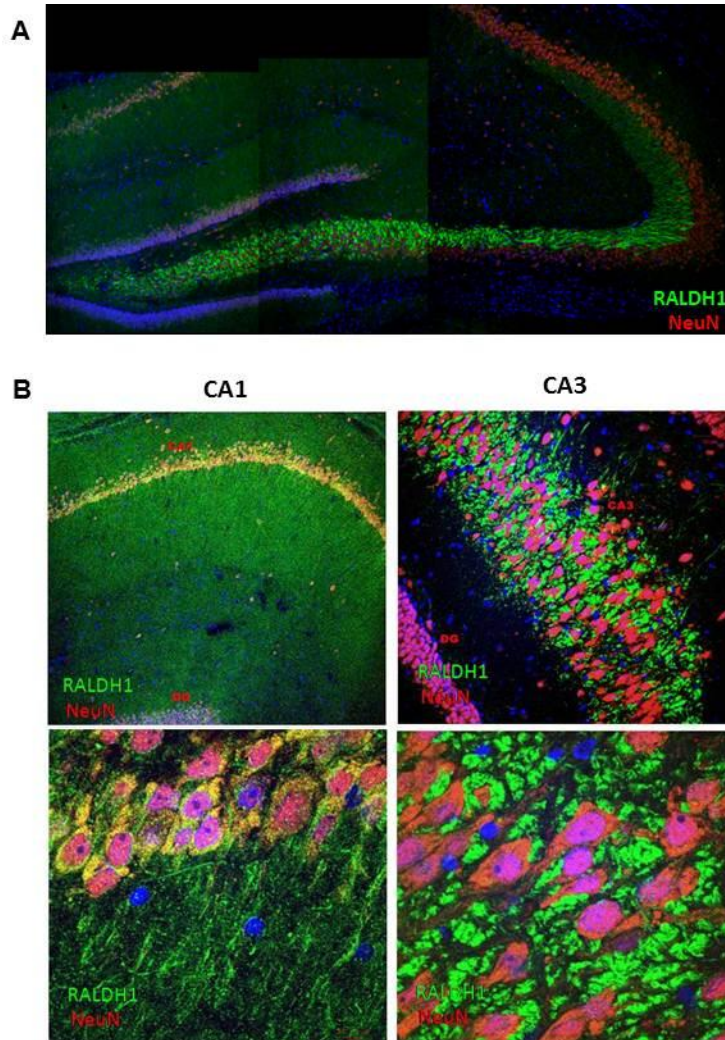


Figure III-2 RALDH1 expression in adult rat hippocampus. (A) 2 month old adult Sprague-Dawley rat hippocampus slice were fixed and stained with RALDH1 (green), NeuN (red) and DAPI (blue). (B) Higher magnification of representative CA1 and CA3 region of hippocampus with same stain as in A. upper panel, 25x; lower panel, 100x.

Figure III-3

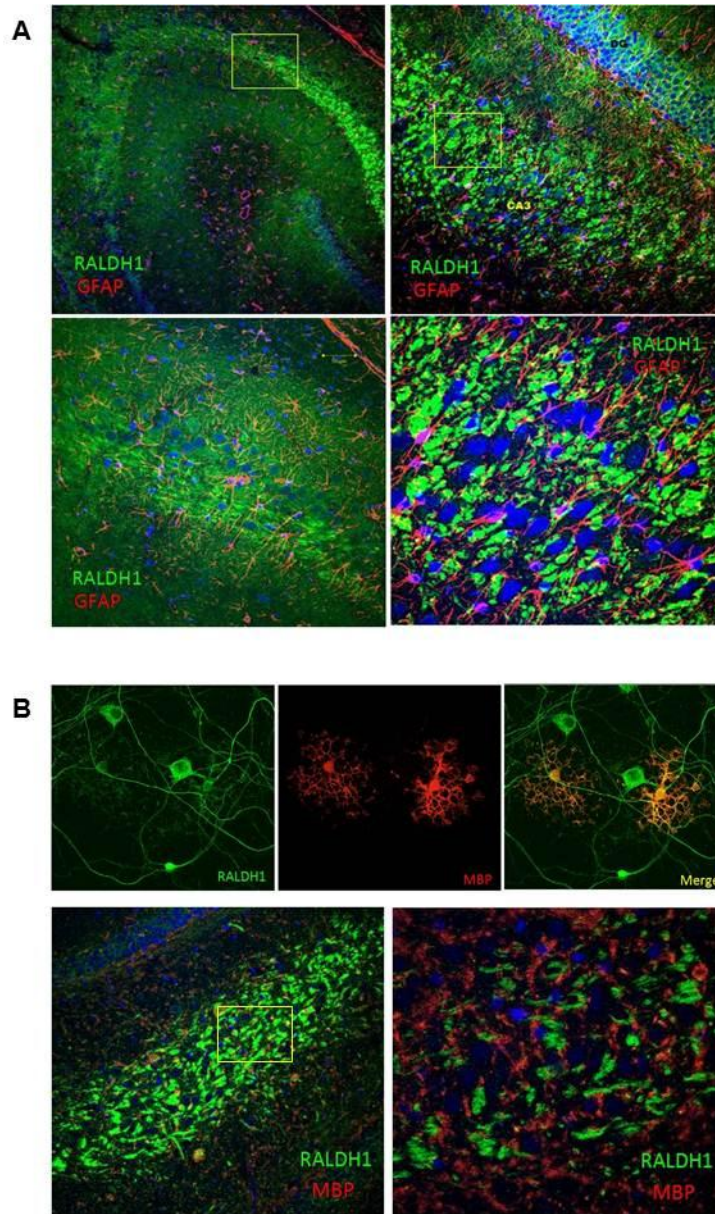


Figure III-3 RALDH1 expression in astrocytes and oligodendrocytes in hippocampus. (A) upper panel, two representative regions of adult rat hippocampus were stained with RALDH1 (green), GFAP (red) and DAPI (blue). Lower panel, higher magnification of selected regions in upper panel with same staining. (B) upper panel, mixed cultured neuron-glia were fixed and stained with RALDH1 (green) and MBP (red). The three channels of each set were merged (merged). Lower panel left, CA3 region of adult rat hippocampus were stained with RALDH1(green), MBP(red) and DAPI (blue); lower panel right, higher magnification of selected region with same staining.

Figure III-4

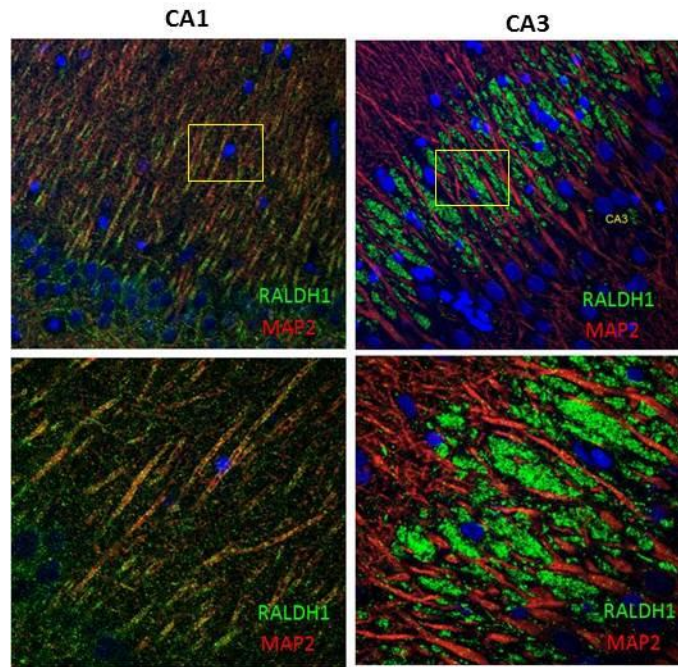


Figure III-4 RALDH1 is expressed in dendrites of CA1 but not CA3 neurons. Upper panel, representative CA1 (left) and CA3 (right) region of adult rat hippocampus fixed and stained with RALDH1 (green), MAP2 (red) and DAPI (blue). Lower panel, higher magnification of selected regions with same staining.

Figure III-5

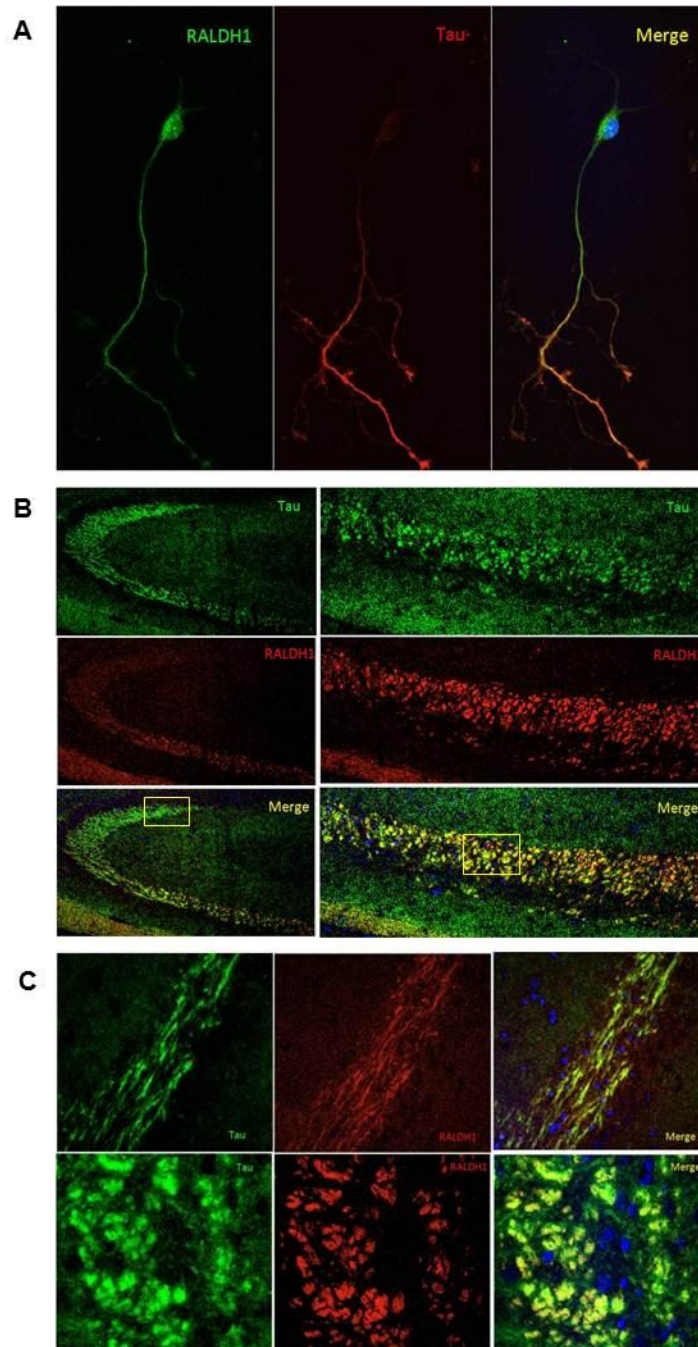


Figure III-5 RALDH1 is co-localized with axons present in CA3 region. (A) Cultured individual neuron was fixed and stained with RALDH1 (green), Tau (red) and DAPI (blue). (B) Representative CA3 region of adult hippocampus were fixed and stained with Tau (green), RALDH1 (red) and DAPI (blue). (C) Higher magnification of selected regions in B with same staining. The three channels were merged (merged).

Figure III-6

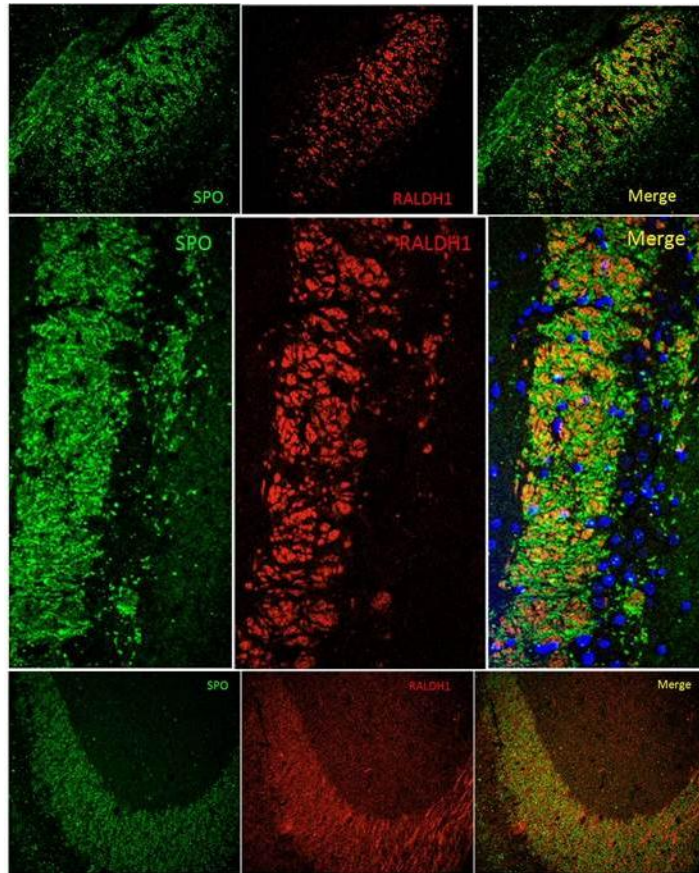


Figure III-6 RALDH1 is expressed on mossy fibers. Three representative areas of CA3 region of adult hippocampus were fixed and stained with synaptoporin (green), RALDH1 (red) and DAPI.

Figure III-7

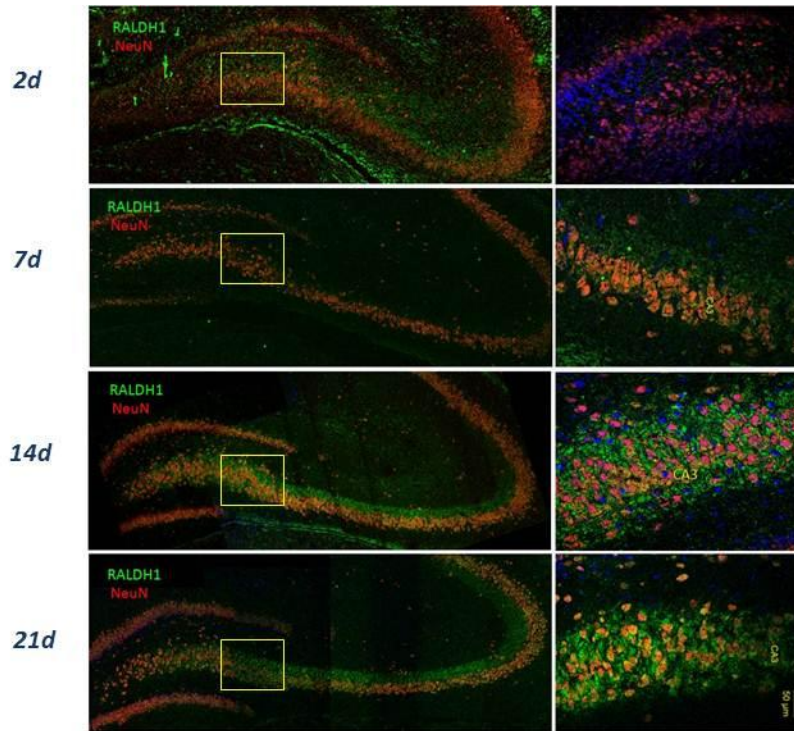


Figure III-7 Developmental changes of RALDH1 expression in adult rat hippocampus. Left panel, hippocampus from P2, P7, P14 and P21 Sprague-Dawley rats were fixed and stained with RALDH1 (green), NeuN (red) and DAPI (blue). Right panel, higher magnification of selected regions in left panel with same staining.

References

- Aoto, J., C. I. Nam, *et al.* (2008). "Synaptic signaling by all-trans retinoic acid in homeostatic synaptic plasticity." Neuron **60**(2): 308-320.
- Asson-Batres, M. A. and W. B. Smith (2006). "Localization of retinaldehyde dehydrogenases and retinoid binding proteins to sustentacular cells, glia, Bowman's gland cells, and stroma: potential sites of retinoic acid synthesis in the postnatal rat olfactory organ." J Comp Neurol **496**(2): 149-171.
- Bradl, M. and H. Lassmann (2010). "Oligodendrocytes: biology and pathology." Acta Neuropathol **119**(1): 37-53.
- Chen, C. and S. Tonegawa (1997). "Molecular genetic analysis of synaptic plasticity, activity-dependent neural development, learning, and memory in the mammalian brain." Annu Rev Neurosci **20**: 157-184.
- Douville, J., R. Beaulieu, *et al.* (2009). "ALDH1 as a functional marker of cancer stem and progenitor cells." Stem Cells Dev **18**(1): 17-25.
- Ginestier, C., M. H. Hur, *et al.* (2007). "ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome." Cell Stem Cell **1**(5): 555-567.
- Gross, C. G. (1993). "Hippocampus minor and man's place in nature: a case study in the social construction of neuroanatomy." Hippocampus **3**(4): 403-415.
- Jiang, F., Q. Qiu, *et al.* (2009). "Aldehyde dehydrogenase 1 is a tumor stem cell-associated marker in lung cancer." Mol Cancer Res **7**(3): 330-338.
- Kempf, M., A. Clement, *et al.* (1996). "Tau binds to the distal axon early in development of polarity in a microtubule- and microfilament-dependent manner." J Neurosci **16**(18): 5583-5592.
- Kobayashi, K. (2010). "Hippocampal mossy fiber synaptic transmission and its modulation." Vitam Horm **82**: 65-85.
- Markiewicz, I. and B. Lukomska (2006). "The role of astrocytes in the physiology and pathology of the central nervous system." Acta Neurobiol Exp (Wars) **66**(4): 343-358.
- McBain, C. J. (2008). "Differential mechanisms of transmission and plasticity at mossy fiber synapses." Prog Brain Res **169**: 225-240.
- Mey, J. and S. Hammelmann (2000). "OLN-93 oligodendrocytes synthesize all-trans-retinoic acid in vitro." Cell Tissue Res **302**(1): 49-58.
- Montague, P., A. S. McCallion, *et al.* (2006). "Myelin-associated oligodendrocytic basic protein: a family of abundant CNS myelin proteins in search of a function." Dev Neurosci **28**(6): 479-487.
- Pereira, F., E. Rosenmann, *et al.* (1991). "The 56 kDa androgen binding protein is an aldehyde dehydrogenase." Biochem Biophys Res Commun **175**(3): 831-838.
- Sun, T., H. S. Xiao, *et al.* (2006). "Differential expression of synaptopodin and synaptophysin in primary sensory neurons and up-regulation of synaptopodin after

- peripheral nerve injury." Neuroscience **141**(3): 1233-1245.
- Witter, M. P., P. A. Naber, *et al.* (2000). "Cortico-hippocampal communication by way of parallel parahippocampal-subicular pathways." Hippocampus **10**(4): 398-410.
- Yamauchi, K. and J. Nakajima (2002). "Effect of coenzymes and thyroid hormones on the dual activities of *Xenopus* cytosolic thyroid-hormone-binding protein (xCTBP) with aldehyde dehydrogenase activity." Eur J Biochem **269**(8): 2257-2264.
- Yamauchi, K., J. Nakajima, *et al.* (1999). "Xenopus cytosolic thyroid hormone-binding protein (xCTBP) is aldehyde dehydrogenase catalyzing the formation of retinoic acid." J Biol Chem **274**(13): 8460-8469.

Chapter IV

TNF α Regulates all-*trans*-RA Biosynthesis in Astrocytes Through Oppositely Changing RALDH1 and RALDH3 Expression

Introduction

Vitamin A deficiency can cause profound reduction of resistance to infection, which indicates that retinoids play an important role in the maintenance and regulation of normal immune system function (Kim, 2008). Overall, RA signaling appears to establish a Th2-type T cell non-inflammatory environment (Pino-Lagos *et al.*, 2010). atRA regulates NO production, enhances IL-1 production and inhibits TNF α production in monocyte/macrophages (Dheen *et al.*, 2005; Kim, 2008; Wang *et al.*, 2007). RA can also regulate migration of dendritic cells (DC) to present antigen to T cells. Not only gut dendritic cells, but also splenic DCs synthesize RA to regulate intestine immunity (Darmanin *et al.*, 2007; Geissmann *et al.*, 2003). Vitamin A also improves antitumor immunity through mechanisms such as induction of cell differentiation and enhancement of migration to lymph nodes (Malkovsky *et al.*, 1983; Mirza *et al.*, 2006). RA synthesis in the immune system is regulated by a wide spectrum of inflammatory mediators, such as IL-4, TLR receptors agonist and atRA acts as an important modulator of immune system (Yokota *et al.*, 2009).

CNS immunity is important for the trauma recovery and for age related neurodegenerative diseases, such as Alzheimer's disease (Hohlfeld *et al.*, 2007). Microglia activation in response to an insult—secretion of a series of inflammatory cytokines, determines the magnitude of neuroinflammatory response (Yang *et al.*, 2010). Glia cells, mainly microglia and astrocytes, become 'activated' or 'reactivated' in response to various insults and secrete proinflammatory cytokines, chemokines, and trophic factors to activate neural immunity (Popovich and Longbrake, 2008). One of the important proinflammatory cytokines secreted in the brain immune response is TNF α (Sriram and O'Callaghan, 2007). TNF α is synthesized as a 26-kDa membrane-bound polypeptide precursor that is cleaved by proteolysis to a 12-kDa subunit. TNF α exerts its effects in autocrine and/or paracrine fashions (Wajant *et al.*, 2003). The biological actions of TNF α are mediated through two distinct cell surface receptors, TNFR1 (p55) and TNFR2 (p75). TNF α exhibits both neurotoxic and neuroprotective roles in the adult CNS, especially during the recovery after CNS injury (Perry *et al.*, 2002).

TNF α contributes to the normal function of the hippocampus. Following transit global ischemia, TNF α was selectively induced in the striatum and hippocampus, but not in other brain areas (Buttini *et al.*, 1996; Meistrell *et al.*, 1997). Furthermore, expression of

TNF α after ischemic brain injury first appeared in neurons and then in astrocytes. TNF α also plays a neuroprotective role against excitotoxic injury in the hippocampus, and mediates neuroprotection in response to acute nitric oxide excitotoxicity (Cheng *et al.*, 1994; Turrin and Rivest, 2006). A lack of highly expressed TNF α is associated with significant chemically induced damage to hippocampus (Little *et al.*, 2002). Mice null of both TNF α receptors are more susceptible to hippocampal excitotoxic and ischemic injury (Bruce *et al.*, 1996).

Astrocytes synthesize and secrete TNF α , whereas TNF α can stimulate proliferation of astrocytes, which is called reactive astrogliosis, a characteristic response of astrocytes to inflammation and trauma of adult CNS (Balasingam *et al.*, 1994). TNF α receptor 2 was quickly and highly induced in astrocytes upon TNF α treatment, which was considered as the potential mechanism that TNF α used to stimulate proliferation of astrocytes.

Because astrocytes can synthesize atRA through multiple retinol dehydrogenases and retinal dehydrogenases, and TNF α is an active regulator of astrocyte activity in the CNS, we tested whether there was any connection between these two important modulators. TNF α performs its regulatory effect through two kinds of receptors TNF R1 and TNF R2 and activates various signaling pathways, such as NF- κ B, c-JNK inducing MAP kinase cascade or p38-MAP kinase cascade (Wajant *et al.*, 2003). Our data show that TNF α can regulate retinal dehydrogenation through oppositely changing RALDH1 and RALDH3 expression. Signaling pathways associated with RALDH1 and RALDH3 regulation by TNF α is different. We also found that TNF α 's regulation on changing atRA biosynthesis is more effective in young astrocytes.

Materials and Methods

Cell culture and treatment--Primary astrocytes were cultured as described in Chapter II. 1 month old primary astrocytes were trypsinized and reseeded in 6-well plates at the concentration of 2×10^5 /well 24 hr before the treatment. Medium were changed the day before the experiments. All of the stimulus, including estrogen (sigma), LPS (sigma), PGE2 (sigma) and TNF α (Invitrogen) were dissolved in water to make the stock solution according to the product manuscript and stored properly. Upon treatment, the stock solution were diluted to desired concentration in the medium and applied on the cultured astrocytes. After 12 hr to 48 hr treatment, new medium containing either retinol or retinal were replaced and incubated for additional 4 hr, retinoids production were assayed and measured as described before.

RT-PCR, Q-PCR and western blot --Same as described in Chapter II, materials and methods

Inhibition of TNF α signal pathways--The inhibitors for TNF α signal pathways were purchased from Enzo life sciences, Inc., including NF- κ B pathway inhibitors IKK-NBD, MAP kinase kinase inhibitor PD98059, P38 inhibitor SB203580 and JNK inhibitor SP600125. All the inhibitors were dissolved in proper solvent as a stock solution according to the product manuscript. Astrocytes medium were changed the day before the experiment. A final concentration of 20 μ M IKK-NBD were pretreated astrocytes for 3 hr, and a final concentration of 20 μ M PD98059, SB203580 and SP600125 were pretreated astrocytes for 1 hr, then 50 ng/ml TNF α were added to the medium for additional 24 hr incubation. Q-PCR of RALDH1 and RLADH3 expression and atRA production from retinol and retinal were detected as described before.

Results

Estrogen, LPS, PGE2 and TNF α don't affect atRA production from retinol in 1 month old astrocytes—To test the effect of different stimuli on atRA production in astrocytes, atRA production from retinol in 1 month old astrocytes were assayed after incremental time incubation with LPS, PGE2 or TNF α . Up to 24 hr incubation of 1 μ M PGE2, 1 μ g/ml LPS or 50 ng/ml TNF α did not change atRA production from retinol (Figure IV-1A). Dose response of these stimuli was also tested. 10 μ M estrogen had no effect on atRA production, but 50 μ M and 100 μ M estrogen slightly decreased atRA biosynthesis from retinol after 3 days treatment (Figure IV-1B). However, this high concentration of estrogen also cause significant cell death, which indicated that such high concentration estrogen might be toxic to astrocytes (data not shown). Up to 10 μ g/ml LPS and up to 100 ng/ml TNF α had no effect on atRA synthesis from retinol (Figure IV-1C and 1D) in 1 month old astrocytes. All these data suggest that atRA homeostasis is fairly stable in 1 month old astrocytes and resistant to these extracellular stimuli.

TNF α treatment impaired atRA production from retinal via inversely regulation of RALDH1 and RALDH3 expression in 1 month old astrocytes--Although TNF α didn't affect atRA biosynthesis from retinol, retinal dehydrogenation activity of astrocytes was significantly reduced after TNF α treatment (Figure IV-2A and 2B). 1 month old astrocytes pre-incubated with 100 ng/ml TNF α for 24 hr synthesized significantly less atRA from retinal compared with control group and the reduction was more robust after 48 hr pre-incubation, which indicated that a translational change or activity impairment of retinal dehydrogenase occurred upon TNF α treatment (Figure IV-2A). Dose response of TNF α treatment on astrocytes revealed that as low as 10 ng/ml TNF α treatment could result in the similar reduction of retinal dehydrogenation activity as 100 ng/ml TNF α treatment (Figure IV-2B). RALDH1 and RALDH2 play key roles in atRA production from retinal in astrocytes. To test whether reduction of retinal dehydrogenation after TNF α treatment is due to the change of RALDH activity, the expression level of RALDHs were measured either after different times of incubation or under different concentrations of TNF α (Figure IV-2C and 2D). A significant TNF α incubation time dependent and dose dependent decrease of RALDH1 expression was found. The time dependent reduction of RALDH1 is consistent with the timeline of decrease retinal dehydrogenation upon TNF α treatment. Surprisingly, TNF α incubation also resulted in a robust increase of RALDH3 expression in the same time. Quantification analysis revealed that decrease of RALDH1 expression and increase of RALDH3 expression depended on the TNF α concentration (Figure IV-2E and 2F). TNF α induced decrease of RALDH1 protein was confirmed by western-blot (Figure IV-2G). The opposite change of RALDH1 and RALDH3 expression resulted in a net decrease of atRA production from retinal, which indicated that increase of RALDH3 activity could not compensate the loss of RALDH1 activity in 1 month old astrocytes. These results were also consistent with previous conclusion that RALDH1 is the major retinal dehydrogenase that contributes to

atRA biosynthesis.

TNF α treatment result in different atRA production in 2 weeks and 3 months astrocytes--There was a significant increase of RALDH1 expression along with extended culture time in primary astrocytes. However, RALDH3 expression didn't change during that period (Chapter II). To test whether TNF α can induce similar gene expression changes in young age astrocytes, we compared RALDH1, RALDH3 expression and atRA production from retinol or retinal in 2 weeks and 3 months old astrocytes. Same as 1 month old astrocyte, 100 ng/ml TNF α up to 48 hr treatment didn't change atRA production from retinol (Figure IV-3A) but caused a time dependent decrease of atRA production from retinal (Figure IV-3B). Opposite expression of RALDH1 and RALDH3 were also observed in 3 months old astrocytes, confirmed by RT-PCR and Q-PCR analysis (Figure IV-3D). TNF α treatment induced similar expression change of RALDH1 and RALDH3 in 2 weeks old astrocytes as 1 month or 3 months old astrocytes (Figure IV-3C). No other genes changes were observed in 2 weeks old astrocytes (data not shown). However, a significant increase of atRA production from retinol and retinal were observed in 2 weeks old astrocytes (Figure IV-3A and 3B). Increase of atRA production from retinol and retinal started as early as 12 hr TNF α treatment, consistent with the RALDH1 and RALDH3 mRNA change (Figure IV-3C and 3D). No additional increase atRA accumulation were detected as elongation of incubation time with TNF α . Interestingly, atRA synthesis from retinol increased around 3 fold while incubation with retinal only increased ~1.5 fold. Q-PCR analysis revealed the relative amount of RALDH1 and RALDH3 expression upon TNF α treatment in 2 weeks or 3 months astrocytes (Figure IV-3C and 3D lower panel). In the absence of TNF α , consistent with previous results, ~500 fold increase of RALDH1 expression were detected in 3 months old astrocytes while RALDH3 didn't change. RALDH1 is always the predominant RALDH according to their relative amount in 2 weeks and 3 months astrocytes. However, in 2 weeks old astrocytes, upon TNF α induction, RALDH3, instead of RALDH1, become the primary RALDH with highest expression level (~100 fold higher than RALDH1). The increase amount of RALDH3 mRNA is much higher than the decrease amount of RALDH1 mRNA. In contrast, in 3 months old astrocytes, though down regulation of RALDH1 upon TNF α treatment, the predominant RALDH is still RALDH1, with ~5 fold higher expression level than the increased RALDH3. The decrease amount of RALDH1 is much higher than the increase amount of RALDH3 mRNA. Besides RALDH1 and RALDH3, we also observed an increased CRBPI expression in both 2 weeks and 3 months astrocytes (Figure IV-3C and 3D, upper panel). All these data indicate that the different change of atRA production upon TNF α treatment in 2 weeks and 3 months astrocytes might be due to the different absolute amount change of RALDH1 and RALDH3.

TNF α regulate RALDH1 and RALDH3 expression through different signal pathways--The opposite regulation of RALDH1 and RALDH3 expression in astrocytes by TNF α raised the question that how the same stimulation can cause inverse effect. As

we know, TNF α executes its different functions through multiple signal pathways. To test whether activation of different signal pathways contribute to the opposite regulation of RALDH1 and RALDH3, NF- κ B, JNK, MEK and P38 signal pathways were blocked by specific inhibitors upon the stimulation of TNF α . The expression of RALDH1 and RALDH3 mRNA were measured and quantified (Figure IV-4A and 3B). Blocking NF- κ B had no effect on either RALDH1 or RALDH3 expression. Blocking JNK and MEK signal pathway significantly reversed the down regulation of RALDH1 mRNA upon TNF α treatment (Figure IV-3A) although neither of this blocking can totally abolish the effect of TNF α . Blocking P38 signal pathway can partially reverse the up regulation of RALDH3 expression which indicate that P38 pathway participates in the regulation of RALDH3 expression upon TNF α treatment, at least in part (Figure IV-3B). Interestingly, block of JNK pathway can significantly further increase RALDH3 expression, indicating that the effect of TNF α on RALDH3 expression is much more complicated. To test whether these RALDH1 and RALDH3 changes will ultimately reflect the overall retinal dehydrogenation activity change, JNK, MEK and P38 signal pathways were blocked by specific inhibitors and atRA production from retinal were measured (Figure IV-3C). Consistent with the blocking effect on RALDH1 and RALDH3 gene changes, blocking JNK and MEK pathways, which reverse the RALDH1 down regulation, resulted in the increase of retinal dehydrogenation compared with the TNF α treatment group. Inhibition of JNK pathway exhibited higher atRA production compared with MEK inhibition, which might be a result of additional increase of RALDH3 expression by blocking JNK pathway. Inhibition of P38 pathway resulted in a slightly further decrease of atRA production from retinal compared with TNF α group, which was also consistent with the partial reverse of RALDH3 up regulation by blocking this signal pathway. No significant increase of atRA production from retinol was found when these signal pathways were blocked except JNK pathway, which may be due to the increase of RALDH3 expression (Figure IV-3D).

Discussion

Retinoic acid is a powerful inducer for cell precursor differentiation. Chen's research also proved that high concentration of RA can stimulate the outgrowth of neurite broadly and quickly (Chen and Napoli, 2008). The concentration of atRA must be tightly regulated in adult hippocampus to maintain the normal function of mature neurons and induce the differentiation of neuron precursors. In previous chapters, we already demonstrated that astrocytes can synthesize atRA in hippocampus but this ability is tightly regulated by either the expression of multiple retinol and retinal dehydrogenases or by feedback regulation of RA degradation and RE formation through atRA itself. However, we haven't found any other factors beyond the atRA itself which participate into the regulation of atRA homeostasis in adult CNS.

In reproductive system, Ong and his colleges have demonstrated that estrogen, a primary female sex hormone, can regulate RALDH2 and CRBP expression to control atRA production in uterus. The RA production ability is fluctuated along with the change of estrogen level in female (Li *et al.*, 2004; Li and Ong, 2003). Since hippocampus is also able to synthesize and secrete estrogen, we hypothesize that the estrogen in the hippocampus can regulate atRA production (Kretz *et al.*, 2004). The other important system that might interact with retinoids signaling is the immune system. Lots of evidence showed that retinoids, especially atRA is an important inducer and regulator of the function of immune system, such as, secretion of different inflammatory cytokines like IL-6 and IFN γ (Pino-Lagos *et al.*, 2010). PGE2, LPS and TNF α also play important functions in regulating the immune response by inducing the expression of these cytokines (Sriram and O'Callaghan, 2007; Zolfaghari *et al.*, 2007). In central nerve system, astrocytes and microglia are two major cell types that respond to environmental insult and regulate the immune response. Since we proved that astrocytes could synthesize and secrete atRA, we are wondering whether these factors that can induce inflammatory cytokines up regulation can also alert atRA synthesis to exhibit their functions.

Our data revealed that none of these factors, including estrogen, LPS, PGE2 and TNF α can affect atRA synthesis from retinol in 1 month old astrocytes. However, TNF α significantly reduced atRA production from retinal in the same age astrocytes which indicated that TNF α could affect retinal dehydrogenation activity in astrocytes. Decrease of RALDH1 upon TNF α treatment can contribute to the decrease of atRA synthesis from retinal. Surprisingly, we also detected a robust increase of RALDH3 expression which should have reversal effect on atRA production from retinal. The up regulation of RALDH3 reached the maximum level earlier than decrease expression of RALDH1—there was no further increased expression of RALDH3 from 12h to 48h whereas a continuous decrease of RALDH1 expression was found upon TNF α treatment. So the response of RALDH3 to TNF α may be much earlier and more sensitive than RALDH1 because as low as 10 ng/ml TNF α can induce dramatic increase of RALDH3 but not on

RALDH. This quick and sensitive response of RALDH3 upon TNF α treatment indicates that RALDH3 might be the enzyme that responds to TNF α in the early inflammation period to regulate atRA production. The longer response time and higher response dose of RALDH1 expression upon TNF α treatment indicate that change of RALDH1 might be a secondary effect of TNF α treatment. Whether increase of RALDH3 is a precondition for down regulation of RALDH1 is unclear and need to be further investigated upon the deletion of RALDH3 in astrocytes.

The net effect of increase of RALDH3 and decrease of RALDH1 in 1 month old astrocytes is to cause a decrease of atRA production from retinal after at least 24 hr treatment. However, this reverse change of two enzymes which have similar catalytic activity sounds biologically inefficient. Considering that RALDH1 is only highly expressed in pure cultured astrocytes but not in mixed culture or in hippocampus *in vivo*, the response we found in these one month old astrocytes might not reflect the real situation *in vivo*. We utilized much younger—2weeks old astrocytes, which exhibit fair low RALDH1 level, to investigate whether the quick increase of RALDH3 has any significant biological effect upon TNF α treatment. Different from 1months and 3months old astrocytes, these 2weeks old, low RALDH1 expression astrocytes showed a dramatic response to TNF α treatment and upon this stimuli, atRA production was significantly increased from both retinol and retinal, indicating that in astrocytes with low expression of RALDH1, TNF α can up regulate atRA biosynthesis through increase RALDH3 expression. In other words, in low RALDH1 expression astrocytes, the increase amount of RALDH3 expression upon TNF α stimulation can overcome the decrease of RALDH1 expression, result in a net increase of atRA production. Vice versa, in high RALDH1 expression astrocytes, such as pure astrocytes older than 1 month, the increase of RALDH3 cannot compensate the decrease of RALDH1 which result in a net decrease of atRA production from retinal.

The 2 weeks old astrocytes not only mimic the low RALDH1 expression astrocytes' response to TNF α treatment, but may also reflect the response of new formed astrocytes to elevated TNF α level in the regeneration after CNS trauma. In this pathological condition, increased TNF α level is usually one of the representative phenomenon indicating the activation of immune response in the injury area (Sriram and O'Callaghan, 2007). Proliferation and activation of newly formed astrocytes is also present after CNS trauma (Balasingam *et al.*, 1994). According to our data, elevated TNF α might quickly stimulate the increase expression of RALDH3, which cause increase secretion of atRA from astrocytes and these atRA is known to play an important role in brain trauma recovery (Maden and Hind, 2003; Mey *et al.*, 2005; Schrage *et al.*, 2006; Yee and Rawson, 2000). The decrease of RALDH1 expression upon TNF α treatment might be a secondary effect responding to increase of atRA production. However, inhibition of TNF α signaling pathway revealed that this change can be directly abolished by blocking JNK and MEK signaling pathway, indicating that TNF α can directly regulate RALDH1 expression. The biological meaning of opposite down regulation RALDH1 remained

unclear. One possibility is to limit the effect of increase of RALDH3 expression to tightly control atRA level. This could be another unknown mechanism that astrocytes utilize to tightly control atRA level. Furthermore, upon TNF α treatment on 1 month or 3 month old astrocytes, though atRA production from retinal significantly decreased, atRA production from retinol didn't change, which confirmed that astrocytes has a strong regulatory mechanism to control the atRA homeostasis, to avoid abnormal atRA production. The increase expression of CRBPI might provide a clue to study the atRA homeostasis upon TNF α treatment. Since this retinol binding protein participate in both retinol dehydrogenation and retinol esterification, the increased level of CRBPI might redirect the proportion of retinol that is used for atRA synthesis and RE formation in astrocytes. Since RALDH1 may perform additional function rather than retinal dehydrogenase in neurons, we cannot exclude the same possibility in astrocytes. The biological significance of down regulation of RALDH1 upon TNF α treatment might be more complex.

A complicated TNF α response system is present in astrocytes. Different signaling pathway is responsible for RALDH1 down regulation and RALDH3 up regulation. Different signaling pathways might also account for different time response of RALDH1 and RALDH3 expression change. JNK and MEK are the two signaling pathways that responsible for TNF α induced reduction of RALDH1 expression, whether a synergistic effect of these two signaling pathways is present need to be further investigated by double blocking. P38 is at least one of the signaling pathway that TNF α utilize to regulate RALDH3 expression. The partial reverse effect by blocking P38 pathway indicates that other pathways and/or mechanisms are required for regulation of RALDH3 expression upon TNF α treatment. The mechanism how these signaling pathways affect RALDH1 and RALDH3 expression is unclear. Further elucidation of the other components of these signaling pathways, including TNF α receptor, transcription factors and co-activators or co-repressors need to be investigated. The inhibition of JNK, MEK and P38 pathway can partially reverse TNF α 's effect on atRA production from retinal, which is consistent with their effect on regulating RALDH1 and RALDH3 expression, further confirm that TNF α do regulate atRA biosynthesis in astrocytes through regulation expression of RALDH1 and RALDH3. Interestingly, JNK inhibition results in increase expression of RALDH3, which indicate that TNF α 's regulation on those genes expression might be more complicated.

Figures and Legends
Figure IV-1

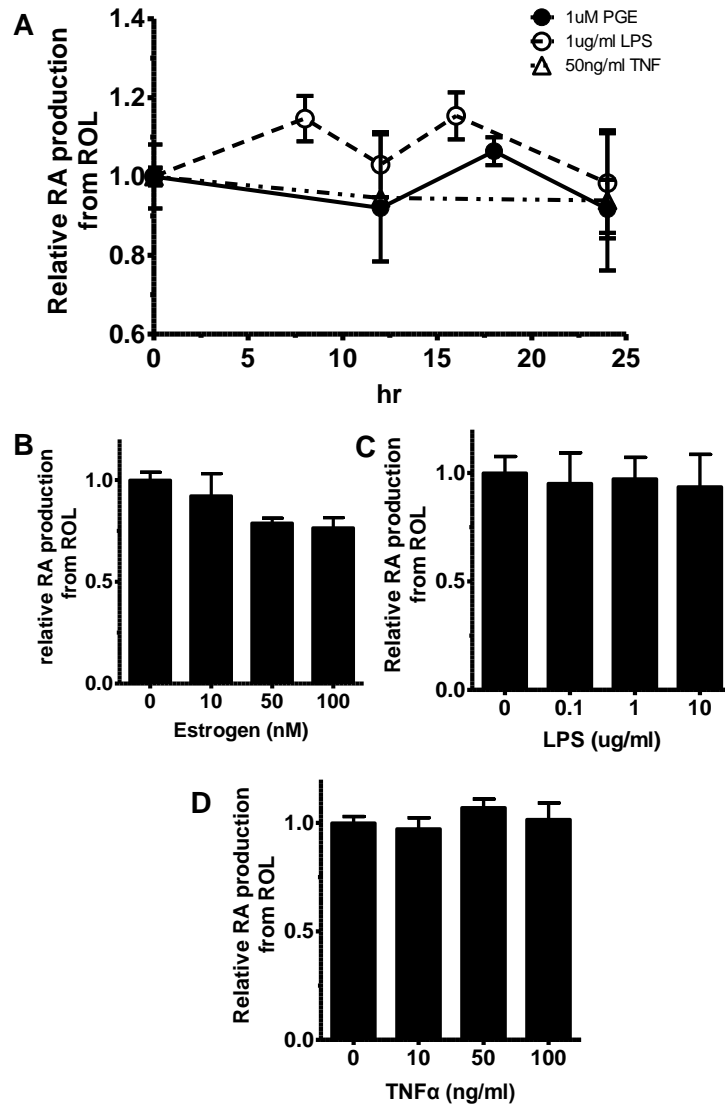


Figure IV-1 atRA production from retinol in 1 month old astrocytes remains stable upon different stimuli. (A) 1 month old primary astrocytes were treated with 1 μ M PGE₂, 1 μ g/ml LPS and 50 ng/ml TNF α for the time indicated, atRA production from 2 μ M ROL for 4 hr incubation were quantified and normalized to control group. (B) Primary astrocytes cultured for 1 month were incubated for 3 days with graded concentrations of estrogen, atRA production were quantified and normalized to non-treated group. (C) Primary astrocytes cultured for 1 month were incubated for 12 hr with graded concentrations of LPS, atRA production from 2 μ M retinol 4 hr incubation were quantified and normalized to non-treated group. (D) Primary astrocytes cultured for 1 month were incubated for 24 hr with graded concentrations of TNF α , atRA production

from 2 μ M retinol 4 hr incubation were quantified and normalized to non-treated group

Figure IV-2

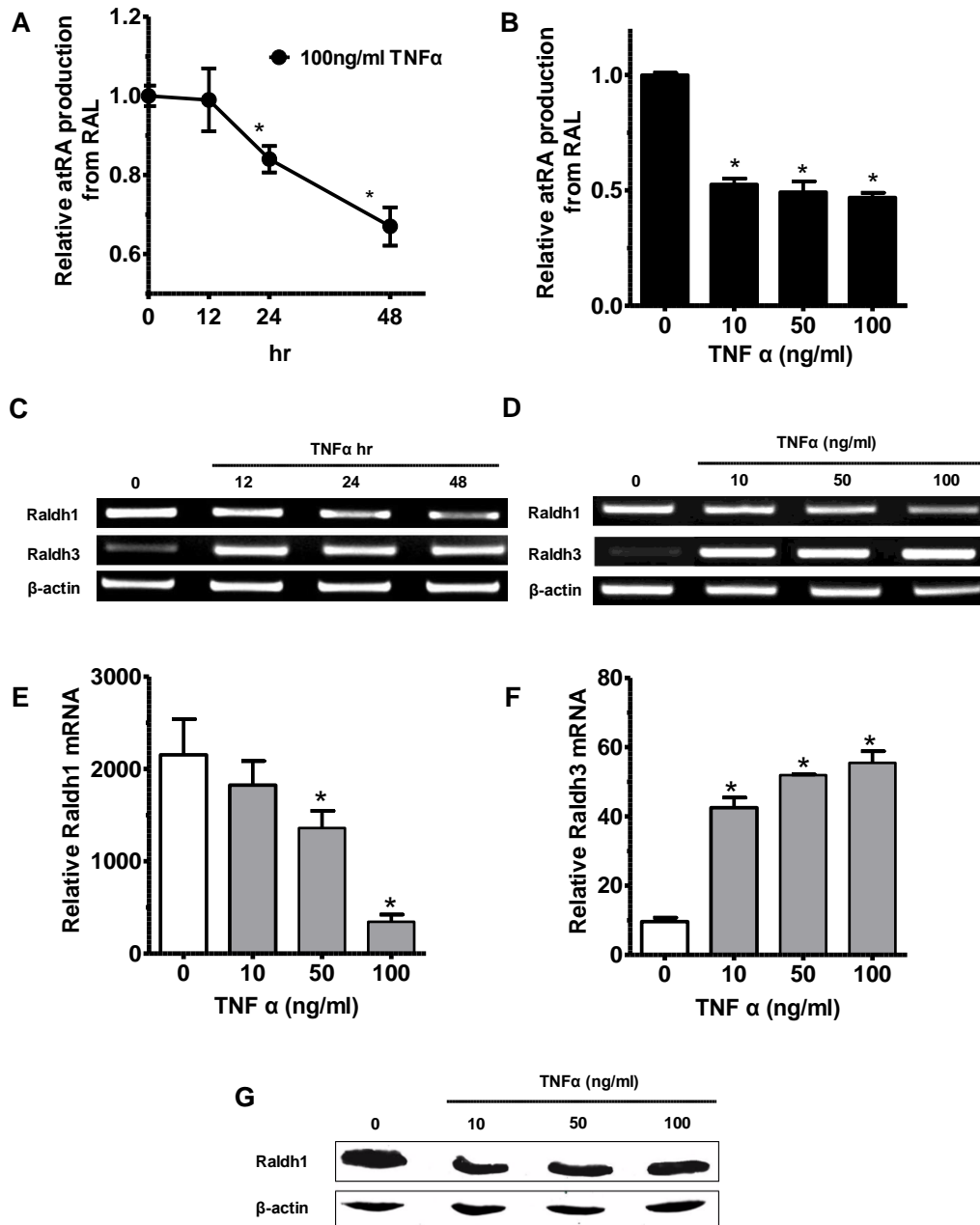


Figure IV-2 TNF α affect retinal dehydrogenation in astrocytes through oppositely changing RALDH1 and RALDH3 expression. 1 month astrocytes were incubated with 100 ng/ml TNF α for the time indicated, atRA production from 1 μ M retinal for 4 hr incubation were quantified and normalized to untreated group (A); mRNA of RALDH1 and RALDH3 expression were measured by RT-PCR (C). Primary astrocytes cultured for 1 month were incubated for 24 hr with graded concentrations of TNF α , atRA production

incubated with 1 μ M retinal were quantified and normalized to untreated group (B); mRNA of Raldh1 and Raldh3 expression were measured by RT-PCR (D) and quantified by Q-PCR (E-F); Protein level of RALDH1 were measured by western-blot(G)

Figure IV-3

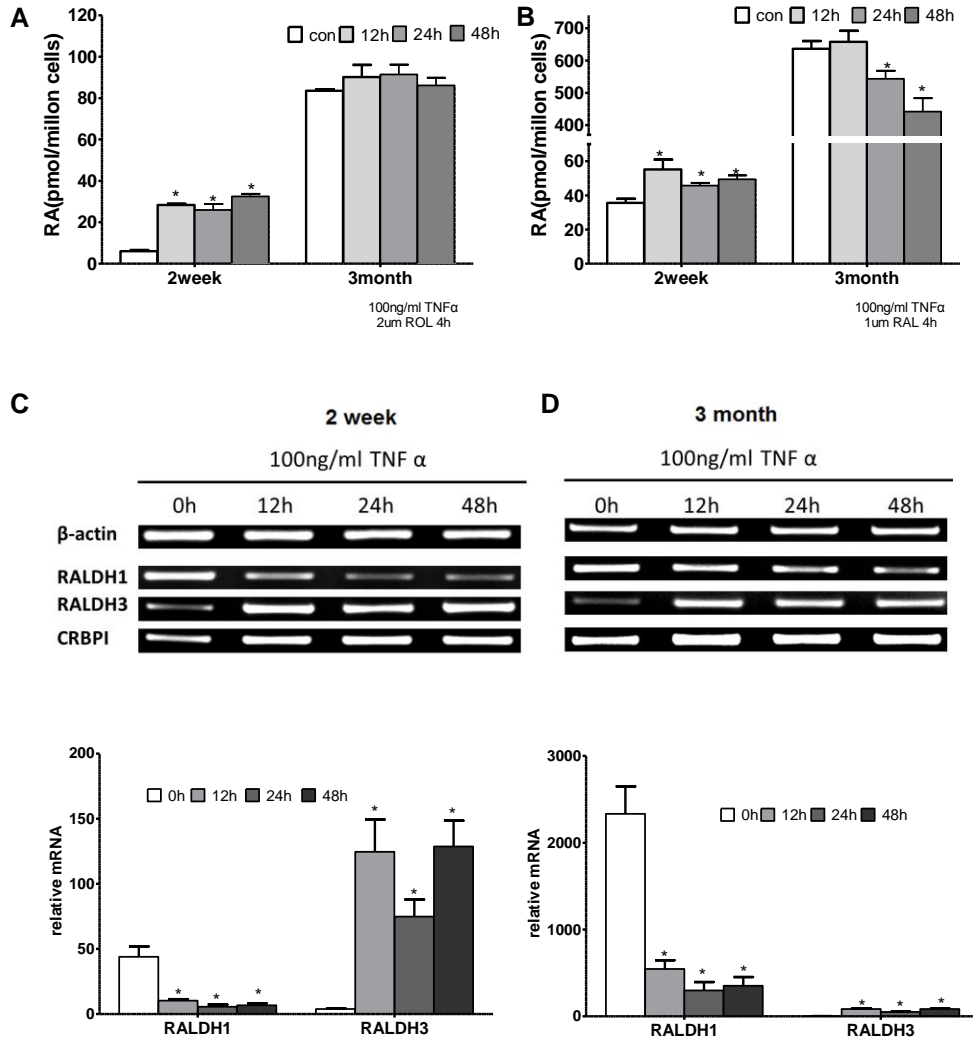


Figure IV-3 TNF α induces similar change of RALDH1 and RALDH3 expression but different atRA production in 2 weeks and 3 months old astrocytes. A and B) 2 weeks or 3 months astrocytes were incubated with 100 ng/ml TNF α for 12 hr, 24 hr and 48 hr, atRA production from 2 μ M retinol or 1 μ M retinol 4 hr incubation were measured. C and D) RALDH1, RALDH3 and CRBPI mRNA were detected by RT-PCR (upper panel), RALDH1 and RALDH3 mRNA were quantified by Q-PCR (lower panel) from 2 weeks old (C) or 3 months (D) astrocytes which received same TNF α treatment as A and B. *P<0.01, n=3

Figure IV-4

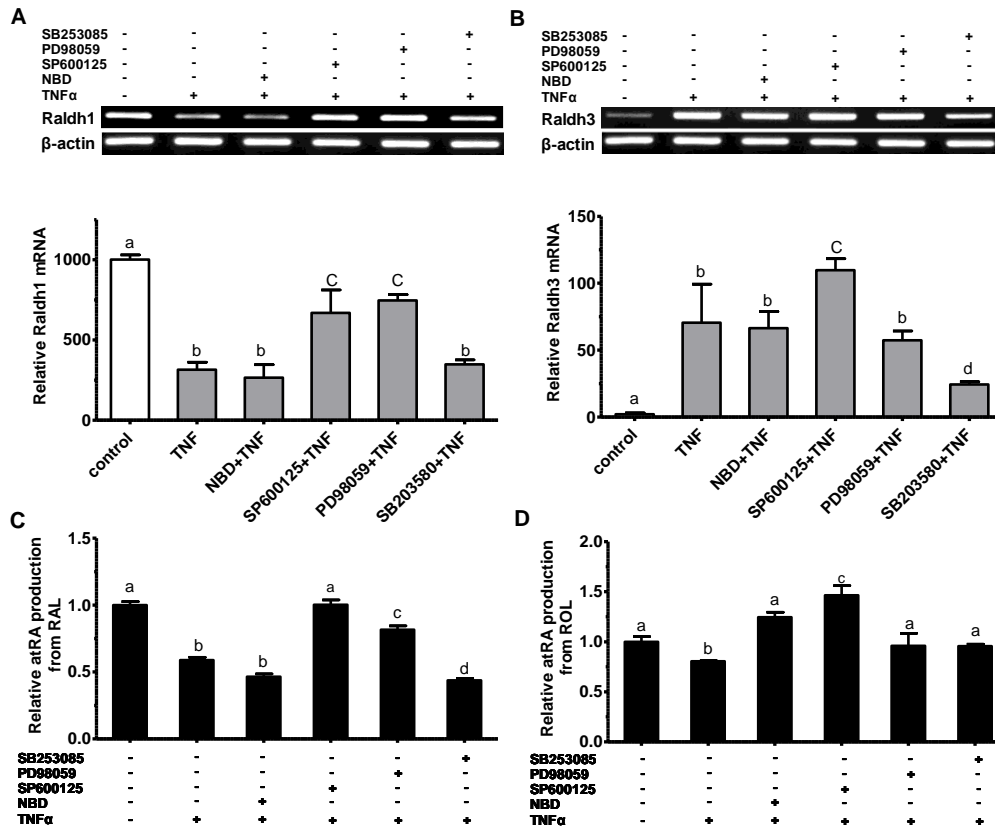


Figure IV-4 TNF α affect RALDH1 expression *via* JNK and MEK pathway; RALDH3 expression partially *via* P38 pathway. 1 month old primary astrocytes were incubated with 50 ng/ml TNF α for 24 hr after pre-treatment with either 20 μ M NBD (NF- κ B inhibitor) for 3 hr, or 20 μ M SP600125 (JNK inhibitor), PD98059 (MEK inhibitor), SB253058 (P38 inhibitor) for 1 hr, separately. (A) mRNA of RALDH1 were detected by RT-PCR (upper panel) and quantified by Q-PCR (lower panel). (B) mRNA of Raldh3 were detected by RT-PCR (upper panel) and quantified by Q-PCR (lower panel). (C-D) Primary astrocytes which were pretreated with same inhibitors as indicated in A were incubated with 50 ng/ml TNF α for 48 hr, atRA production from 1 μ M retinal (C) or 2 μ M retinol (D) were quantified and normalized to untreated group. a,b,c,d indicate statistically different groups; columns labeled with different letters are significant different with each other.

References

- Balasingam, V., T. Tejada-Berges, *et al.* (1994). "Reactive astrogliosis in the neonatal mouse brain and its modulation by cytokines." J Neurosci **14**(2): 846-856.
- Bruce, A. J., W. Boling, *et al.* (1996). "Altered neuronal and microglial responses to excitotoxic and ischemic brain injury in mice lacking TNF receptors." Nat Med **2**(7): 788-794.
- Buttini, M., K. Appel, *et al.* (1996). "Expression of tumor necrosis factor alpha after focal cerebral ischaemia in the rat." Neuroscience **71**(1): 1-16.
- Chen, N. and J. L. Napoli (2008). "All-trans-retinoic acid stimulates translation and induces spine formation in hippocampal neurons through a membrane-associated RARalpha." FASEB J **22**(1): 236-245.
- Cheng, B., S. Christakos, *et al.* (1994). "Tumor necrosis factors protect neurons against metabolic-excitotoxic insults and promote maintenance of calcium homeostasis." Neuron **12**(1): 139-153.
- Darmanin, S., J. Chen, *et al.* (2007). "All-trans retinoic acid enhances murine dendritic cell migration to draining lymph nodes via the balance of matrix metalloproteinases and their inhibitors." J Immunol **179**(7): 4616-4625.
- Dheen, S. T., Y. Jun, *et al.* (2005). "Retinoic acid inhibits expression of TNF-alpha and iNOS in activated rat microglia." Glia **50**(1): 21-31.
- Geissmann, F., P. Revy, *et al.* (2003). "Retinoids regulate survival and antigen presentation by immature dendritic cells." J Exp Med **198**(4): 623-634.
- Hohlfeld, R., M. Kerschensteiner, *et al.* (2007). "Dual role of inflammation in CNS disease." Neurology **68**(22 Suppl 3): S58-63; discussion S91-56.
- Kim, C. H. (2008). "Roles of retinoic acid in induction of immunity and immune tolerance." Endocr Metab Immune Disord Drug Targets **8**(4): 289-294.
- Kretz, O., L. Fester, *et al.* (2004). "Hippocampal synapses depend on hippocampal estrogen synthesis." J Neurosci **24**(26): 5913-5921.
- Li, X. H., B. Kakkad, *et al.* (2004). "Estrogen directly induces expression of retinoic acid biosynthetic enzymes, compartmentalized between the epithelium and underlying stromal cells in rat uterus." Endocrinology **145**(10): 4756-4762.
- Li, X. H. and D. E. Ong (2003). "Cellular retinoic acid-binding protein II gene expression is directly induced by estrogen, but not retinoic acid, in rat uterus." J Biol Chem **278**(37): 35819-35825.
- Little, A. R., S. A. Benkovic, *et al.* (2002). "Chemically induced neuronal damage and gliosis: enhanced expression of the proinflammatory chemokine, monocyte chemoattractant protein (MCP)-1, without a corresponding increase in proinflammatory cytokines(1)." Neuroscience **115**(1): 307-320.
- Maden, M. and M. Hind (2003). "Retinoic acid, a regeneration-inducing molecule." Dev Dyn **226**(2): 237-244.

- Malkovsky, M., C. Dore, *et al.* (1983). "Enhancement of specific antitumor immunity in mice fed a diet enriched in vitamin A acetate." Proc Natl Acad Sci U S A **80**(20): 6322-6326.
- Meistrell, M. E., 3rd, G. I. Botchkina, *et al.* (1997). "Tumor necrosis factor is a brain damaging cytokine in cerebral ischemia." Shock **8**(5): 341-348.
- Mey, J., J. M. D., *et al.* (2005). "Retinoic acid synthesis by a population of NG2-positive cells in the injured spinal cord." Eur J Neurosci **21**(6): 1555-1568.
- Mirza, N., M. Fishman, *et al.* (2006). "All-trans-retinoic acid improves differentiation of myeloid cells and immune response in cancer patients." Cancer Res **66**(18): 9299-9307.
- Perry, S. W., S. Dewhurst, *et al.* (2002). "Tumor necrosis factor-alpha in normal and diseased brain: Conflicting effects via intraneuronal receptor crosstalk?" J Neurovirol **8**(6): 611-624.
- Pino-Lagos, K., Y. Guo, *et al.* (2010). "Retinoic acid: A key player in immunity." Biofactors.
- Popovich, P. G. and E. E. Longbrake (2008). "Can the immune system be harnessed to repair the CNS?" Nat Rev Neurosci **9**(6): 481-493.
- Schrage, K., G. Koopmans, *et al.* (2006). "Macrophages and neurons are targets of retinoic acid signaling after spinal cord contusion injury." Eur J Neurosci **23**(2): 285-295.
- Sriram, K. and J. P. O'Callaghan (2007). "Divergent roles for tumor necrosis factor-alpha in the brain." J Neuroimmune Pharmacol **2**(2): 140-153.
- Turrin, N. P. and S. Rivest (2006). "Tumor necrosis factor alpha but not interleukin 1 beta mediates neuroprotection in response to acute nitric oxide excitotoxicity." J Neurosci **26**(1): 143-151.
- Wajant, H., K. Pfizenmaier, *et al.* (2003). "Tumor necrosis factor signaling." Cell Death Differ **10**(1): 45-65.
- Wang, X., C. Allen, *et al.* (2007). "Retinoic acid enhances the production of IL-10 while reducing the synthesis of IL-12 and TNF-alpha from LPS-stimulated monocytes/macrophages." J Clin Immunol **27**(2): 193-200.
- Yang, I., S. J. Han, *et al.* (2010). "The role of microglia in central nervous system immunity and glioma immunology." J Clin Neurosci **17**(1): 6-10.
- Yee, K. K. and N. E. Rawson (2000). "Retinoic acid enhances the rate of olfactory recovery after olfactory nerve transection." Brain Res Dev Brain Res **124**(1-2): 129-132.
- Yokota, A., H. Takeuchi, *et al.* (2009). "GM-CSF and IL-4 synergistically trigger dendritic cells to acquire retinoic acid-producing capacity." Int Immunol **21**(4): 361-377.
- Zolfaghari, R., C. J. Cifelli, *et al.* (2007). "Lipopolysaccharide opposes the induction of CYP26A1 and CYP26B1 gene expression by retinoic acid in the rat liver in vivo." Am J Physiol Gastrointest Liver Physiol **292**(4): G1029-1036.

Chapter V

Summary and Future Directions

This dissertation established a retinoid homeostasis model in primary cultured astrocytes and neurons, revealed specific RALDH1 expression pattern *in vitro* and *in vivo*, and elucidated effects and mechanisms of TNF α on atRA biosynthesis in astrocytes. To our knowledge, we are the first group to demonstrate that primary cultured astrocytes, but not neurons, from hippocampus origin can synthesize atRA through multiple retinol dehydrogenases and retinal dehydrogenases by an accurate LC/MS/MS technology. Our work also systematically and comprehensively elucidates retinoids metabolism kinetics (both time-course and dose-curve) and atRA feedback regulation on its own homeostasis. Besides that, several novel phenomenon, maybe neural cells specific, were observed in our research, including transportation of atRA from astrocytes to pyramidal neurons, different subcellular locations of Raldh1 and Raldh2, up regulation of Raldh to compensate for impairment of retinol dehydrogenation, culture neurons and mossy fiber enriched Raldh1 expression pattern and opposite regulation of Raldh1 and Raldh3 expression upon TNF α treatment in astrocytes. All these data indicate that retinoid metabolism in adult CNS, especially hippocampal neurons and astrocytes, not only follow the known homeostasis model that has been found in other type of cells, but also exhibit some undiscovered, maybe hippocampus specific, characteristics which may imply CNS specific regulation of retinoids homeostasis model.

Part I Retinoids metabolism in cultured astrocytes and neurons

Determining the source of atRA in the rodent hippocampus was the initial purpose of this research. The strength of our specific LC/MS/MS technology enables us to accurately measure low level of retinoids isomers which is present in small volume of biological samples, such as different loci of adult CNS. Around 3 pmol/g atRA in adult rat hippocampus, plus the presence of high concentration of retinol and RE, suggest that biosynthesis of atRA may be the way that hippocampus acquire the atRA for the normal physiological function, which has been proved by RAR β null mice, VAD mice or aged mice losing spatial learning and memory formation ability. We also considered the possibility of obtaining atRA from circulation. However, the presence of only retinol binding protein, but not CRABP, on blood-brain barrier and radiolabeled retinoids chasing research minimize this possibility (MacDonald *et al.*, 1990; Pardridge *et al.*, 1985; Werner and Deluca, 2002). The meninges is believed to serve as the major source of atRA for early embryonic brain development and differentiation (Romand *et al.*, 2008; Zhang *et al.*, 2003). Highly expressed Raldh2 in meninges and the comprehensive study of

Foxc1 mutant mice which lost meninges in forebrain indicate that atRA from meninges during embryonic development is indispensable for neural precursor cells differentiation (Siegenthaler *et al.*, 2009). However, whether meninges is also an important source for atRA in adult brain remained unclear. One possibility is that during embryonic development, most precursors haven't fully developed and lack the ability to synthesize atRA. Meninges then act as a temporal source to provide atRA to nearby neural precursors to induce differentiation. In adult brain, the presence of functional glia cells which gain the ability to synthesize atRA and the increased volume of the brain make it unnecessary and impossible for the meninges to continue to provide atRA to the neurons or other cell types which need atRA to perform normal function. Loss contact with neurons also make meninges an unlike source for atRA in adult CNS. However, the remaining high expression level of Raldh2 in adult meninges still indicate that it might still play an important function in adult brain, and further investigation need to addressed on this issue (McCaffery *et al.*, 2006).

Since we exclude other possibilities, the most likely source of high atRA we detected in adult hippocampus is from *in situ* biosynthesis. The presence of retinoids metabolism enzymes and binding proteins and retinoic acid receptor in adult hippocampus support this hypothesis. However, as we know, the adult brain, including hippocampus, is highly heterogeneously. Neurons and glia cells are the two major cell types that fully differentiate with different structures and functions. In glia cells, astrocytes account for the majority of total cells and support the normal function of neurons. The objectives of our project are to figure out i) how retinoids are metabolized in these two cell types and which cell can synthesize atRA; ii) what enzymes, and to what extent, participate in retinoids metabolism in these cells; iii) is there any factors that can affect retinoids metabolism in these cells.

Previous research has provided some hints on retinoids metabolism in neurons and astrocytes (See Chapter I). Research in our lab indicates that neurons can quickly respond to atRA stimulation and incorporate RAR α into neurons to suppress translation. Raldh1 was also found to be present in neurons. No direct evidence indicate that neurons can synthesize atRA. In contrast, lots of indirect evidence indicate that astrocytes can synthesize atRA, including coculture astrocytes with RAR reporter cell resulting in positive signal (Korneyi *et al.*, 2007); converting retinol to active form to induce neuron survival only in the presence of astrocytes. However, neither of these evidence directly proved atRA biosynthesis in astrocytes. We utilize the advantages of our LC/MS/MS technology, which has been demonstrated to specifically and sensitively detected all different isoforms of RA, to accurately detect the presence of atRA production from astrocytes incubated with either retinol or retinal, and measure the kinetics parameters of different retinoids metabolism reactions. According to these data, we conclude that: i) atRA biosynthesis is only present in cultured astrocytes; ii) RE formation and retinal reduction occur in both astrocytes and neurons, with similar rates under physiological concentrations of substrate. We also showed cultured neurons can actively take up atRA

from astrocytes without the assistance of cell-cell contact. Besides that, atRA can suppress its own biosynthesis through both up regulation of atRA degradation and RE formation to redirect the flow of retinol in astrocytes. Neurons can also respond to atRA to increase RE formation .

According to these data and the architecture of neurons and astrocytes position, we established a general model how neurons and astrocytes utilize retinol as the substrate and maintain a controlled level of atRA (Figure V-1). In this model, the substrate of atRA biosynthesis is the RBP binding retinol which is circulating in the capillary all over the adult brain. The endfeet of astrocytes covering the whole capillary, which is part of the blood-brain barrier, make sure that only astrocytes get access to the retinol. Stra6, the only known cell membrane receptor for RBP, has been detected in astrocytes (data now shown). The uptake retinol will bind with CrbpI, whose presence was confirmed by RT-PCR detection of messenger RNA. Two known fates of CRBP-retinol (holo-CRBP) are present in astrocytes: esterification and dehydrogenation. The majority of retinol is converted to RE—the storage form of retinoids, for further utilization. Esterification may also serve as mechanism that astrocytes get rid of excess retinol. However, receptor mediated uptake of retinol should control the amount of substrate flow into the astrocytes and RE formation may mainly serve as a backup mechanism in case any vitamin A deficiency. The dramatic increase rate of RE formation under high concentration retinol indicates that astrocyte has a broad capacity to handle large amount of retinol. atRA is synthesized in astrocytes under strict regulation. RE formation is one of the major ways to control the amount of retinol that can be oxidized. The high retinal reduction activity, atRA secretion ability and atRA feedback autoregulation work together to tightly control the concentration of atRA inside of astrocytes. The atRA remained in astrocytes may regulate the some specific gene expression or function of astrocytes. The composition of whole signaling pathway, such as receptors and binding proteins need to be further elucidated in astrocytes.

Secretion of synthesized atRA from astrocytes and quickly induced spine growth of hippocampal pyramidal neurons by atRA indicate that neurons might be able to take up the atRA secreted by astrocytes for utilization. Our co-culture data support this hypothesis and prove that cell-cell contact is not necessary for this atRA uptake. We don't know whether the *in vivo* situation is same as what we observed in primary culture cells. If it is the case, we speculate that atRA are secreted into the micro-environment around the nearby neurons by astrocytes in hippocampus, and then taken up by neurons under a specific, highly regulated mechanism. We don't know whether neurons can feedback regulate the amount of atRA synthesized or secreted by astrocytes, and we also cannot exclude the possibility that atRA is transported through direct neuron-astrocyte contact. The *in vivo* transportation atRA need to be revealed by an accurate chasing method in which atRA can be clearly labeled and visualized.

atRA can increase its own degradation and RE formation in astrocytes and neurons indicate that the common feedback regulation mechanism is also present in CNS cells.

We don't know whether esterification of retinol is also present in neurons *in vivo*, or the RE formation we observed in neurons is just an adaption of *in vitro* culture. However, it at least indicate that neurons can gain the ability to protect themselves from toxic retinol by converting them to RE. How atRA is utilized and metabolized in neurons remains unclear. Knockout and knockdown of retinoids metabolizing enzymes and binding proteins, such as CYP26 enzymes and CRABPs, need to be studied to elucidate the whole atRA metabolism model inside of neurons.

Part II Enzymes that participate in the retinoids homeostasis in CNS

Previous atRA biosynthesis research usually focused on only one specific dehydrogenase, for example Raldh2 during embryonic development. However, our research confirmed that at least three retinol dehydrogenases (Rdh2, Rdh10 and Dhhr9) and three retinal dehydrogenases (Raldh1, 2 and 3) contribute to atRA biosynthesis. We didn't exclude other possible isoenzymes' participation in this process. The contribution of each isoenzyme is different. However, to what extent this result can be applied to *in vivo* status is unknown. We used one month old astrocyte to evaluate the contribution of each enzyme. However, the culture time dependent increased expression of some, but not all of the enzymes indicate that *in vitro* culture condition definitely affect the expression level of these dehydrogenases. It's inappropriate to estimate the *in vivo* contribution of any isoenzyme just according to the *in vitro* knockdown data.

The knockdown results indicate different contributions of each isoenzyme in cultured astrocytes. Knockdown Raldh1 showed the most dramatic effect on decrease of atRA biosynthesis, which is consistent with its highest expression level. However, according to *in vitro* and *in vivo* immunostaining detection of its protein level, we found a fair low expression of Raldh1 in astrocytes in the presence of neurons, either in co-cultured system or in hippocampus slice. Besides that, Raldh1 is also detected in some unexpected location, including the nuclear of astrocytes, all over the cultured neurons and enriched expression on mossy fibers. All these results indicate that Raldh1 in adult hippocampus, not only function as a retinal dehydrogenase, but also have some other specific functions which need to be further addressed. Culture time dependent increase of Raldh1 expression and TNF α induced long term decrease expression of Raldh1 indicate that this is a gene that can respond to environmental stimulation for a long term regulation of retinoids homeostasis.

As an indispensable retinal dehydrogenase during embryonic CNS development, the presence of Raldh2 is usually considered as the powerful evidence to imply the presence of atRA biosynthesis. Our data also support this notion, with significant reduction of retinal dehydrogenation when Raldh2 was knocked down; even its messenger RNA level is about 500-1000 fold lower than Raldh1. Besides that, culture time dependent increased Raldh2 expression also confirms its participation in atRA biosynthesis in astrocytes. The perinuclear enriched expression pattern also suggests Raldh2 might contribute to subcellular specific synthesis of atRA.

Knockdown of Raldh3 didn't result in as much change of atRA biosynthesis as its isoenzymes. Raldh3 also didn't increase its expression level as Raldh1 and Raldh2 when cultured long time *in vitro*. However, increased Raldh3 expression was observed when stimulated by TNF α for less than 12 hr and result in increase of atRA biosynthesis. According to these data, change of Raldh3 expression in astrocytes may serve as a quick response to environmental change, such as different immune cytokines level, to regulate atRA level.

The other possible function of Raldh is to act as a regulator to compensate for any changes that might perturb atRA levels. According to our data, increase in Raldh1 mRNA was observed when Dhhr9 was knocked down in cultured astrocytes; Raldh2 was unregulated in Rdh1 and CrbpI knockout mice astrocytes. Dhhr9, mice Rdh1 (homolog of rat Rdh2) and CrbpI are the enzymes or binding protein that participate in the first step retinol oxidation. It seems like astrocytes will up regulate retinal oxidation to compensate the disturbing of the retinol dehydrogenation. To our knowledge, this is a novel regulation mechanism on atRA homeostasis which has never been reported before. The remaining questions are: i) whether this is an astrocyte, or CNS specific regulation, or this phenomenon is present in other tissues; ii) whether this is an *in vitro* culture dependent phenomenon or up regulation of Raldh can also be detected *in vivo*; iii) The selection of up regulation of Raldh1 or Raldh2 is species (rat *vs.* mouse) specific or linked to the specific first step oxidation components (Dhhr9, Rdh1 and CrbpI); iv) the molecular and cellular mechanism for up regulation of retinal dehydrogenases. As we know, no up regulation of any Raldhs was detected in Rdh1 knockout mice liver. Our research also excludes the possibility that retinal or atRA can regulate Raldh expression. To solve this puzzle, new hypothesis need to be raised and tested.

Both Rdh2 and Rdh10 knockdown result in slightly decrease of atRA production from retinol, and Dhhr9 knockdown result in increase of atRA production which is due to the increase expression of Raldh1. The different response on Rdhs knockdown indicates that they might have different impact on atRA biosynthesis. We didn't detect the subcellular location of these Rdhs due to the lack of specific antibodies. Our results also can't exclude the possibility that other retinol dehydrogenases may also contribute to the first step oxidation. However, our data at least prove that more than one single retinol dehydrogenase contribute to retinol dehydrogenation.

In our research, we only test the enzymes which function as a retinol dehydrogenase. However, because astrocytes showed strong ability to reduce retinal, which is believed as an important regulatory method to control atRA biosynthesis, we speculate that a balance equalization system is present in astrocytes to control the first step oxidation. This system is the bottleneck to limit the atRA synthesis by controlling how much retinal can be utilized for oxidation. This balance system contains multiple retinol dehydrogenases, retinal reductases and retinol/retinal binding proteins. Dhhr9's function in this system needs to be confirmed by knocking it down in Raldh1 null astrocyte. Our research demonstrated that Dhhr9 doesn't function as retinal reductase. Up regulation of CrbpII in

CrbpI knockout astrocytes indicate that both binding proteins are present and have redundant function in astrocytes. Rdh2 and Rdh10 may also reflect the redundancy of retinol oxidation in astrocytes. Future investigation should focus on other possible members of this balance system and their contribution, especially the possible oxidase and reductase and their preferred substrate concentration.

We proved that the major degradation enzyme for atRA degradation is Cyp26B1, which can be induced by atRA itself, consistent with previous report. Cyp26B1, but not Cyp26A1 or C1, has also been reported widely expressed in adult CNS. Consistent with that, low level of Cyp26A1 was detected in cultured astrocytes and it cannot be regulated by atRA itself. Increase Cyp26B1 expression was also observed in astrocytes which exhibited spontaneously increased atRA biosynthesis, such as long time *in vitro* cultured astrocytes or Rdh1 and CrbpI knockout astrocytes. Up regulation of Cyp26B1 in these cells doesn't require exogenous atRA stimulation. If the regulation of Cyp26B1 does require atRA, the subcellular localized atRA biosynthesis, for example, the specific subcellular expression of Raldh1 and Raldh2, might provide a regional high concentration atRA which can induce Cyp26B1 expression. As expected, Lrat was also up regulated by atRA in astrocytes, which is consistent with increased RE formation. These atRA induced gene changes establish the autoregulation of retinoids homeostasis in astrocytes, which is also present in other system though the participants vary. This variation can also be found in neurons. Our data demonstrate that cultured neurons can also respond to atRA stimulation and increase RE formation. However, the only changed component of retinoids signaling is CrbpI in neurons. The mechanism that causes this different response is unclear. atRA delivery, recruiting co-activator or co-repressor, atRA receptor expression pattern could be the directions for further investigation.

Part III Future Directions

Raldh1 is the enzyme broadly studied in our research. However, a lot of unexplained phenomenon makes the follow up research on Raldh1's function in adult CNS a fascinating work. The first unsolved problem is the different expression level of Raldh1 in different astrocytes. Culture time dependent robust increased Raldh1 expression makes it the predominant retinal dehydrogenase in pure cultured astrocytes, which is confirmed by siRNA knockdown. However, fairly low expression of this enzyme in mixed cultured astrocytes with neurons and even lower and rarer expression *in vivo* make us doubt, to what extent this enzyme contribute to atRA biosynthesis in adult hippocampus. Whether the dramatic increase of Raldh1 expression in pure astrocytes is due to the presence of inducer in environment or the lack the inhibition signals from neurons needs to be addressed. If neurons do inhibit Raldh1 expression in astrocytes, figuring out this suppression factors will be the major task which can provide the insight on how neurons can regulate atRA biosynthesis. The studies focused on the regulation of Raldh1 expression will also provide some clues on the mechanism of how Dhhrs9 knockdown can up regulate Raldh1. The TNF α research indicates that JNK and MAPK signaling pathway

might participate in the regulation of Raldh1 expression. Further research focused on these two signaling pathways might help to resolve this problem.

The second unsolved problem is the function other than retinal dehydrogenase of Raldh1. Raldh1 was found highly expressed in cultured astrocytes nucleus, an unusual location that has never been reported before. To further confirm this nucleus specific expression pattern, nucleus protein need to be separated and Raldh1 protein level need to be detected by western blot. Raldh1 was also detected in neurons—throughout cultured neurons and specifically in CA1 neurons and mossy fibers. No evidence showed that Raldh1 in neurons can dehydrogenate retinal though exogenous retinal can be taken up by cultured neurons. The functions of Raldh1 in astrocytes nucleus and neurons remained unclear. Raldh1 knockout mouse is a good model to study the specific function of Raldh1. Cultured astrocytes and neurons from Raldh1 knockout mice will help us to further evaluate its contribution to atRA biosynthesis. Any unusual phenomenon in either cultured neural cells or histology of hippocampus will provide hints for generate new hypothesis and direct followup research.

Raldh2 and Raldh3 are less studied compared with Raldh1. Similar questions are also need to be addressed. For example, the mechanism of Raldh2 up regulation in Raldh1 and CrbpI null astrocytes and TNF α induced P38 signaling dependent Raldh3 expression. Because both Raldh2 and Raldh3 knockout mice are lethal, conditional CNS specific Raldh2 and Raldh3 knockout mice might be an appropriate model to study their functions in adult hippocampus. The elucidation of *in vivo* expression pattern of these two enzymes, especially under different stress like VAD or inflammation, may help to understand the specific functions of these multiple retinal dehydrogenases in adult CNS.

There are also some other projects that are related to retinoids homeostasis model in adult hippocampus. One major project is to elucidate atRA metabolism in neurons. The kinetics of atRA degradation in neurons, the enzymes that participate in atRA metabolism, RE formation and retinal reduction, the mechanism of atRA induced up regulation of CrbpI expression need to be addressed. The other project is to elucidate the functions and contributions of binding proteins in adult CNS retinoids metabolism. One interesting question is that since atRA has to bind with binding proteins all the time, which retinoids binding proteins are essential for atRA secretion from astrocytes and transportation to neurons. Besides binding proteins, are there any other components, such as RAR or Raldh, also involved in atRA secretion and transportation?

Part IV Conclusion

Our research opens the gate towards elucidation of retinoids metabolism in adult hippocampus. Consistent with previous report, our data directly demonstrate astrocytes, but not neurons, can synthesize and secrete atRA, which can be taken up by neurons, through multiple retinol dehydrogenases and retinal dehydrogenases. TNF α may act as a potential regulator to change atRA synthesis through affecting the retinal dehydrogenation. The communication between the first and second step dehydrogenation indicate a novel manner to regulate atRA homeostasis. These data indicate that glia cells

may serve as the major source of atRA in adult hippocampus. Since atRA play an important role in adult brain and is associated with many pathological and age related impairment of brain function, the elucidation of atRA biosynthesis and metabolism in adult hippocampus would greatly help us to find out potential targets to manipulate atRA level, which may contribute to the amelioration and treatment of retinoids related human CNS diseases.

Figures and Legends

Figure V-1

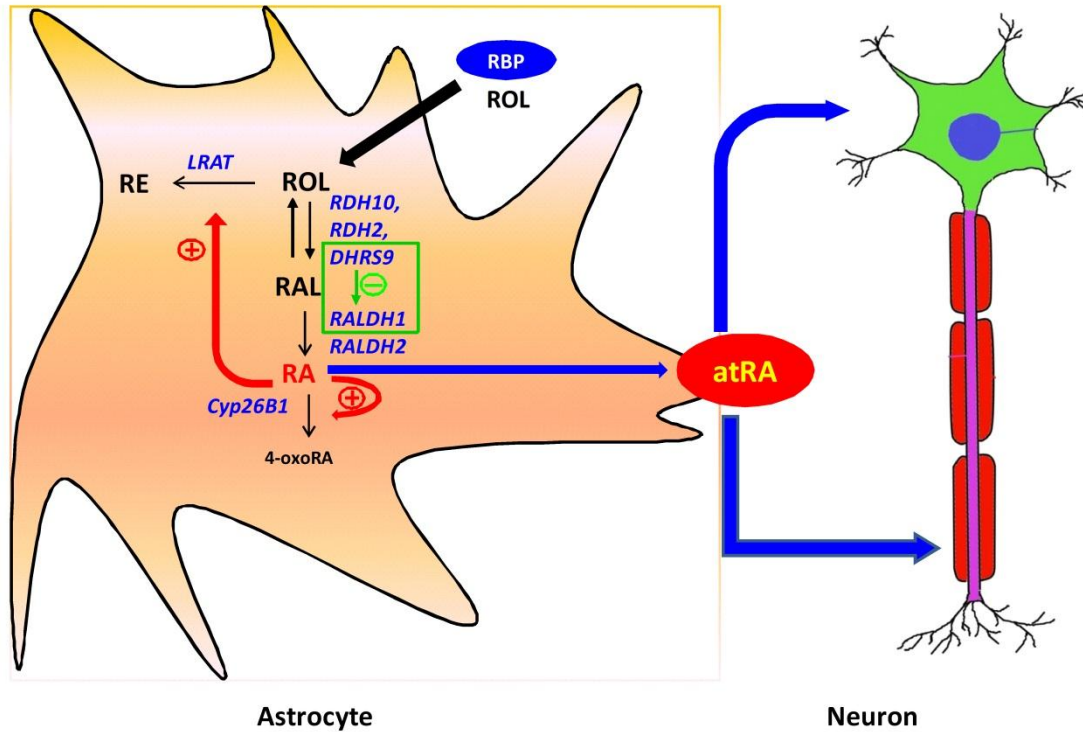


Figure V-1 Retinoids homeostasis model in adult hippocampus. RBP binding retinol is taken up by astrocytes through Stra6 receptor. The intake retinol binding with CRBPI, is either converted to RE for storage by Lrat, or oxidized to atRA through two step dehydrogenation by multiple retinol and retinal dehydrogenases. Synthesized atRA is secreted and sequestered by nearby neurons. atRA can feedback regulate Lrat and Cyp26B1 expression to autoregulated atRA homeostasis. Cross talk between DhRS9 and Raldh1 indicate a novel regulation mechanism on atRA biosynthesis in CNS.

References

- Abu-Abed, S., Dolle, P., Metzger, D., Beckett, B., Chambon, P., and Petkovich, M. (2001). The retinoic acid-metabolizing enzyme, CYP26A1, is essential for normal hindbrain patterning, vertebral identity, and development of posterior structures. *Genes Dev* 15, 226-240.
- Akita, J., Takahashi, M., Hojo, M., Nishida, A., Haruta, M., and Honda, Y. (2002). Neuronal differentiation of adult rat hippocampus-derived neural stem cells transplanted into embryonic rat explanted retinas with retinoic acid pretreatment. *Brain Res* 954, 286-293.
- Alique, M., Herrero, J.F., and Lucio-Cazana, F.J. (2007). All-trans retinoic acid induces COX-2 and prostaglandin E2 synthesis in SH-SY5Y human neuroblastoma cells: involvement of retinoic acid receptors and extracellular-regulated kinase 1/2. *J Neuroinflammation* 4, 1.
- Alliot, F., Delhaye-Bouchaud, N., Geffard, M., and Pessac, B. (1988). Role of astroglial cell clones in the survival and differentiation of cerebellar embryonic neurons. *Brain Res Dev Brain Res* 44, 247-257.
- Alvarez-Buylla, A., and Kirn, J.R. (1997). Birth, migration, incorporation, and death of vocal control neurons in adult songbirds. *J Neurobiol* 33, 585-601.
- Aoto, J., Nam, C.I., Poon, M.M., Ting, P., and Chen, L. (2008). Synaptic signaling by all-trans retinoic acid in homeostatic synaptic plasticity. *Neuron* 60, 308-320.
- Asson-Batres, M.A., and Smith, W.B. (2006). Localization of retinaldehyde dehydrogenases and retinoid binding proteins to sustentacular cells, glia, Bowman's gland cells, and stroma: potential sites of retinoic acid synthesis in the postnatal rat olfactory organ. *J Comp Neurol* 496, 149-171.
- Balasingam, V., Tejada-Berges, T., Wright, E., Bouckova, R., and Yong, V.W. (1994). Reactive astrogliosis in the neonatal mouse brain and its modulation by cytokines. *J Neurosci* 14, 846-856.
- Banker, G.A. (1980). Trophic interactions between astroglial cells and hippocampal neurons in culture. *Science* 209, 809-810.
- Batten, M.L., Imanishi, Y., Maeda, T., Tu, D.C., Moise, A.R., Bronson, D., Possin, D., Van Gelder, R.N., Baehr, W., and Palczewski, K. (2004). Lecithin-retinol acyltransferase is essential for accumulation of all-trans-retinyl esters in the eye and in the liver. *J Biol Chem* 279, 10422-10432.
- Bendich, A., and Langseth, L. (1989). Safety of vitamin A. *Am J Clin Nutr* 49, 358-371.
- Blaner, W.S. (2007). STRA6, a cell-surface receptor for retinol-binding protein: the plot thickens. *Cell Metab* 5, 164-166.
- Blomhoff, R., and Blomhoff, H.K. (2006). Overview of retinoid metabolism and function. *J Neurobiol* 66, 606-630.
- Bradl, M., and Lassmann, H. (2010). Oligodendrocytes: biology and pathology. *Acta Neuropathol* 119, 37-53.
- Brodeur, H., Gagnon, I., Mader, S., and Bhat, P.V. (2003). Cloning of monkey RALDH1

and characterization of retinoid metabolism in monkey kidney proximal tubule cells. *J Lipid Res* 44, 303-313.

Bruce, A.J., Boling, W., Kindy, M.S., Peschon, J., Kraemer, P.J., Carpenter, M.K., Holtsberg, F.W., and Mattson, M.P. (1996). Altered neuronal and microglial responses to excitotoxic and ischemic brain injury in mice lacking TNF receptors. *Nat Med* 2, 788-794.

Bucco, R.A., Zheng, W.L., Wardlaw, S.A., Davis, J.T., Sierra-Rivera, E., Osteen, K.G., Melner, M.H., Kakkad, B.P., and Ong, D.E. (1996). Regulation and localization of cellular retinol-binding protein, retinol-binding protein, cellular retinoic acid-binding protein (CRABP), and CRABP II in the uterus of the pseudopregnant rat. *Endocrinology* 137, 3111-3122.

Buttini, M., Appel, K., Sauter, A., Gebicke-Haerter, P.J., and Boddeke, H.W. (1996). Expression of tumor necrosis factor alpha after focal cerebral ischaemia in the rat. *Neuroscience* 71, 1-16.

Chai, X., Boerman, M.H., Zhai, Y., and Napoli, J.L. (1995). Cloning of a cDNA for liver microsomal retinol dehydrogenase. A tissue-specific, short-chain alcohol dehydrogenase. *J Biol Chem* 270, 3900-3904.

Chambon, P. (1996). A decade of molecular biology of retinoic acid receptors. *FASEB J* 10, 940-954.

Chandrasekaran, V., Zhai, Y., Wagner, M., Kaplan, P.L., Napoli, J.L., and Higgins, D. (2000). Retinoic acid regulates the morphological development of sympathetic neurons. *J Neurobiol* 42, 383-393.

Chen, C., and Tonegawa, S. (1997). Molecular genetic analysis of synaptic plasticity, activity-dependent neural development, learning, and memory in the mammalian brain. *Annu Rev Neurosci* 20, 157-184.

Chen, N., and Napoli, J.L. (2008). All-trans-retinoic acid stimulates translation and induces spine formation in hippocampal neurons through a membrane-associated RARalpha. *FASEB J* 22, 236-245.

Chen, N., Onisko, B., and Napoli, J.L. (2008). The nuclear transcription factor RARalpha associates with neuronal RNA granules and suppresses translation. *J Biol Chem* 283, 20841-20847.

Chen, Y., Sharma, R.P., Costa, R.H., Costa, E., and Grayson, D.R. (2002). On the epigenetic regulation of the human reelin promoter. *Nucleic Acids Res* 30, 2930-2939.

Cheng, B., Christakos, S., and Mattson, M.P. (1994). Tumor necrosis factors protect neurons against metabolic-excitotoxic insults and promote maintenance of calcium homeostasis. *Neuron* 12, 139-153.

Chetyrkin, S.V., Belyaeva, O.V., Gough, W.H., and Kedishvili, N.Y. (2001). Characterization of a novel type of human microsomal 3alpha -hydroxysteroid dehydrogenase: unique tissue distribution and catalytic properties. *J Biol Chem* 276, 22278-22286.

Chiang, M.Y., Misner, D., Kempermann, G., Schikorski, T., Giguere, V., Sucov, H.M.,

Gage, F.H., Stevens, C.F., and Evans, R.M. (1998). An essential role for retinoid receptors RARbeta and RXRgamma in long-term potentiation and depression. *Neuron* *21*, 1353-1361.

Cocco, S., Diaz, G., Stancampiano, R., Diana, A., Carta, M., Curreli, R., Sarais, L., and Fadda, F. (2002). Vitamin A deficiency produces spatial learning and memory impairment in rats. *Neuroscience* *115*, 475-482.

Collins, M.D., and Mao, G.E. (1999). Teratology of retinoids. *Annu Rev Pharmacol Toxicol* *39*, 399-430.

Connor, M.J., and Sidell, N. (1997). Retinoic acid synthesis in normal and Alzheimer diseased brain and human neural cells. *Mol Chem Neuropathol* *30*, 239-252.

Cooper, D.L., Isola, N.R., Stevenson, K., and Baptist, E.W. (1993). Members of the ALDH gene family are lens and corneal crystallins. *Adv Exp Med Biol* *328*, 169-179.

Cowherd, R.M., Lyle, R.E., and McGehee, R.E., Jr. (1999). Molecular regulation of adipocyte differentiation. *Semin Cell Dev Biol* *10*, 3-10.

Crandall, J., Sakai, Y., Zhang, J., Koul, O., Mineur, Y., Crusio, W.E., and McCaffery, P. (2004). 13-cis-retinoic acid suppresses hippocampal cell division and hippocampal-dependent learning in mice. *Proc Natl Acad Sci U S A* *101*, 5111-5116.

Darmanin, S., Chen, J., Zhao, S., Cui, H., Shirkoohi, R., Kubo, N., Kuge, Y., Tamaki, N., Nakagawa, K., Hamada, J., *et al.* (2007). All-trans retinoic acid enhances murine dendritic cell migration to draining lymph nodes via the balance of matrix metalloproteinases and their inhibitors. *J Immunol* *179*, 4616-4625.

Delva, L., Bastie, J.N., Rochette-Egly, C., Kraiba, R., Balitrand, N., Despouy, G., Chambon, P., and Chomienne, C. (1999). Physical and functional interactions between cellular retinoic acid binding protein II and the retinoic acid-dependent nuclear complex. *Mol Cell Biol* *19*, 7158-7167.

Denisenko-Nehrbass, N.I., Jarvis, E., Scharff, C., Nottebohm, F., and Mello, C.V. (2000). Site-specific retinoic acid production in the brain of adult songbirds. *Neuron* *27*, 359-370.

Dev, S., Adler, A.J., and Edwards, R.B. (1993). Adult rabbit brain synthesizes retinoic acid. *Brain Res* *632*, 325-328.

Devaux, Y., Seguin, C., Grosjean, S., de Talance, N., Schwartz, M., Burlet, A., Zannad, F., Meistelman, C., Mertes, P.M., and Ungureanu-Longrois, D. (2001). Retinoic acid and lipopolysaccharide act synergistically to increase prostanoid concentrations in rats in vivo. *J Nutr* *131*, 2628-2635.

Dheen, S.T., Jun, Y., Yan, Z., Tay, S.S., and Ling, E.A. (2005). Retinoic acid inhibits expression of TNF-alpha and iNOS in activated rat microglia. *Glia* *50*, 21-31.

Douville, J., Beaulieu, R., and Balicki, D. (2009). ALDH1 as a functional marker of cancer stem and progenitor cells. *Stem Cells Dev* *18*, 17-25.

Drager, U.C. (2006). Retinoic acid signaling in the functioning brain. *Sci STKE* *2006*, pe10.

Duester, G., Mic, F.A., and Molotkov, A. (2003). Cytosolic retinoid dehydrogenases govern ubiquitous metabolism of retinol to retinaldehyde followed by tissue-specific

metabolism to retinoic acid. *Chem Biol Interact* 143-144, 201-210.

Dupe, V., Matt, N., Garnier, J.M., Chambon, P., Mark, M., and Ghyselinck, N.B. (2003). A newborn lethal defect due to inactivation of retinaldehyde dehydrogenase type 3 is prevented by maternal retinoic acid treatment. *Proc Natl Acad Sci U S A* 100, 14036-14041.

E, X., Zhang, L., Lu, J., Tso, P., Blaner, W.S., Levin, M.S., and Li, E. (2002). Increased neonatal mortality in mice lacking cellular retinol-binding protein II. *J Biol Chem* 277, 36617-36623.

Edwards, R.B., Adler, A.J., Dev, S., and Claycomb, R.C. (1992). Synthesis of retinoic acid from retinol by cultured rabbit Muller cells. *Exp Eye Res* 54, 481-490.

Etchamendy, N., Enderlin, V., Marighetto, A., Pallet, V., Higuieret, P., and Jaffard, R. (2003). Vitamin A deficiency and relational memory deficit in adult mice: relationships with changes in brain retinoid signalling. *Behav Brain Res* 145, 37-49.

Etchamendy, N., Enderlin, V., Marighetto, A., Vouimba, R.M., Pallet, V., Jaffard, R., and Higuieret, P. (2001). Alleviation of a selective age-related relational memory deficit in mice by pharmacologically induced normalization of brain retinoid signaling. *J Neurosci* 21, 6423-6429.

Evans, T.R., and Kaye, S.B. (1999). Retinoids: present role and future potential. *Br J Cancer* 80, 1-8.

Farina, C., Aloisi, F., and Meinl, E. (2007). Astrocytes are active players in cerebral innate immunity. *Trends Immunol* 28, 138-145.

Galter, D., Carmine, A., Buervenich, S., Duester, G., and Olson, L. (2003). Distribution of class I, III and IV alcohol dehydrogenase mRNAs in the adult rat, mouse and human brain. *Eur J Biochem* 270, 1316-1326.

Geissmann, F., Revy, P., Brousse, N., Lepelletier, Y., Folli, C., Durandy, A., Chambon, P., and Dy, M. (2003). Retinoids regulate survival and antigen presentation by immature dendritic cells. *J Exp Med* 198, 623-634.

Ghyselinck, N.B., Bavik, C., Sapin, V., Mark, M., Bonnier, D., Hindelang, C., Dierich, A., Nilsson, C.B., Hakansson, H., Sauvart, P., *et al.* (1999). Cellular retinol-binding protein I is essential for vitamin A homeostasis. *EMBO J* 18, 4903-4914.

Giguere, V., Ong, E.S., Segui, P., and Evans, R.M. (1987). Identification of a receptor for the morphogen retinoic acid. *Nature* 330, 624-629.

Ginestier, C., Hur, M.H., Charafe-Jauffret, E., Monville, F., Dutcher, J., Brown, M., Jacquemier, J., Viens, P., Kleer, C.G., Liu, S., *et al.* (2007). ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell* 1, 555-567.

Goodman, A.B. (1998). Three independent lines of evidence suggest retinoids as causal to schizophrenia. *Proc Natl Acad Sci U S A* 95, 7240-7244.

Goodman, A.B. (2006). Retinoid receptors, transporters, and metabolizers as therapeutic targets in late onset alzheimer disease. *J Cell Physiol* 209, 598-603.

Gottesman, M.E., Quadro, L., and Blaner, W.S. (2001). Studies of vitamin A metabolism

in mouse model systems. *Bioessays* 23, 409-419.

Gould, E., Woolley, C.S., Frankfurt, M., and McEwen, B.S. (1990). Gonadal steroids regulate dendritic spine density in hippocampal pyramidal cells in adulthood. *J Neurosci* 10, 1286-1291.

Gross, C.G. (1993). Hippocampus minor and man's place in nature: a case study in the social construction of neuroanatomy. *Hippocampus* 3, 403-415.

Grun, F., Hirose, Y., Kawauchi, S., Ogura, T., and Umesono, K. (2000). Aldehyde dehydrogenase 6, a cytosolic retinaldehyde dehydrogenase prominently expressed in sensory neuroepithelia during development. *J Biol Chem* 275, 41210-41218.

Gundersen, T.E., and Blomhoff, R. (2001). Qualitative and quantitative liquid chromatographic determination of natural retinoids in biological samples. *J Chromatogr A* 935, 13-43.

Halilagic, A., Ribes, V., Ghyselinck, N.B., Zile, M.H., Dolle, P., and Studer, M. (2007). Retinoids control anterior and dorsal properties in the developing forebrain. *Dev Biol* 303, 362-375.

Hartmann, S., Brors, O., Bock, J., Blomhoff, R., Bausch, J., Wiegand, U.W., Hartmann, D., and Hornig, D.H. (2005). Exposure to retinyl esters, retinol, and retinoic acids in non-pregnant women following increasing single and repeated oral doses of vitamin A. *Ann Nutr Metab* 49, 155-164.

Haselbeck, R.J., Hoffmann, I., and Duester, G. (1999). Distinct functions for Aldh1 and Raldh2 in the control of ligand production for embryonic retinoid signaling pathways. *Dev Genet* 25, 353-364.

Herr, F.M., and Ong, D.E. (1992). Differential interaction of lecithin-retinol acyltransferase with cellular retinol binding proteins. *Biochemistry* 31, 6748-6755.

Hohlfeld, R., Kerschensteiner, M., and Meinel, E. (2007). Dual role of inflammation in CNS disease. *Neurology* 68, S58-63; discussion S91-56.

Jacobs, S., Lie, D.C., DeCicco, K.L., Shi, Y., DeLuca, L.M., Gage, F.H., and Evans, R.M. (2006). Retinoic acid is required early during adult neurogenesis in the dentate gyrus. *Proc Natl Acad Sci U S A* 103, 3902-3907.

Jette, C., Peterson, P.W., Sandoval, I.T., Manos, E.J., Hadley, E., Ireland, C.M., and Jones, D.A. (2004). The tumor suppressor adenomatous polyposis coli and caudal related homeodomain protein regulate expression of retinol dehydrogenase L. *J Biol Chem* 279, 34397-34405.

Jiang, F., Qiu, Q., Khanna, A., Todd, N.W., Deepak, J., Xing, L., Wang, H., Liu, Z., Su, Y., Stass, S.A., *et al.* (2009). Aldehyde dehydrogenase 1 is a tumor stem cell-associated marker in lung cancer. *Mol Cancer Res* 7, 330-338.

Jones-Villeneuve, E.M., Rudnicki, M.A., Harris, J.F., and McBurney, M.W. (1983). Retinoic acid-induced neural differentiation of embryonal carcinoma cells. *Mol Cell Biol* 3, 2271-2279.

Jurukovski, V., Markova, N.G., Karaman-Jurukovska, N., Randolph, R.K., Su, J., Napoli, J.L., and Simon, M. (1999). Cloning and characterization of retinol dehydrogenase

transcripts expressed in human epidermal keratinocytes. *Mol Genet Metab* 67, 62-73.

Kanai, M., Raz, A., and Goodman, D.S. (1968). Retinol-binding protein: the transport protein for vitamin A in human plasma. *J Clin Invest* 47, 2025-2044.

Kane, M.A., Chen, N., Sparks, S., and Napoli, J.L. (2005). Quantification of endogenous retinoic acid in limited biological samples by LC/MS/MS. *Biochem J* 388, 363-369.

Kane, M.A., Folias, A.E., Wang, C., and Napoli, J.L. (2008). Quantitative profiling of endogenous retinoic acid in vivo and in vitro by tandem mass spectrometry. *Anal Chem* 80, 1702-1708.

Kane, M.A., Folias, A.E., Wang, C., and Napoli, J.L. (2010). Ethanol elevates physiological all-trans-retinoic acid levels in select loci through altering retinoid metabolism in multiple loci: a potential mechanism of ethanol toxicity. *FASEB J* 24, 823-832.

Kawaguchi, R., Yu, J., Honda, J., Hu, J., Whitelegge, J., Ping, P., Wiita, P., Bok, D., and Sun, H. (2007). A membrane receptor for retinol binding protein mediates cellular uptake of vitamin A. *Science* 315, 820-825.

Kempf, M., Clement, A., Faissner, A., Lee, G., and Brandt, R. (1996). Tau binds to the distal axon early in development of polarity in a microtubule- and microfilament-dependent manner. *J Neurosci* 16, 5583-5592.

Kim, C.H. (2008). Roles of retinoic acid in induction of immunity and immune tolerance. *Endocr Metab Immune Disord Drug Targets* 8, 289-294.

Klann, R.C., and Marchok, A.C. (1982). Effects of retinoic acid on cell proliferation and cell differentiation in a rat tracheal epithelial cell line. *Cell Tissue Kinet* 15, 473-482.

Kobayashi, K. (2010). Hippocampal mossy fiber synaptic transmission and its modulation. *Vitam Horm* 82, 65-85.

Kornyei, Z., Gocza, E., Ruhl, R., Orsolits, B., Voros, E., Szabo, B., Vagovits, B., and Madarasz, E. (2007). Astroglia-derived retinoic acid is a key factor in glia-induced neurogenesis. *FASEB J* 21, 2496-2509.

Kretz, O., Fester, L., Wehrenberg, U., Zhou, L., Brauckmann, S., Zhao, S., Prange-Kiel, J., Naumann, T., Jarry, H., Frotscher, M., *et al.* (2004). Hippocampal synapses depend on hippocampal estrogen synthesis. *J Neurosci* 24, 5913-5921.

Krezel, W., Ghyselinck, N., Samad, T.A., Dupe, V., Kastner, P., Borrelli, E., and Chambon, P. (1998). Impaired locomotion and dopamine signaling in retinoid receptor mutant mice. *Science* 279, 863-867.

Krezel, W., Kastner, P., and Chambon, P. (1999). Differential expression of retinoid receptors in the adult mouse central nervous system. *Neuroscience* 89, 1291-1300.

Labrecque, J., Bhat, P.V., and Lacroix, A. (1993). Purification and partial characterization of a rat kidney aldehyde dehydrogenase that oxidizes retinal to retinoic acid. *Biochem Cell Biol* 71, 85-89.

Laird, M.D., Vender, J.R., and Dhandapani, K.M. (2008). Opposing roles for reactive astrocytes following traumatic brain injury. *Neurosignals* 16, 154-164.

Lampron, C., Rochette-Egly, C., Gorry, P., Dolle, P., Mark, M., Lufkin, T., LeMeur, M.,

and Chambon, P. (1995). Mice deficient in cellular retinoic acid binding protein II (CRABPII) or in both CRABPI and CRABPII are essentially normal. *Development* *121*, 539-548.

Lane, M.A., and Bailey, S.J. (2005). Role of retinoid signalling in the adult brain. *Prog Neurobiol* *75*, 275-293.

Lei, Z., Chen, W., Zhang, M., and Napoli, J.L. (2003). Reduction of all-trans-retinal in the mouse liver peroxisome fraction by the short-chain dehydrogenase/reductase RRD: induction by the PPAR alpha ligand clofibrate. *Biochemistry* *42*, 4190-4196.

Li, H., Wagner, E., McCaffery, P., Smith, D., Andreadis, A., and Drager, U.C. (2000). A retinoic acid synthesizing enzyme in ventral retina and telencephalon of the embryonic mouse. *Mech Dev* *95*, 283-289.

Li, X.H., Kakkad, B., and Ong, D.E. (2004). Estrogen directly induces expression of retinoic acid biosynthetic enzymes, compartmentalized between the epithelium and underlying stromal cells in rat uterus. *Endocrinology* *145*, 4756-4762.

Li, X.H., and Ong, D.E. (2003). Cellular retinoic acid-binding protein II gene expression is directly induced by estrogen, but not retinoic acid, in rat uterus. *J Biol Chem* *278*, 35819-35825.

Liao, W.L., Tsai, H.C., Wang, H.F., Chang, J., Lu, K.M., Wu, H.L., Lee, Y.C., Tsai, T.F., Takahashi, H., Wagner, M., *et al.* (2008). Modular patterning of structure and function of the striatum by retinoid receptor signaling. *Proc Natl Acad Sci U S A* *105*, 6765-6770.

Lin, M., Zhang, M., Abraham, M., Smith, S.M., and Napoli, J.L. (2003). Mouse retinal dehydrogenase 4 (RALDH4), molecular cloning, cellular expression, and activity in 9-cis-retinoic acid biosynthesis in intact cells. *J Biol Chem* *278*, 9856-9861.

Little, A.R., Benkovic, S.A., Miller, D.B., and O'Callaghan, J.P. (2002). Chemically induced neuronal damage and gliosis: enhanced expression of the proinflammatory chemokine, monocyte chemoattractant protein (MCP)-1, without a corresponding increase in proinflammatory cytokines(1). *Neuroscience* *115*, 307-320.

Lohnes, D., Kastner, P., Dierich, A., Mark, M., LeMeur, M., and Chambon, P. (1993). Function of retinoic acid receptor gamma in the mouse. *Cell* *73*, 643-658.

Lohnes, D., Mark, M., Mendelsohn, C., Dolle, P., Dierich, A., Gorry, P., Gansmuller, A., and Chambon, P. (1994). Function of the retinoic acid receptors (RARs) during development (I). Craniofacial and skeletal abnormalities in RAR double mutants. *Development* *120*, 2723-2748.

MacDonald, P.N., Bok, D., and Ong, D.E. (1990). Localization of cellular retinol-binding protein and retinol-binding protein in cells comprising the blood-brain barrier of rat and human. *Proc Natl Acad Sci U S A* *87*, 4265-4269.

Maden, M. (2002). Retinoid signalling in the development of the central nervous system. *Nat Rev Neurosci* *3*, 843-853.

Maden, M. (2006). Retinoids and spinal cord development. *J Neurobiol* *66*, 726-738.

Maden, M. (2007). Retinoic acid in the development, regeneration and maintenance of the nervous system. *Nat Rev Neurosci* *8*, 755-765.

- Maden, M., and Hind, M. (2003). Retinoic acid, a regeneration-inducing molecule. *Dev Dyn* 226, 237-244.
- Magistretti, P.J. (2006). Neuron-glia metabolic coupling and plasticity. *J Exp Biol* 209, 2304-2311.
- Malkovsky, M., Dore, C., Hunt, R., Palmer, L., Chandler, P., and Medawar, P.B. (1983). Enhancement of specific antitumor immunity in mice fed a diet enriched in vitamin A acetate. *Proc Natl Acad Sci U S A* 80, 6322-6326.
- Mark, M., Ghyselinck, N.B., and Chambon, P. (2009). Function of retinoic acid receptors during embryonic development. *Nucl Recept Signal* 7, e002.
- Markiewicz, I., and Lukomska, B. (2006). The role of astrocytes in the physiology and pathology of the central nervous system. *Acta Neurobiol Exp (Wars)* 66, 343-358.
- McBain, C.J. (2008). Differential mechanisms of transmission and plasticity at mossy fiber synapses. *Prog Brain Res* 169, 225-240.
- McCaffery, P., and Drager, U.C. (1994). High levels of a retinoic acid-generating dehydrogenase in the meso-telencephalic dopamine system. *Proc Natl Acad Sci U S A* 91, 7772-7776.
- McCaffery, P., Koul, O., Smith, D., Napoli, J.L., Chen, N., and Ullman, M.D. (2004). Ethanol increases retinoic acid production in cerebellar astrocytes and in cerebellum. *Brain Res Dev Brain Res* 153, 233-241.
- McCaffery, P., Zhang, J., and Crandall, J.E. (2006). Retinoic acid signaling and function in the adult hippocampus. *J Neurobiol* 66, 780-791.
- Meistrell, M.E., 3rd, Botchkina, G.I., Wang, H., Di Santo, E., Cockcroft, K.M., Bloom, O., Vishnubhakat, J.M., Ghezzi, P., and Tracey, K.J. (1997). Tumor necrosis factor is a brain damaging cytokine in cerebral ischemia. *Shock* 8, 341-348.
- Melhus, H., Michaelsson, K., Kindmark, A., Bergstrom, R., Holmberg, L., Mallmin, H., Wolk, A., and Ljunghall, S. (1998). Excessive dietary intake of vitamin A is associated with reduced bone mineral density and increased risk for hip fracture. *Ann Intern Med* 129, 770-778.
- Mendelsohn, C., Lohnes, D., Decimo, D., Lufkin, T., LeMeur, M., Chambon, P., and Mark, M. (1994). Function of the retinoic acid receptors (RARs) during development (II). Multiple abnormalities at various stages of organogenesis in RAR double mutants. *Development* 120, 2749-2771.
- Mey, J., D, J.M., Brook, G., Liu, R.H., Zhang, Y.P., Koopmans, G., and McCaffery, P. (2005). Retinoic acid synthesis by a population of NG2-positive cells in the injured spinal cord. *Eur J Neurosci* 21, 1555-1568.
- Mey, J., and Hammelmann, S. (2000). OLN-93 oligodendrocytes synthesize all-trans-retinoic acid in vitro. *Cell Tissue Res* 302, 49-58.
- Mey, J., and McCaffery, P. (2004). Retinoic acid signaling in the nervous system of adult vertebrates. *Neuroscientist* 10, 409-421.
- Mirza, N., Fishman, M., Fricke, I., Dunn, M., Neuger, A.M., Frost, T.J., Lush, R.M., Antonia, S., and Gabrilovich, D.I. (2006). All-trans-retinoic acid improves differentiation

of myeloid cells and immune response in cancer patients. *Cancer Res* 66, 9299-9307.

Misner, D.L., Jacobs, S., Shimizu, Y., de Urquiza, A.M., Solomin, L., Perlmann, T., De Luca, L.M., Stevens, C.F., and Evans, R.M. (2001). Vitamin A deprivation results in reversible loss of hippocampal long-term synaptic plasticity. *Proc Natl Acad Sci U S A* 98, 11714-11719.

Molotkov, A., Deltour, L., Foglio, M.H., Cuenca, A.E., and Duester, G. (2002a). Distinct retinoid metabolic functions for alcohol dehydrogenase genes *Adh1* and *Adh4* in protection against vitamin A toxicity or deficiency revealed in double null mutant mice. *J Biol Chem* 277, 13804-13811.

Molotkov, A., Fan, X., Deltour, L., Foglio, M.H., Martras, S., Farres, J., Pares, X., and Duester, G. (2002b). Stimulation of retinoic acid production and growth by ubiquitously expressed alcohol dehydrogenase *Adh3*. *Proc Natl Acad Sci U S A* 99, 5337-5342.

Montague, P., McCallion, A.S., Davies, R.W., and Griffiths, I.R. (2006). Myelin-associated oligodendrocytic basic protein: a family of abundant CNS myelin proteins in search of a function. *Dev Neurosci* 28, 479-487.

Mori, T., Buffo, A., and Gotz, M. (2005). The novel roles of glial cells revisited: the contribution of radial glia and astrocytes to neurogenesis. *Curr Top Dev Biol* 69, 67-99.

Moro, J.R., Iwata, M., and von Andriano, U.H. (2008). Vitamin effects on the immune system: vitamins A and D take centre stage. *Nat Rev Immunol* 8, 685-698.

Nadauld, L.D., Sandoval, I.T., Chidester, S., Yost, H.J., and Jones, D.A. (2004). Adenomatous polyposis coli control of retinoic acid biosynthesis is critical for zebrafish intestinal development and differentiation. *J Biol Chem* 279, 51581-51589.

Nagy, N.E., Holven, K.B., Roos, N., Senoo, H., Kojima, N., Norum, K.R., and Blomhoff, R. (1997). Storage of vitamin A in extrahepatic stellate cells in normal rats. *J Lipid Res* 38, 645-658.

Nakayama, T., Momoki-Soga, T., and Inoue, N. (2003). Astrocyte-derived factors instruct differentiation of embryonic stem cells into neurons. *Neurosci Res* 46, 241-249.

Napoli, J.L. (1996). Retinoic acid biosynthesis and metabolism. *FASEB J* 10, 993-1001.

Napoli, J.L. (1997). Retinoid binding-proteins redirect retinoid metabolism: biosynthesis and metabolism of retinoic acid. *Semin Cell Dev Biol* 8, 403-415.

Napoli, J.L. (1999). Interactions of retinoid binding proteins and enzymes in retinoid metabolism. *Biochim Biophys Acta* 1440, 139-162.

Napoli, J.L. (2010). Vitamin A (Retinoids). In *Encyclopedia of Biological Chemistry* (Elsevier Ltd), p. in press.

Nedergaard, M., Ransom, B., and Goldman, S.A. (2003). New roles for astrocytes: redefining the functional architecture of the brain. *Trends Neurosci* 26, 523-530.

Niederreither, K., Subbarayan, V., Dolle, P., and Chambon, P. (1999). Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. *Nat Genet* 21, 444-448.

Niederreither, K., Vermot, J., Fraulob, V., Chambon, P., and Dolle, P. (2002a). Retinaldehyde dehydrogenase 2 (RALDH2)- independent patterns of retinoic acid

synthesis in the mouse embryo. *Proc Natl Acad Sci U S A* 99, 16111-16116.

Niederreither, K., Vermot, J., Messaddeq, N., Schuhbauer, B., Chambon, P., and Dolle, P. (2001). Embryonic retinoic acid synthesis is essential for heart morphogenesis in the mouse. *Development* 128, 1019-1031.

Niederreither, K., Vermot, J., Schuhbauer, B., Chambon, P., and Dolle, P. (2002b). Embryonic retinoic acid synthesis is required for forelimb growth and anteroposterior patterning in the mouse. *Development* 129, 3563-3574.

Noy, N. (2000). Retinoid-binding proteins: mediators of retinoid action. *Biochem J* 348 Pt 3, 481-495.

O'Byrne, S.M., Wongsiriroj, N., Libien, J., Vogel, S., Goldberg, I.J., Baehr, W., Palczewski, K., and Blaner, W.S. (2005). Retinoid absorption and storage is impaired in mice lacking lecithin:retinol acyltransferase (LRAT). *J Biol Chem* 280, 35647-35657.

Ong, D.E., MacDonald, P.N., and Gubitosi, A.M. (1988). Esterification of retinol in rat liver. Possible participation by cellular retinol-binding protein and cellular retinol-binding protein II. *J Biol Chem* 263, 5789-5796.

Organization, W.H. (1995). Global prevalence of vitamin A deficiency. Geneva.

Paemeleire, K. (2002). The cellular basis of neurovascular metabolic coupling. *Acta Neurol Belg* 102, 153-157.

Pardridge, W.M., Sakiyama, R., and Coty, W.A. (1985). Restricted transport of vitamin D and A derivatives through the rat blood-brain barrier. *J Neurochem* 44, 1138-1141.

Pares, X., Farres, J., Kedishvili, N., and Duester, G. (2008). Medium- and short-chain dehydrogenase/reductase gene and protein families : Medium-chain and short-chain dehydrogenases/reductases in retinoid metabolism. *Cell Mol Life Sci* 65, 3936-3949.

Pasutto, F., Sticht, H., Hammersen, G., Gillessen-Kaesbach, G., Fitzpatrick, D.R., Nurnberg, G., Brasch, F., Schirmer-Zimmermann, H., Tolmie, J.L., Chitayat, D., *et al.* (2007). Mutations in STRA6 cause a broad spectrum of malformations including anophthalmia, congenital heart defects, diaphragmatic hernia, alveolar capillary dysplasia, lung hypoplasia, and mental retardation. *Am J Hum Genet* 80, 550-560.

Penniston, K.L., and Tanumihardjo, S.A. (2006). The acute and chronic toxic effects of vitamin A. *Am J Clin Nutr* 83, 191-201.

Pereira, F., Rosenmann, E., Nylén, E., Kaufman, M., Pinsky, L., and Wrogemann, K. (1991). The 56 kDa androgen binding protein is an aldehyde dehydrogenase. *Biochem Biophys Res Commun* 175, 831-838.

Perry, S.W., Dewhurst, S., Bellizzi, M.J., and Gelbard, H.A. (2002). Tumor necrosis factor-alpha in normal and diseased brain: Conflicting effects via intraneuronal receptor crosstalk? *J Neurovirol* 8, 611-624.

Petkovich, P.M. (2001). Retinoic acid metabolism. *J Am Acad Dermatol* 45, S136-142.

Pfahl, M., Apfel, R., Bendik, I., Fanjul, A., Graupner, G., Lee, M.O., La-Vista, N., Lu, X.P., Piedrafita, J., Ortiz, M.A., *et al.* (1994). Nuclear retinoid receptors and their mechanism of action. *Vitam Horm* 49, 327-382.

Pino-Lagos, K., Guo, Y., and Noelle, R.J. (2010). Retinoic acid: A key player in immunity.

Biofactors.

Popovich, P.G., and Longbrake, E.E. (2008). Can the immune system be harnessed to repair the CNS? *Nat Rev Neurosci* 9, 481-493.

Posch, K.C., Burns, R.D., and Napoli, J.L. (1992). Biosynthesis of all-trans-retinoic acid from retinal. Recognition of retinal bound to cellular retinol binding protein (type I) as substrate by a purified cytosolic dehydrogenase. *J Biol Chem* 267, 19676-19682.

Prins, G.S., Chang, W.Y., Wang, Y., and van Breemen, R.B. (2002). Retinoic acid receptors and retinoids are up-regulated in the developing and adult rat prostate by neonatal estrogen exposure. *Endocrinology* 143, 3628-3640.

Quadro, L., Blaner, W.S., Salchow, D.J., Vogel, S., Piantedosi, R., Gouras, P., Freeman, S., Cosma, M.P., Colantuoni, V., and Gottesman, M.E. (1999). Impaired retinal function and vitamin A availability in mice lacking retinol-binding protein. *EMBO J* 18, 4633-4644.

Quadro, L., Hamberger, L., Colantuoni, V., Gottesman, M.E., and Blaner, W.S. (2003). Understanding the physiological role of retinol-binding protein in vitamin A metabolism using transgenic and knockout mouse models. *Mol Aspects Med* 24, 421-430.

Ransom, B., Behar, T., and Nedergaard, M. (2003). New roles for astrocytes (stars at last). *Trends Neurosci* 26, 520-522.

Rexer, B.N., and Ong, D.E. (2002). A novel short-chain alcohol dehydrogenase from rats with retinol dehydrogenase activity, cyclically expressed in uterine epithelium. *Biol Reprod* 67, 1555-1564.

Rochette-Egly, C., and Germain, P. (2009). Dynamic and combinatorial control of gene expression by nuclear retinoic acid receptors (RARs). *Nucl Recept Signal* 7, e005.

Rodnight, R., Goncalves, C.A., Wofchuk, S.T., and Leal, R. (1997). Control of the phosphorylation of the astrocyte marker glial fibrillary acidic protein (GFAP) in the immature rat hippocampus by glutamate and calcium ions: possible key factor in astrocytic plasticity. *Braz J Med Biol Res* 30, 325-338.

Romand, R., Dolle, P., and Hashino, E. (2006). Retinoid signaling in inner ear development. *J Neurobiol* 66, 687-704.

Romand, R., Kondo, T., Cammas, L., Hashino, E., and Dolle, P. (2008). Dynamic expression of the retinoic acid-synthesizing enzyme retinol dehydrogenase 10 (rdh10) in the developing mouse brain and sensory organs. *J Comp Neurol* 508, 879-892.

Ross, A.C. (2003). Retinoid production and catabolism: role of diet in regulating retinol esterification and retinoic Acid oxidation. *J Nutr* 133, 291S-296S.

Ross, A.C., and Zolfaghari, R. (2004). Regulation of hepatic retinol metabolism: perspectives from studies on vitamin A status. *J Nutr* 134, 269S-275S.

Rothman, K.J., Moore, L.L., Singer, M.R., Nguyen, U.S., Mannino, S., and Milunsky, A. (1995). Teratogenicity of high vitamin A intake. *N Engl J Med* 333, 1369-1373.

Sandell, L.L., Sanderson, B.W., Moiseyev, G., Johnson, T., Mushegian, A., Young, K., Rey, J.P., Ma, J.X., Staehling-Hampton, K., and Trainor, P.A. (2007). RDH10 is essential for synthesis of embryonic retinoic acid and is required for limb, craniofacial, and organ development. *Genes Dev* 21, 1113-1124.

- Schrage, K., Koopmans, G., Joosten, E.A., and Mey, J. (2006). Macrophages and neurons are targets of retinoic acid signaling after spinal cord contusion injury. *Eur J Neurosci* 23, 285-295.
- Schug, T.T., Berry, D.C., Shaw, N.S., Travis, S.N., and Noy, N. (2007). Opposing effects of retinoic acid on cell growth result from alternate activation of two different nuclear receptors. *Cell* 129, 723-733.
- Senoo, H. (2004). Structure and function of hepatic stellate cells. *Med Electron Microsc* 37, 3-15.
- Shapiro, M.L., and Eichenbaum, H. (1999). Hippocampus as a memory map: synaptic plasticity and memory encoding by hippocampal neurons. *Hippocampus* 9, 365-384.
- Shaw, N., Elholm, M., and Noy, N. (2003). Retinoic acid is a high affinity selective ligand for the peroxisome proliferator-activated receptor beta/delta. *J Biol Chem* 278, 41589-41592.
- Shearer, K.D., Goodman, T.H., Ross, A.W., Reilly, L., Morgan, P.J., and McCaffery, P.J. (2010). Photoperiodic regulation of retinoic acid signaling in the hypothalamus. *J Neurochem* 112, 246-257.
- Shudo, K., Fukasawa, H., Nakagomi, M., and Yamagata, N. (2009). Towards retinoid therapy for Alzheimer's disease. *Curr Alzheimer Res* 6, 302-311.
- Siegenthaler, J.A., Ashique, A.M., Zarbali, K., Patterson, K.P., Hecht, J.H., Kane, M.A., Folias, A.E., Choe, Y., May, S.R., Kume, T., *et al.* (2009). Retinoic acid from the meninges regulates cortical neuron generation. *Cell* 139, 597-609.
- Silman, I., and Sussman, J.L. (2005). Acetylcholinesterase: 'classical' and 'non-classical' functions and pharmacology. *Curr Opin Pharmacol* 5, 293-302.
- Song, H., Stevens, C.F., and Gage, F.H. (2002). Astroglia induce neurogenesis from adult neural stem cells. *Nature* 417, 39-44.
- Soref, C.M., Di, Y.P., Hayden, L., Zhao, Y.H., Satre, M.A., and Wu, R. (2001). Characterization of a novel airway epithelial cell-specific short chain alcohol dehydrogenase/reductase gene whose expression is up-regulated by retinoids and is involved in the metabolism of retinol. *J Biol Chem* 276, 24194-24202.
- Sriram, K., and O'Callaghan, J.P. (2007). Divergent roles for tumor necrosis factor-alpha in the brain. *J Neuroimmune Pharmacol* 2, 140-153.
- Steven W. Levison, J.d.V., and James E. Goldman (2005). Astrocyte development. *Developmental Neurobiology*, 4th ed.
- Sun, T., Xiao, H.S., Zhou, P.B., Lu, Y.J., Bao, L., and Zhang, X. (2006). Differential expression of synaptoporin and synaptophysin in primary sensory neurons and up-regulation of synaptoporin after peripheral nerve injury. *Neuroscience* 141, 1233-1245.
- Suzuki, Y., Ishiguro, S., and Tamai, M. (1993). Identification and immunohistochemistry of retinol dehydrogenase from bovine retinal pigment epithelium. *Biochim Biophys Acta* 1163, 201-208.
- Taimi, M., Helvig, C., Wisniewski, J., Ramshaw, H., White, J., Amad, M., Korczak, B.,

and Petkovich, M. (2004). A novel human cytochrome P450, CYP26C1, involved in metabolism of 9-cis and all-trans isomers of retinoic acid. *J Biol Chem* 279, 77-85.

Takahashi, J., Palmer, T.D., and Gage, F.H. (1999). Retinoic acid and neurotrophins collaborate to regulate neurogenesis in adult-derived neural stem cell cultures. *J Neurobiol* 38, 65-81.

Tan, N.S., Michalik, L., Desvergne, B., and Wahli, W. (2004). Peroxisome proliferator-activated receptor-beta as a target for wound healing drugs. *Expert Opin Ther Targets* 8, 39-48.

Thatcher, J.E., and Isoherranen, N. (2009). The role of CYP26 enzymes in retinoic acid clearance. *Expert Opin Drug Metab Toxicol* 5, 875-886.

Theodosiou, M., Laudet, V., and Schubert, M. (2010). From carrot to clinic: an overview of the retinoic acid signaling pathway. *Cell Mol Life Sci* 67, 1423-1445.

Thompson Haskell, G., Maynard, T.M., Shatzmiller, R.A., and Lamantia, A.S. (2002). Retinoic acid signaling at sites of plasticity in the mature central nervous system. *J Comp Neurol* 452, 228-241.

Toresson, H., Mata de Urquiza, A., Fagerstrom, C., Perlmann, T., and Campbell, K. (1999). Retinoids are produced by glia in the lateral ganglionic eminence and regulate striatal neuron differentiation. *Development* 126, 1317-1326.

Turrin, N.P., and Rivest, S. (2006). Tumor necrosis factor alpha but not interleukin 1 beta mediates neuroprotection in response to acute nitric oxide excitotoxicity. *J Neurosci* 26, 143-151.

Unsicker, K., Reichert-Preibsch, H., Schmidt, R., Pettmann, B., Labourdette, G., and Sensenbrenner, M. (1987). Astroglial and fibroblast growth factors have neurotrophic functions for cultured peripheral and central nervous system neurons. *Proc Natl Acad Sci U S A* 84, 5459-5463.

Vaccarino, F.M., Fagel, D.M., Ganat, Y., Maragnoli, M.E., Ment, L.R., Ohkubo, Y., Schwartz, M.L., Silbereis, J., and Smith, K.M. (2007). Astroglial cells in development, regeneration, and repair. *Neuroscientist* 13, 173-185.

Wagner, E., Luo, T., and Drager, U.C. (2002). Retinoic acid synthesis in the postnatal mouse brain marks distinct developmental stages and functional systems. *Cereb Cortex* 12, 1244-1253.

Wajant, H., Pfizenmaier, K., and Scheurich, P. (2003). Tumor necrosis factor signaling. *Cell Death Differ* 10, 45-65.

Wald, G. (1968). The molecular basis of visual excitation. *Nature* 219, 800-807.

Wang, X., Allen, C., and Ballow, M. (2007). Retinoic acid enhances the production of IL-10 while reducing the synthesis of IL-12 and TNF-alpha from LPS-stimulated monocytes/macrophages. *J Clin Immunol* 27, 193-200.

Wang, X., Penzes, P., and Napoli, J.L. (1996). Cloning of a cDNA encoding an aldehyde dehydrogenase and its expression in *Escherichia coli*. Recognition of retinal as substrate. *J Biol Chem* 271, 16288-16293.

Wei, L.N. (2003). Retinoid receptors and their coregulators. *Annu Rev Pharmacol Toxicol*

43, 47-72.

Werner, E.A., and Deluca, H.F. (2002). Retinoic acid is detected at relatively high levels in the CNS of adult rats. *Am J Physiol Endocrinol Metab* 282, E672-678.

White, J.A., Guo, Y.D., Baetz, K., Beckett-Jones, B., Bonasoro, J., Hsu, K.E., Dilworth, F.J., Jones, G., and Petkovich, M. (1996). Identification of the retinoic acid-inducible all-trans-retinoic acid 4-hydroxylase. *J Biol Chem* 271, 29922-29927.

White, J.A., Ramshaw, H., Taimi, M., Stangle, W., Zhang, A., Everingham, S., Creighton, S., Tam, S.P., Jones, G., and Petkovich, M. (2000). Identification of the human cytochrome P450, P450RAI-2, which is predominantly expressed in the adult cerebellum and is responsible for all-trans-retinoic acid metabolism. *Proc Natl Acad Sci U S A* 97, 6403-6408.

Witter, M.P., Naber, P.A., van Haeften, T., Machielsen, W.C., Rombouts, S.A., Barkhof, F., Scheltens, P., and Lopes da Silva, F.H. (2000). Cortico-hippocampal communication by way of parallel parahippocampal-subicular pathways. *Hippocampus* 10, 398-410.

Wolbach, S.B., and Howe, P.R. (1925). Tissue Changes Following Deprivation of Fat-Soluble a Vitamin. *J Exp Med* 42, 753-777.

Wu, B.X., Chen, Y., Fan, J., Rohrer, B., Crouch, R.K., and Ma, J.X. (2002). Cloning and characterization of a novel all-trans retinol short-chain dehydrogenase/reductase from the RPE. *Invest Ophthalmol Vis Sci* 43, 3365-3372.

Wu, B.X., Moiseyev, G., Chen, Y., Rohrer, B., Crouch, R.K., and Ma, J.X. (2004). Identification of RDH10, an All-trans Retinol Dehydrogenase, in Retinal Muller Cells. *Invest Ophthalmol Vis Sci* 45, 3857-3862.

Wuarin, L., Sidell, N., and de Vellis, J. (1990). Retinoids increase perinatal spinal cord neuronal survival and astroglial differentiation. *Int J Dev Neurosci* 8, 317-326.

Wyss, R., and Bucheli, F. (1997). Determination of endogenous levels of 13-cis-retinoic acid (isotretinoin), all-trans-retinoic acid (tretinoin) and their 4-oxo metabolites in human and animal plasma by high-performance liquid chromatography with automated column switching and ultraviolet detection. *J Chromatogr B Biomed Sci Appl* 700, 31-47.

Yamauchi, K., and Nakajima, J. (2002). Effect of coenzymes and thyroid hormones on the dual activities of *Xenopus* cytosolic thyroid-hormone-binding protein (xCTBP) with aldehyde dehydrogenase activity. *Eur J Biochem* 269, 2257-2264.

Yamauchi, K., Nakajima, J., Hayashi, H., Horiuchi, R., and Tata, J.R. (1999). *Xenopus* cytosolic thyroid hormone-binding protein (xCTBP) is aldehyde dehydrogenase catalyzing the formation of retinoic acid. *J Biol Chem* 274, 8460-8469.

Yang, I., Han, S.J., Kaur, G., Crane, C., and Parsa, A.T. (2010). The role of microglia in central nervous system immunity and glioma immunology. *J Clin Neurosci* 17, 6-10.

Yang, Y., Ge, W., Chen, Y., Zhang, Z., Shen, W., Wu, C., Poo, M., and Duan, S. (2003). Contribution of astrocytes to hippocampal long-term potentiation through release of D-serine. *Proc Natl Acad Sci U S A* 100, 15194-15199.

Yashiro, K., Zhao, X., Uehara, M., Yamashita, K., Nishijima, M., Nishino, J., Saijoh, Y., Sakai, Y., and Hamada, H. (2004). Regulation of retinoic acid distribution is required for

proximodistal patterning and outgrowth of the developing mouse limb. *Dev Cell* 6, 411-422.

Yee, K.K., and Rawson, N.E. (2000). Retinoic acid enhances the rate of olfactory recovery after olfactory nerve transection. *Brain Res Dev Brain Res* 124, 129-132.

Yokota, A., Takeuchi, H., Maeda, N., Ohoka, Y., Kato, C., Song, S.Y., and Iwata, M. (2009). GM-CSF and IL-4 synergistically trigger dendritic cells to acquire retinoic acid-producing capacity. *Int Immunol* 21, 361-377.

Yost, R.W., Harrison, E.H., and Ross, A.C. (1988). Esterification by rat liver microsomes of retinol bound to cellular retinol-binding protein. *J Biol Chem* 263, 18693-18701.

Zetterstrom, R.H., Lindqvist, E., Mata de Urquiza, A., Tomac, A., Eriksson, U., Perlmann, T., and Olson, L. (1999). Role of retinoids in the CNS: differential expression of retinoid binding proteins and receptors and evidence for presence of retinoic acid. *Eur J Neurosci* 11, 407-416.

Zetterstrom, R.H., Simon, A., Giacobini, M.M., Eriksson, U., and Olson, L. (1994). Localization of cellular retinoid-binding proteins suggests specific roles for retinoids in the adult central nervous system. *Neuroscience* 62, 899-918.

Zhang, J., Smith, D., Yamamoto, M., Ma, L., and McCaffery, P. (2003). The meninges is a source of retinoic acid for the late-developing hindbrain. *J Neurosci* 23, 7610-7620.

Zhang, M., Chen, W., Smith, S.M., and Napoli, J.L. (2001). Molecular characterization of a mouse short chain dehydrogenase/reductase active with all-trans-retinol in intact cells, mRDH1. *J Biol Chem* 276, 44083-44090.

Zhang, M., Hu, P., Krois, C.R., Kane, M.A., and Napoli, J.L. (2007). Altered vitamin A homeostasis and increased size and adiposity in the rdh1-null mouse. *FASEB J* 21, 2886-2896.

Zhao, D., McCaffery, P., Ivins, K.J., Neve, R.L., Hogan, P., Chin, W.W., and Drager, U.C. (1996). Molecular identification of a major retinoic-acid-synthesizing enzyme, a retinaldehyde-specific dehydrogenase. *Eur J Biochem* 240, 15-22.

Ziouzenkova, O., Orasanu, G., Sharlach, M., Akiyama, T.E., Berger, J.P., Viereck, J., Hamilton, J.A., Tang, G., Dolnikowski, G.G., Vogel, S., *et al.* (2007). Retinaldehyde represses adipogenesis and diet-induced obesity. *Nat Med* 13, 695-702.

Zolfaghari, R., Cifelli, C.J., Lieu, S.O., Chen, Q., Li, N.Q., and Ross, A.C. (2007). Lipopolysaccharide opposes the induction of CYP26A1 and CYP26B1 gene expression by retinoic acid in the rat liver in vivo. *Am J Physiol Gastrointest Liver Physiol* 292, G1029-1036.