PXR IN BRAIN: NOVEL METHODS TO CHARACTERIZE ITS BIOLOGY

By Xunan Shen

Submitted to the graduate degree program in Pharmacology and Toxicology and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Master of Science.

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Chair: Jeff Staudinger, Ph.D.

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Nancy A. Muma, Ph.D.

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Honglian Shi, Ph.D.

Date Defended: April 26th 2017

The thesis committee for Xunan Shen certifies that this is the approved version of the following thesis:

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Chair: Jeff Staudinger, Ph.D.

Date Approved: April 27th 2017

ABSTRACT

Pregnane X receptor (PXR, NR1I2) belongs to the nuclear receptor superfamily of ligand-activated transcription factors and was identified in 1998. PXR is highly expressed in the liver and intestine, and is involved in regulating the expression of genes that encode important drug metabolizing enzymes as well as several key drug transporter proteins. Previous studies have found that ligand-mediated activation of PXR can increase the expression of multidrug resistance protein 1 (Mdr1) in the endothelial cells in the intestine in mice. However, there is no correlation between PXR activation and Mdr1 gene expression levels in liver hepatocytes in these animals. Thus, PXR biology exhibits a curious phenomenon in that there appears to be a tissue-specific role of this nuclear receptor superfamily member. Currently, the molecular mechanism(s) underlying this phenomenon are not known, but we hypothesize here that tissue-specific PXR-binding and co-regulatory/accessory proteins likely play a key role in governing this phenomenon. While liver and intestine express high levels of PXR, other tissues have been found to express lower but significant levels of this nuclear receptor protein including kidney, ovary, stomach, and brain.

Several lines of evidence support the notion of a key role for PXR in regulating brain function. First, the expression of PXR was identified in 2004 in brain capillary endothelial cells. Lower levels of PXR were also detected in several brain regions of different species including rat, rabbit, pig, human, and mouse. Several key endogenous neurosteroidal compounds, including allopregnanolone, have been demonstrated to serve as PXR-ligands to dramatically increase the trans-activation capacity of this nuclear receptor family member. Finally, the proestrous rats infused with PXR anti-sense oligonucleotides to the ventral tegmental area

significantly decreased levels of allopregnanolone, further suggesting an interface between PXR and allopregnanolone metabolism.

Therefore, the purpose of the research described in this thesis is (1) to develop a method for the identification of tissue-specific PXR-binding proteins, and (2) to characterize the potential effects of PXR deletion and PXR activation on the expression of the genes that encode the rate limiting enzymes in the production of allopregnanolone in mice. In the first study, I developed a novel protocol using adenovirus-mediated methods coupled with primary cultures of rat hepatocytes and liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify hepatocyte-specific PXR-binding proteins. In the second study, I tested the hypothesis that PXR can regulate 5α-reductase type 1 (Srd5α1) and type 2 (Srd5α2) gene expression levels in mouse brain. My results reveal that deletion of PXR in mice alters basal expression levels of Srd5α1 and Srd5α2 in a tissue-specific manner. Additionally, Pregnenolone 16α-carbonitrile (PCN) decreased Srd5α1 and Srd5α2 gene expression in the liver and several brain regions in both wild type and PXR knockout mice, suggesting that PCN-mediated decrease of Srd5α1 and Srd5α2 gene expression is in a PXR-independent manner.

Taken together, the data presented in this thesis shed new light upon the role of PXR in regulating the expression of key target genes in the brain. In particular, the data suggest that while PXR plays a role in neurosteroid metabolism, there is also a key PXR-independent role in neurosteroid metabolism in several brain tissue types following exposure to steroidal compounds such as PCN. Finally, the biochemical procedures developed and validated in this thesis should be useful in identifying novel PXR-binding proteins from primary cultures of neuronal cells, as well as other cell types amenable to primary culture methods.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my thesis advisor, Dr. Jeff Staudinger, at the University of Kansas for all the help he gave in my two-year masters training program. His patience, encouragement and advice gave me the direction and led me through the mist towards my graduation. I learned not only science from him, but also life lessons. His care guides and encourages me every day. I am very grateful for what I have learned from him.

I would also like to express my thanks to my committee members for their support and suggestions. Dr. Muma has given me a lot of advice regarding my project and Dr. Shi is always supportive and encouraging.

In addition, I would like to thank Dr. Marco Bortolato's laboratory, specifically, Laura Mosher who trained me on mouse brain dissection. I'd like to give special thanks to my lab member Wenqi Cui for consistent help during this two years. She also trained me on my laboratory skills and provided primary liver hepatocytes to me.

Most importantly, I would like to thank my parents. Without their love and support, I can never make it so far.

This work was supported by the National Institutes of Health National Institute of Digestive, Diabetic, and Kidney Diseases (NIDDK) [Grant R01DK090558] (to J.L.S.), University of Kansas, Strategic Initiative Grant.

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3α-HSD	3α-hydroxysteroid dehydrogenase
AD	Alzheimer's disease
AF2	Activation function 2 region
APL	Acute promyelocytic leukemia
ATRA	Ll-trans-retinoic acid
NPC1	Niemann–Pick type C1
CAR	Constitutive androstane receptor
DSS	Dextran sulfate sodium
ERα	Estrogen receptor α
FXR	Farnesoid X receptor
GR	Glucocorticoid receptor
HDAC1	Histone deacetylase 1
HIV	Human immunodeficiency virus
IBD	Inflammatory bowel disease
IMAC	Immobilized metal affinity chromatography
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDH	Lactate dehydrogenase
LXR	Liver X receptor
MDR	Multidrug resistance
MDR1	Multidrug resistance protein 1
NcoR	Nuclear receptor corepressor
NR	Nuclear receptor

Nup62	Nucleoporin 62
PCN	pregnenolone 16α-carbonitrile
PFC	Prefrontal cortex
PHB1	Prohibitin 1
PHB2	Prohibitin 2
PXR	Pregnane X receptor
Rif	Rifampicin
RINF2	Ring finger protein 2
PIs	Protease inhibitors
RT-PCR	Reverse transcription- polymerase chain reaction
RT-QPCR	Quantitative real-time PCR
SMRT	Silencing mediator for retinoid or thyroid-hormone receptors
SRC-1	Steroid receptor coactivator-1
Srd5α1	5α-reductase type 1
Srd5α2	5α-reductase type 2
Srd5α3	5a-reductase type 3
TBP	TATA box binding protein
VLDL	Very-low density lipoprotein
WT	Wild-type

CHAPTER 1: INTRODUCTION

Pregnane X receptor (PXR, NR1I2) is a ligand-activated transcription factor that belongs to nuclear receptor (NR) superfamily. PXR has a wide range of ligands including lipids, bile acids, and different xenobiotics. Upon activation, PXR binds to a DNA sequence and then regulates its target gene expression.

Similar to other NRs, PXR has a DNA binding domain and a ligand binding domain connected by a hinge region. The DNA binding domain allows PXR to bind to regulatory DNA sequences and therefore regulate transcription process. The ligand binding domain allows PXR to bind to its ligand, dimerize with other nuclear receptors as well as interact with transcriptional co-factors [1]. PXR binds to RXR as a heterodimer to form a transcriptionally active complex [2-3]. When inactive, PXR-RXR forms a complex with corepressor proteins to inhibit transcriptional activity. Activation of PXR by ligands causes a conformational change, resulting in PXR disassociating from its co-repressors. PXR-RXR then recruits coactivators to enhance gene transcription. The main coactivator of PXR is steroid receptor coactivator-1 (SRC-1) and the main co-repressors are silencing mediator for retinoid or thyroid-hormone receptors (SMRT), and nuclear receptor corepressor (NcoR) [4-5].

PXR plays a key role in different kinds of human health problems, including drug-drug interaction, lipid homeostasis, inflammatory based disorders, cancer, and chemotherapeutic resistance.

PXR is highly expressed in the liver and intestine, and can regulate the expression level of phase I P450 enzymes, phase II drug conjugation enzymes and phase III drug transporters [6-11]. Among them are the CYP3A4 enzymes which can metabolize more than 50% of the

clinically used drugs [12] and CYP2B6 which contributes to 10% of the metabolism of clinically used drugs. Therefore, increases in these enzymes, by PXR activation, contributes to the turnover of drugs, and thus, causes drug-drug interactions.

PXR target genes are also involved in lipid metabolism. Activation of PXR chronically increases cholesterol and atherogenic low density lipoprotein (LDL) and very-low density lipoprotein (VLDL) plasma levels in wild-type (WT) mice. This effect was abolished in PXR-knockout (PXR-/-) mice [13], suggesting that PXR plays a role in lipid metabolism. The human immunodeficiency virus (HIV) protease inhibitor (PI) Amprenavir has been shown to increase plasma total cholesterol and atherogenic low density lipoprotein cholesterol levels in wild-type mice, and this effect was totally abolished in PXR-deficient mice. Amprenavir activated PXR and induced CYP3A4, UGT1A1, and MDR1 gene expression in both human hepaRG hepatoma cells and LS180 intestinal cells [14].

PXR additionally contributes to the inflammatory response. Activation of mouse PXR by PCN repressed the NF-κB target genes expression in the dextran sulfate sodium (DSS)-induced inflammatory bowel disease (IBD) mouse model [15]. The interaction between NF-κB p65 and RXR DNA binding domain may inhibit the transactivation by the PXR/RXR complex [16]. Dubrac et al found that PXR expression is increased in T-lymphocytes, a marker of inflammation, following immune activation [17] and activation of PXR inhibits T-lymphocyte proliferation by decreasing the expression of CD25 and IFN-ɣ [17]. These studies indicate that PXR activation can suppress inflammatory responses. Chronic inflammation can precede cancers including hepatocellular carcinoma (HCC), colon cancer (CAC) and gastric cancer [18-19].

The roles of PXR are variable in different cancer tissue types. On the one hand, activation of PXR shows enhanced cell growth and invasion in human colon cancer xenograft mouse models [20], and protects colon cancer LS180 cells from apoptosis [21]. Activation of PXR also protects HepG2 human hepatoma cells from apoptosis [22]. These results suggest that PXR has an anti-apoptotic role in carcinogenesis. On the other hand, PXR shows a pro-apoptotic effect on endometrial and breast cancers [23-24]. Therefore these indicate that PXR has tissue-specific and cancer type-specific functions.

PXR activation can cause chemotherapy resistance. Chemotherapy is one of the major treatments for cancer. However, a large number of the patients experienced multidrug resistance (MDR) during chemotherapy. Jiang et al found that activation of PXR by Rif increased the resistance of colon cancer cells to chemotherapeutics, and shRNA-induced reduction of PXR increased sensitivity of LS174T colon carcinoma cells to chemotherapeutics [25]. In prostate cancer, activation of PXR caused chemotherapeutic resistance and shRNA-based down-regulation of PXR resulted in sensitization toward chemotherapeutic agents, mirroring what was found in colon cancer [26]. All-trans-retinoic acid (ATRA) is a chemotherapeutic agent used for treatment of acute promyelocytic leukemia (APL) [27]. PXR ligands administrated with ATRA can increase ATRA metabolism by increasing CYP3A expression therefore causing ATRA resistance [28]. These results suggest that inhibition of PXR could be a target for reducing resistance to chemotherapeutics.

Recently, a low level of PXR expression has been detected in the brain in different species. PXR was found involved in Alzheimer's disease pathology [29]; activation of PXR delays the progression of neurodegeneration in human Niemann–Pick type C1 (NPC1) disease, a neurodegenerative disease related to the inability to process cholesterol [30]. Moreover, knock

down of PXR decreased levels of allopregnanolone, an endogenous neurosteroid, which led to behavior changes in mice [31]. These indicate that certain type of neurosteroid may activate PXR and regulate PXR function in the brain.

Statement of Purpose

The PXR protein is highly expressed in both liver and intestine where it plays a key role in drug, steroid, and bile acid metabolism. Recently, a low level of PXR expression has been detected in the brain indicating the possibility that PXR may play a role in metabolic brain function, and perhaps in the pathogenesis of neuronal diseases. However, the precise role of PXR in the brain remains unknown. Based on these previous studies, we hypothesize that certain neurosteroids can activate PXR and regulate the expression of vitally important brain-specific PXR-target genes that function in mediating this clinically important phenomenon. Understanding this mechanism may play a key role in finding new drug targets for many neurological diseases.

To date, activation of PXR in the liver and intestines is known to be achieved by numerous compounds including certain steroids, bile acids, natural products, and numerous drugs [32-34]. The increased expression of genes targeted by PXR encodes biochemically linked drug- and steroid-processing proteins that function to regulate the homeostasis of these compounds. For example, PXR activation up-regulates the activity of vital drug and steroid metabolizing enzymes, as well as key drug transporter proteins [35-36]. Drug metabolizing enzymes targeted by PXR include Cytochrome P450 (CYP) proteins CYP3A, CYP2B, and CYP2C [37-38]. PXR activation up-regulates the expression of genes encoding essential drug

and bile acid transporters including key members of the multidrug resistance-associated protein (MRP) and multidrug resistance protein (MDR) families of membrane transporter proteins.

For this proposal, it is noteworthy that PXR activation dramatically increases the expression of Mdr1/P-Gp in the intestine [39]. However, there is no significant correlation between MDR1 expression level and PXR activation in the liver [40]. Taken together, this phenomenon illustrates the now widely accepted notion that PXR has tissue-specific functions as evidenced by the differential and tissue-specific regulation of its target genes. Currently, the molecular mechanism(s) responsible for this phenomenon are unknown. We hypothesize that the tissue-specific PXR co-regulatory protein environment likely mediates the tissue-specific function of PXR and is responsible for the differential regulation of its target genes in different tissues.

Our overall goal is to identify (1) tissue-specific PXR co-regulatory proteins and (2) brain-specific PXR-target genes.

In order to discover PXR-associated proteins, we transduced rat hepatocytes with adenovirus Ad-$(His)_6$-human PXR for 48 hours to achieve a high level of expression of PXR. Together with a non-transduced control group, cells were harvested and the nuclei were extracted in hypotonic buffer. The $(His)_6$-tagged PXR protein and its associated proteins were enriched by immobilized metal affinity chromatography (IMAC). Subsequently, enriched proteins were separated by SDS-PAGE gel and Liquid chromatography-tandem mass spectrometry (LC-MS/MS) detection was performed to identify proteins.

Total RNA was isolated by TRIZOL reagent, and reverse transcription- polymerase chain reaction (RT-PCR) assay was used to detect qualitative expression of genes encoding PXR,

selected CYPs, as well as several key drug transporter proteins in seven mouse brain regions including amygdala, prefrontal cortex, hippocampus, hypothalamus, mid-brain, striatum, and cerebellum. Subsequently, an in vivo study was utilized to understand PXR's role in brain. Mouse PXR ligand PCN or corn oil as a control was injected intraperitoneally into wild-type and PXR-KO mice for 3 consecutive days. On day 4, mice were euthanized and brain regions were dissected and frozen in -80 °C. RNA was isolated from these brain regions. Total RNA isolated from liver was also be used as a positive control group for known PXR-target gene induction. Quantitative real-time PCR (RT-QPCR) was used to quantitatively detect the expression of PXR in brain as well as selected known and potential PXR-target genes including those encoding key 5α-reductase enzymes Srd5α1 and SrR5α2.

CHAPTER 2: A NOVEL METHOD TO IDENTIFY PXR BINDING PARTNERS

Introduction

Cancer is one of the major health problems in the United States. In 2016, an estimated 1,685,210 people were diagnosed as new cancer cases in the United States and around 595,690 people died from cancer [41]. Chemotherapy is one of the most common treatments for cancer. However, the efficacy of chemotherapy is limited because of the multidrug resistance (MDR) phenomenon [42], which is characterized as decreased drug concentrations in the cells as well as a decreased cancer cell response [43]. MDR often causes the failure of chemotherapy and eventually leads to the death. An estimated of 500,000 new cases of cancer will experience MDR each year [44].

MDR can be developed by multiple pathways, including decreased drug influx, increased drug efflux and activated drug detoxification metabolism pathways [45]. Multidrug resistance protein 1 (MDR1, also known as P-glycoprotein 1, P-gp) is an ATP-dependent transmembrane drug efflux transporter. MDR1 is expressed not only in the liver, intestine, kidney, but also in the brain. MDR1 can functionally protect the human body from xenobiotics by pumping them back into the bile ducts, urine, intestinal lumen as well as capillaries in the brain. However, this function can also cause failure of chemotherapy treatment in cancer. Thus, MDR1 overexpression is considered as a major cause of multidrug resistance leading to insufficient drug concentrations in cells [46]. This phenomenon was confirmed in many tumor cells including colon, kidney, breast, liver and pancreatic cells [47]. This suggests that the inhibition of MDR1 could be a strategy for overcoming drug resistance.

First generation inhibitors of MDR1 including quinine, cyclosporine A, and verapamil are already approved by the Food and Drug Administration (FDA), but show toxic side effects at

sufficient MDR1 inhibition doses [48-49]. Second generation inhibitors of MDR1, such as PSC-833, decreased the toxic side effects; however, also decreased the drug clearance systemically and increased the exposure of both normal tissue and cancer tissue to toxic drugs at the same time [50]. Therefore, the investigation of second generation inhibitors of MDR1 has been stopped due to high mortality rates [51]. Third generation inhibitors of MDR1 focused on increasing the affinity of MDR1 binding as well as decreasing the toxicity [52]. The investigations of third generation inhibitors are still ongoing. However, universal inhibition of MDR1 may still cause undesired and uncontrolled toxicity in the human body. Thus, it is necessary to find an alternative mechanism to decrease MDR1 expression or activity in order to overcome MDR.

MDR1 is known to be regulated by nuclear receptor PXR. Upon activation, PXR heterodimerizes with RXR to form an active complex to regulate target genes transcription processes [53]. Several groups found that PXR activation can significantly increase the expression of MDR1 in the intestine, but not in the liver [54]. The mechanism of this phenomenon remains unknown. However, it provides a new basis to regulate tissue-specific MDR1 expression, thus may offer new therapies for overcoming MDR.

PXR has a DNA-binding domain, a ligand-binding domain containing an activation function 2 region (AF2). The structure of AF2 allows PXR to recruit different transcriptional coregulatory proteins, including coactivators and co-repressors, which play critical roles in gene regulation processes. Thus we hypothesized that the tissue-specific PXR co-regulatory protein environment likely mediates the tissue-specific function of PXR and is responsible for the differential regulation of its target genes in different tissues. Here we reported a novel method to identify PXR co-regulators in primary cultured rat hepatocytes.

Materials and Methods

Plasmids and chemicals

The human PXR adenovirus was generated as previously described [55]. Briefly, PXR STOP codon was removed and EcoRV and Xho I restriction enzyme sites were introduced by PCR using $(His)_6$-tagged PXR as a template. PCR product was inserted into the multiple cloning sites in pShuttle IRES-hrGFP expression vector. Then human PXR adenovirus was generated as previously described [56].

Isolation and culturing of primary rat hepatocytes

Adult male rats were allowed water and maintained on standard laboratory chow for one week. Primary hepatocytes were isolated from live rats using a standard collagenase perfusion procedure and the medium was replaced with serum-free Williams E medium four hours later as previously described [57].

Adenovirus infection and GFP fluorescence detection

Four 15-cm plates of non-transduced hepatocytes were used as a control group and four 15-cm plates of hepatocytes were infected with Ad-$(His)_6$-hPXR (0.8 µl/plate). After 48 hours, GFP expression was observed and recorded using a fluorescence microscope.

Nuclei and cytosol fraction separation

Each plate of cells were harvested with 1.5 mL PBS containing protease inhibitors (Fisher, A32965) and centrifuged at 240 x g for 10min at 4 °C. Cell pellets were lysed by 5 pellet volumes of hypotonic buffer (**Table** 1) and centrifuged at 106 x g for 10 min at 4°C. The supernatant was saved as cytosol fraction, and the pellet as nuclei fraction.

Immobilized metal affinity chromatography assay

Nuclear fraction was re-suspended in 1.5 mL lysis buffer (**Table** 1) containing protease inhibitors and homogenized to mix thoroughly. Cobalt beads were pre-washed with lysis buffer and were added to each tube, agitated for 2 hours at 4°C to allow the cobalt beads to bind to $(His)_6$-tagged proteins. Then samples were centrifuged at 425 x g for 1 min at 4°C, and washed with 1 mL wash buffer (**Table** 1) for 3 times by centrifuging at 425 x g for 1 min each time. 30 µL 2x SDS sample buffer (with 50 mM DTT) was added to each sample tube.

Coommassie blue staining, silver staining and western blot analysis

Proteins were resolved on 4-15% gradient SDS-polyacrylamide gel electrophoresis gels and coommassie blue staining was performed by adding coommassie blue dye and agitating for 30 min. Then the gel was de-stained in ddH_2O for 10 min and saved for LC-MS/MS assay. Proteins were also resolved on 4-15% gradient SDS-polyacrylamide gel electrophoresis gels and transferred to polyvinylidene difluoride microporous membranes (Millipore Bioscience Research Reagents) that were probed with anti-PXR antibody (H-11, Santa Cruz). Silver staining was performed as previously described [58].

Liquid chromatography-tandem mass spectrometry analysis

The LC–MS/MS analysis for identifying PXR binding proteins was performed as described previously [59]. Briefly, bands were cut and digested by trypsin, and then analyzed by LC-MS/MS.

Results

PXR overexpression by adenoviral infection

In order to increase hPXR expression in cells and limit adenovirus toxicity, time course and tittering experiments of Ad-$(His)_6$-hPXR were performed in HepG2 and Hepa1-6 cells (data

not shown) to determine adenovirus amount and transduction time. Primary rat hepatocytes were isolated and cultured as described and the experiment design is shown in **Figure** 1. In order to achieve high expression of human PXR to exceed the LC-MS/MS detection limitation, we used 4 15-cm plates of hepatocytes as an experimental group, and another 4 plates as control. Cells in experimental group were infected with Ad-$(His)_6$-hPXR for 48 hours. Together with control group, cell morphology and GFP protein expression were observed and recorded using a fluorescence microscope. Because of the characteristics of internal ribosome entry site (IRES) in adenovirus expression vector, after translation, the expression of PXR protein should be more than 10 times the GFP protein expression. We achieved high expression levels of human PXR in hepatocytes (**Figure** 2). In addition, there was no significant difference in cell viability between the experimental group and control group, which suggests that our transduction method is efficient and non-toxic to primary hepatocytes.

Enrich PXR and binding proteins by IMAC

The string of histidine residues can bind to cobalt beads under specific buffer conditions and can be used to enrich $(His)_6$-tagged proteins. We tried to perform immobilized metal affinity chromatography assays using whole cell lysates. However, the coomassie blue staining showed that numerous background proteins were also pulled down with cobalt beads (data not shown), indicating high level of non-specificity as well as high background for LC-MS/MS analysis assay. Upon activation, PXR is mainly located in the nuclei to regulate target gene transcription, so our proteins of interest should be located mainly in nuclei. We decided to use a biochemical method to extract the nuclei fraction from whole cell lysate and therefore to reduce background signals. Fractions were resolved and separated on 4-15% gradient SDS-PAGE gel, and coomassie blue staining showed that we significantly reduced background signal (**Figure** 3). We

used nuclei marker, nucleoporin 62 (Nup62), and cytosol marker, lactate dehydrogenase (LDH), to further confirm the efficiency of biochemical separation. Western blotting showed a successful separation between nuclei and cytosol fraction (**Figure** 3).

Mild buffer conditions (Imidazole and Tween-20) were used in lysis and washing buffers and all the samples were kept at 4 °C or on ice during the procedure to preserve the binding between PXR and its associated proteins. Immobilized metal affinity chromatography was performed with 25 µL sample of each group resolved and separated on 4-15% gradient SDS-PAGE gel. Compared with control group, the PXR overexpression group showed a significant enrichment of PXR proteins (**Figure** 4) in coomassie blue staining, and western blotting with anti-PXR antibody H-11 further confirmed this result (**Figure** 4). Many more bands appeared in the PXR overexpression group as compared to the control group in coomassie staining gel, which we think were potential PXR associated proteins.

The sensitivity limit of coomassie blue, in this case, instant blue, is 5 ng. Thus, proteins less than 5 ng cannot be visualized with coomassie blue staining. Sliver staining, however, is much more sensitive than coomassie blue, and can detect proteins as low as 0.25 ng/ µl. Therefore, we used silver staining to visualize lower concentrations of proteins as a guide for the LC-MS/MS assay. As expected, there were some bands detected with silver staining but not with coomassie blue, especially proteins larger than 180 kDa (**Figure** 5). Based on these results, we decided to use LC-MS/MS assay guided by silver staining to detect potential PXR associated proteins. LC-MS/MS assay was performed and multiple candidate PXR-binding proteins were identified (**Table** 2).

Discussion

In this study, we designed a novel method to identify PXR-binding partners that uses adenovirus-mediated overexpression of PXR in primary cultured rat hepatocytes. Non-denaturing IMAC-mediated purification methods are coupled with LC-MS/MS to identify co-purified PXR-binding partners. Coomassie blue and silver staining showed high levels of enrichment of exogenously added PXR and its likely binding proteins, thus our buffer conditions are useful tools for this novel non-denaturing IMAC assay. Using this method, we identified Retinoid X receptor α (RXRα) as a heterodimeric partner of PXR. This is important because RXRα serves as the protein partner of numerous liver-enriched nuclear receptors including CAR, LXR, PPAR, as well as PXR [60]. The RXRα protein is expressed at high levels in liver, intestine, and kidney tissues [61]. Hence, this result lends a high level of confidence that our experimental approach is valid.

Of note, the TATA box binding protein (TBP)-associated factors (TAF) including TAF2, TAF5, and TAF6 were also identified as potential PXR binding partners in this study. TBP-associated factors contribute to the RNA polymerase II pre-initiation complex when bound with TBP [62]. Previously, nuclear receptors such as RXRα [63], progesterone receptor [64], and vitamin D receptor [65] were found to interact with this complex to increase the rate of transcription initiation. TAFs function as mediators between nuclear receptors and basal transcription factors in this process. Thus, it is highly likely that we isolated these proteins as a part of a multi-protein complex using PXR as a bait protein in this assay. These data lend further confidence in the validity of our methods.

Several novel candidate PXR-binding proteins including Prohibitin 1, Prohibitin 2, and Jun D, were also identified (**Table** 2). Prohibitins are involved in cell proliferation [66], survival, apoptosis [67], and signaling. Prohibitins, also known as PHB, are ubiquitously expressed and

divided into two classes called PHB1 and PHB2. They have been suggested to interact with different nuclear receptor proteins to modulate their transcriptional activity. While PHB2 binds to estrogen receptor α (ERα) to repress its transactivation capacity [68], PHB also has a repressive effect on the androgen receptor (AR) [69]. In addition, PHB interacts with the important nuclear receptor co-regulatory proteins Nuclear Co-Repressor (NCoR), and histone deacetylase 1 (HDAC1). Additionally, PHB interacts with retinoblastoma (Rb) to repress E2F family transcriptional activities [70-71]. As discussed previously, NCoR and HDAC1 are also well-known PXR co-regulatory binding proteins. Thus, it is highly possible that PHB is part of a multi-protein complex to regulate the transactivation capacity of PXR.

JunD is a transcription factor that belongs to JUN family. JunD functions as a coactivator of the nuclear receptor AR to form a complex that can bind to AR target gene to regulate its transactivation capacity [72]. It is therefore possible that JunD could also interact with the nuclear receptor PXR to modulate its transactivation capacity. Further research is necessary to determine the extent to which this is true.

Another potential binding partner identified is Ring finger protein 2 (RNF2), which is expressed in different cancer types including breast cancer [73], ovarian cancer [74], and pancreatic cancer [75]. To date, there is no evidence showing that RNF2 can interact with any nuclear receptors. It would therefore be interesting to investigate RNF2's biological function in terms of binding to and regulating PXR activity.

In comparison with other methods used to identify protein partners such as yeast two-hybrid system, the method we developed here has several distinct advantages. First, our method utilized primary hepatocytes, or other primary cell types, with an appropriate cellular background. This fact allows for the identification of endogenous co-regulatory proteins from a

pertinent repertoire of potential protein partners. In other words, binding partners are identified from a wild type context. Second, our method utilized full length PXR, as opposed to the artificial fusion of the GAL4-DBD and the PXR-LBD as occurs in the yeast two-hybrid system. Finally, our system allows for the use of PXR ligands, both agonists and antagonists, in the context of a wild type milieu.

In comparison with another commonly used method, transfection activity based enrichment of PXR and its binding proteins, our method using adenoviral infection assay has a high efficiency in terms of overexpression of PXR proteins. Instead of using HeLa cells, our method allows the use of wild type primary cultured cell types and therefore is useful for identifying tissue-specific PXR binding proteins.

Taken together, these data indicate that our method is valid for the identification of PXR-associated proteins from primary cultured hepatocytes, and can likely be used to identify tissue-specific PXR-binding partners in primary cultured cells isolated from other tissue types such as the primary cultures of enterocytes and neuronal cells. Future studies should commence with co-immunoprecipitation methods to further confirm binding between PXR and candidate PXR-binding proteins. Besides, the human PXR ligand, rifampicin, could be added to this assay in order to identify ligand-dependent PXR binding proteins. In addition, PXR co-regulatory proteins that can respond to inflammation can also be identified using stimulated cells under stress condition.

Table 1 Buffer condition

Lysis buffer pH = 8.0	NaH_2PO_4	50 mM
	NaCl	300 mM
	Imidazole	10 mM
	Tween 20	0.05%
Wash buffer pH = 8.0	NaH_2PO_4	50 mM
	NaCl	300 mM
	Imidazole	20 mM
	Tween 20	0.05%
Hypotonic Buffer	Tris (pH=7.7)	10 mM
	$MgCl_2$	1.5 mM
	DTT (before use)	1 mM

Table 2 Identified candidate PXR-binding proteins

Identified Proteins	**Peptides Identified**
heat shock protein 70	34
Cyclin-dependent kinase 9	21
Prohibitin 1	11
Retinoid X receptor alpha	7
TAF5 RNA polymerase II, TATA box binding protein (TBP)-associated factor	6
Cyclin-dependent kinase 12 isoform X1	5
Prohibitin 2	4
Jun D proto-oncogene	4
pre-mRNA-splicing regulator WTAP isoform a	4
Rbmx2 protein	4
Chaperonin containing Tcp1, subunit 3 (gamma)	4
TAF2 RNA polymerase II, TATA box binding protein (TBP)-associated factor	3
Ring finger protein 2	3
TAF6 RNA polymerase II, TATA box binding protein (TBP)-associated factor, isoform CRAa	3
Chaperonin containing TCP1, subunit 2 (beta)	2
Zinc finger protein 644 isoform X2	2

Figure 1

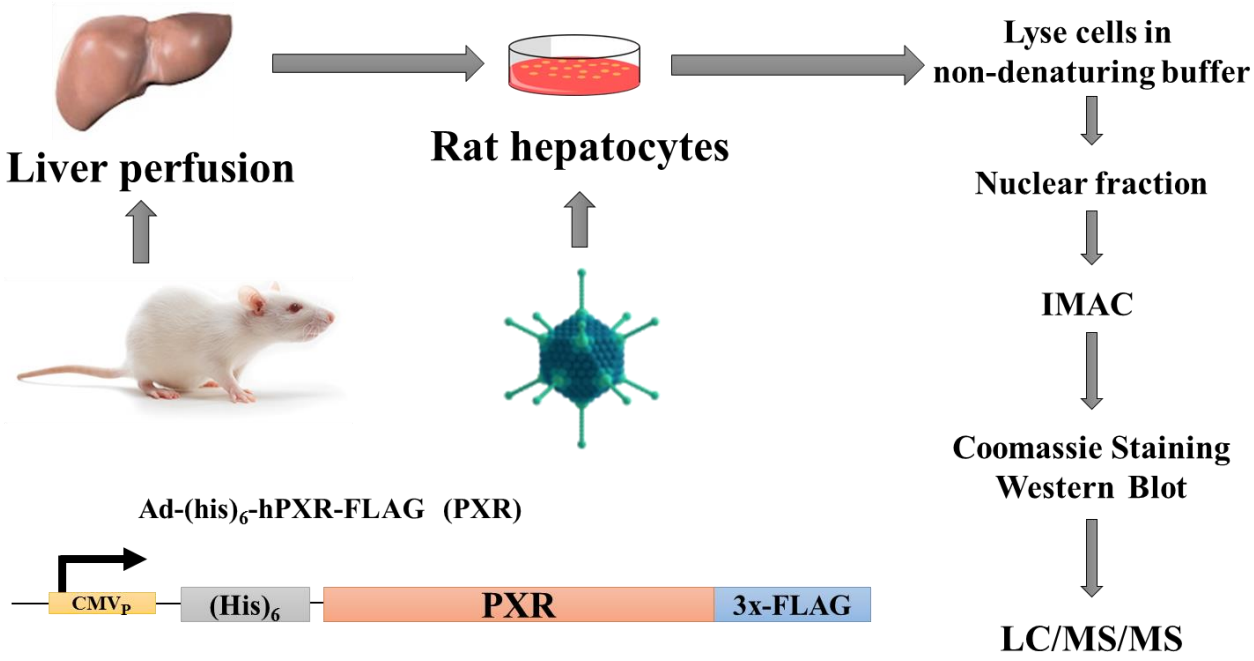


Figure 1. Experimental design. Liver perfusion was performed in live rats to get rat hepatocytes. Cells were infected with Ad-$(His)_6$-hPXR-FLAG adenovirus for 48 hours, and lysed with non-denaturing lysis buffer. Nuclear fraction was extracted using a biochemical method, followed by immobilized metal affinity chromatography. Coomassie staining and Western blotting were performed to visualize PXR and its binding proteins. Liquid chromatography-tandem mass spectrometry (LC/MS/MS) was performed to identify PXR binding partners.

Figure 2

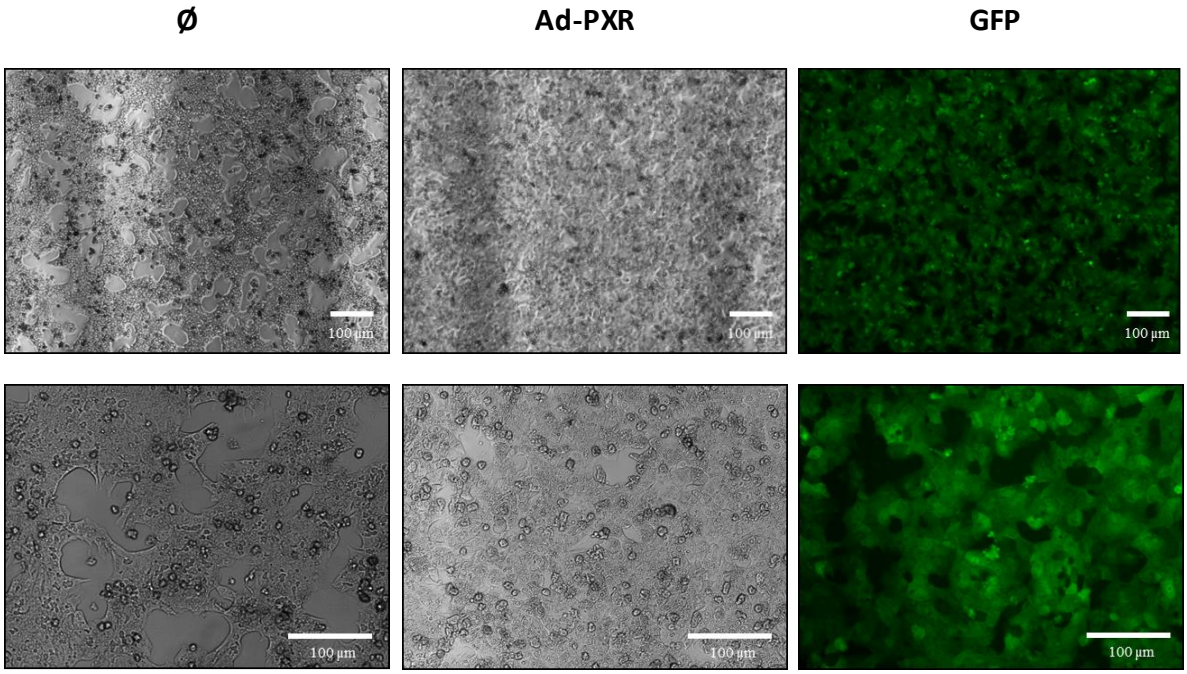


Figure 2. Adenovirus infection assay. Primary rat hepatocytes were isolated and transduced with Ad-hPXR for 48 hours; cell morphology and GFP expression were detected using fluorescence microscope. Ø: Non-transduction control group, Ad-PXR: adenoviral infection group, GFP: GFP fluorescence in adenoviral infection group. Compared with the left panel, the middle panel showed that PXR adenovirus didn't cause significant cell damage, therefore is not toxic to hepatocytes in this dosage. GFP fluorescence showed a high overexpression of PXR protein.

Figure 3

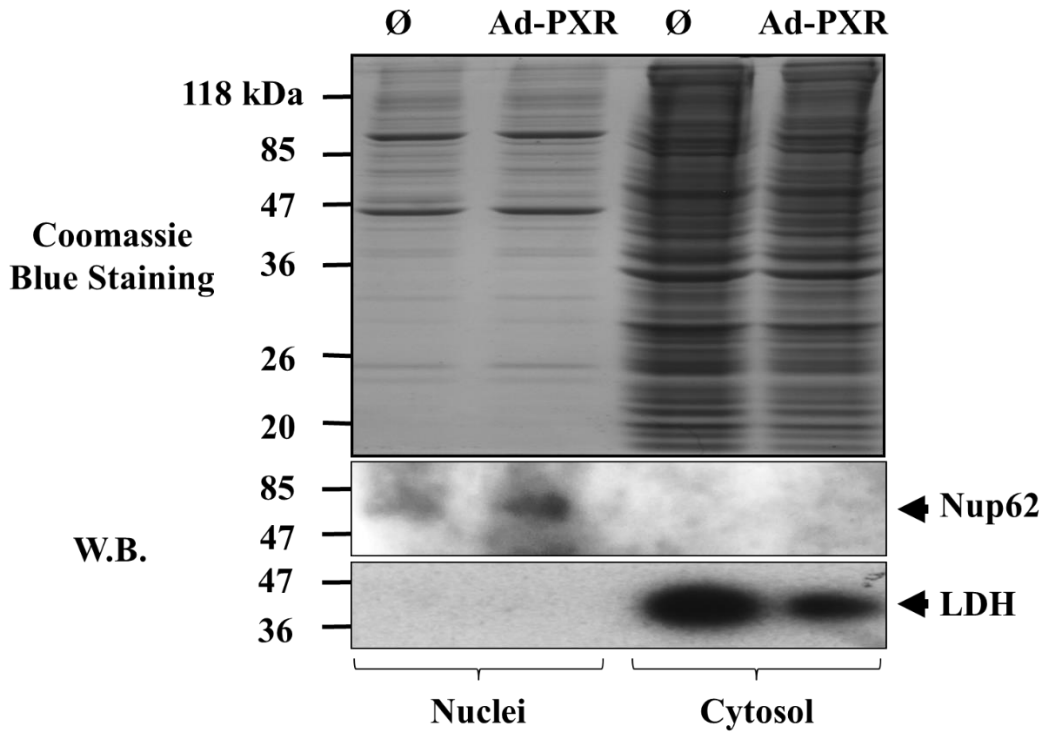


Figure 3. Biochemical separation validation. Hypotonic buffer was used to separate nuclei and cytosol fractions after cell lysis under native condition. Coomassie staining and western blotting were used to validate the efficiency of nuclear and cytosol fraction biochemical separation before immobilized metal affinity chromatography. Ø: no treatment control group, ad-hPXR: PXR infection group, Nup62: Nucleoporin 62, a nuclei marker; LDH: Lactate dehydrogenase, a cytosol marker.

Figure 4

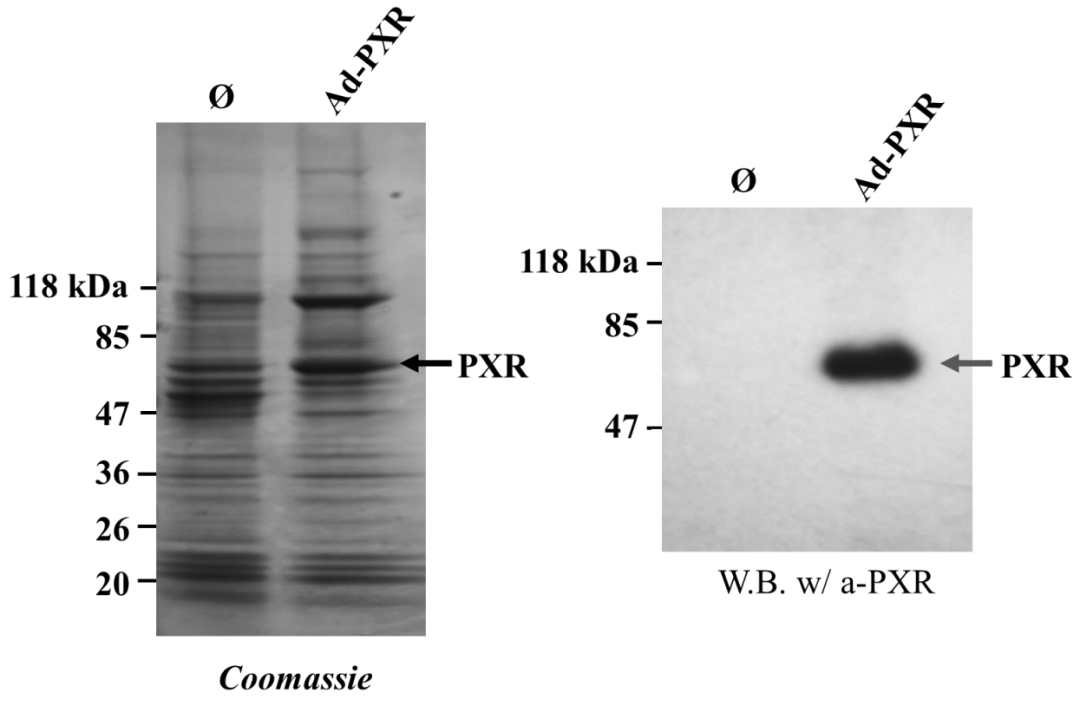


Figure 4. Coomassie staining and Western blotting. Immobilized metal affinity chromatography was performed under native condition in nuclear fraction. Coomassie Staining and Western Blotting were used after IMAC to visualize PXR and its binding protein before LC/MS/MS. Ø: control group, Ad-hPXR: adenoviral infection group. Compared with control group, Ad-PXR group showed a strong enrichment of PXR proteins (58kDa). Western blotting with anti-PXR antibody (H-11) further confirmed this result.

Figure 5

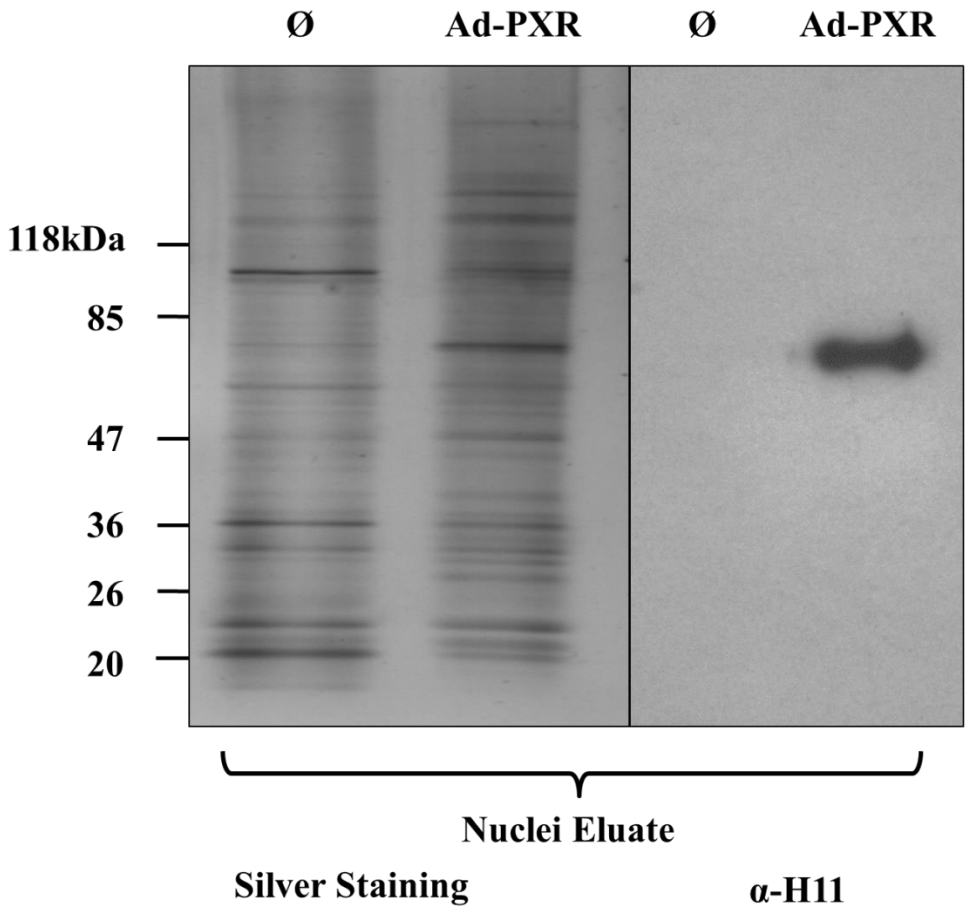


Figure 5. Silver staining and Western Blotting. Silver staining and Western Blotting were performed after IMAC to visualize PXR and its binding proteins before LC/MS/MS. Ø: control group, Ad-PXR: adenoviral infection group. Compared with control group, more bands were appeared in Ad-PXR group with silver staining, especially proteins larger than 118kDa, suggesting an efficient enrichment and purification of PXR-binding proteins. Western blotting with H-11 antibody showed a successful enrichment of PXR proteins.

CHAPTER 3: THE EFFECTS OF PXR ACTIVATION AND DELETION ON 5α-REDUCTASE GENE EXPRESSION

Introduction

PXR was initially identified as a nuclear receptor highly expressed in the liver and intestine, with low levels of expression in the kidney and stomach. However, recently PXR has been identified in the brain of multiple different species. Bauer et al. detected PXR expression in rat whole-brain homogenates and in isolated brain capillaries using RT-PCR [76]. They further confirmed this result by immunostaining rat brain capillaries with a PXR antibody. This is the first evidence showing the existence of PXR in the brain. The expression of PXR was confirmed in the mid brain of rats [77], human thalamus, medulla, pons and spinal cord [78], rabbit cortex [79], and mouse cerebellum, hypothalamus, and thalamus [80]. The role of PXR in the brain remains unknown; however, several studies found that PXR is involved in neurosteroid homeostasis.

Allopregnanolone is an endogenous neurosteroid which can bind to the GABAA receptor and regulate neurotransmission. At low concentration, allopregnanolone potentiates GABAA receptor currents, but does not cause direct activation. However, at high concentrations, it can directly activate the receptor [81-82]. There are two different GABAA receptors: synaptic GABAA receptor and extrasynaptic GABAA receptor. Activation of these two receptors can cause phasic inhibition and tonic inhibition respectively. Allopregnanolone can modulate both synaptic and extrasynaptic GABAA receptors to potentiate phasic and tonic inhibition and has large effects on extrasynaptic δ-subunit GABAA receptors [83-85]. The extrasynaptic GABAA receptors are distributed within the hippocampus, neocortex, thalamus, striatum, hypothalamus, and cerebellum [86]. Increasing of the biosynthesis of allopregnanolone can be used to treat

posttraumatic stress disorder, anxiety and depression [87]. Down-regulation of allopregnanolone biosynthesis in the midbrain is responsible for anxiety, aggression and reduced reproductive behavior in socially isolated mice [88].

Mice treated with allopregnanolone subcutaneously for 24 hours showed an increased PXR mRNA level in hypothalamus and thalamus in the brain [89]. Female mice infused of PXR antisense oligonucleotides (AS-ODNS) to the ventral tegmental area significantly suppressed estradiol-enhanced reproductive behavior [90], demonstrating an important role of PXR in regulating reproductive behavior in the mice. Frye et al found that knocking down of PXR by AS-ODNS in the midbrain significantly reduced allopregnanolone levels in the midbrain and hippocampus, suggesting that PXR may be involved in the formation of allopregnanolone in the brain [91-92].

Allopregnanolone is synthesized from progesterone by cortical and hippocampus pyramidal neurons and pyramidal-like neurons of the basolateral amygdala in the brain [93]. Progesterone is reduced by 5α-reductase to form 5α-dihydroprogesterone, which can later be converted to allopregnanolone by 3α-hydroxysteroid dehydrogenase (3α-HSD). 5α-reductase is the rate-limiting enzyme in this synthesis process. The family of 5α-reductases contains three isoenzymes: 5a-reductase type 1 (SRD5α1), 5a-reductase type 2 (SRD5α2) and 5a-reductase type 3 (SRD5α3). To date, SRD5α1 and SRD5α2 are present in all species examined and well characterized [94]. However, knowledge of SRD5α3 enzymatic activity is limited and its natural steroid substrates are largely unknown. Recently, SRD5α3 has been found lacking steroid 5a-reductase activity and cannot reduce progesterone to 5α-dihydroprogesterone in either human or hamster [95]. Thus, the reduction of progesterone is mainly regulated by SRD5α1 and SRD5α2.

Here we hypothesized that PXR may regulate allopregnanolone levels through regulating Srd5α1 and Srd5α2 gene expression in brain.

Materials and methods

Drug preparation

Pregnenolone 16α-carbonitrile (PCN) (Sigma) was dissolved in corn oil (Sigma) and the final concentration is 40 mg/ml. The drug was stored at 4 °C, and vigorously shake or vortex before use.

Animal treatment administration

WT and PXR-KO male mice (6-week old) were divided into 4 groups, named as WT Vehicle, WT PCN, PXR-knock out (PXR-KO) Vehicle and PXR-KO PCN. Each group has 6 mice. Mice were administered vehicle (corn oil 0.11 cc/10g) or PCN (PCN 40 mg/ml, 0.11 cc/10g) via intraperitoneal injection for consecutive three days. On day four, mice were euthanized and liver as well as seven brain regions (hypothalamus, amygdala, prefrontal cortex, hippocampus, striatum, mid brain and cerebellum) were isolated and frozen in -80°C.

RNA isolation

We combined two mice together for a total of three samples for each brain region. Tissues were dissolved in 300 µl (for brain) or 500 µl (for liver) TRIZOL (Invitrogen) respectively and homogenized. After centrifuging at 4 °C at 20817 x g for 10 min, the supernatant was carefully transferred into a new tube and 180 µL chloroform was add into each tube. After shaking vigorously, sample was centrifuged at 4 °C at 20817 x g for 15 min. Then the upper aqueous phase of each sample was carefully collected and an equitable amount of isopropanol (around 100 µl) was added to each sample. After centrifugation, the pellet was washed by 70% ethanol twice. The tubes were then placed in a 37°C incubator for 5 - 10 minutes

to dry. Then the RNA was dissolved in 20μL ddH_2O via pipetting. The absorbance value at 260nm was used to quantify RNA.

1 % RNA agarose gel

1% RNA agarose gel electrophoresis was performed to assess the integrity of RNA. Agarose (0.6g) was added into 48 mL ddH_2O and microwaved for 1 min until the agarose was fully dissolved. 37% formaldehyde (6 mL) and 6 mL 10X MOPS (20 mM EDTA, 200 mM MOPS, 50 mM Sodium acetate, pH = 7.0) were added into solutions and mixed thoroughly. Gel solution was poured and stabilized in gel electrophoresis set for at least 30 min.

Each sample contains 6.5 μl ddH_2O, 2.5 μl 4x RNA loading buffer (50% glycerol, 1mM EDTA, 0.4% bromophenol blue, 1mg/ml ethidium bromide) and around 1 μg RNA. Samples were heated in 60 °C for 5 min before loading. Electrophoresis was performed in 90V for 60 min, and 2X MOPS (4 mM EDTA, 40 mM MOPS, 10 mM Sodium acetate, pH = 7.0) was used as running buffer.

Reverse Transcription

1 μg of RNA was reversed-transcribed by using random primers following the manufacturer's instruction (Promega). Samples were first incubated at room temperature for 10 minutes. Then samples were heated at 42°C for 60 minutes and followed by heating at 95°C for 5 minutes. The final products were stored at 4°C for later use.

Polymerase chain reaction

Equal amounts of cDNA were used in polymerase chain reactions (PCR). Reactions contained 300 nM primers specific for mouse PXR (**Table** 3). The PCR assay was performed as previously described [96].

Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction was performed as previously described [97]. Briefly, reactions contained 1X SYBR green (Lonza Rockland, Inc., Rockland, ME) and 300 nM primers specific for β-actin, Cyp3a11, Oatp2, Srd5α1 and Srd5α2 (Table 3) respectively. Each sample was analyzed in triplicate. The mRNA levels of Cyp3a11, Oatp2, Srd5α1 and Srd5α2 were normalized to a housekeeping gene β-actin using the formula as following: ΔCT (test gene) = CT (test gene) - CT (β-actin). Then ΔΔCT of each gene was used to represent the relative mRNA induction levels using the formula as following: ΔΔCT (test gene) = ΔCT (test gene) - ΔCT (test gene in WT Veh group). For Cyp3a11 and Oatp2, the fold changes of gene expression were represented by the value of 2- ΔΔCT (test gene). For Srd5α1 and Srd5α2, the percentage changes of gene expression were represented by the value of 100*2- ΔΔCT (test gene) (%).

8% DNA polyacrylamide gel

8% DNA polyacrylamide gel electrophoresis [98] was performed to visualize PXR amplification as well as detect Srd5α1 and Srd5α2 primer sets (**Table** 3) specificity. Each sample contained 9 µl of PCR or quantitative real-time PCR products and 1 µl of 10X DNA loading buffer [98]. Electrophoresis was performed in 120V for 80 min. Then gel was stained in ethidium bromide (EB) for at least 20 min followed by visualization of the amplification bands under UV light.

Data analysis and statistics

All quantitative real-time RT- PCR data were analyzed by two-way analysis of variance (ANOVA) using a statistical program (GraphPad Prism, version 6.02) followed by Tukey's multiple comparisons post-hoc test. A p value of < 0.05 was considered significant. The data are

presented as mean of fold change ± standard error of the mean (SEM) for Cyp3all and Oatp2 in liver. For Srd5α1 and Srd5α2, the data are presented as mean of percent change ± SEM.

Results

PXR mRNA level in wild type mice brain

We examined RNA integrity using 1% RNA agarose gel, which showed high quality of RNA (**Figure 6A**). Then RT-PCR was performed and PXR mRNA expression was detected in 8% DNA polyacrylamide gel. Using liver and PXR plasmid (pCMV-tag2B-mPXR-fl) as positive controls and ddH_2O as negative control, we detected PXR mRNA in all seven brain regions (**Figure 6B**). This result indicates that PXR is expressed in seven brain regions in mRNA level.

The effects of PXR activation and deletion on Cyp3all, Oatp2, Srd5α1 and Srd5α2 expression in liver

We used PCN, a well-known selective mouse PXR ligand, to activate PXR in mice to see if PXR activation has any effects on Srd5α1 and Srd5α2 gene expression in the liver and brain. WT and PXR-KO mice were treated with either corn oil or PCN daily for three days. On day four, mice were sacrificed and the liver and different brain regions were dissected and RNA was isolated. First, we looked at RNA integrity of total 48 samples. Our result showed high quality of RNA (**Figure 7**).

Using quantitative real-time RT-PCR assay, we detected two prototypical PXR target genes, Cyp3a11 and Oatp2, in the liver. As expected, we found that the treatment of PCN dramatically increased Cyp3all (**Figure** 8A) and Oatp2 (**Figure** 8B) gene expression in the liver in WT, and this effect was totally abolished in PXR-KO group. These data showed that PCN can increase Cyp3a11 and Oatp2 gene expression in the liver in a PXR-dependent manner, and are consistent with previous studies [99].

Srd5α1 and Srd5α2 primer sets were also validated, as shown in **Figure 8C-D**. Quantitative real-time RT-PCR of Srd5α1 and Srd5α2 was performed in WT mice in the hypothalamus and liver, and 8% DNA polyacrylamide gel was used to visualize amplification of the result. Our result showed high specificity of Srd5α1 and Srd5α2 primer sets.

We then detected Srd5α1 and Srd5α2 gene expression in the liver. Our results showed that deletion of PXR altered Srd5α1 basal level in liver, and PCN treatment decreased Srd5α1 expression in both WT and PXR-KO group (**Figure** 8E). Treatment of PCN reduced Srd5α2 gene expression level in the PXR-KO group but not in the WT group, and the deletion of PXR also altered Srd5α2 basal level (**Figure** 8F). These data suggests that Srd5α1 and Srd5α2 are not PXR target genes in the liver and PCN-induced decrease of Srd5α1 and Srd5α2 gene expression is in a PXR-independent manner.

The effects of PXR activation and deletion on Srd5α1 and Srd5α2 expression in brain

The quantitative real-time RT-PCR assays were performed in seven brain regions: hypothalamus, amygdala, PFC, hippocampus, striatum, mid brain and cerebellum. As shown in **Figure 9A-G**, no significant change of Srd5α1 gene expression was seen with the activation or deletion of PXR in the seven brain regions. Thus, Srd5α1 gene expression is not regulated by PXR in the seven brain regions.

We found that activation or deletion of PXR didn't cause any significant change in Srd5α2 expression in the amygdala (**Figure** 10B), PFC (**Figure** 10C), hippocampus (**Figure** 10 D), striatum (**Figure** 10E), or mid brain (**Figure** 10F). In the hypothalamus, deletion of PXR altered the Srd5α2 gene basal level, however the activation of PXR did not cause any significant

change (**Figure** 10A). In the cerebellum, treatment of PCN in PXR-KO mice dramatically increased Srd5α2 gene expression in comparison with the PXR-KO vehicle group (**Figure** 10 G).

In summary, our data showed that 1) PXR mRNA is expressed in the hypothalamus, amygdala, PFC, hippocampus, striatum, mid brain and cerebellum in the mouse; 2) knockout of PXR increased Srd5α1 basal level in the liver, and the treatment of PCN decreased Srd5α1 mRNA level in both wild type and PXR-KO group in the liver, However, there is no significant change of Srd5α1 in the brain in these groups; 3) knockout of PXR increased Srd5α2 basal level in both the liver and hypothalamus. The treatment of PCN decreased Srd5α2 expression in PXR-KO in liver, on the contrary, increased Srd5α2 expression in PXR-KO group in cerebellum.

Discussion

Our study showed for the first time that PXR was expressed in seven brain regions at a low mRNA level. However, we didn't look at the protein levels of PXR in these brain regions. In the future studies, immunohistochemistry can be used to determine PXR protein expressions in these brain region tissues.

Previous studies showed that knocking down of PXR decreased allopregnanolone level in the midbrain and hippocampus. However, from our results, we found that deletion of PXR can alter the basal levels of Srd5α1 and Srd5α2 in the liver, and the basal Srd5α2 in the hypothalamus. Thus, Srd5α1 and Srd5α2 are not PXR target genes in either the liver or brain and other mechanisms should be responsible for PXR knockdown-induced decrease of allopregnanolone level. As discussed previously, two major enzymes involved in the biosynthesis of allopregnanolone from progesterone are 5α-reductase and 3α-Hydroxysteroid dehydrogenase (3α-HSD). Previously study found that activation of PXR by rifampicin in human PXR-transgenic mice can significantly increase the expression of 3β-HSD, another member of

hydroxysteroid dehydrogenase enzymes, in the adrenal glands [100]. Therefore, it is possible that PXR can regulate 3α-HSD expression in the brain to regulate allopregnanolone levels. Future studies should commence with testing this hypothesis.

According to our results, PXR activation or deletion did not have any effects on Srd5α1 expression in seven brain regions. Interestingly, treatment of PCN, a prototypical mouse PXR ligand, led to reduced Srd5α1 gene expression in both WT and PXR-KO groups in the liver. These two different results from the brain and the liver suggest that 1) Srd5α1 is not a PXR target gene in the brain and 2) PCN-induced decrease of Srd5α1 in the liver is carried out in a PXR-independent manner. We hypothesized that PCN may have some feedback suppression effects on Srd5α1 and Srd5α2, which could explain PCN-induced decrease of Srd5α1 and Srd5α2 in the liver. It is known that PCN is a synthesized steroid and shares the same core structure with allopregnanolone (**Figure** 11). As discussed previously, Srd5α1 and Srd5α2 are rate-limiting enzymes of the allopregnanolone synthesis process. Thus, it is possible that allopregnanolone and PCN is responsible for some feedback suppression on Srd5α1 and Srd5α2 expression.

Surprisingly, in PXR-KO group, PCN treatment in the cerebellum significantly increased Srd5α2 gene expression compared with the vehicle-treated group, indicating other mechanisms may be involved in this phenomenon. For decades, PCN has been considered as a selective mouse PXR ligand. However, recently, PCN was found to have immunosuppressive activity independent of PXR activation in mouse liver [101]. Although the mechanism underlying this phenomenon is unknown, it is noteworthy that other nuclear receptors such as Glucocorticoid receptor (GR), liver X receptor (LXR), Farnesoid X receptor (FXR) and Constitutive androstane receptor (CAR) are also involved in the liver immune system. Thus, PCN might interact with

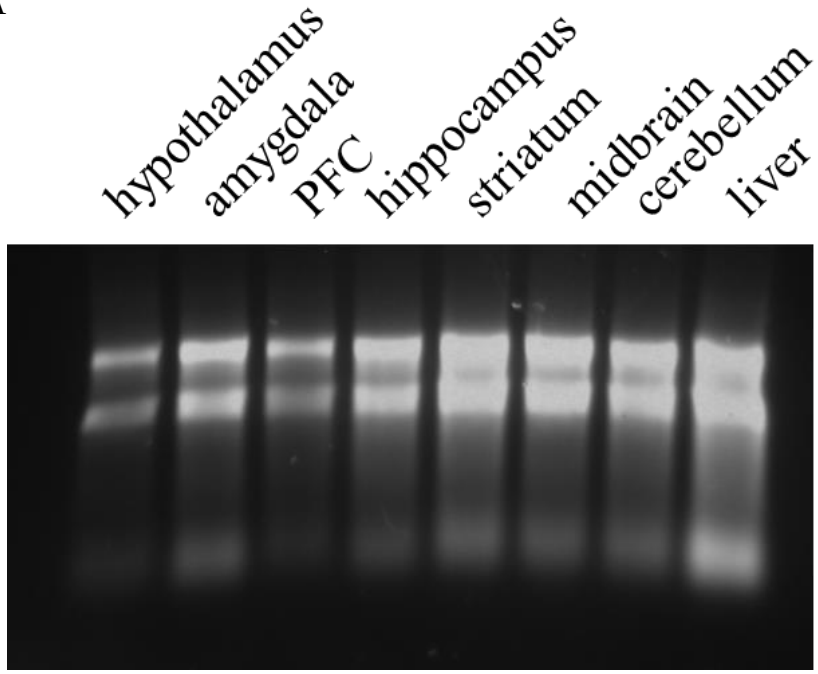
other nuclear receptors to suppress immune activity. Among many NRs, thyroid hormone receptor alpha (THRA), thyroid hormone receptor beta (THRB), and LXRs were found to be highly expressed in the cerebellum [102]. LXRs are master regulators of cholesterol homeostasis in the brain [103], and progesterone can be synthesized from cholesterol. Therefore, it is possible that other nuclear receptor, such as LXR, may interact with PCN to regulate Srd5α2 gene expression in the cerebellum. Further studies are necessary to test this possibility. Due to the fact that some of the quantitative real-time RT-PCR data have large error bars, it is hard to draw reliable conclusions from these data. Therefore, in the feature study the same experiments need to be performed again with a large sample size in order to get more reliable results and conclusions.

Table 3. Primer sets sequences.

Srd5α1	Left primer: 5' gAg TTg gAT gAg TTg CgC CTA 3' Right primer: 5' ggA CCA CTg CgA ggA gTA g 3'
Srd5α2	Left primer: 5' gAT CCT gTg CTT Tgg gAA ACC 3' Right primer: 5' gCA TCC CTA CCg ACA CCA C 3'
Cyp3a11	Left primer: 5' CAA ggA gAT gTT CCC TgT CA 3' Right primer: 5' CCA CgT TCA CTC CAA ATg AT 3'
PXR	Left primer: 5' gAT ggA ggT CTT CAA ATC TgC C 3' Right primer: 5' ggC CCT TCT gAA AAA CCC CT 3'
Oatp2	Left primer: 5' TTg CTg ACT gCA ACA CAA Ag 3' Right primer: 5' Tgg TTC CAg TTC CAA CAg AC 3'
β-actin	Left primer: 5' CAA gAT CAT TgC TCC TCC Tg 3' Right primer: 5' TAA CAg TCC gCC TAg AAg CA 3'

Figure 6

A


hypothalamus
amygdala
PFC
hippocampus
striatum
midbrain
cerebellum
liver

B

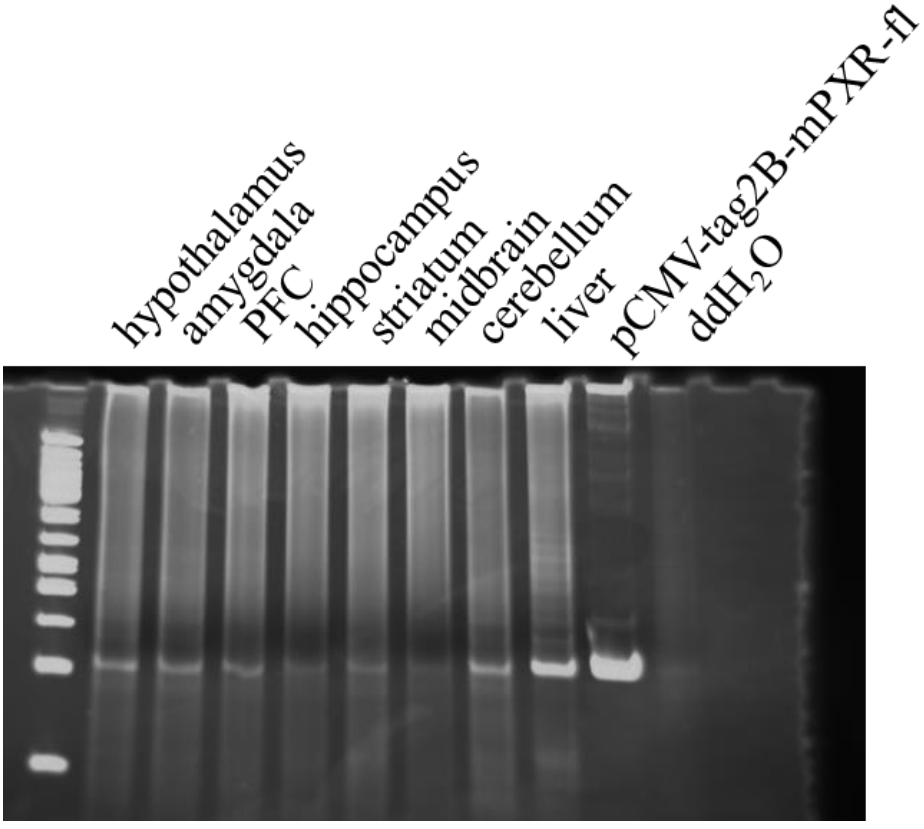

hypothalamus
amygdala
PFC
hippocampus
striatum
midbrain
cerebellum
liver
pCMV-tag2B-mPXR-fl
ddH2O

Figure 6. PXR mRNA level in wild type mice brain. Wild type male mice were sacrificed and seven brain regions as well as liver were isolated. RNA was extracted and RT-PCR was performed. **(A)** 1% RNA agarose gel electrophoresis was performed with RNA isolated from brain regions and liver. **(B)** 8% DNA polyacrylamide gel was used to visualize amplification products from RT-PCR.

Figure 7

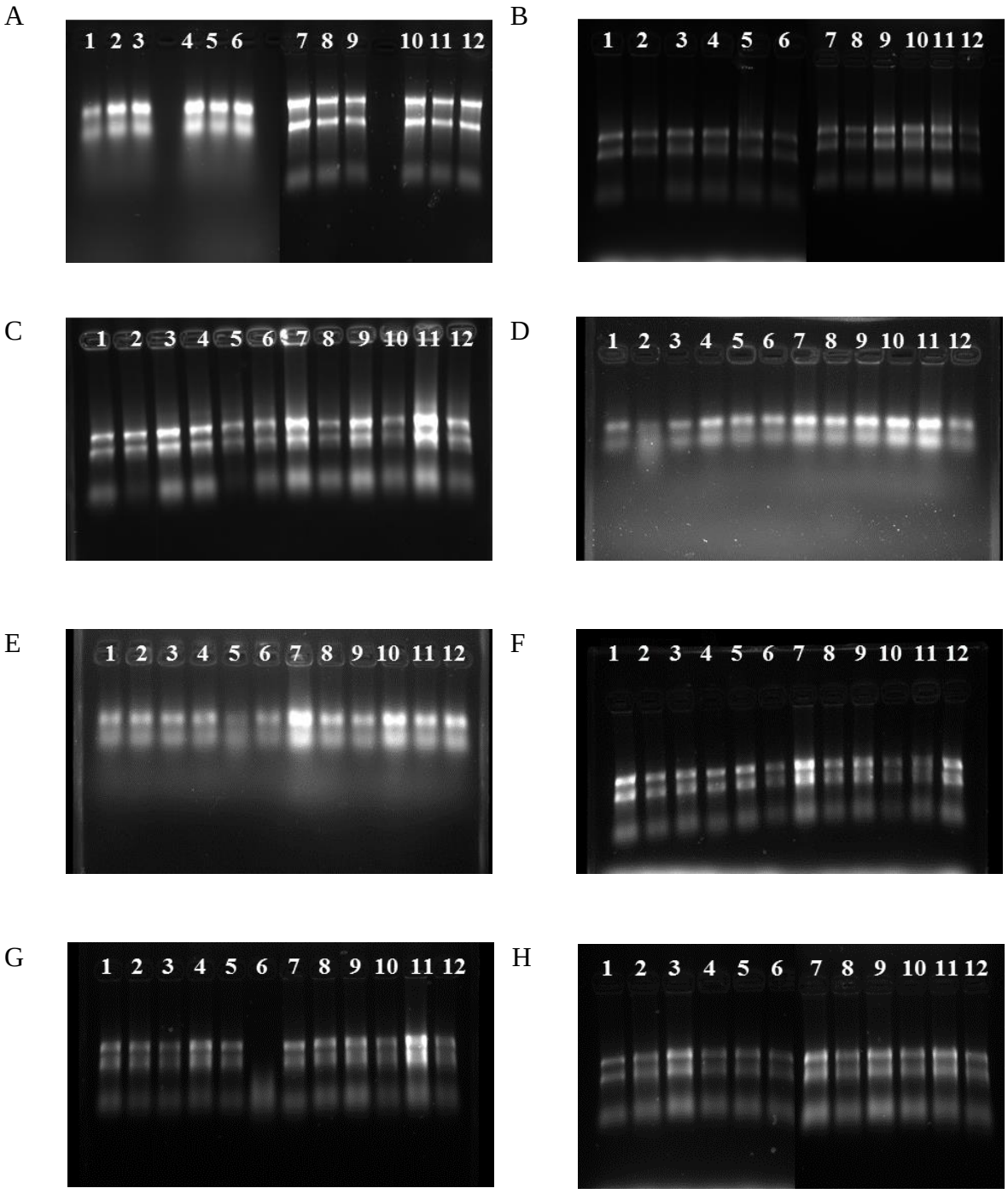


Figure 7. RNA integrity of brain regions and liver samples. Mice were treated with PCN or corn oil as control respectively. RNA were extracted and examined in 1% RNA agarose gel. **(A):**

liver, (**B)**: hypothalamus, (**C)**: amygdala, (**D)**: PFC, (**E)**: hippocampus, (**F)**: striatum, (**G)**: mid brain, and (**H**) cerebellum. 1-3: WT Veh; 4-6: WT PCN; 7-9: PXR-KO Veh; 10-12: PXR-KO PCN.

Figure 8

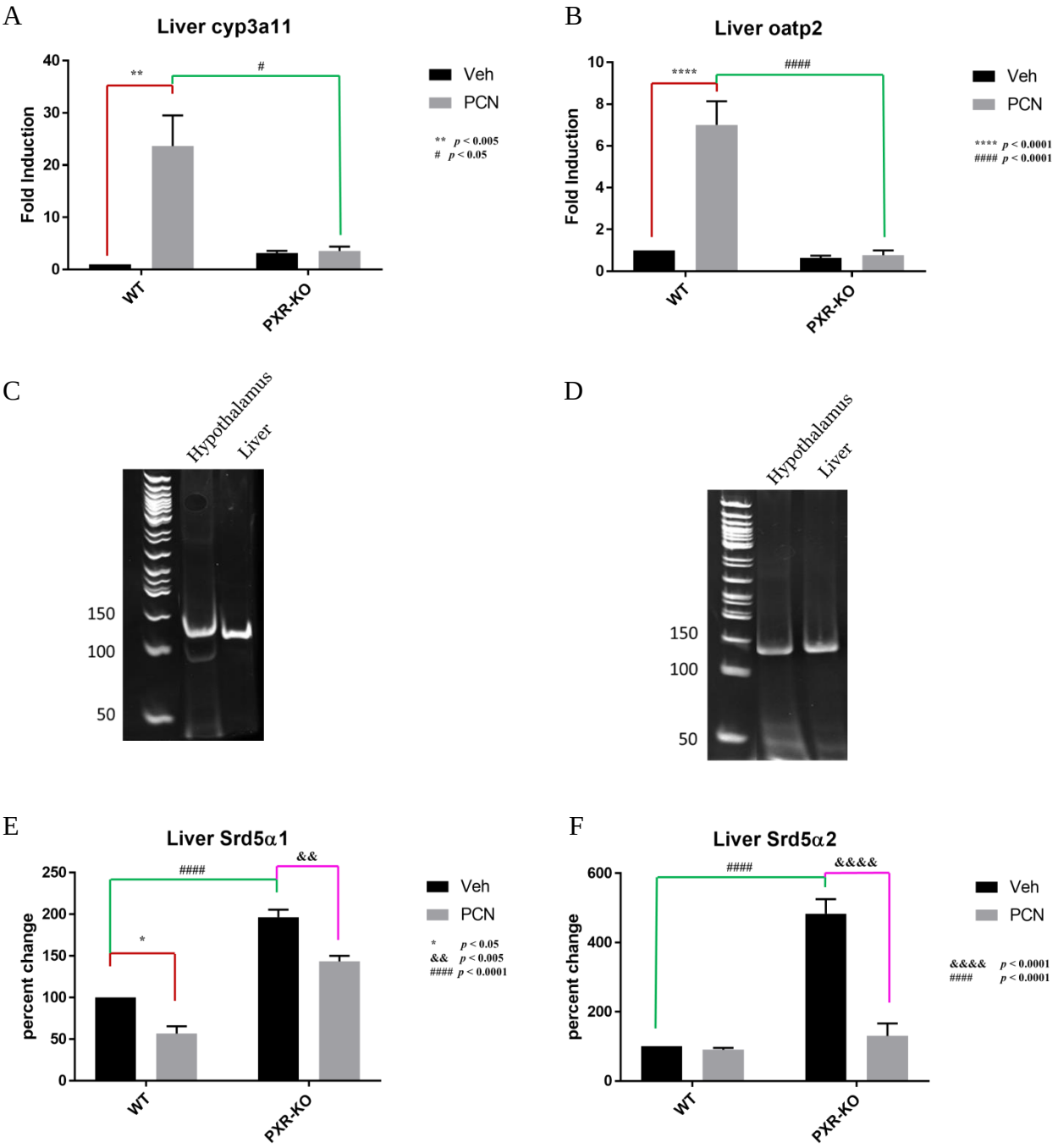


Figure 8. The effects of PXR activation and deletion on Cyp3all, Oatp2, Srd5α1 and Srd5α2 expression in liver. Quantitative real-time RT-PCR was performed in liver samples. **(A)** Cyp3all gene expression in liver. Two-way ANOVA shows a significant main effect of gene deletion:

F(1,8)=9.075, p=0.0167, a significant main effect of treatment (PCN): F(1,8)=14.92, p=0.0048 and a significant interaction between treatment and gene deletion: F(1,8)=13.9, p=0.0058. Post hoc by Tukey's multiple comparisons test indicates treatment of PCN in WT significantly increased Cyp3all level (**p<0.005) and treatment of PCN in KO significantly decreased Cyp3all compared with WT ($^{\#}$p<0.05). n=3 **(B)** Oatp2 gene expression in liver. Two-way ANOVA shows a significant main effect of gene deletion: F(1,8)=96.32, p<0.0001, a significant main effect of treatment (PCN): F(1,8)=83.18, p<0.0001 and a significant interaction between treatment and gene deletion: F(1,8)=76.11, p<0.0001. Post hoc by Tukey's multiple comparisons test indicates treatment of PCN in WT significantly increased Oatp2 level (****p<0.0001) and treatment of PCN in KO significantly decreased Oatp2 compared with WT ($^{\#\#\#\#}$p<0.0001). n=3 **(C)** Validation of Srd5α1 primer set. Quantitative real-time RT-PCR was performed in wild type mouse hypothalamus and liver. Amplification size: 123bp. **(D)** Validation of Srd5α2 primer set. Quantitative real-time RT-PCR was performed in wild type mouse hypothalamus and liver. Amplification size: 134bp. **(E)** Srd5α1 gene expression in liver. Two-way ANOVA shows a significant main effect of gene deletion: F (1, 8) = 168.1, P<0.0001, a significant main effect of treatment (PCN): F (1, 8) = 46.72, P=0.0001 and interaction between treatment and gene deletion: F (1, 8) = 0.5, P=0.4996. Post hoc by Tukey's multiple comparisons test indicates knockout of PXR significantly increased basal level of Srd5α1 ($^{\#\#\#\#}$p<0.0001) and treatment of PCN significantly decreased Srd5α1 gene expression in both WT (*p<0.05) and PXR-KO ($^{\&\&}$p<0.005). n=3 **(F)** Srd5α2 gene expression in liver. Two-way ANOVA shows a significant main effect of gene deletion: F (1, 8) = 58.23, P<0.0001 a significant main effect of treatment (PCN): F (1, 8) = 42.89, P=0.0002 and a significant interaction between treatment and gene deletion: F (1, 8) = 38.3, P=0.0003. Post hoc by Tukey's multiple comparisons test indicates

deletion of PXR significantly increased basal level of Srd5α2 ($^{\#\#\#\#}p<0.0001$) and compared with Veh, treatment of PCN significantly decreased Srd5α2 gene expression in PXR-KO ($^{\&\&\&\&}p<0.0001$). n=3

Figure 9

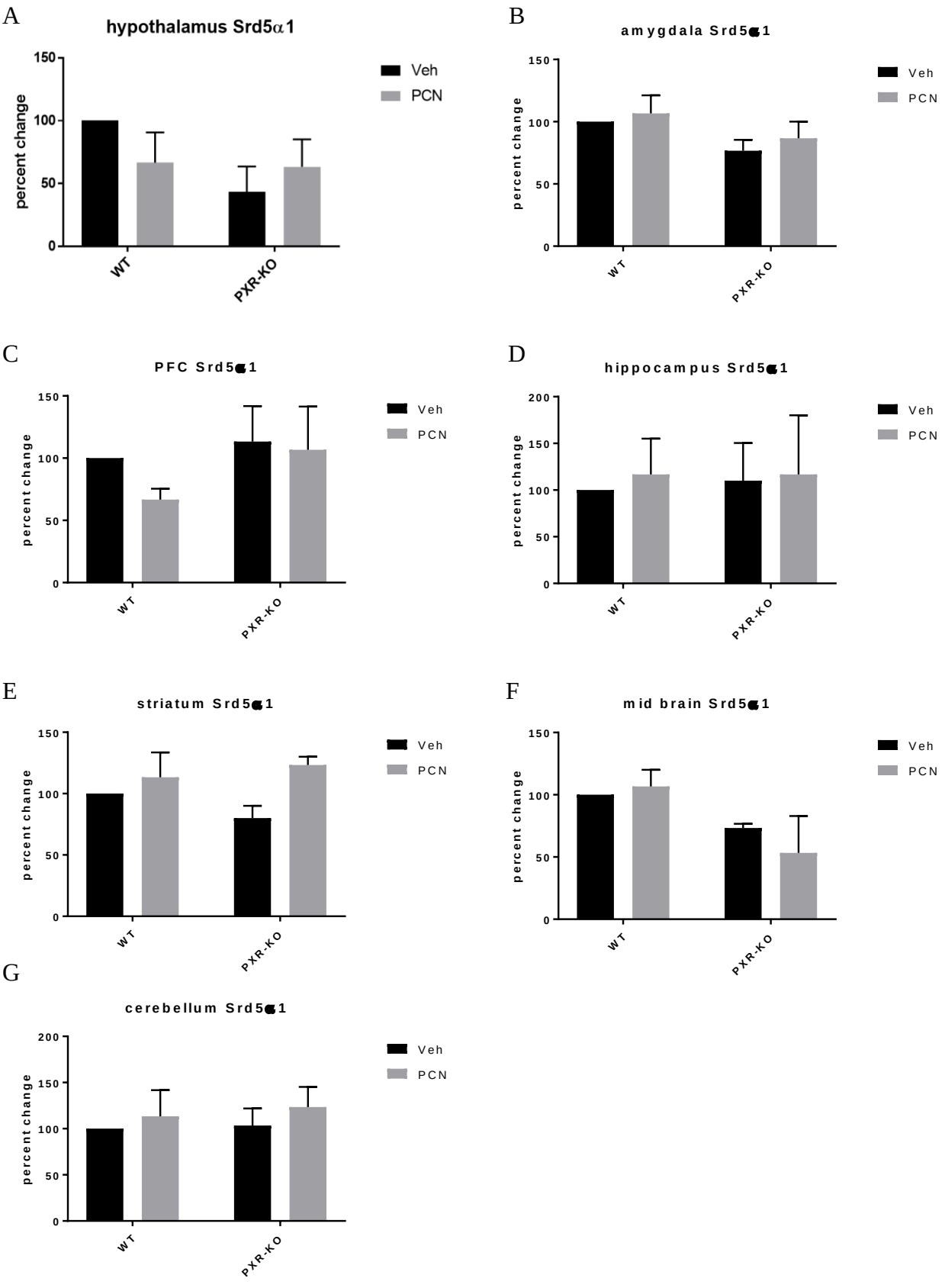
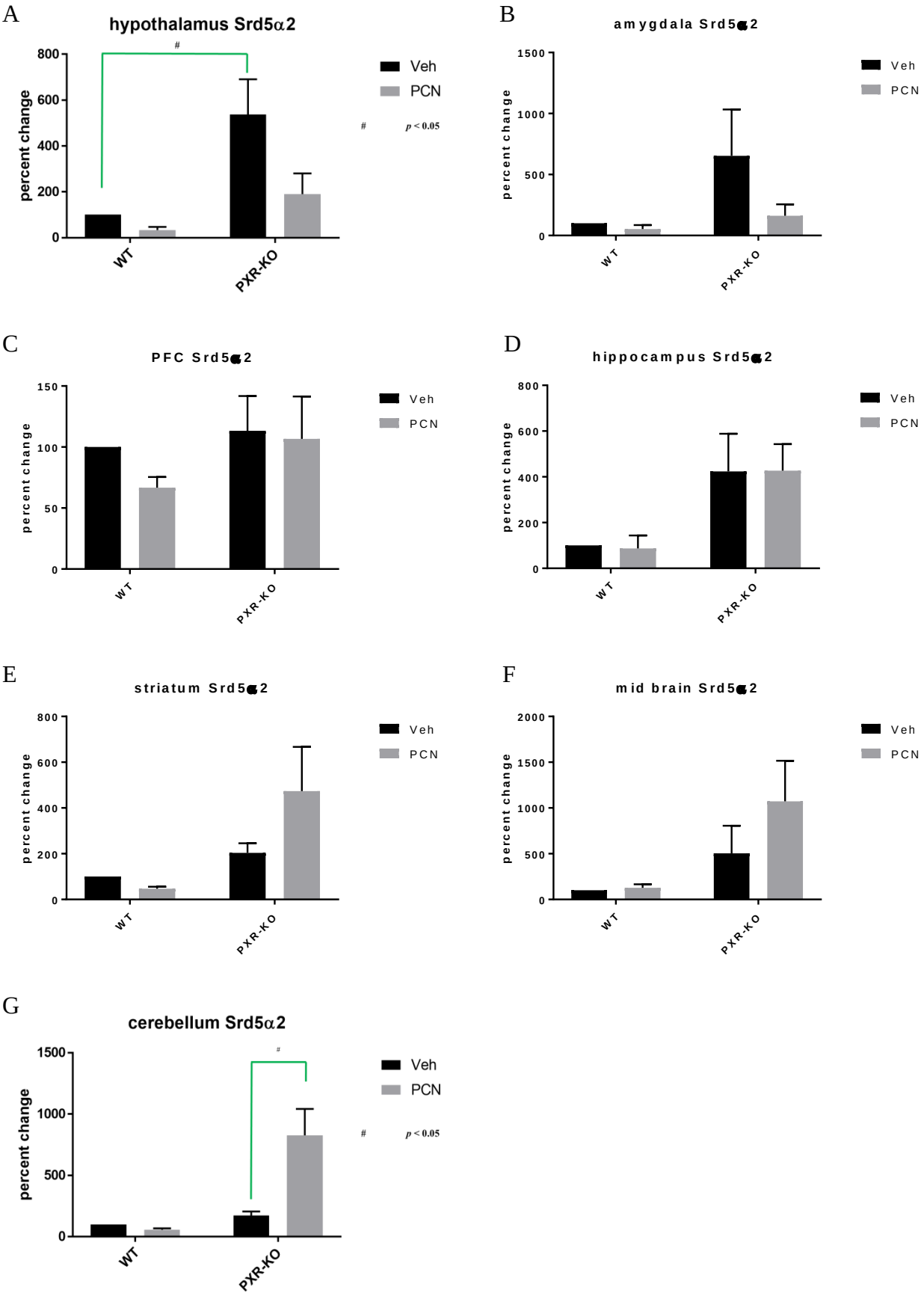

A
hypothalamus Srd5α1
percent change
150
100
50
0
WT
PXR-KO
Veh
PCN
B
amygdala Srd5α1
percent change
150
100
50
0
WT
PXR-KO
Veh
PCN
C
PFC Srd5α1
percent change
150
100
50
0
WT
PXR-KO
Veh
PCN
D
hippocampus Srd5α1
percent change
200
150
100
50
0
WT
PXR-KO
Veh
PCN
E
striatum Srd5α1
percent change
150
100
50
0
WT
PXR-KO
Veh
PCN
F
mid brain Srd5α1
percent change
150
100
50
0
WT
PXR-KO
Veh
PCN
G
cerebellum Srd5α1
percent change
200
150
100
50
0
WT
PXR-KO
Veh
PCN

Figure 9. The effects of PXR activation and deletion on Srd5α1 gene expression in brain. Quantitative real-time RT-PCR was performed in seven brain regions as described previously. **(A)** hippocampus Two-way ANOVA shows no significant main effect of gene deletion: $F(1,8)=2.455$, $p=0.1558$, no significant main effect of treatment (PCN): $F(1,8)=0.1212$, $p=0.7367$ and no significant interaction between treatment and gene deletion: $F(1,8)=1.939$, $p=0.2012$. n=3 **(B)** amygdala Two-way ANOVA shows no significant main effect of gene deletion: $F(1,8)=4.024$, $P=0.0798$, no significant main effect of treatment (PCN): $F(1, 8) = 0.5952$, $P=0.4626$ and no significant interaction between treatment and gene deletion: $F(1, 8) = 0.02381$, $P=0.8812$. n=3 **(C)** PFC Two-way ANOVA shows no significant main effect of gene deletion: $F(1, 8) = 1.354$, $P=0.2780$, no significant main effect of treatment (PCN): $F(1, 8) = 0.7619$, $P=0.4082$ and no significant interaction between treatment and gene deletion: $F(1, 8) = 0.3386$, $P=0.5767$. n=3 **(D)** hippocampus Two-way ANOVA shows no significant main effect of gene deletion: $F(1, 8) = 0.01404$, $P=0.9086$, no significant main effect of treatment (PCN): $F(1, 8) = 0.07644$, $P=0.7892$ and no significant interaction between treatment and gene deletion: $F(1, 8) = 0.01404$, $P=0.9086$. n=3 **(E)** striatum Two-way ANOVA shows no significant main effect of gene deletion: $F(1, 8) = 0.18$, $P=0.6826$, a significant main effect of treatment (PCN): $F(1, 8) = 5.78$, $P=0.0429$ and no significant interaction between treatment and gene deletion: $F(1, 8) = 1.62$, $P=0.2388$. Post hoc by Tukey's multiple comparisons test shows no significance. n=3 **(F)** mid brain Two-way ANOVA shows a significant main effect of gene deletion: $F(1, 8) = 6$, $P=0.0400$, no significant main effect of treatment (PCN): $F(1, 8) = 0.1667$, $P=0.6938$ and no significant interaction between treatment and gene deletion: $F(1, 8) = 0.6667$, $P=0.4379$. Post

hoc by Tukey's multiple comparisons test shows no significance. n=3 **(G)** cerebellum Two-way ANOVA shows no significant main effect of gene deletion: $F(1, 8) = 0.1088$, $P=0.7499$, no significant main effect of treatment (PCN): $F(1, 8) = 0.6803$, $P=0.4334$ and no significant interaction between treatment and gene deletion: $F(1, 8) = 0.02721$, $P=0.8731$. n=3

Figure 10


A
hypothalamus Srd5α2
percent change
800
600
400
200
0
#
WT
PXR-KO
Veh
PCN
# p < 0.05
B
amygdala Srd5α2
percent change
1500
1000
500
0
WT
PXR-KO
Veh
PCN
C
PFC Srd5α2
percent change
150
100
50
0
WT
PXR-KO
Veh
PCN
D
hippocampus Srd5α2
percent change
800
600
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200
0
WT
PXR-KO
Veh
PCN
E
striatum Srd5α2
percent change
800
600
400
200
0
WT
PXR-KO
Veh
PCN
F
mid brain Srd5α2
percent change
2000
1500
1000
500
0
WT
PXR-KO
Veh
PCN
G
cerebellum Srd5α2
percent change
1500
1000
500
0
#
WT
PXR-KO
Veh
PCN
# p < 0.05

Figure 10. The effects of PXR activation and deletion on Srd5α2 gene expression in brain. Quantitative real-time RT-PCR was performed in seven brain regions as described previously. **(A)** hippocampus Two-way ANOVA shows a significant main effect of gene deletion: $F(1, 8) = 10.95$, $P=0.0107$, a significant main effect of treatment (PCN): $F(1, 8) = 5.313$, $P=0.0501$ and no significant interaction between treatment and gene deletion: $F(1, 8) = 2.438$, $P=0.1570$. Post hoc by Tukey's multiple comparisons test indicates deletion of PXR significantly increased Srd5α2 basal level ($^{\#}p<0.05$). n=3 **(B)** amygdala Two-way ANOVA shows no significant main effect of gene deletion: $F(1, 8) = 2.841$, $P=0.1304$, no significant main effect of treatment (PCN): $F(1, 8) = 1.885$, $P=0.2070$ and no significant interaction between treatment and gene deletion: $F(1, 8) = 1.288$, $P=0.2892$. n=3 **(C)** PFC Two-way ANOVA shows no significant main effect of gene deletion: $F(1, 8) = 1.354$, $P=0.2780$, no significant main effect of treatment (PCN): $F(1, 8) = 0.7619$, $P=0.4082$ and no significant interaction between treatment and gene deletion: $F(1, 8) = 0.3386$, $P=0.5767$. n=3 **(D)** hippocampus Two-way ANOVA shows a significant main effect of gene deletion: $F(1, 8) = 10.01$, $P=0.0133$, no significant main effect of treatment (PCN): $F(1, 8) = 0.002274$, $P=0.9631$ and no significant interaction between treatment and gene deletion: $F(1, 8) = 0.006318$, $P=0.9386$. Post hoc by Tukey's multiple comparisons test indicates no significance. n=3 **(E)** striatum Two-way ANOVA shows a significant main effect of gene deletion: $F(1, 8) = 7.129$, $P=0.0284$, no significant main effect of treatment (PCN): $F(1, 8) = 1.191$, $P=0.3068$ and no significant interaction between treatment and gene deletion: $F(1, 8) = 2.653$, $P=0.1420$. Post hoc by Tukey's multiple comparisons test indicates no significance. n=3 **(F)** mid brain Two-way ANOVA shows a significant main effect of gene deletion: $F(1, 8) = 6.32$, $P=0.0361$, no significant main effect of treatment (PCN): $F(1, 8) = 1.235$, $P=0.2988$ and no significant interaction between treatment and gene deletion: $F(1, 8) = 1.024$, $P=0.3413$. Post hoc

by Tukey's multiple comparisons test indicates no significance. n=3 **(G)** cerebellum Two-way ANOVA shows a significant main effect of gene deletion: $F(1, 8) = 14.97$, $P=0.0047$, a significant main effect of treatment (PCN): $F(1, 8) = 7.834$, $P=0.0232$ and a significant interaction between treatment and gene deletion: $F(1, 8) = 10.22$, $P=0.0127$. Post hoc by Tukey's multiple comparisons test indicates treatment of PCN significantly increased Srd5α2 gene expression in PXR-KO compared with Veh (($^{\#}p<0.05$). n=3

Figure 11

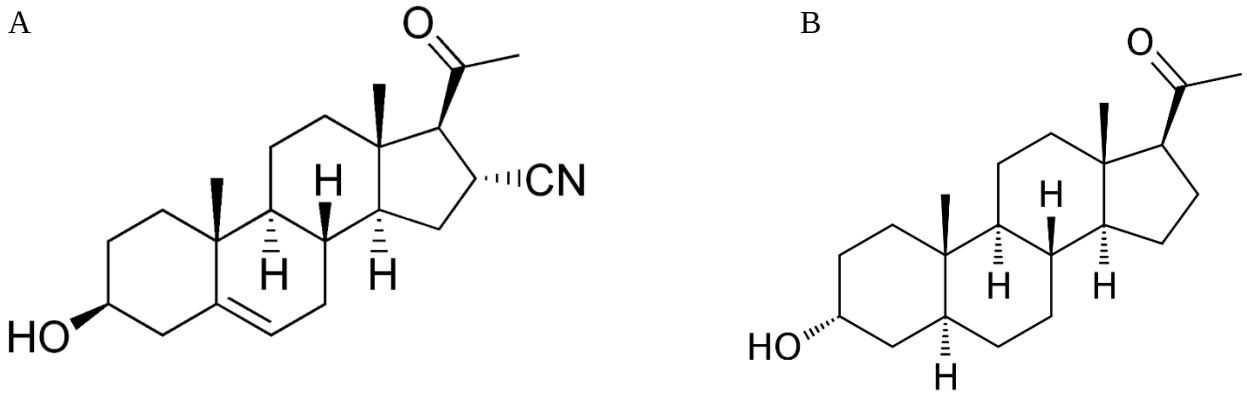

Figure 11. Structures of (A) PCN and (B) allopregnanolone

CHAPTER 4: DISSCUSSION

Previous studies have reported that PXR showed tissue-specific functions in terms of regulating its target genes. Activation of PXR can significantly increase MDR1 gene expression as well as protein level in the intestine. However, PXR activation didn't cause any change of MDR1 gene expression in the liver. Recently, PXR was also found to express in the brain in different species, and showed brain-specific functions. PXR is involved in the regulation of neurosteroid levels and therefore involved in the pathology of different neurodegenerative diseases and mental disorders. Although the precise mechanism of PXR performing tissue-specific functions is currently unknown, it is notable that PXR function as a transcription factor through binding to co-regulatory proteins and form a complex to initiate a transcription process. To date, different co-activators and co-repressors of PXR have been discovered. Thus we hypothesized that tissue-specific co-regulatory proteins may lead to tissue-specific PXR functions.

In the present study, I mainly focused on two parallel projects. In my first project, I used a novel method to identify PXR co-regulatory proteins in the liver. PXR protein were overexpressed in primary cultured rat hepatocytes using adenoviral infection assay, coupled with immobilized metal affinity chromatography assay in native condition to enrich PXR and its binding proteins. LC-MS/MS assay was used to identify protein peptides. Besides a well- known PXR binding partner RXRα, several potential binding partners were also identified, including prohibitin 1, prohibitin 2, Ring finger protein 2, zinc finger protein and different TATA box binding protein (TBP)-associated factors. In this study, I designed and validated buffer conditions that are specific for IMAC in non-denaturing conditions, which can be used in the

future for studying protein-protein interactions. This study provides a new basis for identifying PXR-binding partners in primary cells.

In future studies, co-immunoprecipitation should be used to further confirm the interaction between PXR and different potential binding partners. Additionally, in order to explain PXR tissue-specific functions, the LC-MS/MS experiment can be performed in primary cells isolated from other tissue types such as primary cultured enterocytes or neuronal cells. Then by comparing with the result we generated from the liver, tissue-specific binding partners might also be identified. Ligand-dependent PXR co-regulatory proteins can also be identified by adding PXR ligands in this assay. Moreover, PXR co-regulatory proteins that can respond to inflammation can also be identified using stimulated primary cultured cells under stress condition.

While the first project identified PXR binding partners in the rat liver, my second project characterized PXR function in the brain, and specifically, the effects of PXR on Srd5α1 and Srd5α2 gene expression in different brain regions. In this study, WT and PXR-KO mice were treated with either corn oil as vehicle control, or PCN daily for consecutive three days. On day 4, mice were euthanized and the liver and brain regions were isolated. RT-PCR and quantitative real-time RT-PCR were performed with RNA isolated from these samples respectively. My results showed that Cyp3all and Oatp2 expression increased dramatically upon PXR activation, and PCN regulates Cyp3all and Oatp2 in a PXR-dependent manner, which is consistent with the results previously generated from our lab [104]. Our studies have demonstrated for the first time that 1) PXR is expressed in mRNA level in the hypothalamus, amygdala, PFC, hippocampus, striatum, midbrain and cerebellum in the mouse brain. 2) Srd5α1 and Srd5α2 are not PXR target genes in either liver or brain in the mouse and PCN-mediated decrease of Srd5α1 and Srd5α2

gene expression in the liver is likely through other mechanisms. 3) PCN can induce an increase of Srd5α2 gene expression in the cerebellum. Although the mechanism underlying this phenomenon is unknown, we hypothesized that other nuclear receptors such as LXR may be involved in this regulation process.

In the future, the role of PXR in terms of regulating 3α-HSD gene expression in the brain can be investigated to further explain the mechanism of knocking down PXR-mediated decrease of allopregnanolone level in the midbrain and hippocampus. In addition, the role of LXR in this process should also be investigated.

While in the first project, we mainly focused on the PXR binding partners in the liver, our second project focused on studying the PXR function in the brain. Our two projects characterized PXR biology across different tissues and through different methods, thus, providing scientists with a further basis to study PXR.

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