Published in final edited form as:
Int J Dev Neurosci. 2014 November ; 0: 59–67. doi:10.1016/j.ijdevneu.2014.07.008.

IUGR Disrupts the PPARγ-Setd8-H4K20me[1] and Wnt Signaling Pathways in the Juvenile Rat Hippocampus

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Abstract

Intrauterine growth restriction (IUGR) programs neurodevelopmental impairment and long-term neurological morbidities. Neurological morbidities in IUGR infants are correlated with changes hippocampal volume. We previously demonstrated that IUGR alters hippocampal cellular composition in both neonatal and juvenile rat pups in association with altered hippocampal gene expression and epigenetic determinants. PPARγ signaling is important for neurodevelopment as well as epigenetic integrity in the brain via the PPARγ-Setd8-H4K20me[1] axis and Wnt signaling. We hypothesized that IUGR would decrease expression of PPARγ, Setd8, and H4K20me[1] in juvenile rat hippocampus. We further hypothesized that reduced PPARγ-Setd8-H4K20me[1] would be associated with reduced Wnt signaling genes Wnt3a and β-catenin, and wnt target gene Axin2. To test our hypothesis we used a rat model of uteroplacental insufficiency-induced IUGR. We demonstrated that PPARγ localizes to oligodendrocytes, neurons and astrocytes within the juvenile rat hippocampus. We also demonstrated that IUGR reduces levels of PPARγ, Setd8 and H4K20me[1] in male and female juvenile rat hippocampus in conjunction with reduced Wnt signaling components in only male rats. We speculate that reduced PPARγ and Wnt signaling may contribute to altered hippocampal cellular composition which, in turn, may contribute to impaired neurodevelopment and subsequent neurocognitive impairment in IUGR offspring.

Keywords

Intrauterine growth restriction; brain development; PPARγ; epigenetics

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Conflict of Interest Statement
None of the authors have any conflicts of interest.

1. Introduction

Intrauterine growth restriction (IUGR) occurs when a fetus fails to reach optimal growth potential *in utero*. In developed countries, IUGR frequently occurs secondary to maternal **hypertensive** disorders and uteroplacental insufficiency. Insults such as IUGR, which occur during developmentally plastic periods, program neurodevelopmental impairment and long-term neurological morbidities (Geva et al. 2006, von Beckerath et al. 2013).

In IUGR infants, neurological impairments include both short and long-term cognitive disabilities evident as early as 2 years of age and persisting beyond school entry (Sung et al. 1993). Long-term neurological morbidities in IUGR infants are correlated with changes in brain connectivity and reduced volume of the hippocampus, a brain region key for the formation of certain types of memory (Olton et al. 1978, Morris et al. 1982, Zola-Morgan et al. 1986, Batalle et al. 2012, Padilla et al. 2014). However, mechanisms for the programming of neurodevelopmental impairment in IUGR are not well understood.

In order to understand the mechanisms driving the programming of neurodevelopmental impairment following IUGR, our group utilizes a rat model of uteroplacental insufficiency induced IUGR. In our model, IUGR results from multiple components including nutrient restriction and hypoxia. We previously demonstrated that IUGR rat pups have cognitive impairment as adults (D. Caprau 2012). We also demonstrated that IUGR alters hippocampal cellular composition in both neonatal and juvenile rat pups (Schober et al. 2009, Fung et al. 2012). Importantly, alterations in cellular composition are accompanied by changes in both hippocampal epigenetic determinants and changes in hippocampal gene expression (Ke et al. 2006, Ke et al. 2010, Ke et al. 2011). Our previous results suggest that IUGR disrupts pathways regulating hippocampal phenotype, epigenetics and gene expression in the rat.

An important pathway unexplored in the IUGR rat hippocampus is the peroxisome proliferator-activated receptor gamma (PPARγ) pathway. PPARγ is a transcription factor that belongs to the PPAR subfamily of nuclear receptors. The PPARγ gene is well conserved between the human and rat and gives rise to multiple mRNA variants, and two protein isoforms, PPARγ1 and PPARγ2 (Zhu et al. 1995, Fajas et al. 1997, Ershov and Bazan 2000). The PPARγ pathway is involved in hippocampal repair and plasticity (Zhao et al. 2009). PPARγ is expressed throughout the developing brain, however, hippocampal PPARγ variant expression patterns remain unknown (Cullingford et al. 1998, Moreno et al. 2004). PPARγ activation improves hippocampus-dependent cognitive function in neurodegenerative disorders in both human and animals (Watson et al. 2005, Pedersen et al. 2006, Risner et al. 2006, Escribano et al. 2009, Rodriguez-Rivera et al. 2011, Denner et al. 2012). In addition, neural PPARγ knockout mice show that postnatal reduction of PPARγ in neurons heightens sensitivity to ischemia (Zhao et al. 2009).

A novel means by which PPARγ may contribute to brain development is the transcriptional regulation of epigenetic modifying enzymes. Epigenetic modifying enzymes affect developmental processes by altering expression patterns of target genes. The genes coding for several chromatin modifying enzymes contain PPAR response elements (PPRE) and are

demonstrated transcriptional targets of PPARγ (Wakabayashi et al. 2009). One of these PPARγ responsive genes is the set domain containing histone methyltransferase, Setd8, which puts a monomethyl (me^1) group on lysine (K) 20 of Histone (H) 4.

Accumulating evidence shows that $H4K20me^1$ can function as a transcriptional activator in canonical Wnt signaling (Li et al. 2011). Canonical Wnt signaling plays an important role in a wide range of biological and pathophysiological processes involving central nervous system development (Logan and Nusse 2004, Clevers 2006, Willert and Jones 2006). A transcriptional target of active Wnt signaling is Axin inhibition protein 2 (Axin2). Axin2 is an essential regulator of normal myelination and remyelination (Fancy et al. 2011).

Despite the importance of PPARγ and Wnt signaling in hippocampal outcomes in IUGR, the effects of IUGR on PPARγ-Setd8-$H4K20me^1$ and wnt signaling components is unknown. We hypothesized that IUGR would decrease expression of PPARγ and its target gene Setd8, as well as subsequent levels of $H4K20me^1$ in juvenile rat hippocampus. We further hypothesized that reduced PPARγ-Setd8-$H4K20me^1$ would be associated with reduced expression of Wnt signaling genes Wnt3a and β-catenin, as well as wnt target gene Axin2. To test our hypothesis we used a well characterized rat model of IUGR.

2. Methods

2.1 Rat Model of IUGR

All animal procedures were approved by the University of Utah Animal Research Committee and are in accordance with the American Physiological Society's guiding principles (Society 2002). Surgical methods have been described previously (Ke et al. 2005, Ke et al. 2006, Joss-Moore et al. 2010) and are briefly described below. IUGR was induced in rat pups by ligation of both uterine arteries on day 19 of gestation, following anesthesia. Dams for control pups received identical anesthesia. IUGR and control rat dams delivered spontaneously and litters were culled to six in order to normalize postnatal nutrition. At postnatal day 21 (d21), IUGR and control rats were separated from their dams for 4 hours, anesthetized, and killed. We chose to assess the effects of IUGR on the PPARγ and Wnt signaling pathways at postnatal d21 (juvenile) because this time point follows key maturational processes such as myelination and synaptogenesis in the rat brain (Rice and Barone 2000). Brains were quickly removed, and hippocampi dissected and flash frozen in liquid nitrogen and stored in −80°C.

Whole brain from one male and one female rat pup from each litter were individually fixed via intra-cardiac perfusion as previously described (Fung et al. 2012). Dissected whole brains were postfixed at 4 °C overnight, cryoprotected with 15% and 30% sucrose at 4 °C overnight, and embedded in 2% gelatin, 0.9% NaCl, 0.05% NaN3 (Sigma-Aldrich) under freezing conditions. Brains were sectioned coronally at 12 μm per section (Microm HM550; Microm International, Walldorf, Germany). Sections containing dorsal hippocampus were collected sequentially on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA).

All experiments used 6 non-sibling rat pups per group (male control, male IUGR, female control, female IUGR).

2.2 Immunofluorescent staining for PPARγ localization

Immunofluorescent (IF) double labeling was used to co-localize PPARγ in neurons, oligodendrocytes and astrocytes in the hippocampus as previously described (Fung et al. 2012). 49,6-diamidino-2-phenylindole (DAPI) was used for nuclei counter staining. The following primary antibodies were used: anti-PPARγ (#2435, Cell Signaling) labeled in red, anti-neuron marker NeuN antibody (MAB377; Millipore, Billerica, MA); anti-oligodendrocyte marker APC antibody (ab16794, abcam) and anti-astrocyte marker GFAP antibody (IF30L-100ug; Millipore, Billerica, MA). Negative control sections underwent similar staining procedures with the omission of primary antibody.

Sections were imaged with the Nikon's A1 Nikon's A1 multiphoton laser scanning confocal microscope (Nikon Instruments Inc./Americas) and taken at ×20 and ×60 magnification to encompass all subregions of the hippocampus (CA1, CA3, and DG).

2.3 Real-Time RT-PCR

Real-time reverse transcriptase polymerase chain reaction (RT-PCR) was used to evaluate mRNA abundance of hippocampal PPARγ variants, Setd8, Wnt3a, β-catenin and Axin2 as previously described (Ke et al. 2006, Joss-Moore et al. 2010). The following Assay-on-demand primer/probe sets were used: PPAR γ1 – Rn01492275_m1, PPARγ 2 – Rn00440940_m1, Setd8 – Rn01477383_g1, Wnt3a-RN01470643_m1, β-catenin-00584431_g1, Axin2-Rn00577441_m1 (Applied Biosystems, Foster, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. GAPDH primer and probe sequences; Forward: CAAGATGGTGAAGGTCGGTGT; Reverse: CAAGAGAAGGCAGCCCTGGT; Probe: GCGTCCGATACGGCCAAATCCG.

2.4 Immunoblot

Hippocampal levels of PPARγ, Setd8, Wnt3a and γ-catenin protein were quantified using immunoblot as previously described (Joss-Moore et al. 2010, Ke et al. 2010). GAPDH was used as a loading control. The following primary antibodies were used: PPARγ (H-100, sc-7196, Santa Cruz Biotechnology), Setd8 (#2996, Cell Signaling Technology), Wnt3a (ab28472, abcam), β-catenin (H102, sc-7199, Santa Cruz Biotechnology) and GAPDH (rabbit polyclonal, Cell Signaling)

Global hippocampal H4K20me^1 was quantified by immunoblot with acid-extracted histones, prepared as previously described (Ke et al. 2006, Joss-Moore et al. 2010). Levels of histone H4 monomethyl Lys20 (H4K20Me pAb, #39727, Active Motif) was quantified relative to total H4 (#61299, Active Motif).

2.5 Statistics

All data presented are expressed as mean ± SD. Effects of IUGR on PPARγ-Setd8-H4K20me1 and wnt signaling in male and female rats were analyzed by Mann Whitney U test. Statistical significance was accepted as $p<0.05$.

3. Results

3.1 Weights of IUGR Rat Pups

IUGR rat pups weighed significantly less than age- and sex-matched control pups at day of life 1 and at day of life 21 (Table 1).

3.2 PPARγ co-localizes to oligodendrocytes, neurons and astrocytes in d21 rat hippocampus

To determine the cellular distribution of PPARγ in the d21 rat hippocampus, PPARγ co-immunofluorescence was used with markers for oligodendrocytes, neurons and astrocytes. All 3 cell types stained positive for PPARγ **(Figure 1A-D)**. Oligodendrocytes qualitatively displayed greater PPARγ expression than neurons and astrocytes. Neurons expressing PPARγ were primarily localized to the CA3 region of the hippocampus.

3.3 IUGR decreases Rat Hippocampal PPARγ-Setd8-H4K20me^1

The effect of IUGR on hippocampal expression of PPARγ, and downstream target Setd8, was assessed in female and male d21 rats. IUGR decreased PPARγ1 mRNA and protein levels, relative to sex-matched controls, in both female **(mRNA p=0.026, protein p=0.015)** and male **(mRNA p=0.009, protein p=0.026)** rat hippocampus (Figure 2A-B). The PPARγ2 variant was undetectable in d21 male or female rat hippocampus. Similarly, IUGR decreased Setd8 mRNA and protein abundance in both female **(mRNA p=0.026, protein p=0.004)** and male **(mRNA p=0.002, protein p=0.015)** hippocampus (Figure 3A-B).

Because Setd8 places the H4K20me^1 mark, we measured global levels of H4K20me^1 in d21 rat hippocampus. IUGR reduced global H4K20me^1 both female **(p=0.026)** and male **(p=0.041)** hippocampus (Figure 4).

3.4 IUGR Decreases Hippocampal Wnt3a and β-catenin expression, and Axin2 mRNA

Because the regulation of Wnt signaling is a downstream effect of H4K20me^1, we measured the effect of IUGR on expression of Wnt signaling components, Wnt3a and β-catenin, as well the Wnt signaling target, Axin2. In female hippocampus, IUGR did not affect Wnt3a mRNA or protein abundance (Figure 5A-B). However, IUGR did decrease β-catenin protein abundance **(p=0.015)** relative to female control (Figure 6B). Wnt target gene, Axin2 mRNA was not altered by IUGR in female rat hippocampus (Figure 7). In contrast, in male hippocampus, IUGR decreased mRNA and protein levels of Wnt3a **(mRNA p=0.026, protein p=0.015)**, β-catenin protein levels **(p=0.015)** as well as mRNA levels of Axin2 **(p=0.041)** (Figure 5,6,7).

4. Discussion

The results of our study demonstrate that IUGR disrupts the PPARγ-Setd8-H4K20me^1 axis and Wnt signaling in the juvenile rat hippocampus. Our novel data show that PPARγ is expressed in oligodendrocytes, neurons, and astrocytes in juvenile rat hippocampus. While overall decreases in the PPARγ-Setd8-H4K20me^1 axis were observed in both male and female IUGR rat hippocampus, effects on Wnt signaling components and Axin2 were

predominantly seen in male rat hippocampus. Given the importance of PPARγ and Wnt signaling in appropriate neural development, decreased PPARγ-Setd8-H4K20me^1 and Wnt signaling may contribute to the IUGR-induced alterations in hippocampal phenotype previously observed in the model.

In our study, we identified PPARγ expression in different cell types within the hippocampus. We observed PPARγ expression in hippocampal neurons, consistent with previous studies (Inestrosa et al. 2005, Di et al. 2009). Interestingly, we also observed that PPARγ is primarily localized to neurons in CA3 region of the hippocampus. The hippocampal CA3 subregion plays a significant role in spatial memory formation (Handelmann and Olton 1981, Sutherland et al. 1983, Stubley-Weatherly et al. 1996). Our study also showed significant PPARγ expression in hippocampal oligodendrocytes. In oligodendrocytes, PPARγ is involved in lipid metabolism and differentiation in vitro (Roth et al. 2003). Oligodendrocytes are the myelinating cells of the central nervous system (CNS) that enable formation of myelin and saltatory nerve conduction, and provide a supporting role for neurons in the CNS. We speculate that decreased PPARy expression and its downstream signaling may account for reduced oligodendrocyte density previously reported in IUGR male rats (Schober et al. 2009).

In the past, PPARγ has been investigated for its action in ameliorating the development and progression of a number of CNS diseases. PPARγ activation has been shown to increase neuron survival and decrease lesion sizes in animal models of Parkinson's disease, central inflammation, intracerebral hemorrhage, and cerebral ischemia (Heneka et al. 2000, Breidert et al. 2002, Dehmer et al. 2004, Ou et al. 2006, Victor et al. 2006, Zhao et al. 2006). PPARγ agonists have been shown to improve impaired hippocampus-dependent cognitive function in neurodegenerative disorders in both human and animals (Watson et al. 2005, Pedersen et al. 2006, Risner et al. 2006, Escribano et al. 2009, Rodriguez-Rivera et al. 2011, Denner et al. 2012). Our finding of IUGR-induced reductions in hippocampal PPARγ expression suggests that PPARγ contribute to impaired hippocampal development and impaired learning and memory function seen in IUGR rats. Further investigations into the causative nature of PPARγ levels on the IUGR phenotype are warranted.

Specific roles of PPARγ in hippocampal development and function are currently unknown. The discovery that PPARγ regulates the expression of chromatin modifying enzymes (Wakabayashi et al. 2009) provides a novel mechanism by which PPARγ may impact hippocampal development. Epigenetic regulation provides a way to selectively express information contained within the genome. This is particularly important in the context of developmentally specific gene expression. One mode of epigenetic regulation is methylation of histones by methyltransferase enzymes. The PPARγ target gene, Setd8 is a histone lysine methyltransferase, which places the H4K20me^1 mark.

Roles of H4K20me^1 are varied and include transcriptional repand transcriptional activation repression (Nishioka et al. 2002, Talasz et al. 2005, Vakoc et al. 2006). One pathway that is activated by H4k20me^1 is canonical Wnt/β-catenin signaling (Li et al. 2011). Setd8 mediates Wnt/β-catenin signaling and Wnt signaling stimulates H4K20me^1 enrichment at target gene promoters (Li et al. 2011). One of the Wnt signaling genes Wnt3a is crucial for normal

growth of the hippocampus. Wnt3a acts locally to regulate the expansion of the caudomedial cortex, from which the hippocampus develops (Lee et al. 2000). Moreover, the Wnt signaling target gene Axin2 is essential for myelination and remyelination in brain development (Fancy et al. 2011). In this study, we found that IUGR-induced decreased hippocampal PPARγ expression is associated with decreased Setd8 expression and H4K20me^1 abundance, as well as reductions of Wnt3a, β-catenin and Axin2, in male rat hippocampus.

Our finding of IUGR-induced decreased Wnt3a and Axin2 expression in male rats, but not in female rats, suggests a gender-specific response to brain Wnt signaling. This is important in the context of IUGR because gender-specific molecular changes are often observed (Joss-Moore et al. 2010, Ke et al. 2010, Ke et al. 2011, Fung et al. 2012). Mechanisms driving the gender-specific molecular responses are not fully elucidated. However, cross-talk between Wnt and estrogen signaling pathways is known to occur via a functional interaction between β-catenin and estrogen receptor alpha (ERα) (Kouzmenko et al. 2004). Furthermore, we previously demonstrated that IUGR reduces expression of the estrogen synthase, aromatase and ERα mRNA levels in male newborn rat hippocampus (O'Grady et al. 2010, Numpang et al. 2013). Collectively, we speculate that reduction of aromatase and ERα expression may play a role in decreased Wnt3a and subsequent axin2 levels in male IUGR rat hippocampus.

Our study is not without limitations. Our molecular findings are limited to global hippocampus and are not specific to any hippocampal subregion or specific cell type. In our study, we only examined the juvenile IUGR brain. In order to separate prenatal effects (IUGR) from postnatal effects (growth rate) on PPARγ-Setd8-H4K20me1 and wnt signaling, newborn rats will need to be studied. Our study is also descriptive in nature. Future studies examining the effect of PPARγ activators and antagonists on downstream targets will be an important next step in establishing cause and effect relationships.

In conclusion, IUGR decreases hippocampal PPARγ expression with an associated decreased in PPARγ downstream target Setd8 and H4K20me^1 abundance. Reduction in H4K20me^1 is further associated with decreased expression of Wnt signaling genes Wnt3a and β-catenin, as well as axin2 in male pups. We speculate that reduced PPARγ and Wnt signaling may contribute to altered hippocampal cellular composition which, in turn, may contribute to impaired neurodevelopment and subsequent neurocognitive impairment seen in IUGR offspring.

Acknowledgements

We would like to acknowledge the Division of Neonatology for support. This study was supported by the NIH (K01-DK080558 (LJM) and R03-DK095970 (LJM)).

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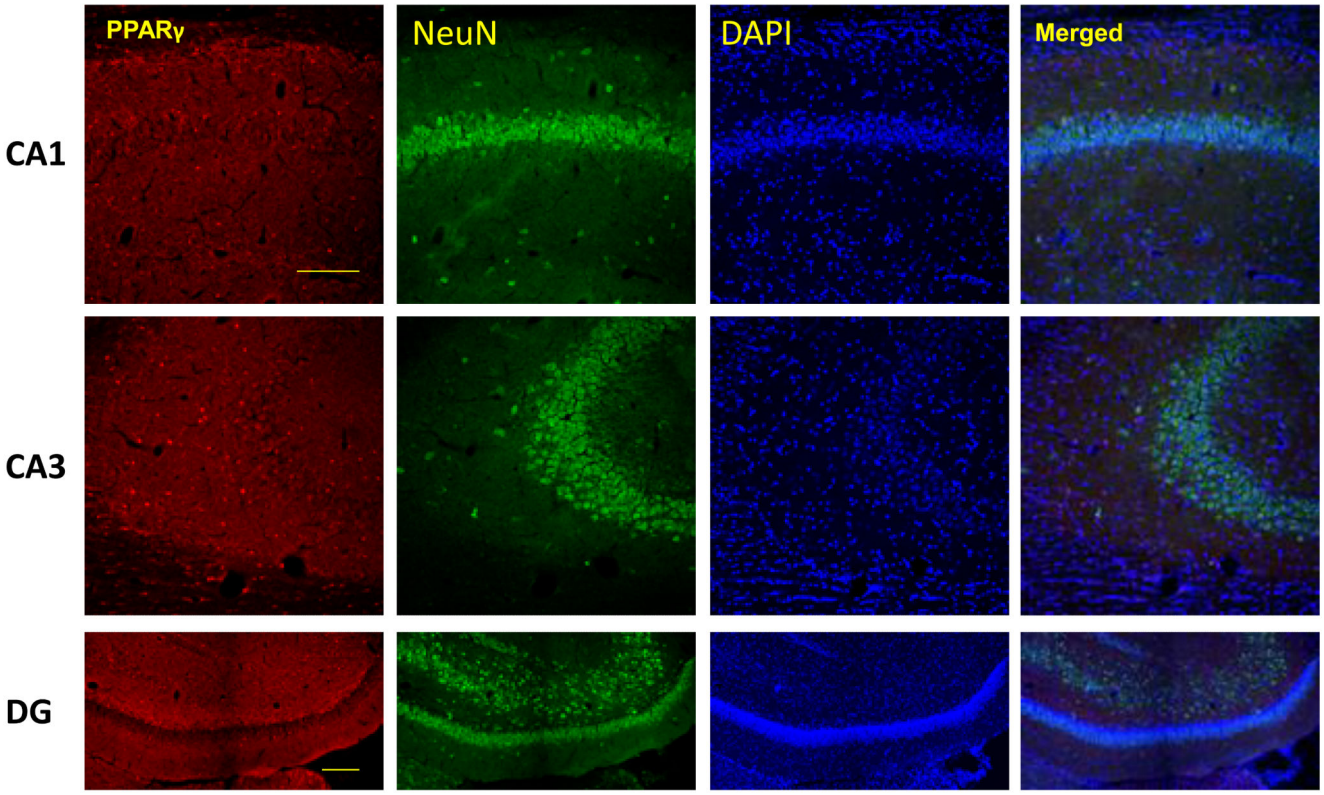
Highlights

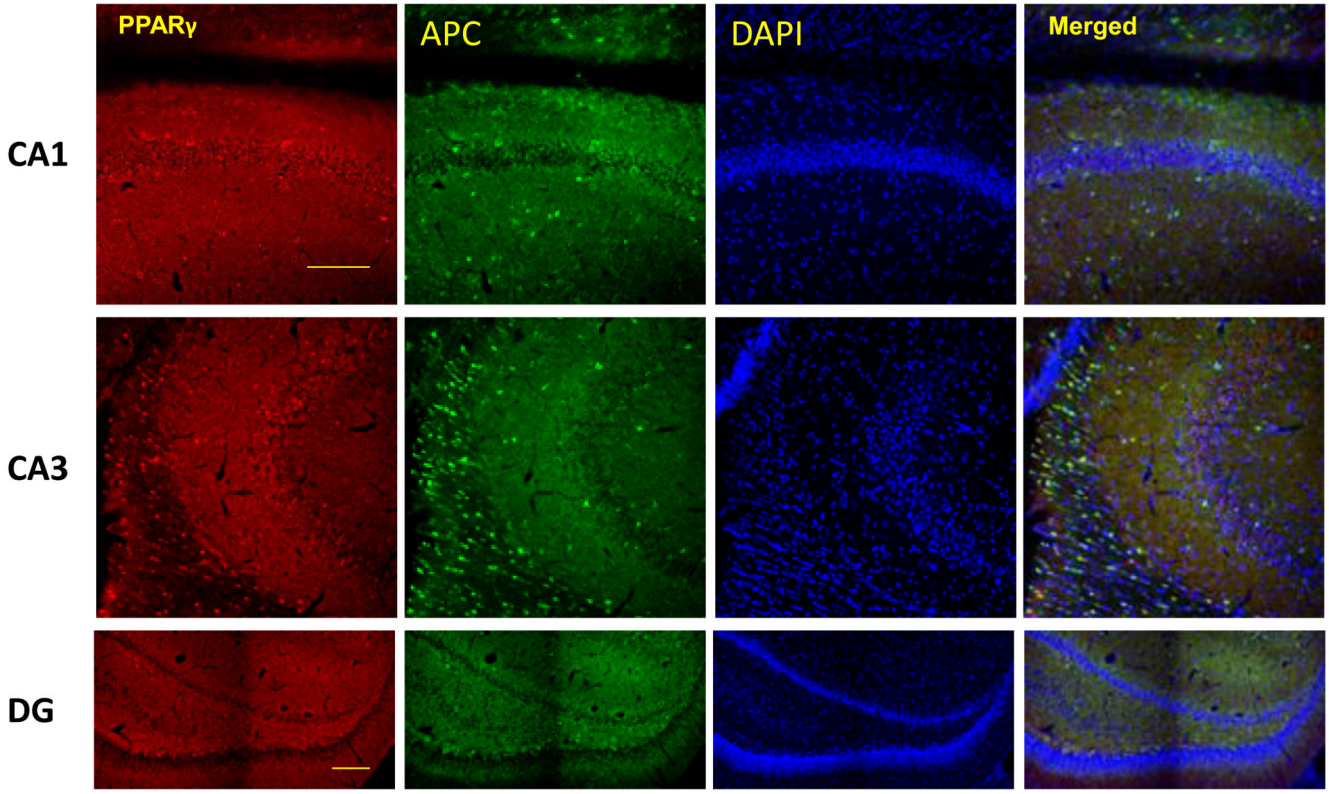
Intrauterine growth restriction programs neurodevelopmental impairment and long-term neurological morbidities.

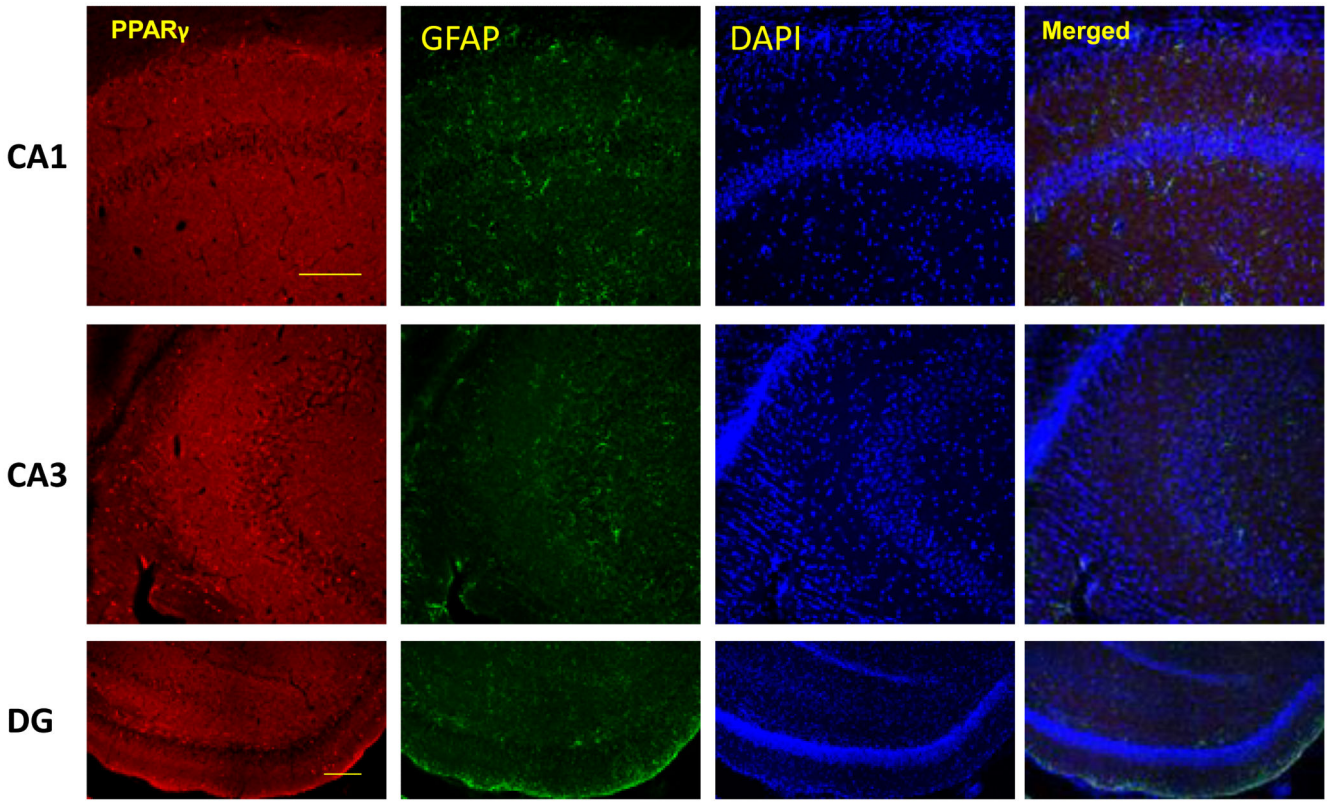
We use a rat model to test the effects of IUGR on two developmentally important pathways; PPARγ-Setd8-H4K20me^1 and Wnt signaling.

IUGR reduces mRNA and protein components of both pathways as well as mRNA of final target gene Axin2.

IUGR programming of neurodevelopmental impairment likely involves disruption to the PPARγ-Setd8-H4K20me^1 and Wnt signaling pathways.







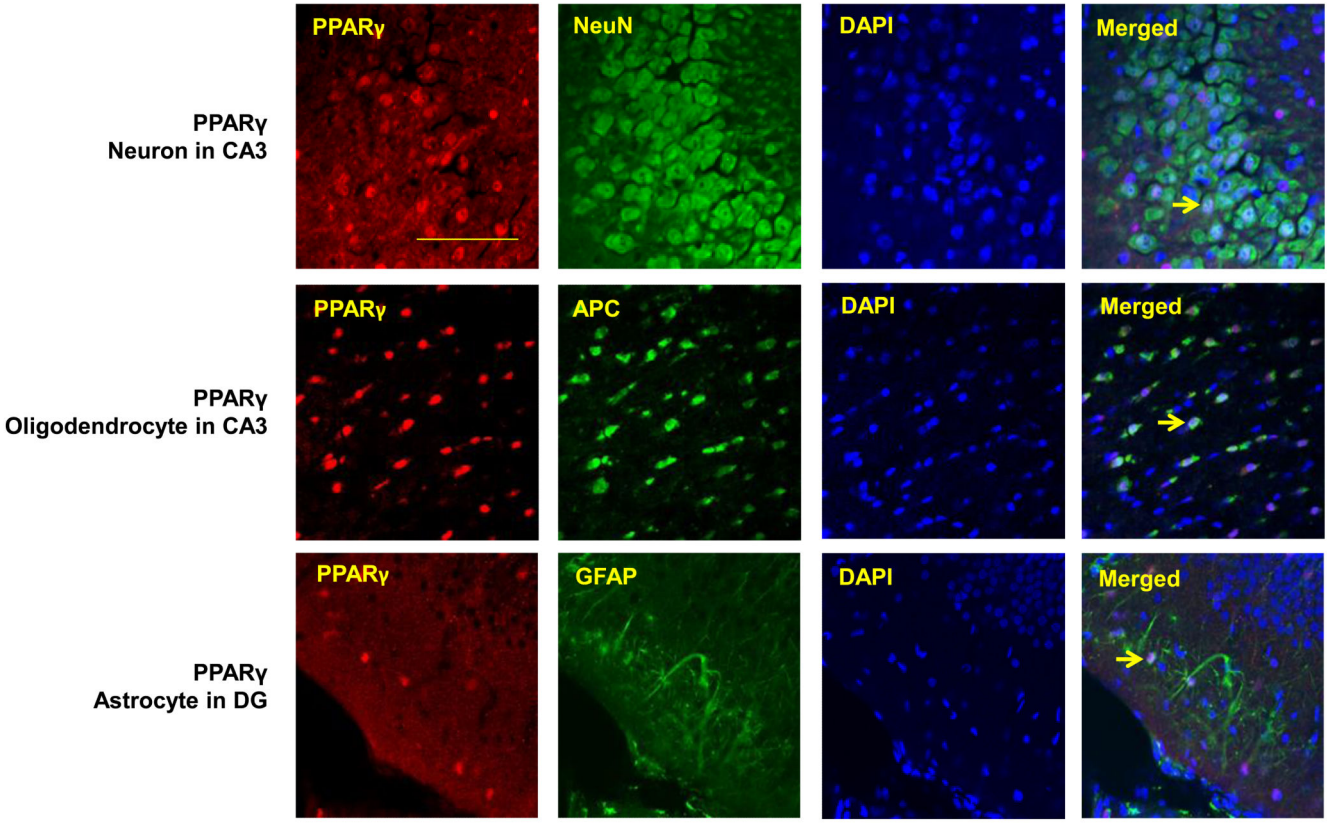


Figure 1.

PPARγ is expressed in neurons, oligodendocytes and astrocytes of the rat hippocampus. Representative images of PPARγ (red) co-immunoflorescent staining with A) NeuN for neurons(green), B) APC for oligodendrocytes (green), and GFAP for astrocytes(green) in CA1, CA3 and dentate gyrus (DG) subregions of the rat hippocampus. Nuclei were counterstained with DAPI (blue). Three colors equally merged shows in white. D) Arrows show representative PPARγ positive cells in each case. Scale bar=100μm.

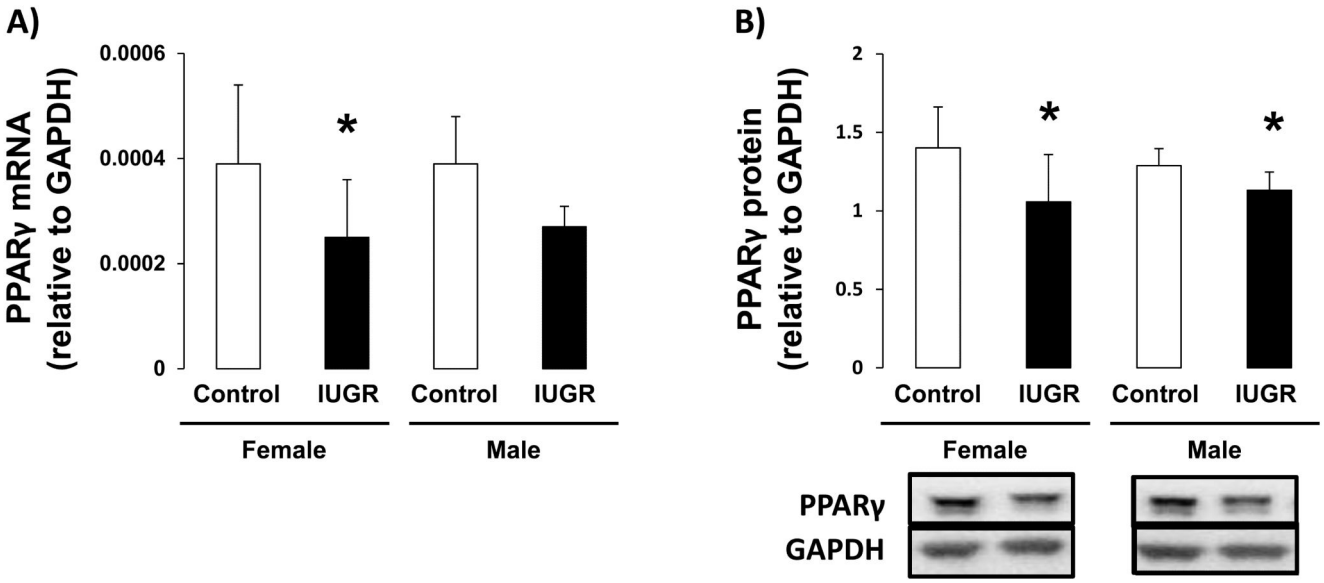


Figure 2.

PPARγ mRNA and protein levels in d21 hippocampus. IUGR significantly decreased PPARγ mRNA (A) and protein levels (B) in both female and male hippocampus n=6. *p<0.05

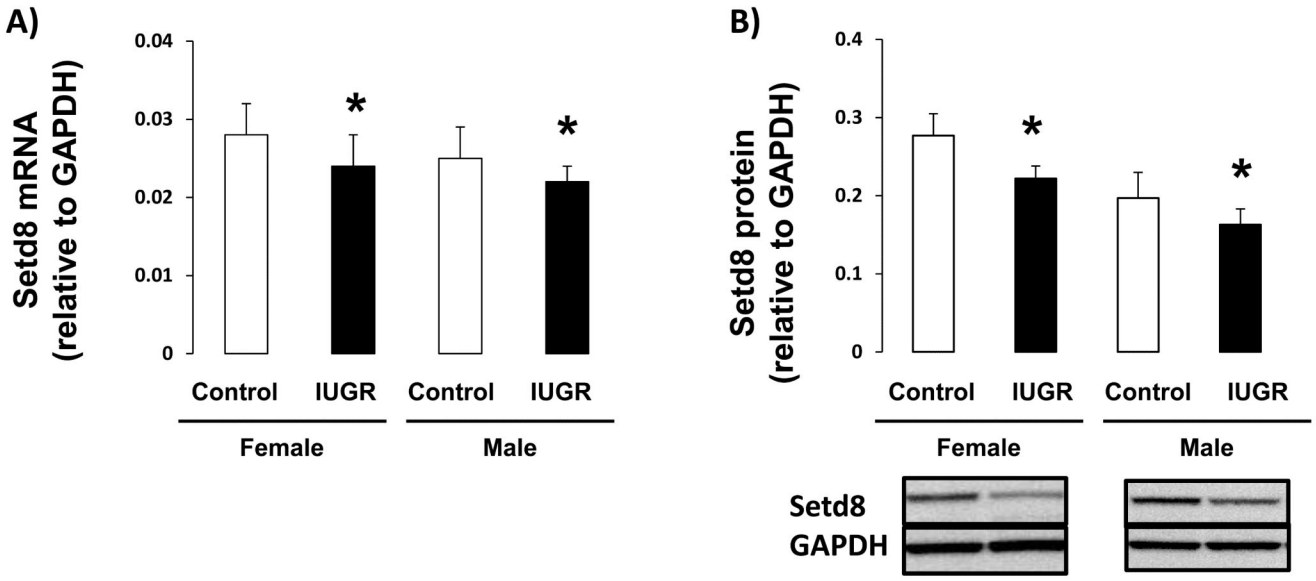


Figure 3.

Setd8 mRNA and protein levels in d21 hippocampus. IUGR significantly decreased Setd8 mRNA levels (A) and protein levels (B) in both female and male hippocampus n=6. *p<0.05

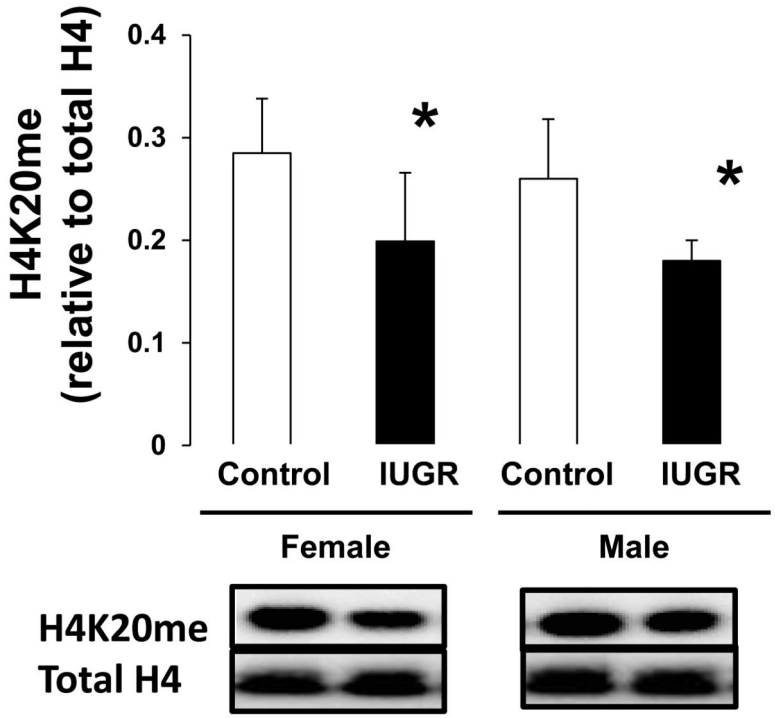


Figure 4.

H4K20me protein levels in d21 hippocampus. IUGR significantly reduced H4K20me protein abundance in both female and male hippocampus. n=6. *p<0.05

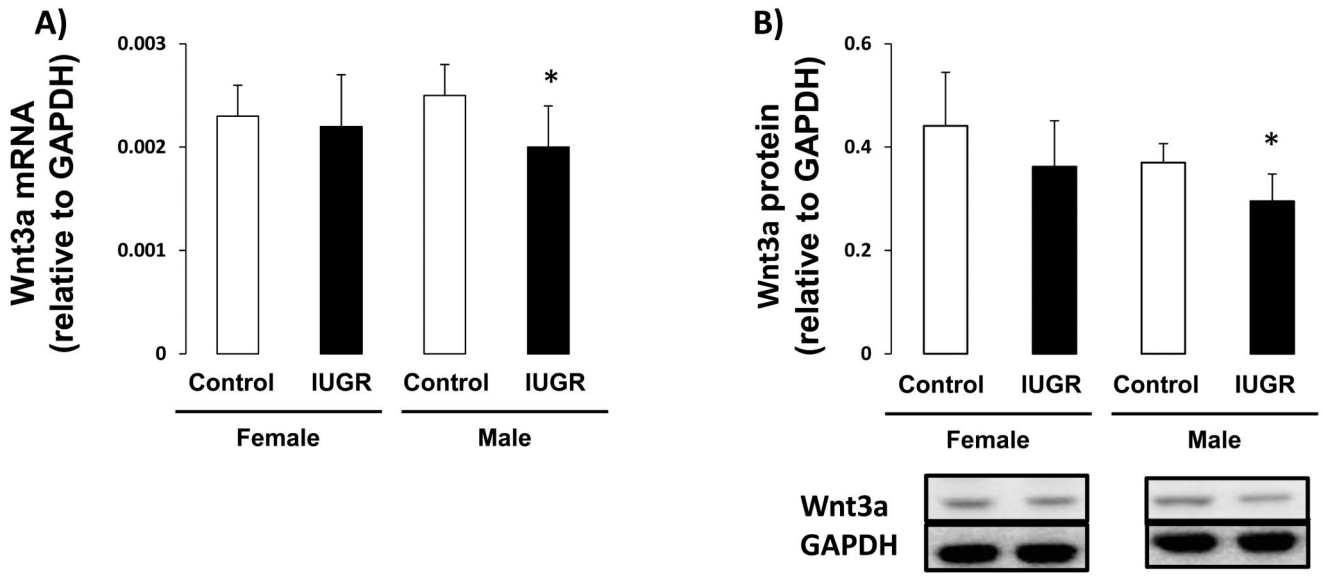


Figure 5.

Wnt3a mRNA and protein levels in d21 hippocampus. A-B: IUGR significantly decreased Wnt3a mRNA and protein abundance in male rat hippocampus but not in female rat hippocampus. n=6. *p<0.05

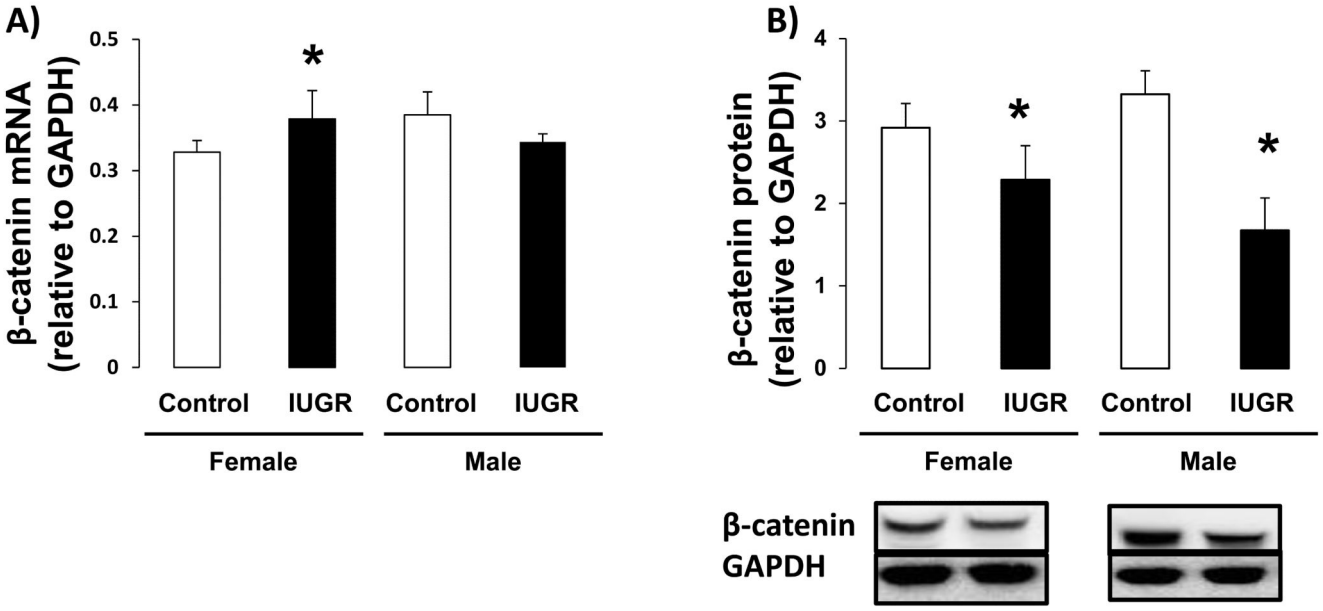


Figure 6.

β-catenin mRNA and protein levels in d21 hippocampus. A-B: IUGR significantly decreased β-catenin protein abundance in male rat hippocampus. IUGR also decreased β-catenin protein levels in female rat hippocampus. n=6. *p<0.05

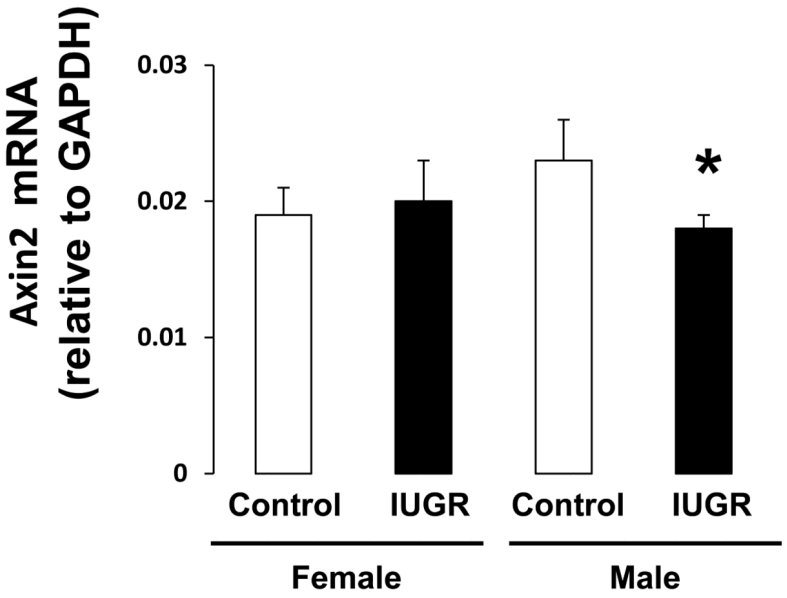


Figure 7.

Axin 2 mRNA levels in d21 hippocampus.: IUGR significantly decreased Axin2 mRNA levels in male rat hippocampus without affecting female rat hippocampus. n=6. *p<0.05

Table 1

IUGR Decreased Body Weight (gm) in Rat Pups at day of life 1 and 21 (mean ± SD)

Postnatal	Male		Female	
Age (days)	Control	IUGR	Control	IUGR
DOL1	7.1 ± 0.4	5.1 ± 0.5*	6.7 ± 0.3	5.4 ± 0.3*
DOL21	68.3 ± 3.7	55.3 ± 7.7*	63.4 ± 1.9	54.6 ± 4.2*

*Different from age- and sex-matched control group, $p<0.05$.