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# Regulation of Glucose Transporter 1 (Slc2a1) in the Pituitary Gonadotrope of Mice after Puberty

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## Abstract

Glucose provides fuel for cell metabolism and plays an important role in normal reproductive function. Glucose transporters such as (SLC2A1) facilitate entry of glucose into cells. We have previously shown that gonadotropin releasing hormone (GnRH) stimulates *Slc2a1* expression in LβT2 gonadotrope cells and in the pituitary gland of pre-pubertal mice. In this study we examined changes in *Slc2a1* expression in the pituitary gland of mice after puberty and in LβT2 gonadotrope cells treated with sex steroids. There were no detectable changes in *Slc2a1* gene expression in the pituitary gland of pre-pubertal and adult male and female mice using real-time PCR analysis. In contrast, Western blot analysis detected higher levels of SLC2A1 protein in the pituitary gland of adult male and female mice compared to pre-pubertal male and female mice, respectively. In addition, peak levels of SLC2A1 protein as determined by Western blot analysis were detected in the pituitary gland of female mice during the proestrus and oestrus stages of the oestrous cycle. The proestrus stage corresponds to the surge of luteinizing hormone in the gonadotrope, which then triggers ovulation. Additional studies using immunofluorescent analysis of pituitary glands revealed increased co-localization of SLC2A1 with luteinizing hormone β(LHβ) in the gonadotrope cell of adult male and female mice compared to pre-pubertal mice. *In vitro* studies in LβT2 cells did not detect any effects of estradiol and/or progesterone on *Slc2a1* expression by real-time PCR analysis, but estradiol alone increased SLC2A1 protein levels in LβT2 cells as determined by Western blot analysis. Furthermore, combined treatment of estradiol and progesterone induced glucose uptake in LβT2 cells. Together, our findings show that regulation of *Slc2a1* in the gonadotrope of adult mice likely reflects changes in sex steroids that are also important for pubertal development and fertility.

**Keywords:** Gonadotrope; Glucose transporter 1; Sex steroids; Puberty

## Introduction

Pubertal development and normal reproductive function are regulated by the hypothalamic-pituitary-gonadal (HPG) axis, which consists of gonadotropin releasing hormone (GnRH) neurons, the pituitary gonadotrope cells, and the gonad [1-3]. The hypothalamus contains specialized neurons that secrete GnRH, which binds to its receptor located on the gonadotrope cell in the anterior pituitary gland to stimulate the synthesis and secretion of the gonadotropins; luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH and FSH are composed of two subunits: a common α-subunit and β-subunit that is unique to each hormone. LH and FSH then bind to their respective receptors in the gonad to stimulate the synthesis and secretion of sex steroids, maturation of the oocyte, and induction of spermatogenesis. Estrogen, progesterone and testosterone are the main sex steroids produced by the gonads, and these steroids regulate the HPG axis through negative feedback inhibition at the level of the hypothalamic GnRH neuron and the pituitary gonadotrope cells [3-5]. Estrogen also has positive feedback regulation of the HPG axis, which is important for the preovulatory surge of LH and FSH in various animal models and in humans [6-12]. Thus, regulation of HPG axis by GnRH and sex steroids is important for puberty and fertility.

Glucose is an important source of energy for cellular metabolism, and disorders that impair glucose homeostasis are associated with pubertal and reproductive abnormalities due to dysregulation of the HPG axis [13]. Hyperglycemia impairs GnRH neuronal cell function, and impairment of glucose availability reduces LH secretion *in vivo* [14-18]. Glucose enters cells via the solute carrier-2 (SLC2) family proteins also called glucose transporters (SLC2s), which are important for tissue-specific nutrient sensing and utilization of glucose [19,20]. Some SLC2s are regulated in different tissues of the reproductive axis, and studies have shown that *Slc2a1* is expressed in the testis, uterus, ovary, as well as endometrial and breast cancer cells [21-25]. FSH, interleukin1b (IL1b), and basic fibroblast growth factor (bFGF) stimulate *Slc2a1* gene expression and glucose uptake in rat Sertoli cells [26]. In endometrial stromal cells *Slc2a1* gene expression and glucose uptake are stimulated by progesterone and inhibited by estradiol [22]. In oocytes and granulosa cells, *Slc2a1* mRNA and protein levels are regulated by IGF1 and estradiol [25]. Therefore, regulation of *Slc2a1* gene expression is detected throughout tissues of the reproductive axis.

We have previously shown that Slc2a1 is regulated by GnRH in the gonadotrope cell both in vitro in LβT2 cells and in vivo in mice, and that GnRH increases glucose uptake in LβT2 gonadotrope cells [27]. We also showed that treatment of pre-pubertal mice with exogenous GnRH increased Slc2a1 mRNA expression but not Slc2a2, Slc2a4, or Slc2a8 in the mouse pituitary gland, which suggests that Slc2a1 may be important for glucose metabolism in the gonadotrope compared to other glucose transporters. In this study, we investigated the regulation

of Slc2a1 in the pituitary gland of pre-pubertal and adult mice. We also examined changes in Slc2a1 in the pituitary gland of female mice during the estrous cycle. To correlate with our in vivo studies in mice, we investigated effects of estradiol and progesterone on Slc2a1 and glucose uptake in LβT2 gonadotrope cells.

## Materials and Methods

### Animals

Pre-pubertal (4 weeks of age) and adult (2 months of age or older) male and female C57BL/6J mice were identified. Mice were kept on a standard diet with access to food and water ad libitum, and a 12 hour day/night cycle. Vaginal smears were performed with 0.9% saline solution on adult C57BL/6J female mice daily for 1-2 weeks to identify different stages (diestrus, proestrus, and estrus) in the estrous cycle [28]. Vaginal washings were examined under a light microscope with 10X magnification to identify different cell types that are representative of the estrous cycle stages. Mice were euthanized by isofluorane inhalation followed by cervical dislocation prior to harvesting tissues. All protocols were submitted to, approved by, and conducted in accordance with the Wayne State University Institutional Animal Care and Use Committee.

### Cell culture

LβT2 cells [29]; [30] were grown in DMEM (4.5 mg/L of glucose, 110 mg/L pyruvate, and 548 mg/L L-glutamine) supplemented with 10% FBS and 1% penicillin-streptomycin and incubated at 37°C with 5% $CO_2$ supply. For the time course experiments, cells were plated in 6 well plates at a concentration of $1.5-2.0 \times 10^6$. Cells were subsequently changed to phenol-free media with 2% heat-inactivated charcoal dextran-stripped calf serum, and 1% penicillin/streptomycin and then stimulated with estradiol (10 nM) or progesterone (100 nM) for 2, 8, and 24 hours or vehicle alone (ethanol, 0 hour). At the end of the time course of steroid induction, RNA or whole cell extracts (WCE) were isolated. Steroid induction experiments were performed three times for RNA and protein isolation.

### RNA isolation and Real-Time PCR

Tissues were harvested from euthanized mice as described above and RNA was isolated from the pituitary for cDNA synthesis to examine changes in *Slc2a1* and *Lhb* gene expression via real-time PCR. TATA box binding protein (*Tbp*) gene was used as an endogenous control. The cDNA (representing 50 or 100 ng of starting RNA) was used for real-time PCR analyses via Taqman Gene Expression Assays for *Slc2a1* (Mn00441473_m1), *Lhb* (Mm00656868_m1) and *Tbp* (Mm00446973_m1). PCR reactions were conducted using the Step One plus Real-time PCR System as previously described [27]. Cycle threshold was obtained for each sample. A corrected Ct (Delta Ct) was calculated by subtracting the *Tbp* Ct from the target genes (*Slc2a1* and *Lhb*) Ct for each sample. Relative differences from the control sample were then calculated by using the formula:

$$\text{fold change} = 2^{(\text{Control Delta Ct minus Sample Delta Ct})}$$

Pre-pubertal males n=7-9, and adult males n=12, pre-pubertal females n=9-12 and adult females in diestrus n=7, proestrus n=6-7, and estrus n=8 were used for *Slc2a1* and *Lhb* gene expression.

### Western immunoblot analysis

Whole cell extracts (WCE) were prepared from mouse pituitary glands, testes, and ovaries using RIPA buffer containing 1X Phosphate buffered saline, 0.1% sodium dodecyl sulfate and 1% Nonidet P40 with 1X Complete Protease Inhibitor cocktail as previously described [27]. Protein concentration was determined by using Coomassie Plus Protein Assay Reagent, and Western blot analysis was performed on protein extracts (20-50 ug/lane) with a rabbit polyclonal antibody against SLC2A1 and polyclonal antibody against α-Tubulin. Immunodetection of protein was performed using Super Signal West Pico Chemiluminescent reagent. Western blots were quantified by analyzing the film with Image J 1.47 program. When quantitating the Western blots, images of multiple exposures were examined to ensure that the films were not over exposed. SLC2A1 protein levels in the pituitary gland of pre-pubertal male mice n=6 were compared to adult male mice n=7. SLC2A1 protein levels in the pituitary gland of pre-pubertal female mice n=7 compared to adult female mice in diestrus n=7, proestrus n=7, and estrus n=7. SLC2A1 protein levels in the testis of pre-pubertal male mice n=5 were compared to pubertal male mice n=7. SLC2A1 protein levels in the ovary of pre-pubertal female mice n=7 compared to adult female mice in diestrus n=4, proestrus n=4, and estrus, n=3.

### Histology and immunofluorescent analysis

Pituitary gland of two pre-pubertal mice and three pubertal mice C57/BL6 mice were isolated and fixed in 10% formalin overnight prior to paraffin embedding. Paraffin embedding was performed by the University Pathology Research Services at Wayne State University School of Medicine. Paraffin-embedded pituitaries were subsequently sectioned (4 micron) using a rotatory microtome and then the tissue was mounted on slides. The tissues were rehydrated and then subjected to antigen retrieval buffer (10 mM citrate, pH 6.2, 2 mM EDTA, and 0.05% Tween 20) as previously described [31]. Primary rabbit-polyclonal LHβ antibody was diluted at 1:200 and goat-polyclonal SLC2A1 antibody was diluted at 1:200 and then incubated overnight at 40°C in a humidified chamber. Subsequently, tissues were incubated with secondary antibodies, Alexa Fluor® 488 donkey anti-goat, and Alexa Fluor® 568 goat anti-rabbit for 2 hours at RT. After washing with PBS, mounting media with the nuclear marker DAPI was added to the tissues. Immunofluorescent analysis of paraffin-embedded stained pituitary sections and LβT2 cells was performed with Nikon Eclipse Ti Confocal Imaging System (Nikon Instruments Inc.) equipped with an S Fluor 40X oil objective lens (numerical aperture 1.30). Images were taken by Roper Scientific–CoolSNAP HQ2 camera system (resolution: 1392x1040) and analyzed using NIS-Elements AR imaging software (version 3.22.00), which also has a quantification feature to identify and count cells in the images. To determine specificity of the SLC2A1 antibody in these studies, SLC2A1 antibody was preincubated with vehicle (dH20) or SLC2A1 peptide prior to performing immunofluorescent analysis and a no primary antibody control was also used (Supplementary figures 1-3).

### Glucose uptake assay

LβT2 cells were grown in DMEM (4.5 mg/L of glucose, 110 mg/L pyruvate, and 548 mg/L L-glutamine) supplemented with 10% FBS and 1% penicillin-streptomycin and incubated at 37°C with 5% $CO_2$. Cells were plated in 96 well black clear bottom plates at a concentration of $1.5 \times 10^5$ cells/well. Cells were subsequently changed to phenol-free media with 2% heat-inactivated charcoal dextran-stripped

calf serum, and 1% penicillin/streptomycin, and then stimulated with vehicle alone (ethanol, 0 hr) or 17β-estradiol (10 nM) or progesterone (100 nM) for 2, 8, and 24 hours. Glucose uptake was performed using the Glucose Uptake Cell-Based Assay Kit from Cayman. Media was changed to glucose free and phenol free media before the addition of 2-deoxy-2-[(7-nitro-2,1,3-benzoxadioazol-4-yl) amino]-D-glucose (2-NBDG) for 15 minutes. At the end of the treatment, cells were centrifuged for 5 minutes at 400xg at room temperature, and then each well was washed with 200ul of cell-based Assay Buffer, and the plate was centrifuged for 5 minutes at 400xg at room temperature. The supernatant was aspirated, and 100 ul of cell based assay buffer was added to each well before measuring the amount of 2-NBDG taken up by the cells. Fluorescence was detected with a plate reader using wavelengths of excitation/emission=485/535 nm. After fluorescence reading was obtained, 50 μl of sodium hydroxide (0.1 M) was added to each well and protein concentration was determined by using Coomassie plus Protein Assay Reagent. Fluoresce readings were corrected for protein concentration and basal uptake for each sample, and then control samples were used to determine fold change. The glucose uptake time course was repeated twice (which gives a total of 3 separate experiments).

## Reagents and chemicals

High Capacity cDNA Reverse Transcript Kit, Glucose and phenol free media, Taqman gene expression assay, StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA); TRIzol, Alexa Fluor® 488 donkey Anti-goat, Alexa Fluor® 568 goat Anti-rabbit, DAPI (Invitrogen Co., Carlsbad, CA); Estradiol and Progesterone (Sigma-Aldrich Co., LLC, St. Louis, MO); Complete Protease Inhibitor cocktail (Roche Diagnostics, Germany); Super Signal West Pico Chemiluminescent Substrate and Coomassie Plus Protein Assay Reagent (Thermo Scientific, Rockford, IL); citrate, EDTA, HEPES, methanol, sodium chloride, sodium dodecyl sulfate, Tris base, Triton-X, and Tween 20, (Fisher Scientific, Fair Lawn, NJ); Glucose Uptake Cell-Based Assay Kit ( Cayman Chemical Company, Ann Arbor, MI); DMEM (Mediatech, Manassas, VA); LHβ antibody (anti-rat LHβ - IC-3, AFP571292393 from the National Hormone and Peptide Program-National Institute of Diabetes and Digestive and Kidney Diseases, Dr. A.F. Parlow); goat-polyclonal SLC2A1 antibody N-20, polyclonal antibody against A-Tubulin (Santa Cruz Biotechnology, Santa Cruz, CA); a rabbit polyclonal antibody against SLC2A1 (Abcam Inc., Cambridge, MA ).

## Statistical analysis

Data are presented as means+SEM. Statistical analysis was performed with GraphPad 5.04 (San Diego, CA). Significance was determined by ANOVA with Bonferroni's or Dunnett's multiple comparison test or Student's (unpaired) t- test.

## Results

### Slc2a1 gene expression in the murine pituitary gland

We examined the regulation of *Slc2a1* in the pituitary gland of pre-pubertal and adult male and female C57BL/6J mice. RNA was isolated from the pituitary gland and cDNA was made for real-time PCR analysis. There were no significant changes detected in *Slc2a1* mRNA levels in pre-pubertal and adult mice in males (Figure 1A). In contrast, *Lhb* mRNA levels increased 2.4-fold in the pituitary gland of adult

male mice compared to pre-pubertal male mice (P<0.05, Figure 1A). We next examined the regulation of *Slc2a1* gene expression in pre-pubertal and adult female mice. We identified adult female mice in different stages of the estrous cycle (diestrus, proestrus, and estrus) by performing vaginal smears [28]. Similar to observations in male mice, there was no detectable difference in *Slc2a1* mRNA levels in the pituitary of pre-pubertal and adult female mice (Figure 1B). In contrast, *Lhb* mRNA levels increased 1.4-fold in adult females during proestrus compared to the estrus (P<0.001, Figure 1B). These results show that there is no significant change in *Slc2a1* gene expression in the pituitary gland after puberty.
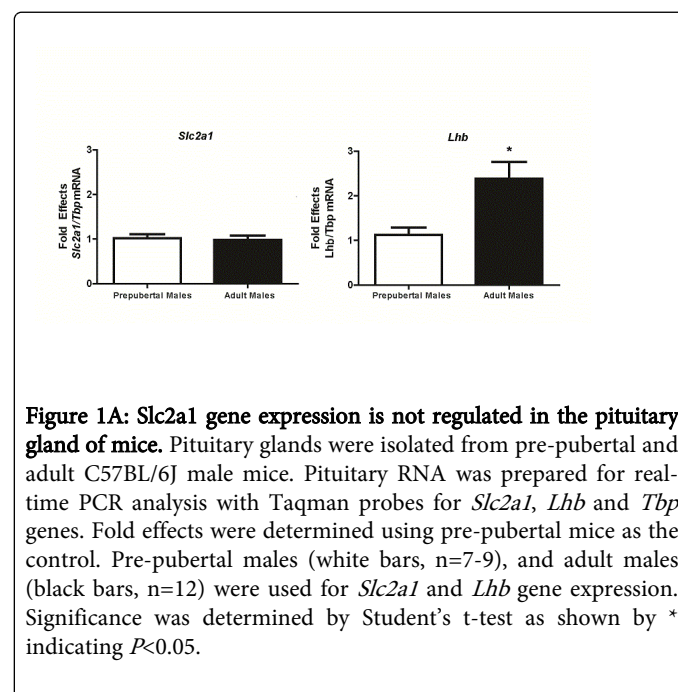


**Figure 1A: Slc2a1 gene expression is not regulated in the pituitary gland of mice.** Pituitary glands were isolated from pre-pubertal and adult C57BL/6J male mice. Pituitary RNA was prepared for real-time PCR analysis with Taqman probes for *Slc2a1*, *Lhb* and *Tbp* genes. Fold effects were determined using pre-pubertal mice as the control. Pre-pubertal males (white bars, n=7-9), and adult males (black bars, n=12) were used for *Slc2a1* and *Lhb* gene expression. Significance was determined by Student's t-test as shown by * indicating *P*<0.05.
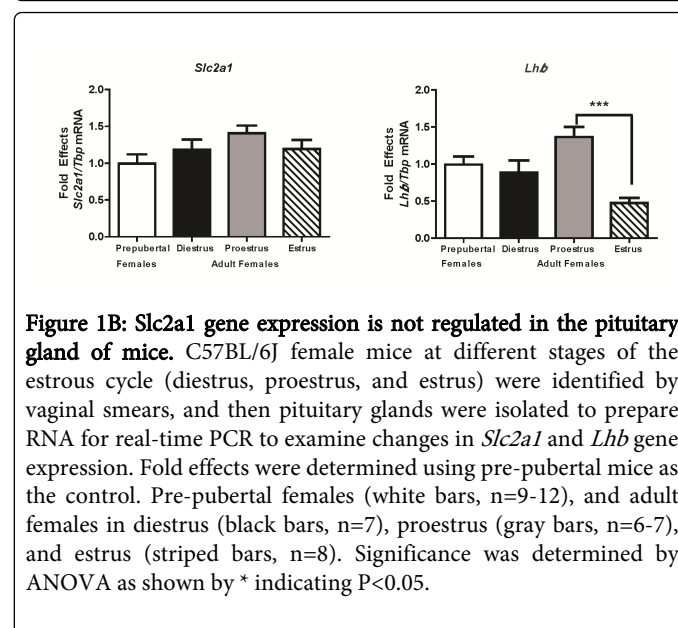


**Figure 1B: Slc2a1 gene expression is not regulated in the pituitary gland of mice.** C57BL/6J female mice at different stages of the estrous cycle (diestrus, proestrus, and estrus) were identified by vaginal smears, and then pituitary glands were isolated to prepare RNA for real-time PCR to examine changes in *Slc2a1* and *Lhb* gene expression. Fold effects were determined using pre-pubertal mice as the control. Pre-pubertal females (white bars, n=9-12), and adult females in diestrus (black bars, n=7), proestrus (gray bars, n=6-7), and estrus (striped bars, n=8). Significance was determined by ANOVA as shown by * indicating P<0.05.

## SLC2A1 protein regulation in the murine pituitary gland

Since changes in *Slc2a1* mRNA levels were not detected in the pituitary glands of male and female mice, we investigated if there is regulation of SLC2A1 protein in the pituitary gland of mice. Whole cell extracts (WCE) were isolated from the pituitary glands of pre-pubertal and adult male mice and Western blot analysis was performed with SLC2A1 antibody. SLC2A1 protein level was increased by 3.0-fold in the pituitary gland of adult compared to pre-pubertal male mice ($P<0.01$, Figure 2A). SLC2A1 protein was also induced in adult female mice (Figure 2A). Compared to pre-pubertal female mice, SLC2A1 protein level increased 1.8-fold in diestrus, 9.4-fold in proestrus, and 11.8-fold in estrus ($P<0.001$ and $P<0.0001$ proestrus and estrus, respectively). Therefore, SLC2A1 protein levels increase in the pituitary gland of adult mice.
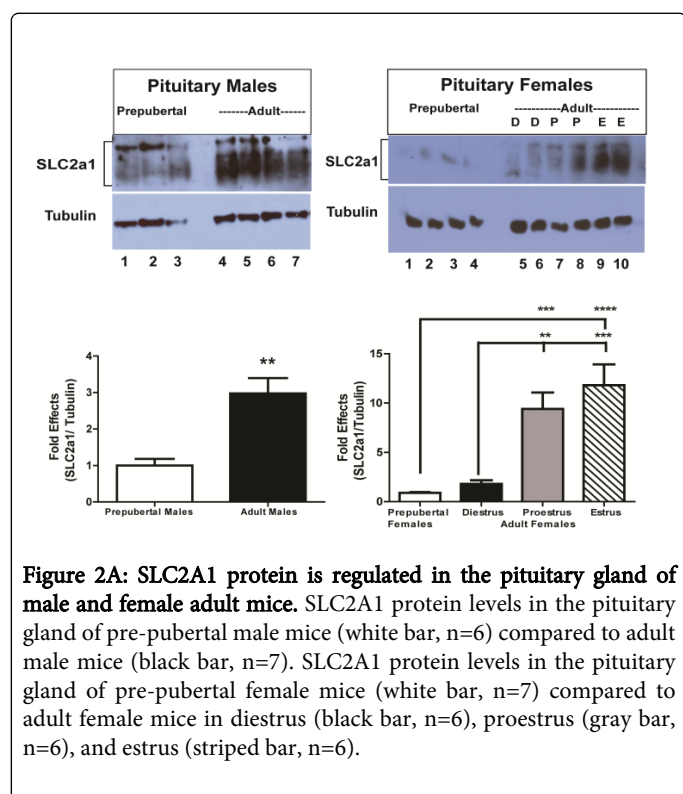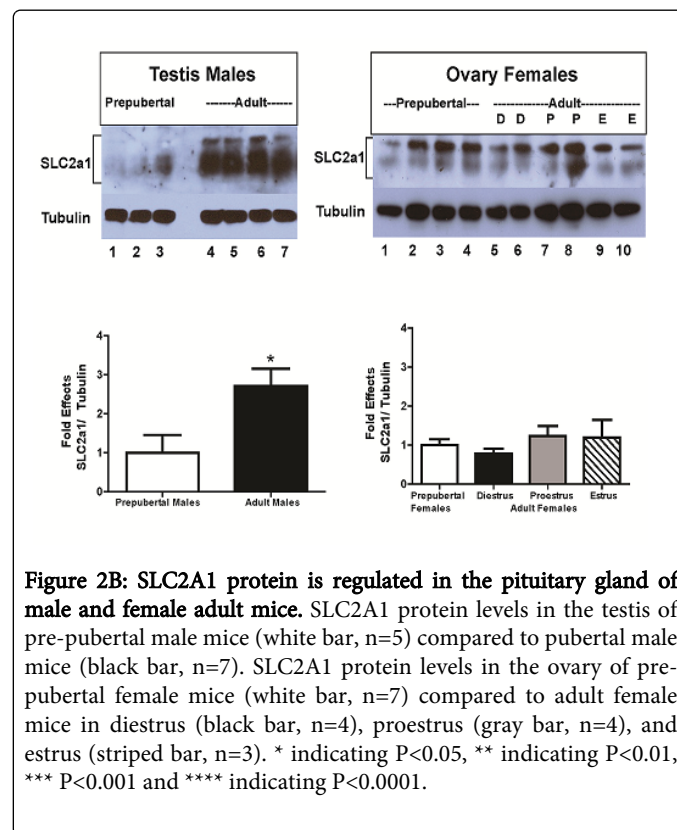


**Figure 2A: SLC2A1 protein is regulated in the pituitary gland of male and female adult mice.** SLC2A1 protein levels in the pituitary gland of pre-pubertal male mice (white bar, n=6) compared to adult male mice (black bar, n=7). SLC2A1 protein levels in the pituitary gland of pre-pubertal female mice (white bar, n=7) compared to adult female mice in diestrus (black bar, n=6), proestrus (gray bar, n=6), and estrus (striped bar, n=6).

Whole cell extracts (WCE) were isolated from pituitary glands, brain (cerebral cortex), and gonads from pre-pubertal and adult mice. Tissues were isolated from female adult mice at different stages of the estrous cycle. Western blot analysis was performed with SLC2A1 and α-tubulin antibodies. The α-Tubulin antibody was used as a loading control to normalize protein. Autoradiographs are shown with each lane representing WCE (20-50 µg) prepared from a single mouse. Representative Western blot analyses are shown. Densitometry analysis of SLC2A1 protein was normalized to α-Tubulin protein and then fold effects were determined using pre-pubertal SLC2A1 protein level as the control, and the mean+S.E.M is shown in each graph. Significance was determined using Student's t test for male mice and ANOVA for female mice.

To examine whether changes in SLC2A1 protein are specific to the pituitary gland, protein extracts were isolated from gonads of pre-pubertal and adult mice and Western blot analysis was performed. SLC2A1 protein level increased 3.2-fold in the testis of adult male mice

compared to pre-pubertal male mice ($P<0.01$ Figure 2B). In contrast, there were no significant changes in SLC2A1 protein level in the ovary of pre-pubertal and adult female mice (Figure 2B). Thus, induction of SLC2A1 protein in the pituitary gland occurs after puberty in both adult male and female mice; whereas, gonadal changes in SLC2A1 protein after puberty appear to be gender-specific.
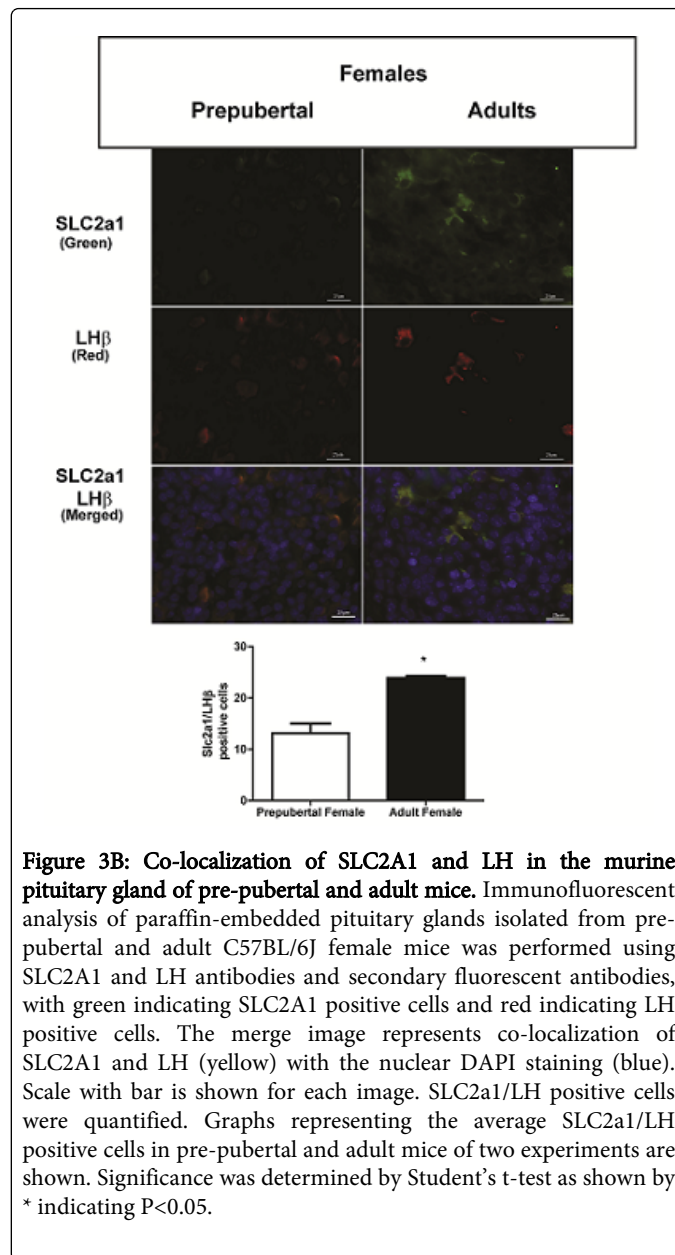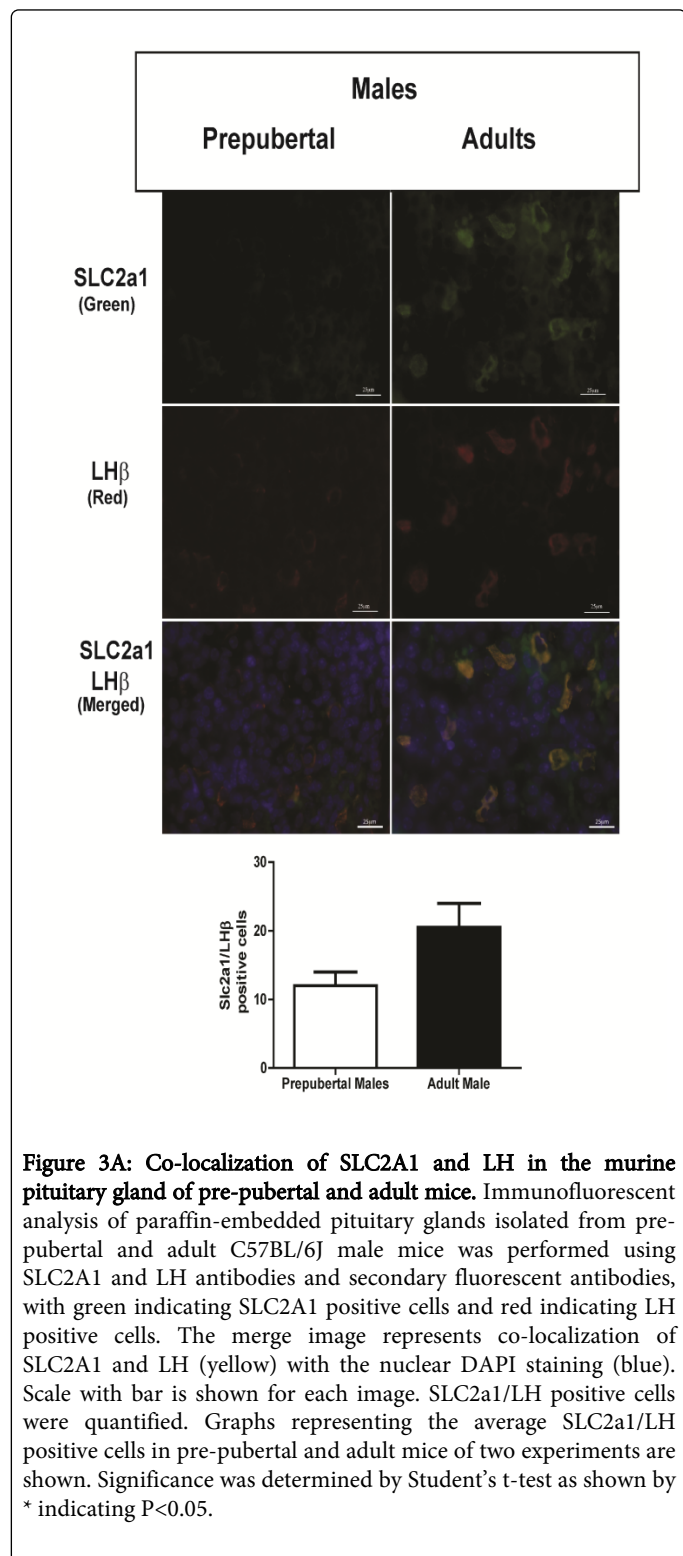


**Figure 2B: SLC2A1 protein is regulated in the pituitary gland of male and female adult mice.** SLC2A1 protein levels in the testis of pre-pubertal male mice (white bar, n=5) compared to pubertal male mice (black bar, n=7). SLC2A1 protein levels in the ovary of pre-pubertal female mice (white bar, n=7) compared to adult female mice in diestrus (black bar, n=4), proestrus (gray bar, n=4), and estrus (striped bar, n=3). * indicating P<0.05, ** indicating P<0.01, *** P<0.001 and **** indicating P<0.0001.

## Co-localization of SLC2A1 and LH in the murine pituitary gonadotrope

To determine if stimulation of SLC2A1 protein in the pituitary gland of adult mice primarily reflects changes in the gonadotrope cell, immunofluorescent analysis was performed using LHβ as a marker for the gonadotrope. Pituitary glands were isolated from pre-pubertal and adult mice, paraffin-embedded, and then subjected to immunofluorescent analysis with SLC2A1 and LHβ antibodies. In pre-pubertal mice, low levels of SLC2A1 and LHβ proteins co-localized to the gonadotrope cell in males (Figure 3A). After puberty, there was increased fluorescent intensity of both LHβ and SLC2A1 proteins that co-localize to the gonadotrope of adult male mice (Figure 3A). Although there was a 2-fold increase in the number of SLC2a1/LHβ positive cells detected in the pituitary glands of adult male mice compared to pre-pubertal male, this difference was not statistically significant (Figure 3A). We next performed immunofluorescent analysis of paraffin-embedded pituitary glands isolated from pre-pubertal female mice and adult female mice in the proestrus stage. Similar to findings in males, there was induction of SLC2A1 protein that co-localized predominantly with LHβ protein in the gonadotrope cell of adult female mice compared to pre-pubertal female mice (Figure 3B). There was a 1.8-fold increase in the number of SLC2A1/LHβ positive cells detected in the pituitary glands of adult female mice compared to pre-pubertal female ($P<0.05$, Figure 3B).

Therefore, the induction of SLC2A1 in the pituitary gland of adult mice reflects changes primarily in the gonadotrope cell.



**Figure 3A: Co-localization of SLC2A1 and LH in the murine pituitary gland of pre-pubertal and adult mice.** Immunofluorescent analysis of paraffin-embedded pituitary glands isolated from pre-pubertal and adult C57BL/6J male mice was performed using SLC2A1 and LH antibodies and secondary fluorescent antibodies, with green indicating SLC2A1 positive cells and red indicating LH positive cells. The merge image represents co-localization of SLC2A1 and LH (yellow) with the nuclear DAPI staining (blue). Scale with bar is shown for each image. SLC2a1/LH positive cells were quantified. Graphs representing the average SLC2a1/LH positive cells in pre-pubertal and adult mice of two experiments are shown. Significance was determined by Student's t-test as shown by * indicating P<0.05.



**Figure 3B: Co-localization of SLC2A1 and LH in the murine pituitary gland of pre-pubertal and adult mice.** Immunofluorescent analysis of paraffin-embedded pituitary glands isolated from pre-pubertal and adult C57BL/6J female mice was performed using SLC2A1 and LH antibodies and secondary fluorescent antibodies, with green indicating SLC2A1 positive cells and red indicating LH positive cells. The merge image represents co-localization of SLC2A1 and LH (yellow) with the nuclear DAPI staining (blue). Scale with bar is shown for each image. SLC2a1/LH positive cells were quantified. Graphs representing the average SLC2a1/LH positive cells in pre-pubertal and adult mice of two experiments are shown. Significance was determined by Student's t-test as shown by * indicating P<0.05.

## Regulation of SLC2A1 by sex steroids in LβT2 gonadotrope cells

Since it is well known that sex steroids have both positive and negative effects on the gonadotrope, we investigated if sex steroids regulate SLC2A1 in gonadotrope cells, which may account for changes in SLC2A1 protein levels detected in adult female pituitary gland especially during the estrous cycle. We examined effects of estradiol and progesterone on SLC2A1 in LβT2 cells, an immortalized gonadotrope cell line. LβT2 cells were incubated with vehicle alone (ethanol 0 hr) or progesterone (100 nM) and/or 17β-estradiol (10 nM) for 2, 8, and 24 hr, and then RNA was isolated for real-time PCR and whole cell extract was also prepared for Western blot analysis. Estradiol and/or progesterone had no effect on *Slc2a1* gene expression (Figure 4A). In contrast, SLC2A1 protein increased 1.3-fold at 2 hours, 2.3-fold at 8 hours, and 5.5-fold at 24 hours of exposure to 17β-

estradiol, and the 17β-estradiol-induced SLC2A1 level at 24 hours compared to cells with vehicle alone was significant (Figure 4B, lane 4 compared to lane 1, $P<0.05$).
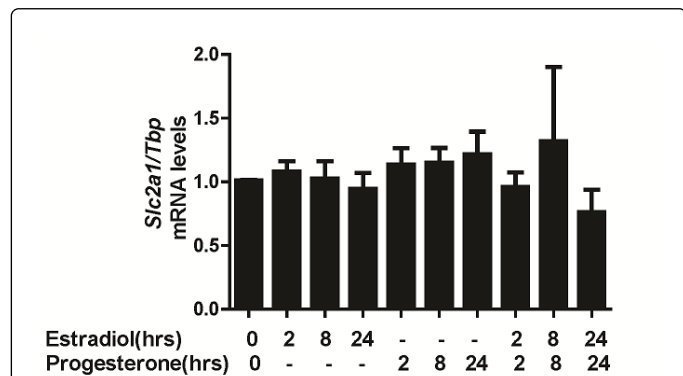


**Figure 4A: SLC2A1 protein is regulated by estradiol in gonadotrope cells.** RNA was isolated cells for real-time PCR analysis to examine changes in Glut1 gene expression. Fold effects were determined by comparing SLC2A1 levels in vehicle alone (ethanol, 0 hr) compared to steroid-treated cells. The graph represents the mean±S.E.M (n= 2 for each time point).
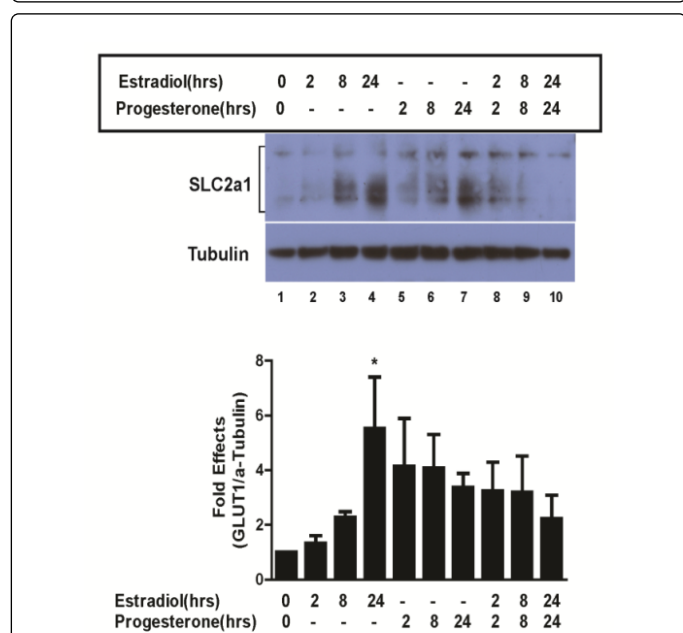


**Figure 4B: SLC2A1 protein is regulated by estradiol in gonadotrope cells.** After LβT2 cells were treated with estradiol and/or progesterone as described above, whole cell extracts were isolated for Western blot analysis with SLC2A1 and α-tubulin antibodies. The α-tubulin antibody was used as a loading control to normalize protein. A picture of a representative autoradiograph is shown. Densitometry analysis was performed and SLC2A1 protein was normalized to α-Tubulin protein and then to control (unstimulated, 0hr) cells to determine fold effects. The mean ±S.E.M (n=4) is shown in the graph. Significance was determined using ANOVA and * indicates P<0.05.

Cells were incubated with phenol free media supplemented with 2% charcoal stripped fetal bovine serum and stimulated with progesterone (100 nM) or estradiol (10 nM) for 2, 8, and 24 hours or vehicle alone (ethanol).

Although SLC2A1 protein increased 4.1-fold at 2 hours, 4.1-fold at 8 hours, and 3.4-fold at 24 hours of progesterone treatment, these changes in SLC2A1 were not statistically significant compared to cells with vehicle alone (Figure 4B, lanes 5-7 compared to lane 1). Combined treatment of 17β-estradiol and progesterone induced a down regulation of SLC2A1 at 24 hours compared to the induction of SLC2A1 at 24 hours of 17β-estradiol treatment alone (Figure 4B, lane 10 compared to lane 4). These results show that SLC2A1 protein is induced by estradiol but suppressed by combined treatment of 17β-estradiol and progesterone in LβT2 gonadotrope cells.

### Glucose Uptake in LβT2 gonadotrope cells

Since 17β-estradiol regulates SLC2A1 protein levels in LβT2 cells, we examined effects of sex steroids on glucose uptake in these cells. Glucose uptake assays were performed in LβT2 cells after treatment with vehicle alone (ethanol, 0 hr) or 17β-estradiol (10nM) and/or progesterone (100 nM) for 2, 8 and 24 hours using 2-NBDG, a fluorescent glucose analog. Progesterone alone or 17β-estradiol alone did not stimulate glucose uptake (Figure 5). In contrast, combined treatment of 17β-estradiol and progesterone increased glucose uptake 2.3- and 2.0-fold at 2 and 8 hours, respectively compared to vehicle alone (Figure 5, $P<0.01$ for 2 hr compared to vehicle alone, and $P<0.05$ and 8 hours compared to vehicle alone). Combined treatment of 17β-estradiol and progesterone at 24 hours did not stimulate glucose uptake. Therefore, combined treatment of 17β-estradiol and progesterone modulates glucose uptake in a time-dependent manner in LβT2 gonadotrope cells.
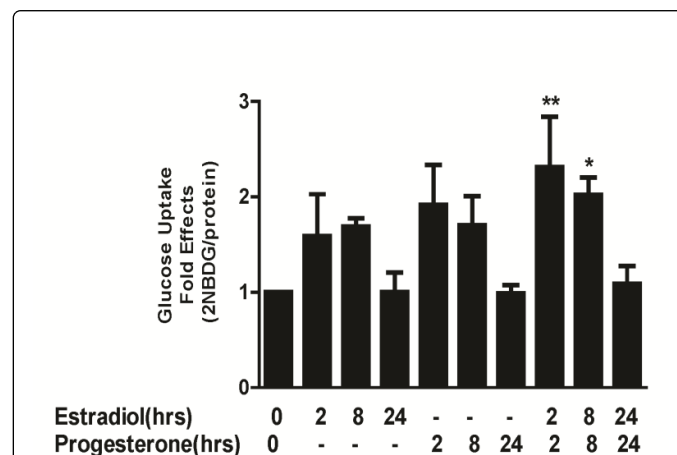


**Figure 5:** Glucose Uptake

Glucose uptake was performed using the fluorescent glucose analog, 2-NBDG. Cells were incubated with phenol-free media supplemented with 2% charcoal stripped fetal bovine serum and stimulated with vehicle alone (ethanol, 0 hr) or progesterone (100 nM) or estradiol (10 nM) for 2, 8, and 24 hours and then treated with 2-NBDG for 15 minutes, and fluorescence was measured using a plate reader. Wavelength excitation/emission=485/535 nm. Fluoresce readings were corrected for basal fluorescence and protein concentration, then the

steroid-treated samples were corrected with the control sample (vehicle alone) and fold effects were calculated. The mean±S.E.M (n=4) is shown in the graph. Fold effects of estradiol and progesterone combination at 2 hr and 8 hr were 2.4 and 1.9 respectively. Significance was determined using ANOVA and * indicates P<0.05, and ** indicates P< 0.01.

## Discussion

This is the first report showing that SLC2A1 is regulated at the post-transcriptional level in the murine pituitary gonadotrope after puberty. Immunofluorescent analysis of the pituitary gland shows co-localization of SLC2A1 with LHβ in the gonadotrope of both male and female mice with increased intensity of SLC2A1 protein detected in the gonadotrope of adult mice. These findings indicate that there is a gonadotrope-specific induction of SLC2A1 protein after puberty in mice.

Changes in SLC2A1 protein level in the pituitary gland of adult female mice were also detected during the estrous cycle. Peak levels of SLC2A1 were detected during proestrus and estrus stages compared to the diestrus stage of the estrous cycle. It is well known that ovarian sex steroids rise and fall during the estrous cycle. Estradiol peaks during the morning of proestrus and then subsequently decreases as progesterone reaches its peak during late proestrus before the surge of gonadotropins [9,32,33]. In addition a smaller increase in progesterone also occurs during diestrus, when estradiol levels are low [9]. Estradiol induces a positive feedback at the level of the hypothalamus and pituitary in preparation for the preovulatory surge of gonadotropins, and progesterone has also been shown to be important in this process [9]. In our study, an initial increase in SLC2A1 protein was first detected during proestrus and was sustained during estrus but then subsequently decreased in diestrus. This pattern of induction of SLC2A1 correlates most with changes in estradiol although we cannot completely exclude that progesterone, GnRH, or other factors also contribute to the changes in SLC2A1. To further investigate direct effects of estradiol and progesterone on SLC2A1, we performed studies with sex steroids in LβT2 gonadotrope cells. We found that estradiol but not progesterone increased SLC2A1 protein levels. Therefore, we conclude that changes in SLC2A1 levels during the estrus cycle in mice primarily reflect changes in estradiol, which also correlates with changes in the gonadotrope during proestrus before ovulation occurs. Changes in SLC2A1 during the estrous cycle is not limited to the pituitary gland, since others have shown that *Slc2a1* is regulated in the endometrium during the estrous and menstrual cycle, respectively [34,35]. Therefore, regulation of SLC2A1 in the pituitary gonadotrope during the estrous cycle correlates with changes in sex steroids.

A common observation in our study is that *Slc2a1* is regulated at the post-transcriptional level in the gonadotrope and this effect appears to involve sex steroids. We detected post-transcriptional induction of SLC2A1 levels in the pituitary gland of mice after puberty and during the estrous cycle and this correlated with estradiol-induced SLC2A1 levels in LβT2 cells. Others have shown that various pathophysiological processes and hormonal stimuli modulate *Slc2a1* gene expression at the post-transcriptional level via mechanisms involving interactions between *trans*-regulatory proteins and *cis*-acting elements in the Slc2a1 3'-untranslated region [36,37]. Ovarian sex steroids have been shown to regulate *Slc2a1* at the posttranscriptional level in endometrial cancer cells, but the precise mechanism for this effect is unknown [38]. Therefore, we propose that

post-transcriptional regulation of *Slc2a1* may provide an important mechanism for rapid changes in gene expression and glucose metabolism during the estrous cycle in preparation for ovulation. Further investigation is needed to determine post-transcriptional mechanisms in the pituitary gonadotrope regulating *Slc2a1* after puberty and during the estrous cycle.

In this study we show that ovarian steroids induce glucose uptake in LβT2 gonadotrope cells. Combined treatment with estradiol and progesterone was required to stimulate glucose uptake in LβT2 cells although estradiol alone increased SLC2A1 protein in these cells. This combined effect of estradiol and progesterone on glucose uptake was independent of changes in SLC2A1 protein levels and was limited to acute exposure to hormonal treatment (2-8 hours). These findings suggest that induction of SLC2A1 protein by estradiol could prime the gonadotrope for this combined effect of estradiol and progesterone on glucose uptake to prepare for changes required for ovulation. The mechanism explaining time-dependent effects of combined sex steroid treatment on glucose metabolism in LβT2 cells requires further investigation. Others have reported that acute exposure to progesterone at 30 minutes increases GnRH-induced LH release from estrogen-primed pituitary cells, but chronic exposure to progesterone at 48 hours decreases GnRH-induced LH release from estrogen-primed pituitary cells [9]. Although we did not use GnRH in our *in vitro* studies, it is well known that the preovulatory surge of LH is a time-dependent phenomenon that is regulated by estradiol, progesterone, GnRH, and other neuroendocrine factors [9]. Glucose is an important source of energy for cellular metabolism, and so we propose that the gonadotrope needs more glucose acutely in preparation for the preovulatory surge of LH. Subsequently, there is a reduction in glucose uptake after this acute increase in glucose metabolism occurs. This explanation correlates with our findings that glucose uptake increases in LβT2 cells after 2-8 hours of combined treatment with 17β-estradiol and progesterone, but then glucose uptake decreases after 24 hours of combined treatment with 17β-estradiol and progesterone. Since there is time-dependent regulation of the preovulatory surge of LH, we propose that there is also time-dependent regulation of glucose uptake in the gonadotrope that is controlled by sex steroids. Furthermore, limiting the duration of stimulated-glucose uptake in the gonadotrope may allow cellular processes to recover and prepare for the next preovulatory surge of LH. More studies are needed to investigate specific mechanisms underlying combined effects of 17β-estradiol and progesterone signaling on glucose metabolism in the gonadotrope. Still, our observations suggest that both 17β-estradiol and progesterone together are important for the regulation of glucose metabolism in the gonadotrope. One study has reported changes in glucose metabolism in the pituitary gland of postmenopausal women induced by an infusion of estradiol and progesterone without any detectable changes in the hypothalamus [39]. Therefore, regulation of SLC2A1 and glucose transport in the gonadotrope by ovarian sex steroids suggests that changes in glucose metabolism play an important in gonadotrope function.

Our previous study showed that GnRH increases *Slc2a1* mRNA and SLC2A1 protein levels in LβT2 gonadotrope cells [**27**]. In our current study, estradiol regulates SLC2A1 protein but not *Slc2a1* mRNA levels in LβT2 cells. These observations suggest effects of GnRH and estradiol on *Slc2a1* are mediated via different mechanisms that may be critical for differential activation of cellular processes in the gonadotrope that are important for puberty and fertility.

We detected some gender differences in SLC2A1 protein in males and female mice that appear to be tissue-specific. In particular, adult male mice had increased levels of SLC2A1 in the pituitary gland and testis compared to pre-pubertal male mice. In contrast, adult female mice had increased levels of SLC2A1 in the pituitary gland but no differences were detected in the ovaries of pre-pubertal and adult female mice. These observations suggest that changes in SLC2A1 at different levels of the HPG axis in male mice may be a general indicator of the post- pubertal state. In contrast, changes in SLC2A1 in female mice correlates with changes indicative of the gonadotrope reaching pubertal maturation that is required for full reproductive capacity. Additional investigation is needed to further understand the regulation of the *Slc2a1* gene expression in the pituitary gland and other reproductive tissues during other physiological states important for pubertal development and reproduction.

In summary, regulation of *Slc2a1* in the pituitary gonadotrope occurs at the post-transcriptional level in adult mice and during the estrous cycle. In LβT2 gonadotrope cells estradiol induces post-transcriptional changes in *Slc2a1* expression and estradiol together with progesterone stimulate glucose uptake. Therefore, these findings provide new insights into the regulation of *Slc2a1* and glucose metabolism in the gonadotrope that is dependent on sex steroids and changes associated with puberty and fertility.

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## Author Contributions

**Colleen Buggs-Saxton:** conception and design of research, revised manuscript

**Francina Gonzalez De Los Santos, Ahmad El -Yaman El-Dandachli and Colleen Buggs-Saxton:** performed experiments and analyzed data, prepared figures, approved final version of manuscript

**Francina Gonzalez De Los Santos and Colleen Buggs-Saxton:** interpreted results of experiments, drafted manuscript

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