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Estradiol Regulation of Lipocalin-Type Prostaglandin D Synthase Promoter Activity: Evidence for Direct and Indirect Mechanisms

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Abstract

In the CNS, lipocalin-type prostaglandin D synthase (L-PGDS) is predominantly a non-neuronal enzyme responsible for the production of PGD₂, an endogenous sleep promoting substance. We have previously demonstrated that estradiol differentially regulates L-PGDS transcript levels in the rodent brain. In hypothalamic nuclei, estradiol increases L-PGDS transcript expression, whereas in the ventrolateral preoptic area L-PGDS gene expression is reduced after estradiol treatment. In the present study, we have used an immortalized glioma cell line transfected with a L-PGDS reporter construct and estrogen receptor (ER) α and ER β expression plasmids to further elucidate the mechanisms underlying estradiol regulation of L-PGDS gene expression. We found that physiologically relevant concentrations of estradiol evoked an inverted U response in cells expressing ER α . The most effective concentration of estradiol (10^{-11} M) increased the promoter activity 3-fold over baseline. Expression of ER β did not increase activity over control and when ER β was co-expressed with ER α there was a significant attenuation of the promoter activity. While ER α significantly increased L-PGDS promoter activity, our previous *in vivo* studies demonstrate a greater magnitude of change in L-PGDS gene expression in the presences of estradiol. This led us to ask whether estradiol is signaling via a paracrine factor released by the neighboring neurons. Conditioned media from estradiol treated neurons applied to the glioma cell line resulted in a significant 7-fold increase in L-PGDS promoter activity supporting the possibility that neuronal-glia interaction are involved in estradiol regulation of L-PGDS.

Lipocalin-Prostaglandin D₂ Synthase (L-PGDS) catalyzes the conversion of Prostaglandin (PG)H₂ to PGD₂. In the adult brain, PGD₂ is the most abundant prostanoid and has been implicated in a variety of neurological functions including olfaction, nociception, thermoregulation and sleep (for review [13]). In fact, PGD₂ is one of the most potent endogenous somnogen identified to date [14]. L-PGDS is predominantly expressed in non-

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neuronal cells that include the leptomeninges, choroids plexus and parenchymal oligodendrocytes [1, 19, 28]. Numerous organs outside of the CNS also express the functional protein [7, 25, 27, 31] suggesting that L-PGDS gene expression may be regulated by tissue and cell-specific factors. Notwithstanding this fact, regulators of L-PGDS gene expression in the CNS are not well understood.

We have previously demonstrated region-specific alterations in L-PGDS gene expression and protein levels in the CNS of ovariectomized female rodents receiving estradiol replacement [11, 19, 20]. Estradiol increases expression in MBH nuclei (arcuate nucleus and the ventromedial nucleus of the hypothalamus) while markedly down-regulating its expression in the ventrolateral preoptic area (VLPO) [11, 19].

In the present study, we are interested in further investigating the regulation of L-PGDS gene expression by estradiol. Classically, estradiol effects are mediated by two nuclear hormone receptors, estrogen receptor alpha (ER α) and estrogen receptor beta (ER β). Both receptor proteins belong to a class of ligand-activated proteins that when bound to specific sequences of DNA (i.e. estrogen response element; ERE) either activate or repress transcription within the cell nucleus. Thus, the aim of this study was to address whether ER α and/or ER β are capable of mediating transcriptional activity of the L-PGDS gene. We used the immortalized U251 human glioma cell line that was transfected with a L-PGDS reporter construct and ER α and ER β expression plasmids. We chose the U251 cell line as our model because of its non-neuronal complement of cells and ease of transfection. Additionally, low protein expression of estrogen receptors (supplemental figure 1 a-c) was advantageous in testing the individual contributions of ER α and β via transfection of the specific expression constructs.

The U251 cell line was maintained in DMEM (Invitrogen, Carlsbad, California) containing 10% FBS (Bioreclamation, New York), 100units/ml penicillin and 50 μ g/ml streptomycin. For transfection experiments, the cells were plated in 6-well plates at a density of 0.2×10^6 cells/well. For transfection, the cells were cultured in steroid and phenol-red free media supplemented with 5% charcoal dextran-stripped FBS (Gemini Biotech, Alachua, FL) plus antibiotics. Cells were transfected at 50-80% confluence, approximately 48 hours after plating, via Effectene (Qiagen, Valencia, CA) according to the manufacturer's instructions. In addition to the reporter constructs described below, all cells were transfected with pSV- β gal (80ng/well; Promega; Madison, WI) and pBSSKII+ (to a final concentration of 400ng/well). The pSV- β gal plasmid was used as an internal control for the normalization of transfection efficiency and lysate preparation. The pBSSKII+ plasmid was used as a means of standardizing the total amount of DNA transfected. Following transfection, the cells were washed, replaced with fresh phenol-red free media and 17 β -estradiol (10^{-7} M dissolved in ethanol; Sigma, St. Louis, MO) was added directly to the culture well. Following 24h of exposure, the cells were lysed with Glo Lysis Buffer (Promega) and lysate from each well was used for both luciferase and β -gal assays according to the manufacturer's instructions. Luciferase activity was normalized per total protein and reported as activity (fold induction over vehicle).

We first tested whether the U251 glioma cell line was supported ER mediated transcriptional activity. The cells were transfected with 150ng/well of a 3xERE Reporter Plasmid only (non-transfected; NT) or co-transfected with the expression plasmids encoding ER α (pSG-hER α ; 80ng) or ER β (pCMV-hER β ; 80ng) or both together (160ng total). The 3xERE reporter construct (pGL2-TATA-Inr-Luc plasmid construct) was a kind gift from Donald McDonnell and has three-tandem consensus EREs upstream from the luciferase reporter gene [12]. The plasmids encoding human ER α (pSG-hER α) and human ER β (pCMV-hER β)

were kind gifts from Dr. P. Chambon (Strasbourg, France), and Dr. J.-Å. Gustafsson (Huddinge, Sweden), respectively.

Enhanced immunocytochemical detection of ER α and ER β protein in the transfected cell lines compared to non-transfected cells was seen following transfection (supplemental Figure 1a-d). The transfected cells were incubated with 10⁻¹⁰M estradiol for either 2 or 8 h. Previous work has shown this time course is sufficient to elicit transcriptional activity from the 3xERE reporter [30]. Following hormone treatment the cells were lysed and luciferase activity measured as described above.

The next set of experiments tested whether ER α or ER β or both initiated L-PGDS promoter activity in the U251 cells. The cells were transfected with the L-PGDS -1250/+77 luciferase-reporter construct alone (NT) or co-transfected with either the ER α or ER β expression plasmids or both simultaneously as described above. Construction of the L-PGDS promoter/luciferase gene plasmid was previously described [8]. Following transfection, individual wells were treated with increasing concentrations of estradiol and incubated for 24 h. The cells were then lysed and luciferase activity measured as described above.

Finally, neuron-glia interactions have been implicated in the modulation of glial function. To test whether an estrogen-induced, neuron-derived factor was sufficient to initiate L-PGDS promoter activity, SKN cells were treated with 17 β -estradiol (10⁻⁷M) for either 0 or 2 hr. Following 17 β -estradiol exposure, the cells were washed and placed in fresh media for approximately 16 hours. U251 cells transfected with the L-PGDS -1250/+77 luciferase-reporter construct were then treated with the neuronally conditioned media for 24 h. Following hormone treatment the cells were lysed and luciferase activity measured as described above.

Results from all replicate experiments (n=8 per treatment group in each experiment) were plotted as fold induction over vehicle for that group. All values represent the mean \pm SEM of samples in a treatment group from the replicate experiments. Data gathered from experiments 1 and 2, were analyzed using a two-way ANOVA with independent variables estrogen receptor expression and estradiol concentration or time. Bonferroni post hoc analysis was used to determine significant differences between groups. The alpha level was set at 0.05. For experiment 3, statistical analysis was performed with a two-tailed t-test. Statistical tests were conducted using GraphPad Prism 4 (San Diego, CA) on a Macintosh computer.

The U251 glioma cell line was demonstrated ER mediated transcription from the 3xERE reporter supporting its use as a potential model system for investigating ER action at the L-PGDS promoter. Following 2 and 8 hr of estradiol treatment, U251 cells expressing ER α resulted in an approximate 17-fold and 7.5-fold induction of activity, respectively, (Figure 1; two-way ANOVA; $F_{(6, 36)}=5.40$, $p<0.0005$). The ER α induced activity was significantly greater than the activity induced by ER β alone or ER α and ER β together (Figure 1; two-way ANOVA; $F_{(8, 27)}=8.056$, $p<0.0002$). Independent one-way ANOVAs for the ER β and ER α and β transfected groups revealed that the 2 and 8 h treatments of estradiol significantly increased the promoter activity over their respective controls albeit to a lesser degree than ER α (ER β ; $F_{(2,11)}=18.93$; $p<0.0006$, ER α/β ; $F_{(2,11)}=31.64$; $p<0.0001$). While some U251 cells endogenously expressed ER α and ER β protein (Supplemental Figure 1a-c), estradiol treatment did not significantly increase transcriptional activity over the control in the NT group (NT; $F_{(2,11)}=0.2438$) suggesting that endogenous expression of ERs may not be sufficient for estradiol-initiated transcription.

Since the previous experiment suggested that U251 cells were capable of supporting ER mediated transcription, we next investigated whether ERs were capable of initiating L-

PGDS promoter activity in U251 cells. ER α and ER β expression plasmids were transiently co-transfected, separately and together, with the L-PGDS reporter construct in the U251 cell line. The transfected cells were incubated with a range of concentrations of estradiol for 24 h. In cells over expressing ER α only, a maximum 3-fold stimulation was produced by 10⁻¹¹M of estradiol and the induction in this group was significantly greater than all other transfection groups except for the ER α cells treated with 10⁻¹⁰M estradiol (Figure 2; two-way ANOVA; F_(16,112)=2.945; p<0.004). The highest concentration of estradiol had no effect. Curiously, this inverted U response curve was also seen in the NT group where 10⁻¹⁰M estradiol produced a maximum 1.7-fold stimulation that was significantly greater than the control, but the highest concentration of estradiol (10⁻⁹M) was not (Figure 2; NT group one-way ANOVA; F_(3,31)=3.07, p<0.05). Expression of ER β did not significantly change L-PGDS promoter activity over control for the respective groups. However, co-expression of ER α and ER β led to a significant attenuation of the activity at the highest concentrations of estradiol (Figure 2; α and β group; one-way ANOVA; F_(3,31)=6.51, p<0.05).

The results from the above experiments suggested that, while estradiol acting primarily through ER α resulted in significant changes in L-PGDS promoter activity, these changes were of relatively small magnitude that were not consistent with our previous results found *in vivo* [19]. One explanation is that estradiol may also mediate L-PGDS promoter activity through indirect signaling mechanism. Evidence for estradiol mediated neuronal-glia interaction exists [18]. To test whether the L-PGDS promoter activity is affected by an estradiol mediated factor secreted from neurons, we treated the SKN neuroblastoma cell line with 10⁻⁷M estradiol for either 0 or 2 h, removed the treatment media, and allowed the SKN cells to condition fresh media for approximately 16 hr. The addition of neuronally conditioned media to U251 cell transfected with the L-PGDS reporter construct resulted in a 6-fold induction of the promoter activity that was significantly different from the control (Figure 3; two tailed t-test; t₍₁₄₎=3.37, p<0.005).

The purpose of this study was to investigate the role of ER α and ER β in the regulation of L-PGDS gene expression by estradiol. In a glial cell line expressing ER α , physiologically relevant concentrations of estradiol evoked an inverted U response where the most effective concentration, 10⁻¹¹M, increased the promoter activity approximately 3-fold over baseline. Expression of ER β did not increase activity over control for the respective groups and when ER β was co-expressed with ER α there was a significant attenuation of promoter activity. While ER α significantly increased L-PGDS promoter activity, the level of activity was weak in comparison to the level of activity initiated at the 3xERE construct in the same cell line. In fact, our previous *in vivo* studies demonstrate a greater magnitude of change in L-PGDS gene expression in the presences of estradiol [19]. Taken together, this led us to posit estradiol via its cognate receptors may influence L-PGDS promoter activity by direct and indirect interactions. In support of this assertion, conditioned media from estradiol treated neurons applied to the glioma cell line resulted in a significant 6-fold increase in L-PGDS promoter activity supporting the possibility that neuronal-glia interaction are involved in estradiol regulation of L-PGDS.

Our study suggests two potential mechanisms through which estradiol may be regulating L-PGDS gene expression *in vivo*. First, ER α induced L-PGDS promoter activity whereas ER β , when co-expressed with ER α , blocked this action and even further decreased L-PGDS promoter activity. Second, estradiol may be coordinating the communication via a releasable signaling factor, between neighboring cells such as the neurons and the non-neuronal cells that express L-PGDS (oligodendrocytes and leptomeninges).

Glial cells, in general, are remarkably sensitive to changes in their extracellular environment and are capable of responding to neuronally derived factors leading to responses that include but are not limited to changes in gene and protein expression [26, 29]. While the suspected estradiol-induced paracrine factor in this study is unknown, interleukin-1 β (IL-1 β) may be a potential candidate. Mounting evidence has implicated cytokines and in particular IL-1 β in the production and release of PGD₂ [5, 8, 10, 15]. In fact, IL-1 β upregulates L-PGDS gene expression in cultured leptomeningeal cells to maximum levels by 24hr via activation of two NF- κ B response elements on the L-PGDS promoter [8]. In the CNS, estrogens have been implicated in the regulation of proinflammatory cytokine expression including as IL-1 β , but thus far only, in the context of an immune challenge [2-4, 16]. At present, it is unclear what role estrogens may have in the normal expression and release of cytokines in the CNS.

Another exciting finding in this study was that ER β acted as a suppressor of L-PGDS promoter activity. ER α and ER β are separate gene products that most likely evolved from a gene duplication event and thus share some similarities in amino acid sequences especially in the ligand binding and DNA binding domains. However, there is a significant divergence in the amino acid sequence of the activation domains (A/B and F domain) and hinge region [6, 21]. Consequently, ER α exhibits greater transcriptional activity than ER β at certain target genes [6, 21]. When both receptors are co-expressed, ER β has been reported to attenuate the transcriptional ability of ER α presumably through actions as a dominant negative when the two receptors heterodimerize [12, 17]. At present, it is not known whether these heterodimers are present in our *in vitro* model system or *in vivo*.

Interestingly, the preoptic area is one of the few regions that demonstrate similar numbers and distribution of ER α and ER β expressing cells [23] as well as co-expression of ER α and ER β [24]. This is in contrast to mediobasal hypothalamic (MBH) nuclei, like the arcuate nucleus and the ventromedial nucleus of the hypothalamus, where ER α is expressed in higher levels, while there is little ER β expression [23, 24]. We have previously reported, *in vivo*, a dual regulation of L-PGDS mRNA expression by estradiol. In the arcuate nucleus and the ventromedial nucleus of the hypothalamus, estradiol significantly increases L-PGDS mRNA expression, whereas, there is a highly specific down-regulation in the presence of estradiol in the VLPO [11, 19]. This raises an interesting possibility that the limited distribution of cells that co-express ER α and ER β may enable that population to respond differently to estradiol when compared to cells expressing ER α or ER β alone.

In light of our current data, the co-expression of ER α and ER β in preoptic area nuclei may have a broader implication on sleep-wake behavior. Suppression of L-PGDS protein by estradiol in the VLPO of female rodents is one potential molecular mechanism through which estradiol may be acting to reduce sleep via a concomitant decrease in PGD₂ levels leading to a reduction in VLPO neuronal activity [11]. In a recent study, estradiol regulation of L-PGDS gene expression in preoptic area extracts follows a U-shaped response curve where physiologically relevant doses of estradiol decrease L-PGDS expression. More interestingly, the estradiol-mediated changes in L-PGDS expression are correlated with increases in running wheel behavior [22]. In light of our current findings, it is tempting to speculate that, in the VLPO, ER β in the presences of estradiol may be acting as a repressor at the L-PGDS promoter resulting in a more aroused state.

The current study suggests two potential mechanisms through which estradiol may be regulating L-PGDS gene expression *in vivo*. However, because glioma cell lines are typically heterogeneous in their glial complement, it is difficult to know whether oligodendrocytes, the main cell type expressing L-PGDS in the brain parenchyma, respond in a similar manner as the gliomas. Future studies will begin to explore whether one or both of these mechanisms affect *in vivo* L-PGDS expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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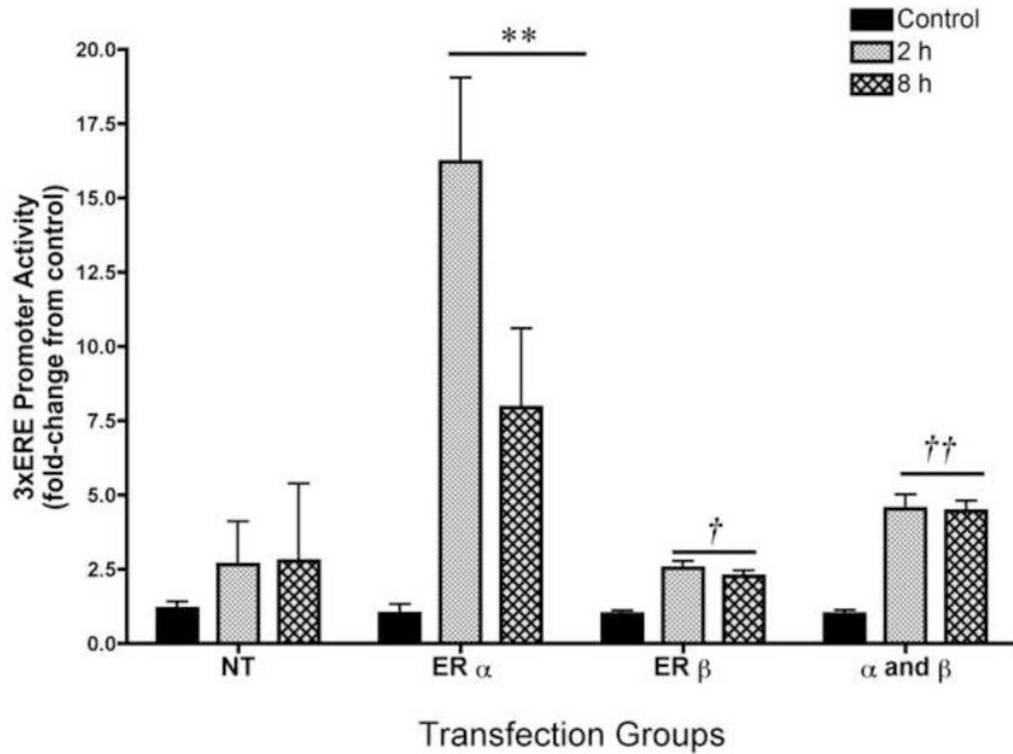


Figure 1. Estrogen response element (ERE) mediated promoter activity in U251 glial cell line
 The addition of 10^{-10} M estradiol for 2 and 8 h to U251 cell co-transfected with the 3xERE reporter construct and the ER α expression plasmid resulted in an approximate 17-fold and 7.5-fold induction of the 3xERE promoter activity, respectively (** $p < 0.0001$, compared to NT, ER β alone, and ER α /ER β together; $n = 8$ for all groups). Independent one-way ANOVAs for the ER β and ER α and β transfected groups revealed that the 2 and 8 h treatments of estradiol significantly increased the promoter activity over their respective controls albeit to a lesser degree than ER α ($\dagger p < 0.0006$ and $\dagger\dagger p < 0.0001$). Estradiol treatment did not induce ER activity in the U251 cell not transfected with ER expression plasmids (NT). Basal expression of the pGL3-Enhancer vector was unchanged in all groups (data not shown). Data are the mean \pm SEM.

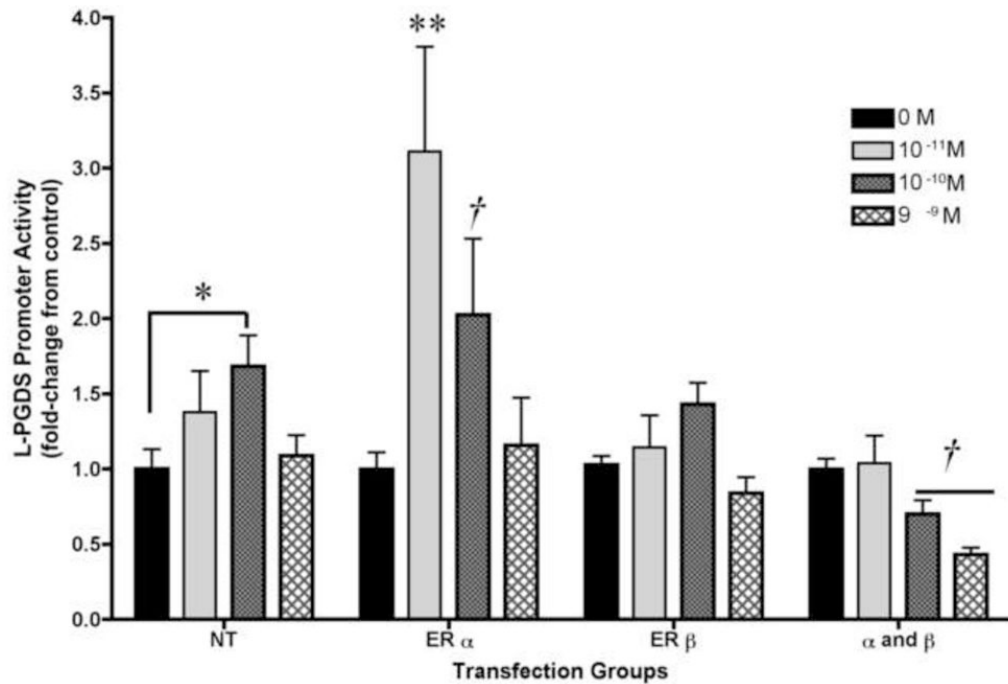


Figure 2. Exogenous estrogen receptor mediated L-PGDS promoter activity in U251 glial cell line

In cells over-expressing ER α -only, 10⁻¹¹M and 10⁻¹⁰M of estradiol produced 3-fold and 2-fold stimulation over control (two-way ANOVA; **p<0.0001, and *p<0.05, respectively). In the non-transfected cells (NT), produced 1.7 fold stimulation over control (*p<0.05). Co-expression of ER α and ER β led to a significant attenuation of the activity at the highest concentration of estradiol (one-way ANOVA; †p<0.05). Basal expression of the pGL3-Enhancer vector was unchanged in all groups (data not shown). Data are the mean \pm SEM (n=8 for all groups).

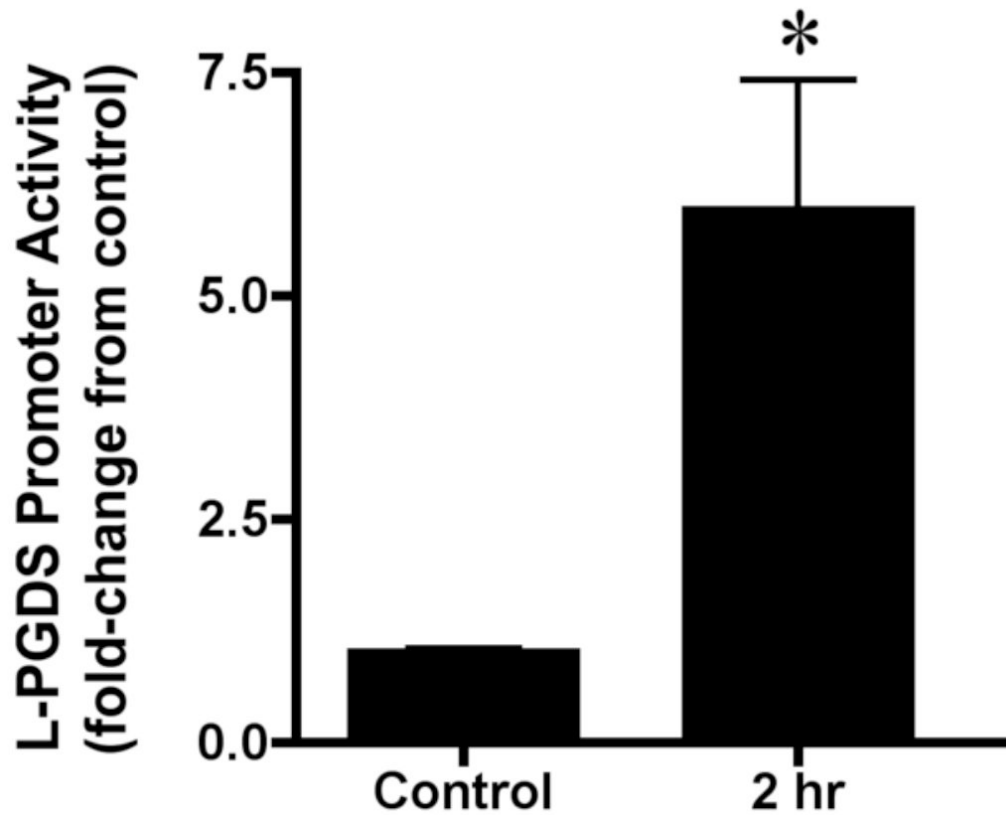


Figure 3. Neuron-conditioned medium

The SKN neuroblastoma cell line was treated with 17β -estradiol (10^{-7}M) for either 0 (control) or 2 hours ($n=8$ for both conditions). Following 17β -estradiol exposure, the cells were washed and placed in fresh media for approximately 16 hours. The resulting conditioned media was used to treat the U251 cells transfected with the L-PGDS -1250/+77 luciferase-reporter construct for 24 hours. The neuronally conditioned media induced L-PGDS reporter activity approximate 6-fold over control, $*p<0.005$. Basal expression of the pGL3-Enhancer vector was unchanged in all groups (data not shown). Data are the mean \pm SEM.