Probing Circadian Clock-Steroid Receptor Interactions in GnRH Neurons

by

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ABSTRACT

Endogenous circadian clock regulation is essential to normal rhythmicity, particularly the timing of hormone release in the brain. In the context of mammalian reproduction, a surge of a specific hormone, gonadotrophin-releasing hormone (GnRH), initiates a surge of luteinizing hormone (LH) from the pituitary gland, which is required for ovulation in females. Ordinarily, GnRH is secreted in a pulsatile pattern distinct from the surging that promotes ovulation. These surges occur with an approximately 24-hour release pattern and require elevated levels of ovarian estradiol (E2), the most common form of estrogen in human mammals, originating from the granulosa cells of the developing follicle. GnRH neurons express the estrogen receptor isoform estrogen receptor β (ERβ). Currently, little is known about mechanisms underlying GnRH/LH surge timing and how E2 acts directly on GnRH neurons. To better understand how endogenous clocks interact with sex-steroid hormone signalling, we explored protein-protein interactions between ERβ and the circadian clock transcription factor BMAL1 in multiple representative cell lines, in both the absence and presence of E2. It was hypothesized that direct protein-protein interactions may exist among clock components and ERβ in immortalized GnRH neurons (GT1-7 cell lines), exhibiting altered interactions in the presence of E2. Co-Immunoprecipitation (Co-IP) and Western blot procedures provided unclear results as some data supported the hypothesis, while other data contradict it.

Key Words: GnRH, circadian, estradiol, Bmal, Steroid hormone receptor

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Introduction

Background

Research within the Chappell lab focuses on the timing of mammalian reproduction, particularly the timing of hormone release from specialized neurons in the hypothalamus of the brain. The hypothalamus comprises part of the hypothalamic–pituitary–gonadal (HPG) axis, an important system regulating many bodily responses to external factors within the mammalian body. Approximately 2000 gonadotrophin-releasing hormone (GnRH) neurons lie within the hypothalamus. Ordinarily GnRH is secreted in a pulsatile manner; however, in the presence of elevated oestradiol (E2) levels, a surge of GnRH necessary for ovulation is secreted. Surges resulting in ovulation occur with a 24-hour release pattern, but only in the presence of elevated E2 levels, inducing a positive feedback response in GnRH neurons, leading to ovulation. E2-replacement in ovariectomized (OVX) female mice demonstrated LH surges in 24-hour intervals on consecutive days, providing evidence of E2 and circadian clock influence on GnRH surges(1,2).

Circadian rhythm and the SCN

Circadian rhythms are defined as endogenous biological processes occurring with an approximate twenty-four hour oscillation. This internal clock system regulates many autonomous body functions, such as the sleep-wake cycle, hormonal secretion rhythms, core body temperature, hunger perception via feeding time, and heart rate (3). These patterns occur throughout many taxonomic levels, including prokaryotes and basic eukaryotic organisms, as well as plants and animals, demonstrating a highly conserved evolutionary mechanism. This rhythmic circadian oscillation is under the control of autonomous cellular clocks. For decades, it

was thought that the only clock in vertebrates, was located in the suprachiasmatic nuclei (SCN) of the hypothalamus. Composed of a heterogeneous mixture of neuronal cell types, the SCN secretes several neuropeptides, stimulating multiple endocrine axes (4). Oscillation occurs on multiple levels, ranging from molecular and cellular rhythmicity to rhythms extending through entire body systems. Transcriptional-translational feedback loops at the molecular level regulate gene expression, hormone synthesis, and protein degradation. Despite the SCN maintaining primary duties as the circadian pacemaker, in the past two decades a surprising discovery has been made characterizing oscillations of clock gene expression in peripheral tissues independently of the SCN (5). Many independent oscillators are located in neuroendocrine cell types, including GnRH neurons (6,7,8). Gene expression patterns are likely constructed to influence the timing of secretions of various neuropeptides and hormones, important to successful physiological function and survival. When circadian signalling is lost, many problems arise, including reproduction problems, perturbed sleep-wake cycles, altered food consumption, and cancer. Many *in vivo* models with mutated, nonfunctional circadian genes develop uncontrolled cellular proliferation, leading to tumors and cancer- predominantly breast and prostate cancers. Also, circadian clock gene *Bmal1* plays a role in mammalian reproductive physiology regulation as both male and female homozygous *Bmal1* gene knockout (KO) mice are infertile (9).

Clock Components

CLOCK and BMAL1 are basic helix-loop-helix PAS domain transcription factors, comprising the positive regulatory piece of the core molecular clock. Together CLOCK and BMAL dimerize to form protein complexes, which bind to E-box elements within the *Period* (Per) and *Cryptochrome* (Cry) promoters, stimulating transcription of these genes. Per and Cry then

function as negative feedback components, returning to the nucleus to inhibit their own transcription (10). An additional regulatory loop controls antiphasic *Bmal1* expression, involving nuclear response receptors competing at response elements on the *Bmal1* promoter (11). The antcillary loop is regulated by two orphan nuclear receptors, repressor Rev-erb α and transcriptional activator RORα, maintaining an antiphasic rhythm of *Bmal1* with *m*Cry and *m*Per (12). These two molecular clock “arms” are found in the SCN, GnRH neurones, pituitary, and reproductive organs (13). Figure 1 illustrates key components of the circadian loop.

Estrogen Receptor Alpha and Beta

E2 exerts its effects via two distinct estrogen receptors (ER), ERα and ERβ, which act as ligand-inducible transcription factors. GnRH neurones do not express estrogen receptor α (ERα), an isoform believed essential to fertility. However, they do express estrogen receptor β (ERβ). E2 binds both ER isoforms with equal affinity, and through estrogen response elements (ERE) these receptors target gene promoters, such that gene expression is activated or repressed, dependent on the presence of cofactors. Estrogen binding appears to down-regulate ERβ expression, contributing to negative feedback of GnRH secretion. Though the precise mechanisms regarding how E2 may stimulate GnRH surges remain unclear, recent data suggest that within GnRH neurones, E2 may interact with endogenous circadian oscillators to modulate neuronal excitability in a rhythmic manner (14). The exact interactions, however, remain unclear. While the SCN is important for synchronizing endogenous clocks throughout the organism with ambient light signals, timing of reproductive function is also modulated by endogenous clocks in cells and tissues of the reproductive axis. As GnRH neurones possess autonomous clocks, coordination of oscillation in response to E2 is of interest.

GnRH Regulation

Regulation of GnRH surge secretion is likely mediated by many hypothalamic nuclei cells and their constituent neuropeptides, including neurones secreting the recently characterised 54-amino acid peptide Kisspeptin. Kisspeptin acts via its receptor, G protein-coupled receptor 54 (GPR54) or Kiss-1 receptor (Kiss-1R). Kisspeptin is a powerful stimulator of GnRH release, and is essential for pubertal progression and feedback effects of steroid hormones (15). In most mammals, it is estimated that greater than 85% of GnRH neurones express Kiss-1R (16). Beginning at birth, levels of Kiss-1R expression in GnRH neurones gradually increase as the neurones mature. Abundant levels coincide with the onset of sexual maturity at puberty. Data supports the importance of both Kiss-1 and Kiss-1R: mutations inactivating Kiss-1R in humans prevents puberty and are linked to hypogonadotropic hypogonadism; genetic knockout of *Kisspeptin* results in infertility and hypogonadism; the administration of Kiss-1R antagonists delays puberty onset; and Kisspeptin administration increases LH secretion and early puberty onset (17).

Previous research in the Chappell laboratory demonstrated that rhythmicity of Kiss-1r expression in an in vitro culture of immortalized GT1-7 GnRH neurones can be induced upon exposure to elevated levels of E2, with intracellular ERβ receptors appearing to facilitate these responses (14). While evidence for interaction among circadian clock components and ERs has been shown in breast cancer cells, (18), it is currently unknown whether these factors interact in a normal physiological context or how these two may come together to regulate the Kiss-1 receptor promoter. Previous work in the Chappell lab (manuscript in progress) demonstrated that *Clock* and *Bmal1* overexpression increases Kiss-1R transcriptional activity, and that E2 decreases this effect, but it is unclear how. Figure 2 below shows levels of Kiss-1R luciferase activity when co-transfected with *Clock* and *Bmal1*, both exposed to E2 or vehicle. Quantitation

of promoter activity using luminometry demonstrates a decrease of Kiss1-R expression after exposure to E2, regardless of co-transfection (19). We have found E2 response elements (ERE) within the Kiss-1 receptor promoter sequence, as well as E-boxes, which are binding sites for CLOCK and BMAL1. The presence and proximity of these sites implies that E2 could affect the timing of Kiss-1 receptor expression directly or indirectly, by interacting with clock components, thus allowing for maximal stimulation of GnRH secretion at the appropriate time.

A Possible Interaction among Estrogen Receptors and Clock Mechanisms

A previous study demonstrated that E2 and ERα expression in breast cancer cell lines induces Per2 mRNA levels (18). Transcriptional regulation of target genes is directed by ERα through interactions with ERE. Presence of E2 in the ERE promoter region stimulates the expression of Per2, which in turn indirectly regulates E2 transcriptionally. Thus, induced Per2 transcription functions as a negative feedback mechanism, modulating the effects of E2 on transcriptional activity of ERα. Binding of E2 to the already short-lived protein ERα further accelerates its degradation (20), and while Per2 overexpression produces no effect on ERα mRNA levels, it downregulates ERα protein levels in ERα expressing breast cancer cell line MCF-7 (18). This indicates an essential role of Per2 in ERα degradation, and thus the importance of clock-controlled genes in regulating ERα effects, including proliferation. Whereas ERα expression exhibits a lack of circadian oscillation in normal cells, *Clock* mutant mice demonstrate ERα gene downregulation (21), suggesting that CLOCL proteins effect ERα expression levels. GT1-7 cells, (an immortalized, hypothalamic, GnRH neuronal cell line) lack ERα, but they express the isoform ERβ (22). Though these isoforms maintain some differing characteristics, both function as estrogen receptors, and clock mechanisms effect expression of each. This regulatory effect implies that Clock proteins CLOCK and BMAL1 directly affect transcription levels in cells

expressing ERβ. However, Per2 translocates from the cytoplasm to nucleus, whereas BMAL1 remains in the nucleus the majority of the time. Therefore, protein-protein interactions are likely more detectable between positive elements and ERβ. In a previous experiment using mouse lung, results suggested that ERβ displays an endogenous circadian oscillatory protein expression pattern and possible circadian output, further supporting ERβ as a direct target of the CLOCK-BMAL1 heterodimer in lung (23). GT1-7 cells express ERβ and endogenous circadian oscillatory patterns (unpublished data), making this cell line a prime candidate for direct ERβ-BMAL1 experimentation.

Experimental Goal and Hypothesis

The primary goal of this study was to identify protein-protein interactions between ERβ and BMAL1 in multiple representative cell lines in the absence and presence of E2. SCN 2.2 cell lines have confirmed circadian capabilities and were employed as a "positive control" as circadian pacemakers and a GT1-7 cell line was used to experimentally determine the extent of circadian regulation in GnRH neurones. We hypothesized that direct protein-protein interactions exist between clock components and ERβ in both GT1-7 and SCN cell lines, and exhibit limited interactions in the presence of E2. These protein-protein interactions would suggest a novel mechanism of how steroid hormone levels modulate circadian clock control.

Materials and Methods

Cell lines

GT1-7 and SCN cells were rapidly thawed, centrifuged at 12,000xg, and plated on 10cm poly-L-lysine treated cell culture plates re-suspended in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine-serum.

Protein extraction and quantification

Protein lysates were extracted from both the GT1-7 and SCN 2.2 cell cultures, which had been grown to confluency in a 10cm culture plate (Greiner Bio-one). The cells were first washed in 1X PBS, then lysate in 1000µL IP/lysis buffer containing proteinase inhibitor cocktail (Fermentas). Bicinchoninic Acid (BCA) assay was used to quantify protein concentration using a plate reader with absorbance at 562nm.

Western Blot

Fifteen µg of protein quantified by BCA assay were loaded into a 25% polyacrylamide gel and run at 140V for approximately an hour. Separated proteins were then transferred via electrophoresis to nitrocelluslose membranes. Once the transfers were completed, the membranes were blocked with condensed milk for 30 minutes at 4°C, and then washed 3 times with PBS-tween. Primary antibodies (directed against either ERβ or BMAL1) were incubated on blots for 12 hours, at 4°C, then washed 3 times with PBS-tween before addition of a secondary antibody conhugated to IR dye (Li-cor). Following completion of Co-Immunoprecipitation, Western blot was performed again upon these enriched protein fractions, and then re-probed with BMAL1 and/or ERβ antibodies. Primary antibodies utilized included 5µg (1:1000

concentration) of Invitrogen rabbit estrogen-receptor β and rabbit Bethyl BMAL1. Each primary antibody remains exposed to the Western blot membrane for approximately 12 hours, at $4^{o}C$ on a shaker, then washed in PBS-tween for three cycles at 15, 5, and 5 minutes. Li-cor anti-rabbit secondary antibody was next applied to the membrane at a 1:10,000 concentration, and incubated for 40 minutes at room temperature, followed by a PBS-tween wash of three cycles at 15, 5, and 5 minutes. Visualisation occurred on a Li-cor Odyssey imager, which scanned the membrane for protein bands illuminated by the light sensitive secondary antibody.

Co-Immunoprecipitation

A Pierce Co-Immunoprecipatation Kit was used for all Co-IP procedures. Antibody coupling first associated 10-75µg (we used 15µg) of either Invitrogen rabbit estrogen receptor β primary antibody or Bethyl rabbit BMAL1 primary antibody with resin on the interior of a plastic spin column. As shown in Figure 3 below, lysates from each cell line were applied to the column, and the corresponding antigen and connected proteins within the lysate interacted with the primary antibody, becoming attached to the column walls. A washing step ensured the elution of all non-specific proteins, leaving only the specific antigen-protein complex within the column. Elution of the bound protein occurred next, which released the protein previously bound to the resin into a collection tube below. This solution contained only protein specific to the binding antigen, and isolated the specific protein of interest as well as any other bound protein/complex directly associated with it (24). The primary antibodies were used to target ERβ and BMAL1 associated proteins from GT1-7 and SCN 2.2 cells and isolated proteins bound to this steroid hormone receptor. For each replication and new study, another column was coupled with antibody to ensure reproducibility and viability of results. GT1-7 lysates (E2-absent) were exposed to both ERβ and BMAL1 coupled columns, and SCN and E2 exposed GT1-7 lysates to ERβ. These elutions

were probed using western blotting to confirm the existence and functionality of both primary antibodies within our neuronal cell lines upon completion of each Co-IP.

During the Co-IP procedures, 200ug of protein (SCN and GT1-7 cell lines was pre-cleared with 15uL control resin. After incubation and centrifugation, this flow-through was diluted with 150uL-200uL Co-IP lysis/wash buffer, as per instructions, and incubated in an antibody coupled column for 2 hours at room temperature, allowing the protein and its conjugates to couple with the antibody containing column. Then, the column was washed and eluted, producing unbound proteins in the wash and those strongly interacting with the antibody and/or another protein associated with that antibody in the elution. Western blot analysis was utilized to view the results of the Co-IP, the amount of each solution loaded determined by the control lysate.

Results

Protein extracted from different cell lines in Co-IP lysis/wash buffer contained different concentration of protein based on the specific culture the lysate was extracted from, and none expressed a concentration lower than 1,400μg/mL. The size of ERβ is referenced as 53 kilodaltons (kDa), and BMAL1 as 69kDa. Thus, BMAL1 should run slower than ERβ, its band appearing higher up the western blot than ERβ.

Preliminary Western Blot Test

A preliminary western blot preceded co-immunoprecipitation procedures. Protein extracts from both neuronal and non-neuronal cell lines were analyzed by Western blot. Interestingly, when incubated with ERβ antibody, bands appeared at approximately 70kb and 53kb in the lanes containing lysate from GT1-7 cells, GT1-7 subclones in which ERβ was stably overexpressed, and GT1-7 transiently overexpressing BMAL1 (Figure 4). Though bands at 53kDa are expected in ERβ lanes, the higher band was unexpected. Unfortunately, Figure 4 did not demonstrate a band in the SCN lysate lane at 53kDa, which did appear in the other blots. In order to verify the positive results, the procedure was repeated three times and each produced the same response, with the exception of the SCN lane. Also, to eradicate the possibility of lysate from another line spilling into the BMAL1 protein lane, this lane was preceded and flanked by negative controls as demonstrated below. Observance of these specific blots motivated subsequent studies, further probing protein-protein interactions suggested by this preliminary experiment.

SCN Co-IP and Blot

When a Co-IP column was coupled with ERβ primary antibody and SCN 2.2 lysate bound, and then eluted, the western blot membrane was then incubated with ERβ primary antibody and imaging of the product produced Figure 5a. Protein bands occurred in five of the eight lanes: antibody verification, unbound protein, both elutes, and cell lysate when coupled with ERβ and incubated with both ERβ and Bmal1 during western blot analysis. The antibody verification lane contained excess antibody remaining unbound to the column after the antibody coupling step, the unbound protein was nonspecific and excess protein washed from the column following the Co-IP incubation step, elution lanes contained undiluted Co-IP column elute of protein complexes directly interacting with antibody coupled column, and the cell lysate was the same SCN 2.2 lysate introduced to the antibody coupled Co-IP column.   Each lane demonstrating positive protein results possessed a band at just above 50kDa. However, the elution lane bands were slightly above the unbound protein and SCN protein bands. Figure 5b demonstrated the results of ERβ coupling and BMAL1 incubation. Placement of bands appeared nearly identical to Figure 5a. However, a band in Figure 5b SCN protein lane appeared at approximately 70kDa, and a much less distinct band appeared at 53kDa than Figure 5a. Also, the unbound protein lanes depicting non-specific and non-binding protein differed, as Figure 5a had two bands at just above 50kDa, while Figure 5b demonstrated protein of 53kDa, 69kDa, and 74kDa sizes.

GT1-7 Co-IP and Blot

A western blot of GT1-7 protein coupled with ERβ primary and exposed to ERβ primary antibody, anti-rabbit secondary demonstrated bands at 53kDa in all lanes except for the negative control wash and blank (Figure 5a). As demonstrated in Figure 5b below, a light band

occurred at approximately 55kDa in the lanes labelled elution #1, elution #2, lysate, and darker at antibody verification. Figure 5b shows protein bands after coupling with ERβ and BMAL1 primary incubation. Results seemed similar in the elution and antibody verification lanes, yet fewer bands appeared in the unbound protein and lysate lanes than in the preceding blots. Lastly, Figure 5b demonstrated a light band at approximately 55kDa in the lanes labelled elution #1, elution #2, lysate, and darker at antibody verification. Figure 5c shows the same cell line in a western blot coupled with BMAL1, then probed with ERβ primary antibody and anti-rabbit secondary. At approximately 55 kDa and 70kDa, bands appeared in lanes labelled antibody verification, protein wash, and lysate, indicating positive results for antibody and protein, respectively. In the lanes titled elution #1 and #2, visible bands appeared at approximately 53kDa. In each figure, the lane labelled "Negative control" was an elution from a Co-IP "control column," which underwent the Co-IP procedure in tandem with the experimental tube, with the exception of antibody coupling step. Presence of protein bands at appropriate heights in the lysate lanes and absence of visible bands in the negative control lanes indicated that contamination did not occur during the Co-IP and that the western blot procedure was carried out successfully.

Oestradiol Exposed GT1-7 Co-IP and Blot

Finally, a 10cm plate of 90% confluent GT1-7 cells was exposed to 1nM E2 for twenty-four hours. Co-IP procedures were repeated as above, and results revealed a lack of association. Bands appeared in Figure 7a in the antibody verification, unbound protein, and E2 exposed GT1-7 protein lanes. However, only one band is present in the antibody verification lane compared with multiple bands in previous western blots. Also, the unbound protein and E2 exposed GT1-7 protein lanes exemplified less protein than any other blots, with bands at approximately 50kDa

and 75kDa. The blot probed with polyclonal antibody recognizing BMAL1 also exhibited bands in only the antibody coupling verification, unbound protein, and E2 exposed GT1-7 protein lanes. Both the unbound protein and lysate possessed multiple lines, most notably at roughly 50kDa, 70kDa, and 75kDa. However, the elution lanes remained devoid of any bands in both cases.

Discussion

Originally, we hypothesized that direct protein-protein interactions exist between BMAL1 in GT1-7, SCN cell lines and ERβ in the absence of E2, and existed in a reduced state in the presence of E2. According to data consistently produced by the Co-IP and Western blot, this hypothesis was only partially supported. Though each lysate deriving directly from each cell line showed positive protein results when exposed to BMAL1 primary during western blot analysis, the Co-IP elution solutions did not demonstrate the same positive protein results from the Co-IP column elution and primary antibody during the western blot. The procedure of Co-immunoprecipitation isolates protein strongly interacting with the antibody coupled within the tube, inhibiting its movement through the tube until the elution steps. However, the specific antigen binding to the column does not ensure only one protein will be obtained in the elution; in fact, any other protein directly connected to the antibody target binds and ultimately elutes. This property is the fundamental reason why Co-IP was used in order to better understand protein-protein interactions in complicated pathways. Such proteins remain associated until boiled with β mercaptoethanol (BME) prior to western blotting.

A wash removing unassociated protein was performed during the Co-IP, immediately following protein introduction and incubation in the antibody coupled tube. Thus, this wash step will contain the unspecific pre-cleared lysate solution as well as excess protein. Though an amount consistent with the protocol recommendations was present in the pre-cleared mixture, not all was bound to the column. This indicates two possibilities: 1) the lane containing the wash solution should be positive for the protein of interest and 2) the Co-IP procedure could be modified for optimization, as introducing less protein will be both efficient and cost effective.

Both the lysate and elution from the control column served as experimental controls. The control column established during the Co-IP underwent nearly identical steps as the

experimental column, with the exception of antibody coupling. Without coupling a column, the protein had nothing to adhere to upon incubation, and merely washed out directly after incubation. This illustrated the importance of proper coupling in isolating the protein(s) of interest, and drove home the concept that bands should not exist in the negative control column lane. The lysate served as a positive control, as the entirety of a cell extract contained individual target proteins. Though the same lysate utilized in the Co-IP was used as a positive western blot control, the amount of BMAL1 and ERβ protein was not clearly identified. This made it difficult to interpret other positive results, as the control had an unknown amount itself and a discrepancy in concentration existed between the elution and lysate. Co-IP elution targeted one specific protein and its affiliate(s), including BMAL1 and ERβ, while those proteins were only one among many in the lysate. Thus, only the presence or absence of each protein should accurately be determined by band visualization.

Antibody verification lanes contain solution derived from the Co-IP antibody coupling procedure, primarily the run-off of antibody used to couple the tube. This lane is always expected to show a protein band at its specific height if the Co-IP coupling antibody running through the gel originates from the same species as the secondary antibody. While the presence of the antibody can serve as a positive control, it also may negate the credibility of results. Both the Invitrogen ERβ and Bethyl BMAL1 commercial antibodies originate from rabbit, which was a source of possible error. The anti-rabbit secondary antibody has the potential to pick up signal from antibody used in the coupling step. Although the wash and elution solutions should not possess antibody from the coupled column, residual antibody may have dissociated into these solutions during the Co-IP, inviting a false positive response. Also, the lower than expected band placement during BMAL1 western blot primary antibody incubation could be attributed to this. In order to reduce error, two different BMAL1 antibodies were used in addition to the Bethyl

rabbit. The first (Santa Cruz produced) antibody originated from goat rather than rabbit, and second was produced in guinea pig specifically for the Chappell lab. The intention was eliminating the possibility of interaction with the ERβ antibody, except in the case of legitimate protein-protein interactions. Unfortunately, both antibodies proved a failure, demonstrating a lack of protein bands, and consequently results. The lack of viable antibody is not unique to BMAL1 protein, as a general shortage of effective circadian clock protein antibodies exists.

The SCN cell line elution demonstrated BMAL1 protein bands at approximately 55-60kDa in one experiment. The height of the line was lower than the prescribed 69kDa, yet was possibly within an acceptable range. However, those results were not consistently produced. In fact, they only appeared in the earliest of experiments. As demonstrated in Figures 5a,6a, and 6b, protein bands did not appear at the correct 69kDa height in the elution lanes of any cell line when BMAL1 was utilized as the western blot primary antibody. However, when the converse GT1-7 experiment was carried out and Co-IP column coupled with BMAL1 and western blot probed with ERβ, results supporting the hypothesis were produced. The bands at 53kDa in Figure 6c correspond with ERβ protein, which was eluted from the BMAL1 coupled tube. This could only occur if a BMAL1-ERβ complex existed in the GT1-7 cell lysate. Despite the rather confusing data produced by the other blots depicting ERβ coupling and BMAL1 western blot incubation, this conversely coupled and probed blot may be the most accurate. Though certain primary antibodies may exhibit perfect functionality for certain assays, their functionality may fail when applied to other assays. Thus, while the ERβ antibody proved functional when applied to western blotting, it may not have worked properly in Co-IP procedures, and the bands in ERβ coupled, ERβ probed blots were false positives. And although the BMAL1 antibody produced less than stellar western blot results, it may have successfully coupled with the column during the Co-IP.

After running multiple experiments and experiencing unclear data, Co-IP procedure optimization adjustments occurred. Initial results of bands at heights lower than normal inspired further investigation and troubleshooting. When utilizing a BMAL1 antibody produced in a species distinct of the ERβ antibody failed, another method to eliminate false-positive responses was employed. Antibody from the coupled column inappropriately dissociated during the elution step coupled with secondary antibody during the Western blot stage, produced a false positive band at the height of the coupled antibody. In order to negate this effect, the column was washed twice as many times during the oestradiol exposed GT1-7 Co-IP, as initially prescribed after the coupling stage, successfully removing loosely bound antibody within the column. After this adjustment, however, bands appeared in only the antibody verification, unbound protein, and oestradiol exposed GT1-7 protein lanes in both ERβ coupled, ERβ probed and ERβ coupled, BMAL1 probed blots. Lack of band(s) present in the elution lane for the ERβ coupled, ERβ probed as well as reduced number in the other lanes normally showing multiple bands suggests inaccurate results. The additional wash steps after antibody coupling most likely dissociated the antibody from the column, leading to a loss of antibody-antigen interactions. Although oestradiol has been demonstrated to down-regulate ERβ expression, the presence of 53kDa size protein in the E2 exposed GT1-7 lysate lane contradicts the complete absence of protein in the ERβ coupled, ERβ probed elution lanes.

In order to confirm and expound upon the results of this study, further assays must be employed. Ideally, generation of an effective BMAL1or ER beta antibody in a species other than rabbit would produce clearer results in regard to the nature of ERbeta-BMAL1 interactions. However, because such an antibody does not exist, other measures must be relied upon for verification. Chromatin Immunoprecipitation (ChIP) assay investigates the relationship between protein and DNA within a cell. This procedure determines whether a specific genomic region

interacts with the protein of interest, at transcription factors on promoters or other DNA binding sites. Chromatin from lysate and corresponding protein are bound together, and then sheared so that DNA fragments of interest can be immunoprecipitated using antibodies from the protein of interest. Next, the immunoprecipitated complexes can be collected and purified, then DNA separated from protein and sequenced. If an interaction does exist, such a specific assay possesses the potential to further elucidate important questions including: 1) where on the Kiss1R promoter ERβ and BMAL1 are binding, 2) what domain of each transcription factor is/are required for this interaction, and 3) if the ERβ and BMAL1 binding sites are close enough to facilitate direct protein-protein interactions. Despite unclear evidence, it is possible that direct circadian clock-steroid receptor interactions, as ERβ-BMAL1 interactions, occur in GnRH neurones. This suggests the possibility of steroid hormone modulation of the clock, and is exciting as it suggests a novel method of transcriptional regulation.

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LEGENDS

1. CLOCK/BMAL circadian loop. CLOCK/BMAL heterodimer bind to Per/Cry E-box, activating Per/Cry mRNA transcription, which is translated to protein, complexing outside of the nucleus. The PER/CRY protein then translocates into the nucleus and binds the CLOCK/BMAL heterodimer, inactivating it and blocking its own transcription. Two orphan nuclear receptors, repressor Rev-erbα and transcriptional activator RORα, maintain antiphasic rhythm with mCry and mPer, competing with one another at the Bmal1 promoter.

2. GPR54- luciferase expression dependent on oestradiol exposure. Gpr54-luciferase expression is depressed when treated with E2, even when co-transfected with CLOCK/BMAL1. Kiss-1r mRNA levels when co-transfected with CLOCK/BMAL1. The data of two experiments are shown, and each graph demonstrates mRNA levels detected by qPCR without E2 treatment and with E2 treatment (14).

3. Co-Immunoprecipitation assay flow diagram. Schematic summary of a standard co-immunoprecipitation assay (20). Cell lysate is first incubated in an antibody coupled Co-IP column, then spun and washed, then the binding protein complex eluted and analyzed.

4. Preliminary western blot ERβ probed western blot. The western blot above demonstrates proteins specific to ERβ primary rabbit Invitrogen antibody. 15µg of GT1-7, ERβ overexpressed Gt1-7, BMAL1, SCN, LNCAP, PC-3, and MCF-7 lysate was transferred to a membrane and incubated in a 1:1000 concentration of primary antibody and 1:10,000 concentration of anti-rabbit secondary antibody.

5a. SCN coupled, ERβ probed Co-IP and western blot. Column coupled with 15µ ERβ rabbit primary antibody, then incubated with 1:1000 concentration ERβ rabbit antibody during western blot analysis. Prominent bands appeared in the antibody verification, unbound protein, both elution, and SCN 2.2 protein lanes. 15µg SCN 2.2 lysate was used during the blot, and the same volume of all other solutions for consistency

5b. SCN coupled ERβ, probed BMAL1 Co-IP western blot. Column coupled with 15µg ERβ primary antibody, incubated with 1:1000 concentration BMAL1 rabbit primary antibody during western blot analysis. Bands perceived in antibody verification, unbound protein, elution, and SCN protein lanes. However, bands unexpectedly appeared just above 50kDa in the elution lanes.

6a. GT1-7 ERβ coupled, ERβ probed Co-IP western blot. GT1-7 cell line protein bound in ERβ rabbit primary coupled column (15µg , then incubated in 1:1000 concentration ERβ rabbit primary antibody. ERβ protein present in elution lanes, with band at approximately 53kDa. Antibody verification, unbound protein, elution, and GT1-7 lysate lanes all showed bands within range of ERβ size.

6b. GT1-7 ERβ coupled, BMAL1 probed Co-IP western blot. GT1-7 cell line protein bound in ERβ coupled column (15µg primary rabbit antibody), then eluted and incubated in 1:1000 BMAL1 primary rabbit antibody. Bands were present in the same lanes as Figure 6a; protein of approximately 53kDa size was demonstrated by bands in antibody verification, unbound protein, both elution, and GT1-7 lysate lanes. A band of approximately 75kDa size was also shown in the GT1-7 lysate lane.

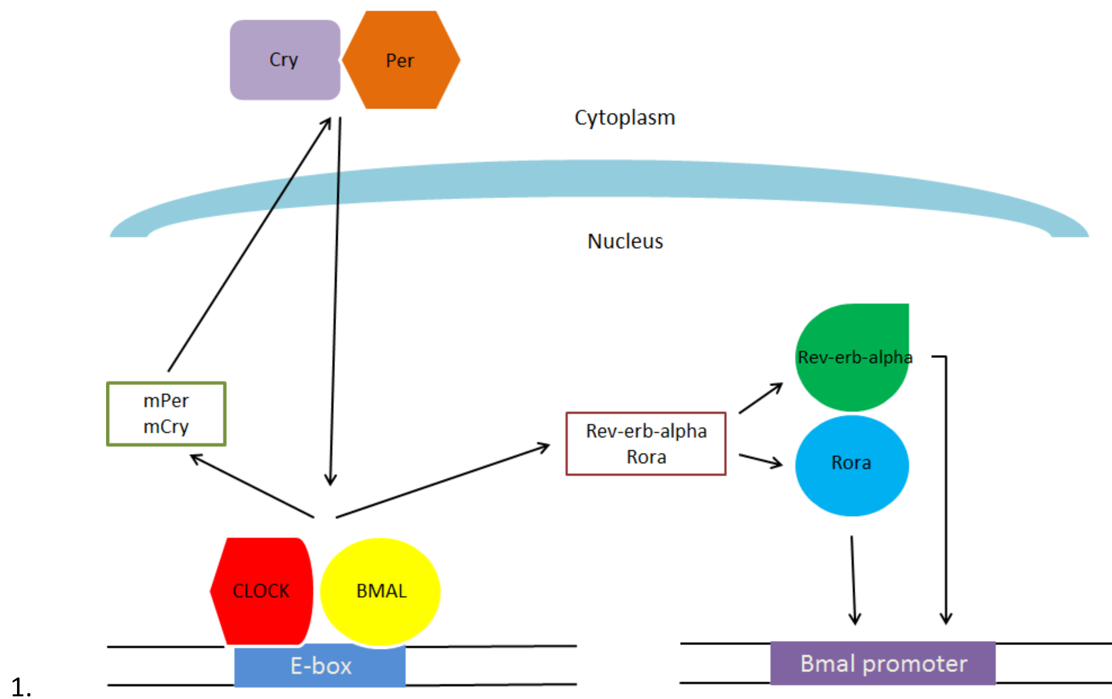
6c. GT1-7 BMAL1 coupled, ERβ probed Co-IP western blot. GT1-7 cellular protein bound to BMAL1 coupled column (15µg primary rabbit antibody), then incubated with 1:1000 ERβ primary rabbit antibody. Protein bands of approximately 53kDa size in antibody verification, unbound protein, elution, and GT1-7 lysate lanes appeared.GT1-7 protein lane had two bands, the first faint protein band was 53kDa and the second protein was approximately 75kDa.

7a. E2 GT1-7 ERβ coupled, ERβ probed Co-IP western blot. E2 exposed GT1-7 cell protein was introduced to a column coupled with 15µg of ERβ primary rabbit antibody, then incubated (probed) with a concentration of 1:1000 ERβ primary rabbit antibody. Though strong bands existed in three lanes of antibody verification, unbound protein, and E2 GT1-7 protein, none appeared in the elution lanes.

7b. E2 GT1-7 ERβ coupled, BMAL1 probed Co-IP western blot. 1nM E2 exposed to GT1-7 cell protein for 24 hours was introduced to a coupled column of 15µg ERβ, then incubated (probed) with 1:1000 concentration BMAL1 primary rabbit antibody. Strong bands appeared in antibody verification, unbound protein, and E2 GT1-7 protein, yet no indication of protein in either elution lane existed.

TABLES AND FIGURES

1.



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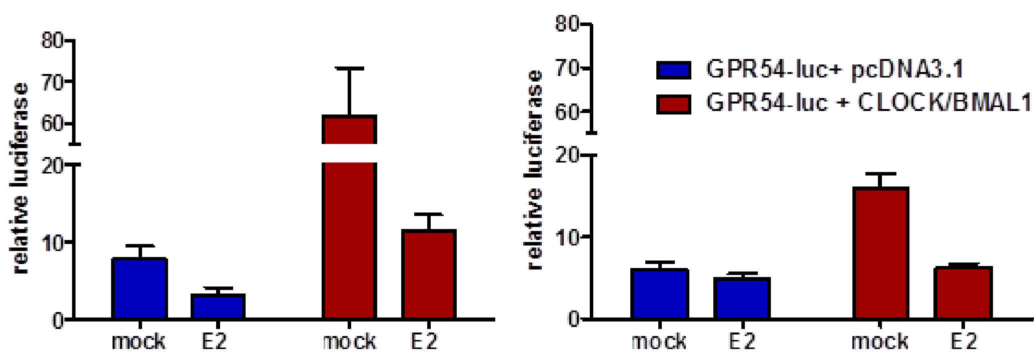

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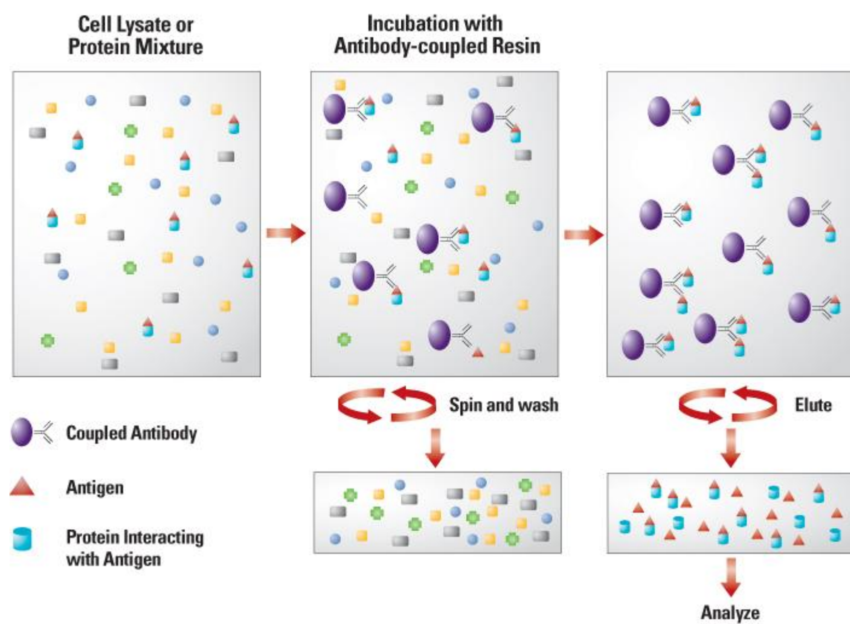

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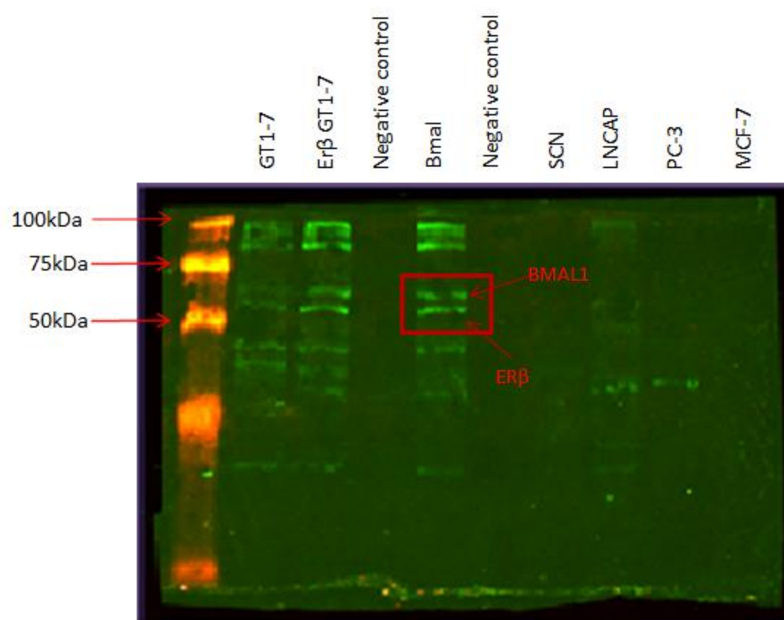

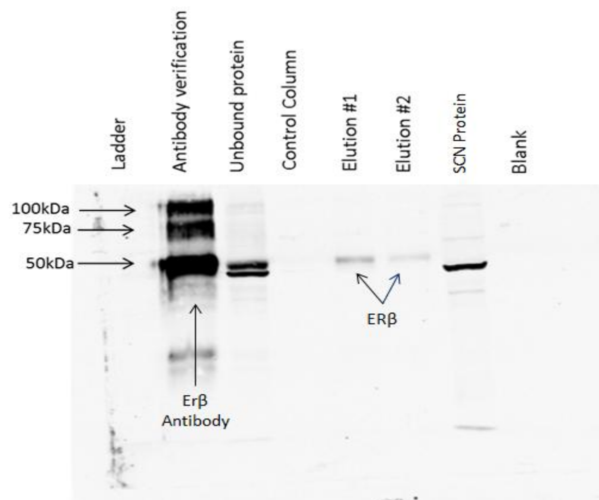

SCN Erβ coupled, Erβ probed

5a.

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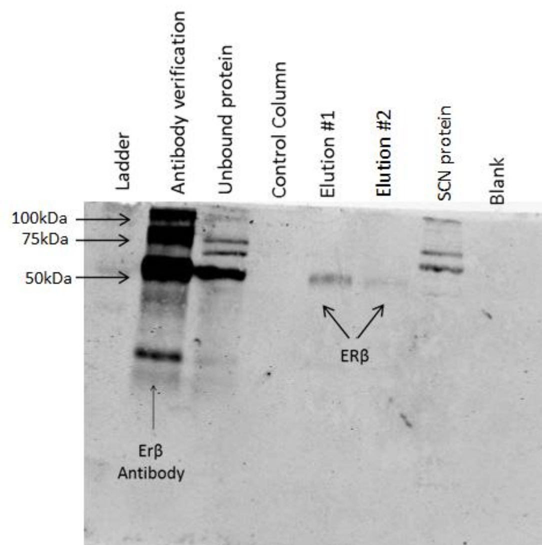
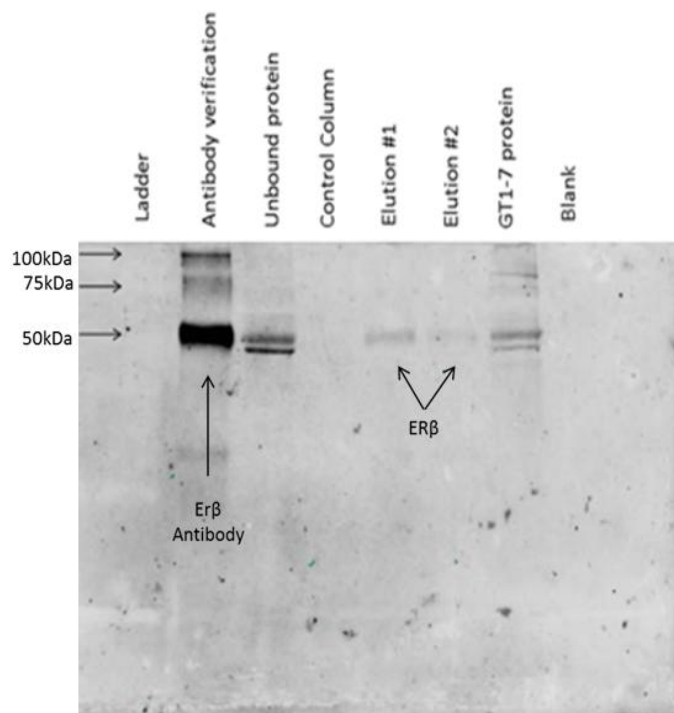
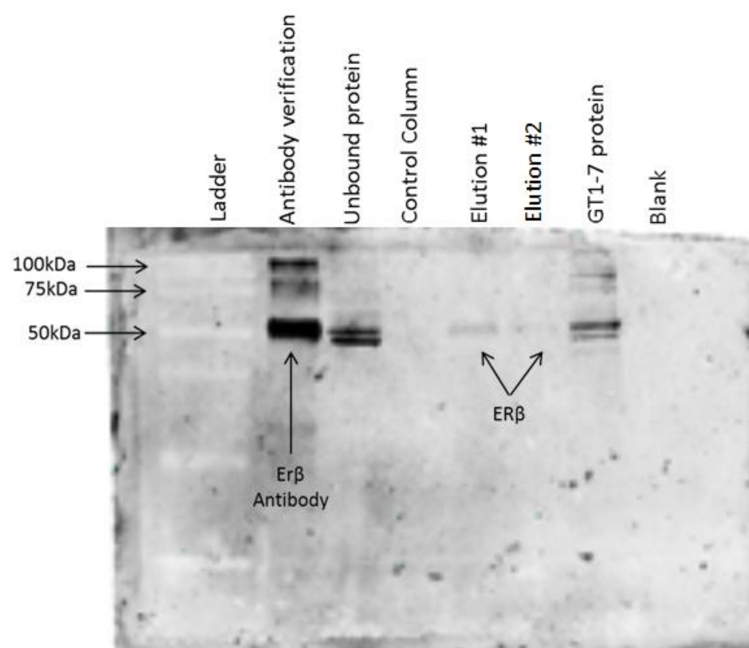


SCN coupled Erβ, probed BMAL

5b.



6a.



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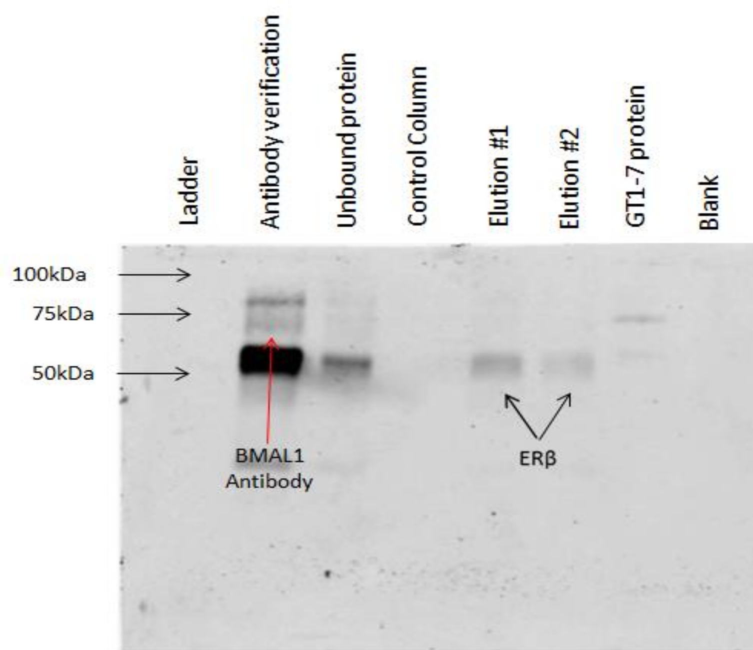
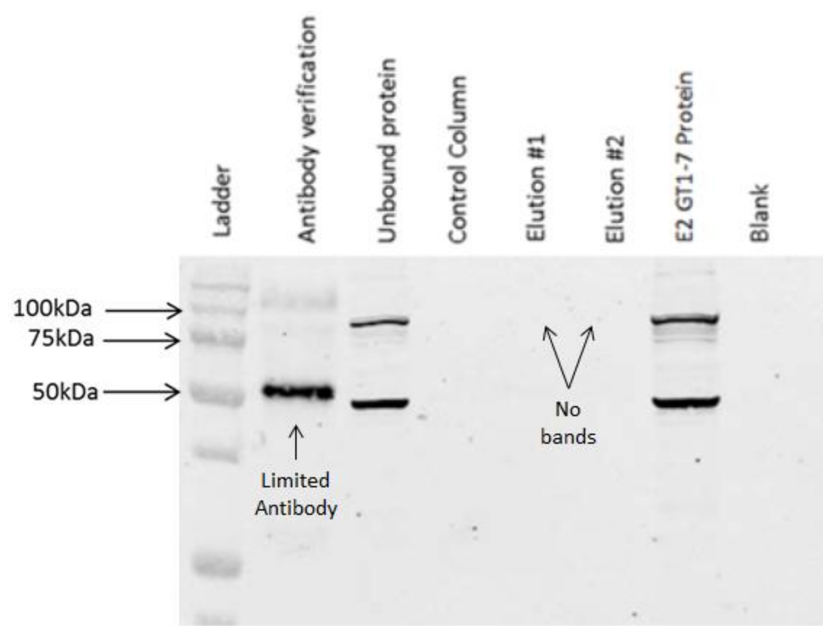


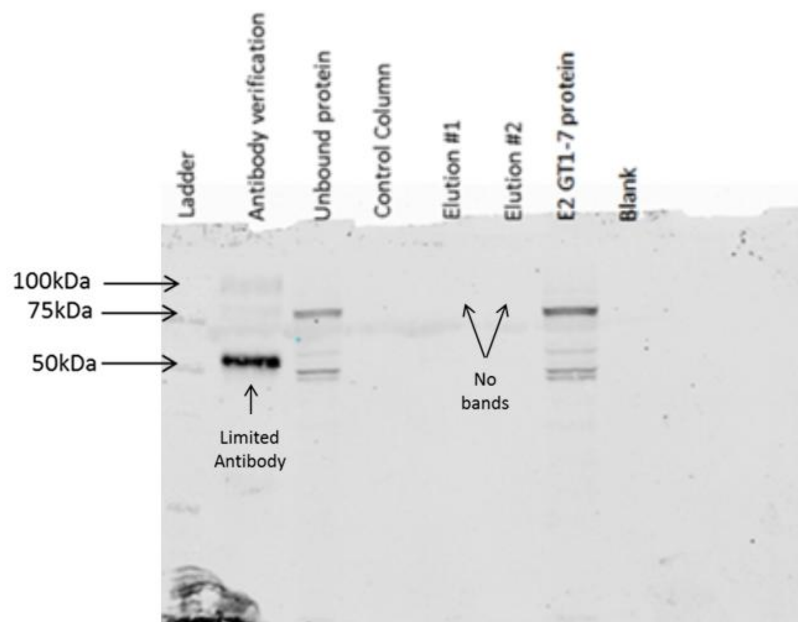
GT1-7 Bmal1 coupled, Erβ probed

6c.



E2 GT1-7 Erβ coupled, Erβ probed

7a.



E2 GT1-7 ERβ coupled, BMAL1 probed

7b.