Summer November 2014

THE ESTRADIOL-INDUCED TRANSCRIPTOME OF THE FEMALE MOUSE ANTEROVENTRAL PERIVENTRICULAR NUCLEUS: MORE THAN JUST A KISS

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University of Massachusetts - Amherst

THE ESTRADIOL-INDUCED TRANSCRIPTOME OF THE FEMALE MOUSE ANTEROVENTRAL PERIVENTRICULAR NUCLEUS: MORE THAN JUST A KISS

A Dissertation Presented

by

LEAH K. AGGISON

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

September 2014

Molecular and Cellular Biology

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DEDICATION

To my mother, father and the memory of my grandparents.

ACKNOWLEDGMENTS

I would like to thank Dr. Sandra L. Petersen, who has been most supportive through the entirety of this process. She has been a dedicated teacher and mentor and a precious friend. She has been fiercely attentive to both my professional and personal development, while allowing me the space needed to grow as both a scientist and a teacher. Thank you for all that you have done for me, both in the lab and outside.

I wish to thank fellow members of the Petersen lab: Ted Hudgens, Sudha Krishnan, Jinyan Cao, Dianne Baker, Lan Ji, Justyne Ogdahl, Maristela Poletini, Javier del Pino, Kay Son, Daniel Brewer and Hillary Adams. I wish to extend a very special thank you to labmates Karlie Intelkofer and Paula Moura-Conlon, and very close friends Emily Merchasin and Katie Maher, whose care and support has been unceasing.

I am also tremendously grateful to committee member Dr. R. Thomas Zoeller for his scientific insight and teaching mentorship. In addition, I wish to thank the members of the Zoeller lab: Ruby Bansal, Dave Sharlin, Stephanie Giera and Judy Brewer.

I am thankful to the Molecular and Cellular Biology Program for the opportunity and environment to complete this research, with special recognition to Doreen Fifield and Sarah Martell. Thank you to Kathleen Arcaro and Sallie Smith-Schneider for their time and patience as committee members. Also, many thanks go to the Northeast Alliance for Graduate Education & the Professoriate, for providing a framework for graduate success.

Finally I wish to thank my parents, Sally and Lee, and my stepmother, Angela; my brother, Lee A. Aggison Jr., PhD and his wife, Ruth A. Washington, PhD; and my sister, Kimberly Kemp, BSN, RN. They have been beacons of support and encouragement, as well as examples of the benefits of perseverance.

ABSTRACT

THE ESTRADIOL-INDUCED TRANSCRIPTOME OF THE FEMALE MOUSE ANTEROVENTRAL PERIVENTRICULAR NUCLEUS: MORE THAN JUST A KISS

SEPTEMBER 2014

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Estradiol (E_2) is critical in the reproductive mechanisms of mammals. In female rodents E_2 acts through the neurons of the anteroventral periventricular nucleus (AVPV) to exert neuroendocrine control over ovulation, via synaptic activation of the gonadotropin releasing hormone (GnRH) neurons. The neurocircuitry of the AVPV is complex, receiving input from the suprachiasmatic nucleus and ventral premammillary nucleus and the as well as projecting to organum vasculosum of lamina terminalis and the arcuate. This suggests a broader role for the AVPV as a center of multisignal-integration in regards to ovulation. I used full genome expression microarrays to assess the E_2-induced transcriptome in the female mouse AVPV and further investigated several targets using mouse neuronal cells. I discovered that within the AVPV, E_2 regulates several genes important for energy balance. Additionally, I found that E_2 regulates transcription factor v-ets avian erythroblastosis virus E26 oncogene homolog 2 (Ets2), which in turn regulates estrogen receptor α and is necessary in the E_2-dependent regulation of kisspeptin. Together these findings support a broader role for AVPV function and identify a novel mechanism by which E_2 mediates transcription.

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Female ovulation is an essential component of mammalian reproduction. It is a result of estradiol (E_2) -mediated activation of gonadotropin releasing hormone (GnRH) neurons within the brain. The robust release of GnRH stimulates the production of a luteinizing hormone (LH) surge that in turn provokes ovulation. Information garnered from rodent models has implicated the estrogen receptor alpha (ERα) -expressing neurons within the anteroventral periventricular nucleus (AVPV) as critical in transmitting the E_2 signal to the GnRH neurons (Simonian et al., 1999, Petersen et al., 2003, Wintermantel et al., 2006, Mayer et al., 2010).

1.2 LH Surge Mechanisms and the AVPV

In trying to elucidate the mechanisms of neuroendocrine control of ovulation, it was determined that electrostimulation of the preoptic area (POA) could induce ovulation in rodents (Everett and Radford, 1961). Similarly, direct implantation of E_2 within the preoptic area of ovariectomized rats could stimulate an LH surge release from the pituitary (Goodman, 1978). Narrowing down the region mediating these signals even further, lesions within the rostral POA blocked the E_2- and progesterone-dependent LH surge in ovariectomized rodents (Ronnekleiv and Kelly, 1986). Similarly, microimplants of anti-estrogens in this region also blocked the E_2-induced LH surge (Petersen and Barraclough, 1989).

Retrograde tracing combined with *in situ* hybridization identified estrogen receptor α (ERα) -expressing neurons within the AVPV and MPN as providing the most prominent estrogen receptive inputs to the GnRH neurons (Simonian et al., 1999). This suggested ERα as the predominant mediator of the E_2 signal, and indeed this idea was later supported when Wintermantel's group showed that ER beta (ERβ) knockout mice exhibited a normal E_2-dependent preovulatory LH surge; however, it was absent in ERα knockout mice (Wintermantel et al., 2006).

As stated above, the LH surge in mice is also progesterone-dependent. Importantly, E_2 increases the expression of progesterone receptor (PR) in the AVPV (Simerly et al., 1996) whereas anti-estrogens are antagonistic to E_2-induced PR expression in the AVPV (Shughrue et al., 1997). Moreover, blocking PR with a PR antagonist completely blocks both GnRH and LH surges. More specific inhibition of PR in the AVPV with antisense oligonucleotides also blocks the LH surge (Chappell and Levine, 2000). This firmly supports the contention that the AVPV is a critical nucleus for relaying the E_2 and progesterone signals necessary to produce the LH surge, and thus ovulation.

1.3 Specific Signals from the AVPV to the GnRH Neurons

Most of the neurons populating the female AVPV are dual-phenotypic, being both GABAergic and glutamatergic. At the time of the LH surge, GABAergic vesicles decline, while excitatory glutamate vesicles increase in the terminals (Ottem et al., 2004). Importantly, these neurons, which are almost entirely positive for esr1 gene expression (mRNA corresponding to ERα), are also multipeptidergic.

Many of these neurons express neurotensin (Nts) (Axelson et al., 1992), and although E_2 increases *nts* expression in the AVPV, intracerebroventricular injection of Nts failed to activate GnRH neurons or stimulate LH secretion (Dungan Lemko et al., 2010). On the other hand, a more periventricular subpopulation of ERα-expressing neurons in the AVPV also express kisspeptin (Kiss1), a neuropeptide critical for the LH surge release (Smith et al., 2005, Oakley et al., 2009). This is significant because loss of function of the Kiss1 receptor, Kiss1r (formerly gpr54), results in a hypogonadotropic hypogonadism phenotype (Colledge, 2009). Interestingly, virtually none of the Nts-expressing neurons colocalize with the Kiss1-expressing neurons (Dungan Lemko et al., 2010). E_2 increases *kiss1* expression in the AVPV (Smith et al., 2005), and I discovered that all *kiss1* expression in the AVPV colocalizes with GABA neurons (identified by the marker glutamic acid decarboxylase) (Petersen et al., 2012). This further supports the critical nature of E_2 actions in the AVPV.

1.4 AVPV Neuronal Circuitry with Other Nuclei

Although it has been well established that AVPV neurons synapse onto GnRH neurons (Simonian et al., 1999), these are not the only neurons with which they communicate. The AVPV receives inputs from the leptin receptor-rich ventral premammillary nucleus, implicated in mediating adiposity signals contributing to reproductive capability (Donato et al., 2011). There are also inputs from the suprachiasmatic nucleus (SCN) (Watson et al., 1995), suggesting integration of daylight signals, also important for reproduction. Considering that SCN also expresses leptin receptors (Guan et al., 1997) and regulates the secretion of hypocretin (Zhang et al.,

2004), this further implicates the AVPV as a site for the integration of daylight signals and energy balance in regards to reproductive function.

Furthermore, there are also projections from the AVPV to other nuclei. These include a region surrounding the OVLT (Gu and Simerly, 1997), suggestive of a role in thirst management. Additionally, there are projections to a subset of neurons in the arcuate nucleus suggesting a supplemental role in the negative feedback mechanisms of E_2, for which the arcuate is known (Gu and Simerly, 1997, Yeo and Herbison, 2014).

While much has been learned over the last 40 years to delineate both the function of E_2 and its mode of action in the AVPV, it has occurred by way of ever-tightening the focus of the investigation, specifically on kisspeptin and ovulation. Although it has been very valuable, I contend that to better understand the actions of E_2 in the AVPV, a more global approach is warranted. To address this, I have focused my research on identifying novel E_2-regulated gene transcripts within the AVPV.

1.5 Figures

Figure 1.1 Dual-label in situ Hybridization of Kiss1 and GAD

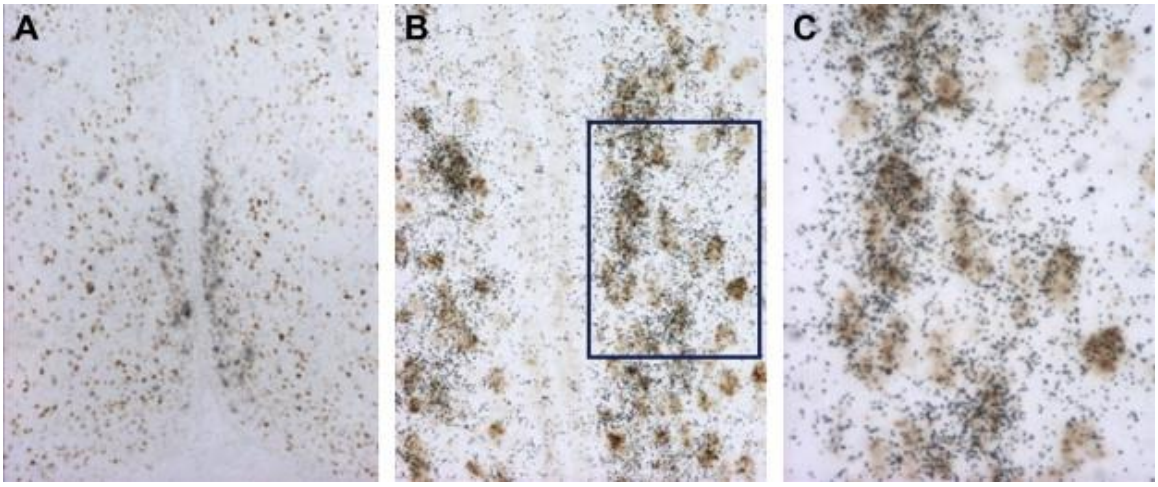


Figure 1.1 Photomicrograph showing results of dual-label *in situ* hybridization histochemistry study colocalizing ^{35}S-labeled cRNA probe for Kiss1 (black sliver grains) and dioxigenin-labeld probes for Gad1 and Gad2.

1.6 Bibliography

Axelson JF, Shannon W, Van Leeuwen FW (1992) Immunocytochemical localization of estrogen receptors within neurotensin cells in the rostral preoptic area of the rat hypothalamus. Neuroscience letters 136:5-9.

Chappell PE, Levine JE (2000) Stimulation of gonadotropin-releasing hormone surges by estrogen. I. Role of hypothalamic progesterone receptors. Endocrinology 141:1477-1485.

Colledge WH (2009) Transgenic mouse models to study Gpr54/kisspeptin physiology. Peptides 30:34-41.

Donato J, Jr., Cravo RM, Frazao R, Gautron L, Scott MM, Lachey J, Castro IA, Margatho LO, Lee S, Lee C, Richardson JA, Friedman J, Chua S, Jr., Coppari R, Zigman JM, Elmquist JK, Elias CF (2011) Leptin's effect on puberty in mice is relayed by the ventral premammillary nucleus and does not require signaling in Kiss1 neurons. The Journal of clinical investigation 121:355-368.

Dungan Lemko HM, Naderi R, Adjan V, Jennes LH, Navarro VM, Clifton DK, Steiner RA (2010) Interactions between neurotensin and GnRH neurons in the positive feedback control of GnRH/LH secretion in the mouse. American journal of physiology Endocrinology and metabolism 298:E80-88.

Everett JW, Radford HM (1961) Irritative deposits from stainless steel electrodes in the preoptic rat brain causing release of pituitary gonadotropin. Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine 108:604-609.

Goodman RL (1978) The site of the positive feedback action of estradiol in the rat. Endocrinology 102:151-159.

Gu GB, Simerly RB (1997) Projections of the sexually dimorphic anteroventral periventricular nucleus in the female rat. The Journal of comparative neurology 384:142-164.

Guan XM, Hess JF, Yu H, Hey PJ, van der Ploeg LH (1997) Differential expression of mRNA for leptin receptor isoforms in the rat brain. Molecular and cellular endocrinology 133:1-7.

Mayer C, Acosta-Martinez M, Dubois SL, Wolfe A, Radovick S, Boehm U, Levine JE (2010) Timing and completion of puberty in female mice depend on estrogen receptor alpha-signaling in kisspeptin neurons. Proceedings of the National Academy of Sciences of the United States of America 107:22693-22698.

Oakley AE, Clifton DK, Steiner RA (2009) Kisspeptin signaling in the brain. Endocrine reviews 30:713-743.

Ottem EN, Godwin JG, Krishnan S, Petersen SL (2004) Dual-phenotype GABA/glutamate neurons in adult preoptic area: sexual dimorphism and function. J Neurosci 24:8097-8105.

Petersen SL, Barraclough CA (1989) Suppression of spontaneous LH surges in estrogen-treated ovariectomized rats by microimplants of antiestrogens into the preoptic brain. Brain Res 484:279-289.

Petersen SL, Krishnan S, Aggison LK, Intlekofer KA, Moura PJ (2012) Sexual differentiation of the gonadotropin surge release mechanism: a new role for the canonical NfkappaB signaling pathway. Frontiers in neuroendocrinology 33:36-44.

Petersen SL, Ottem EN, Carpenter CD (2003) Direct and indirect regulation of gonadotropin-releasing hormone neurons by estradiol. Biology of reproduction 69:1771-1778.

Ronnekleiv OK, Kelly MJ (1986) Luteinizing hormone-releasing hormone neuronal system during the estrous cycle of the female rat: effects of surgically induced persistent estrus. Neuroendocrinology 43:564-576.

Shughrue PJ, Lane MV, Merchenthaler I (1997) Regulation of progesterone receptor messenger ribonucleic acid in the rat medial preoptic nucleus by estrogenic and antiestrogenic compounds: an in situ hybridization study. Endocrinology 138:5476-5484.

Simerly RB, Carr AM, Zee MC, Lorang D (1996) Ovarian steroid regulation of estrogen and progesterone receptor messenger ribonucleic acid in the anteroventral periventricular nucleus of the rat. Journal of neuroendocrinology 8:45-56.

Simonian SX, Spratt DP, Herbison AE (1999) Identification and characterization of estrogen receptor alpha-containing neurons projecting to the vicinity of the

gonadotropin-releasing hormone perikarya in the rostral preoptic area of the rat. The Journal of comparative neurology 411:346-358.

Smith JT, Cunningham MJ, Rissman EF, Clifton DK, Steiner RA (2005) Regulation of Kiss1 gene expression in the brain of the female mouse. Endocrinology 146:3686-3692.

Watson RE, Jr., Langub MC, Jr., Engle MG, Maley BE (1995) Estrogen-receptive neurons in the anteroventral periventricular nucleus are synaptic targets of the suprachiasmatic nucleus and peri-suprachiasmatic region. Brain Res 689:254-264.

Wintermantel TM, Campbell RE, Porteous R, Bock D, Grone HJ, Todman MG, Korach KS, Greiner E, Perez CA, Schutz G, Herbison AE (2006) Definition of estrogen receptor pathway critical for estrogen positive feedback to gonadotropin-releasing hormone neurons and fertility. Neuron 52:271-280.

Yeo SH, Herbison AE (2014) Estrogen-Negative Feedback and Estrous Cyclicity Are Critically Dependent Upon Estrogen Receptor-alpha Expression in the Arcuate Nucleus of Adult Female Mice. Endocrinology 155:2986-2995.

Zhang S, Zeitzer JM, Yoshida Y, Wisor JP, Nishino S, Edgar DM, Mignot E (2004) Lesions of the suprachiasmatic nucleus eliminate the daily rhythm of hypocretin-1 release. Sleep 27:619-627.

CHAPTER 2

FULL GENOME MICROARRAY ANALYSIS OF 17β-ESTRADIOL GENE TARGETS IN THE ANTEROVENTRAL PERIVENTRICULAR NUCLEUS OF THE C57Bl/6 MOUSE BRAIN

2.1 Introduction

Although the AVPV contains one of the densest populations of ERα in the brain (Mitra et al., 2003), many of the mechanisms of estradiol ($E_{2)}$ in this nucleus remain unresolved. This lack of information hinders our ability to fully understand the E_2-dependence of ovulation, and thus fertility (Simonian et al., 1999, Petersen et al., 2003, Wintermantel et al., 2006). Not only that, it is possible that such mediation involves E_2-dependent gene products supplementary to the well-documented kiss1 (de Roux et al., 2003, Seminara et al., 2003). Alternatively, E_2 may have functions in the AVPV beyond that of mediating the E_2 signal to the GnRH neurons to produce the LH surge. It is also probable that E_2 exerts some of its transcriptional effects via non-canonical secondary mechanisms, including inducing reactive oxygen species (Felty et al., 2005a) and phosphorylation events (Micevych and Kelly, 2012). Endeavoring to get a comprehensive grasp of the function of E_2 in the AVPV requires a more global view of the transcriptome.

One of the most robust ways to assess the transcriptome is by employing full genome expression microarrays. No researchers have examined the E_2-dependent transcriptome of the AVPV, but several groups previously performed microarray studies to assess E_2 effects on the whole hypothalamus (Sakakibara et al., 2013), anterior

hypothalamus, posterior hypothalamus (Xu et al., 2008) or medial basal hypothalamus (Blutstein et al., 2006). Considering the volume of the AVPV is miniscule in comparison to the entirety of the hypothalamus (Davis et al., 1996), it has been difficult to extrapolate useful information from these previous array studies regarding signaling specifically originating the AVPV, or its regulation by E_2. Moreover, the hypothalamus contains many other E_2-responsive nuclei, including the sexually dimorphic nucleus (Gorski, 1985, Tsukahara, 2009), ventromedial hypothalamus (Flanagan-Cato et al., 2001, Calizo and Flanagan-Cato, 2003), the periphery of both the organum vasulosum of the lamina terminalis (Somponpun et al., 2004) and subfornical organ (Rosas-Arellano et al., 1999), arcuate nucleus (Shughrue et al., 1992, Dellovade and Merchenthaler, 2004) and paraventricular nucleus (Simonian and Herbison, 1997, Scordalakes et al., 2002). As the effects of E_2 are also dependent on the neuronal inputs into the nucleus, it could result in differential regulation of the same transcript in multiple nuclei (Watson et al., 1995, Polston et al., 2004, Vida et al., 2010). Such contrary regulation could wash out detection of the effects of E_2, with any effects that are observable being nearly impossible to attribute to a particular nucleus.

In order to address the inadequacies of previous studies, I microdissected the AVPV of both E_2-and oil-treated adult mice and then employed full genome expression microarrays and multiple analyses. Here I report numerous novel E_2 targets and suggest a possible new function for the AVPV.

2.2 Materials and Methods

2.2.1 Animals

All protocols were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts and all animals were housed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Eight-week-old female C57Bl/6 mice (Jackson Labs; Bar Harbor, ME) were housed four to a cage in a temperature- and light-controlled room (12:12 light/dark cycle), with standard feed and water provided *ad libitum.* After a minimum of 48h post-arrival, all mice were bilaterally ovariectomized under isofluorane anesthesia. Five days later, mice were injected subcutaneously with sesame oil vehicle or 0.05µg/g b.w. 17β-E_2 dissolved in sesame oil. Twelve hours later, animals were anesthetized with CO_2, brains were collected, rapidly frozen on powdered dry ice, wrapped in Parafilm™ (Pechiney Plastic Packaging Company; Chicago, Illinois) and stored at -80°C in cryotubes. The dosage and twelve hour time point was chosen specifically to capture early transcriptional targets within the AVPV, well before the LH surge event (Figure 2.6.1)

2.2.2 Tissue Preparation and RNA Isolation

Brains were allowed to thaw slowly at -20°C, then coronally cryosectioned at 12 µm using a Leica CM3000 cryostat (Nussloch, Germany), until the early AVPV was reached. The early AVPV was determined by the initial appearance of the optic recess. I took a 300-µm coronal section and immediately excised the AVPV from it using a 1mm circular Harris Uni-Core™ stainless steel tissue micropunch needle (Ted Pella Inc.; Redding, CA) (Figure 2.6.2). I transferred the micropunched tissue to a 1.5-ml

microcentrifuge tube, on powdered dry ice. To obtain enough starting material, I pooled four AVPV micropunches to make one sample.

Total RNA was isolated from each pool using Trizol™ (Invitrogen; Carlsbad, CA) and Qiagen RNeasy Lipid kit (Qiagen; Valencia, CA). Sample concentration was determined via Nanodrop™ (Thermo Scientific; Wilmington, DE) and quality was verified using the Agilent 2100 Bioanalyzer® and RNA 6000 Nano LabChips (Agilent Technologies, Palo Alto, CA). Samples with 260/280 readings ≥1.7 and 260/230 readings ≥ 1.5 were deemed acceptable.

2.2.3 Microarray and Analyses

Pooled AVPV RNA samples were frozen and shipped on dry ice to the Keck Microarray Institute at Yale University (West Haven, CT). They processed the samples and executed the Mouse Gene 1.0 ST Arrays (Affymetrix; Palo Alto, CA). Full genome expression analysis comparing means of AVPV genes from oil-treated (n=3 pools of 4 animals) and E_2-treated (n=3 pools) animals was also performed by the Keck Institute. The analysis reported all transcripts that had both a minimum fold-change ≥1.2 and a *p*-value ≤0.05. In addition to the comparison analysis, the Keck Institute also performed a gene ontology analysis based on all genes with fold-change ≥1.2 and *p*-value ≤0.5. The gene ontology analysis was based on highest gene set enrichment, with an enrichment score above 3 representing significant overexpression.

I used Ariadne Pathway Studio™ software to broader evaluate possible signaling pathways differentially regulated by E_2. Additionally, using the raw fluorescence values, I compiled a list of the highest expressed transcripts within the AVPV of both oil- and E_2-treated mice. The Mouse Gene 2.0 ST Array uses multiple 20mer oligonucleotides to

determine the expression of a single gene. In the case of the highest expressed genes, they were often identified by multiple transcripts on the array, and thus I removed gene duplications from the list.

2.3 Results

2.3.1 Identification of E_2 Gene Targets within the Female Mouse AVPV

The gene expression analysis performed by the Keck Institute identified a total of 269 transcripts differentially regulated by E_2 in the female AVPV: 155 increased and 114 decreased. This was a full genome analysis and many of the transcripts identified have not been well characterized; indeed, 69 (25.7%) of the transcripts consisted of various predicted sequences. These sequences included 28 RIKEN, 8 Ensembl, 4 LOC, 3 OTTMUSG, 7 Genscan, 3 mmu-mir (microRNAs), 12 that have not yet received a gene symbol (only an mRNA assignment ID) and 4 that do not yet have an mRNA assignment ID (Table 2.5.1).

2.3.2 Identification of E_2-Regulated Cellular Functions and Pathways

The Keck Microarray Consortium used the Gene Ontology Term Enrichment technique to interpret functional characteristics of the gene set. Analyzing the 269 differentially expressed genes, there were 457 biological functions significantly regulated by E_2 (Table 2.5.2). Further analysis using Ariadne Pathway Studio™ only identified one significantly regulated pathway, "feeding and drinking behavior."

2.3.3 Highest Expressed Transcripts within the Female AVPV

When viewed in order of highest raw fluorescence values, none of the 300 highest expressed transcripts were differentially regulated by E_2 (Table 2.5.3). Not surprisingly, many of them represented housekeeping genes. However, there were 45 (15%) that are

relatively uncharacterized, not yet having a gene symbol ID assigned. In fact, only 3 of the 10 highest expressed transcripts have been named: phosphatidylinositol glycan anchor, cytochrome B and cytochrome C oxidase.

2.4 Discussion

This set of microarray data represents the first transcriptome evaluation of the mouse AVPV, of any kind. Herein I have identified 269 E_2-regulated transcripts within the female AVPV, generally considered a rather small number of transcripts for a microarray study. Likewise, the highest fold change amount in this study was only 2.36-fold; uncharacteristically low for a microarray. Many expression analyses set the lower threshold for differentially regulated transcripts to 2-fold, however, such a stringency would have only captured four increased transcripts and no decreased transcripts. Furthermore, ignoring more minor fold-changes greatly modifies the gene ontology and pathway analyses (Dalman et al., 2012). Taking into account the more specific excision of the AVPV from surrounding brain nuclei, it is likely that this small gene set is relevant to the functioning of this nucleus, specifically, its regulation by E_2.

The expression analysis revealed many previously unidentified transcripts within the AVPV, some of which are also novel targets of E_2. Remarkably, of the 15 most regulated transcripts (11 increased and 4 decreased), only Pgr (Simerly et al., 1996) and Esr1 (Mitra et al., 2003, Chakraborty et al., 2005), have been previously described as regulated by E_2 in the AVPV. Little is known about the distribution of the remaining 12 transcripts within this brain region, as the expression of only three others, c1ql2, slitrk6 and pgr15l1, have been positively mapped to coronal sections of the AVPV, as depicted in the Allen Brain Atlas (http://www.brain-map.org/). However, the atlas is not complete

in that the coronal sections are 100 μm apart, and some genes have only been screened on sagittal sections, or not at all. What is more, the possible role of these transcripts in E_2-dependant AVPV signaling to the GnRH neurons lies completely unassessed.

The gene ontology analysis includes 457 cellular functions significantly regulated by E_2 in the female AVPV (Table 2.5.2). However, it is difficult to develop a distinct picture of which functions may be of most importance. The gene ontology functional groups are mired in minutiae, with 29 functional groups each comprised of a single gene and 39 groups comprised of only two genes. Considering the gene set enrichment score is largely based on the percentage of genes regulated within a gene group, 100% in a group only represented by one gene may not be as physiologically important as 40% in a group represented by 12 genes. Furthermore, without QPCR validation it is impossible to know which targets are false-positives, and may need to be removed from the data set. Thus this particular gene ontology analysis offers little additional value on its own.

There are no significant differences in any of the 300 highest expressed transcripts (Table 2.5.3). In fact, many of these transcripts are commonly regarded as housekeeping genes. It is noteworthy to mention, but not surprising, that many of the genes within this set are involved in GABA/Glu signaling. As the AVPV represents one of the few nuclei known to contain dual-phenotypic GABAergic/glutamatergic neurons, this was to be expected (Ottem et al., 2004). It is not readily apparent what information may be extracted from this data set; however this may provide a new pool of tissue- and treatment-specific housekeeping genes. Furthermore, it may prove interesting to compare this high-expression analysis with that of the male AVPV. As the male AVPV is roughly half the size of the females, it may be quite informative to identify which of the highest

expressed transcripts are the same and if there are any that are differentially expressed. Likewise, comparing this data set to other microarray sets utilizing E_2-responsive tissue may provide significant insight into tissue-specific E_2 transcriptome regulation.

The Ariadne™ pathway analysis only identified one significantly regulated pathway, feeding and drinking behavior. Despite the fact that there are a substantial number of genes significantly regulated in this pathway, many of which are olfactory receptors, validation by QPCR is necessary to determine if in fact these are genuine targets. It is likely that E_2 significantly regulates many more pathways; however, due to very modest fold-changes and *p*-values just above the 0.05 cutoff, these may not emerge in this type of analysis.

Together, the analyses of this microarray data set not only provide a specific and more complete view of the function of E_2 within the AVPV, but may also provide information regarding E_2 mediation of signals in areas to which these neurons project, specifically, GnRH neurons (Kalra, 1993, Simerly, 1998). This represents a wealth of new information pertaining to brain control of ovulation and quite possibly feeding and drinking behavior, discussed further in Chapter 4.

2.5 Tables

Table 2.1 Gene Enrichment

Genes >1.2x and p<0.05 using all samples						
Transcript ID	**Fold-Change (E_2 vs. oil)**	**p-value**	**Mean (E_2)**	**Mean (oil)**	**Gene Symbol**	**RefSeq**
10538832	2.357	0.0010	610.4	259.0	Mad2l1	NM_019499
10453715	2.189	0.0007	156.1	71.3		---
10349340	2.056	0.0006	548.5	266.7	C1ql2	NM_207233
10583195	2.017	0.0003	1239.9	614.7	ENSMUSG00000022845	ENSMUST00000104915
10487238	1.927	0.0006	202.1	104.9	Hdc	NM_008230
10422244	1.885	0.0095	228.7	121.4	Slitrk6	NM_175499
10461777	1.884	0.0301	36.2	19.2	Olfr1444	NM_146702
10583179	1.807	0.0003	620.8	343.5	Pgr	NM_008829
10426425	1.619	0.0120	366.3	226.3	Pdzrn4	ENSMUST00000035399
10360664	1.611	0.0040	754.9	468.7	ENSMUSG00000056615	ENSMUST00000070201
10386455	1.583	0.0121	493.8	311.9	Rasd1	NM_009026
10485117	1.552	0.0100	175.1	112.8	Creb3l1	NM_011957
10516723	1.540	0.0015	226.9	147.3	Hcrtr1	NM_198959
10416945	1.533	0.0378	47.1	30.7	5033413D16Rik	AK053349
10440406	1.516	0.0004	299.5	197.5	Nrip1	NM_173440
10394674	1.516	0.0153	364.9	240.8	ENSMUSG00000050974	ENSMUST00000052528
10605753	1.499	0.0235	50.2	33.5	4932442L08Rik	BC115707
10546725	1.497	0.0148	134.3	89.7	Pdzrn3	NM_018884
10404264	1.490	0.0433	85.5	57.4	Prl	NM_011164
10436770	1.482	0.0424	22.3	15.0	ENSMUSG00000044227	ENSMUST00000062524
10484569	1.478	0.0373	30.5	20.6	Olfr1045	NM_147017
10441902	1.477	0.0012	86.1	58.3	Smoc2	NM_022315
10598073	1.457	0.0397	1083.9	743.9		---
10399505	1.453	0.0150	101.0	69.5	Greb1	NM_015764
10437160	1.428	0.0024	1075.3	753.1	Ets2	NM_011809
10476935	1.415	0.0317	571.0	403.6	OTTMUSG00000015750	BC147352
10362513	1.414	0.0107	232.5	164.5	Hs3st5	NM_001081208

10459512	1.413	0.0251	255.4	180.8	Mc4r	NM_016977
10545130	1.412	0.0010	326.1	231.0	Gadd45a	NM_007836
10444459	1.398	0.0057	174.7	124.9	Tnxb	NM_031176
10496425	1.396	0.0028	62.2	44.5	Adh7	NM_009626
10530089	1.386	0.0007	252.1	181.9	Cckar	NM_009827
10552311	1.382	0.0015	239.6	173.4	OTTMUSG00000022427	XR_030737
10390080	1.381	0.0147	20.0	14.5	Tmem92	NM_001034896
10360454	1.377	0.0040	324.1	235.3	Opn3	NM_010098
10538783	1.370	0.0259	184.1	134.4	C130060K24Rik	BC119578
10502863	1.365	0.0003	542.5	397.4	Ak5	NM_001081277
10595657	1.358	0.0017	313.8	231.2	AF529169	AF529169
10487269	1.354	0.0369	52.9	39.1	Usp50	NM_029163
10505489	1.352	0.0219	171.5	126.8	Pappa	NM_021362
10403816	1.345	0.0129	9.2	6.8		---
10372069	1.344	0.0159	272.0	202.4	Socs2	NM_007706
10452793	1.343	0.0205	304.1	226.4	Galnt14	NM_027864
10442098	1.342	0.0056	37.8	28.2	Fpr3	NM_008042
10593646	1.341	0.0073	255.8	190.8	Tnfaip8l3	NM_001033535
10439832	1.338	0.0404	35.6	26.6		---
10537296	1.338	0.0269	101.4	75.8		---
10431154	1.334	0.0010	161.5	121.0	Phf21b	NM_001081166
10375432	1.334	0.0362	347.8	260.8	C030019I05Rik	BC104394
10494945	1.332	0.0096	199.6	149.8	Syt6	NM_018800
10554723	1.332	0.0104	16.2	12.2		---
10347115	1.331	0.0430	10.8	8.1		---
10372139	1.330	0.0115	1271.0	955.4	Nts	NM_024435
10429160	1.330	0.0028	204.2	153.5	St3gal1	NM_009177
10603878	1.328	0.0263	57.8	43.5	Uxt	NM_013840
10551282	1.325	0.0078	59.8	45.2	LOC100047728	XR_033870
10604682	1.320	0.0073	26.6	20.2	Gm648	BC147598
10552526	1.320	0.0129	41.6	31.5	Klk5	NM_026806
10594447	1.319	0.0159	2180.7	1653.1	Map2k1	NM_008927
10402981	1.317	0.0449	82.7	62.8	Gm900	ENSMUST00000103414
10553743	1.313	0.0030	64.5	49.1	Oca2	NM_021879
10503992	1.311	0.0046	190.0	144.9	Tmem215	ENSMUST00000049655
10598146	1.308	0.0060	24.9	19.1	Tcstv3	NM_153523
10463997	1.302	0.0074	1418.3	1089.5	Pdcd4	NM_011050
10562486	1.294	0.0390	75.3	58.2	Rgs9bp	NM_145840
10588219	1.293	0.0281	55.5	42.9		---

10543676	1.293	0.0289	109.6	84.8	1700080G18Rik	ENSMUST00000059487
10366346	1.293	0.0042	298.8	231.1	Phlda1	NM_009344
10522530	1.290	0.0383	327.5	253.9	Kit	NM_001122733
10428938	1.288	0.0212	8.7	6.8		---
10405619	1.287	0.0130	655.7	509.4	5133401N09Rik	NM_198004
10428012	1.287	0.0204	75.5	58.7	Ropn1l	NM_145852
10495987	1.286	0.0350	41.4	32.2	EG435755	DQ851564
10545101	1.284	0.0196	153.5	119.5	Ptgds2	NM_019455
10468722	1.283	0.0112	1160.1	903.9	Gfra1	NM_010279
10360666	1.282	0.0043	286.6	223.5	6330403A02Rik	BC120654
10518947	1.281	0.0292	340.8	266.1	Ajap1	NM_001099299
10470412	1.281	0.0363	52.3	40.8	Dbh	NM_138942
10405334	1.277	0.0025	126.9	99.4	Eif4e1b	NM_001033269
10394823	1.276	0.0244	680.3	533.3	546752	XR_035702
10474064	1.275	0.0256	688.4	539.7	Trp53i11	NM_001025246
10491805	1.274	0.0167	76.4	60.0	Plk4	NM_011495
10503334	1.273	0.0476	134.4	105.5	Gem	NM_010276
10428171	1.272	0.0238	1651.1	1297.6	Ankrd46	NM_175134
10399965	1.270	0.0021	117.6	92.5	F730043M19Rik	ENSMUST00000063828
10571655	1.269	0.0345	9.5	7.5		---
10461802	1.267	0.0118	18.3	14.4	Olfr1467	NM_146691
10531556	1.266	0.0222	15.6	12.3	Gk2	NM_010294
10359255	1.265	0.0003	411.9	325.7	6430517E21Rik	NM_207583
10553477	1.265	0.0011	80.4	63.5	Ano5	NM_177694
10479274	1.263	0.0101	332.9	263.6	Cdh4	NM_009867
10416181	1.262	0.0046	273.6	216.7	Stc1	NM_009285
10378568	1.261	0.0337	123.0	97.5		---
10450069	1.260	0.0064	74.0	58.8	EG630499	NR_004446
10598612	1.259	0.0289	25.3	20.1	Otc	NM_008769
10394770	1.259	0.0216	1215.6	965.8	Odc1	NM_013614
10500710	1.258	0.0103	52.0	41.3	BC037703	BC037703
10441601	1.257	0.0330	76.3	60.7	Tagap	NM_145968
10492428	1.250	0.0032	397.7	318.1	Tiparp	NM_178892
10552594	1.247	0.0424	29.6	23.8	Klk1b22	NM_010114
10552604	1.246	0.0301	46.1	37.0	Klk1b24	NM_010643
10606583	1.245	0.0496	20.7	16.6	4932411N23Rik	BC117864
10423647	1.241	0.0355	206.6	166.5	Kcns2	NM_181317
10602772	1.241	0.0454	419.9	338.4	Rps6ka3	NM_148945
10566993	1.240	0.0027	217.3	175.3	Galntl4	NM_173739
10483546	1.238	0.0267	26.6	21.5		---

10509992	1.237	0.0188	60.1	48.6	Hspb7	NM_013868
10567564	1.236	0.0120	505.5	409.0	Cdr2	NM_007672
10523190	1.236	0.0006	493.5	399.4	9130213B05Rik	BC006604
10576054	1.234	0.0160	73.1	59.3	Foxl1	NM_008024
10517060	1.234	0.0443	763.9	619.2	Nudc	NM_010948
10358754	1.231	0.0448	291.9	237.1	EG639787	XR_034437
10438769	1.231	0.0222	82.5	67.0	Cldn1	NM_016674
10597076	1.231	0.0450	33.0	26.8	C85627	BC139081
10473528	1.230	0.0487	15.9	12.9	Olfr1120	NM_147029
10565391	1.230	0.0493	21.9	17.8	Olfr305	NM_146616
10392284	1.230	0.0379	582.6	473.8	Kpna2	NM_010655
10450762	1.228	0.0164	96.9	78.9	H2-M10.2	NM_177923
10467206	1.228	0.0497	434.6	354.0	Ppp1r3c	NM_016854
10369409	1.227	0.0415	55.8	45.5	1700125F08Rik	ENSMUST00000036304
10467489	1.225	0.0023	1523.5	1244.3	627166	NR_002686
10566219	1.222	0.0468	54.3	44.4	Olfr610	NM_147081
10589798	1.221	0.0036	167.0	136.7		---
10404975	1.221	0.0289	1838.0	1505.7	Id4	NM_031166
10538695	1.220	0.0088	17.1	14.0	EG434019	ENSMUST00000101355
10428839	1.219	0.0106	98.2	80.5	EG546638	ENSMUST00000075109
10418898	1.218	0.0056	57.0	46.8	Ppyr1	NM_008919
10469575	1.218	0.0010	1702.7	1398.1	OTTMUSG00000011595	NR_002688
10437684	1.216	0.0376	87.5	72.0	Prm1	NM_013637
10403943	1.216	0.0040	223.6	184.0	Hist1h2bm	NM_178200
10442219	1.215	0.0354	210.3	173.1	Zfp52	NM_144515
10581643	1.215	0.0076	87.1	71.7		---
10542875	1.215	0.0065	51.9	42.7	3010003L21Rik	BC106181
10347117	1.215	0.0256	62.3	51.2	Cps1	NM_001080809
10390974	1.215	0.0475	89.3	73.5	Krt34	NM_027563
10362939	1.213	0.0122	52.8	43.5	EG215974	XM_894477
10517731	1.212	0.0344	350.9	289.6	Igsf21	NM_198610
10391043	1.212	0.0353	44.1	36.4	Krt9	NM_201255
10601988	1.212	0.0057	23.1	19.0	Trap1a	NM_011635
10344620	1.211	0.0258	34.4	28.4	ENSMUSG00000073742	ENSMUST00000097833
10565067	1.211	0.0267	169.1	139.7	Nmb	NM_026523
10490611	1.209	0.0462	48.1	39.8	Ptk6	NM_009184
10465912	1.208	0.0077	161.2	133.4	Fen1	NM_007999

10549932	1.207	0.0190	187.9	155.7	2810047C21Rik	BC071238
10550986	1.206	0.0122	33.2	27.5	BC049730	BC049730
10395684	1.204	0.0299	110.7	92.0	Nubpl	NM_029760
10349637	1.204	0.0485	36.3	30.2	2700049P18Rik	BC138225
10456171	1.203	0.0114	124.1	103.2	Spink10	NM_177829
10428157	1.203	0.0037	630.9	524.3	Rnf19a	NM_013923
10385477	1.203	0.0136	90.0	74.8		---
10550998	1.202	0.0489	39.5	32.9	EG545936	BC100485
10576249	1.202	0.0216	193.4	160.9	4732415M23Rik	NM_177279
10600823	1.202	0.0340	39.9	33.2	LOC675747	ENSMUST00000116173
10540207	1.201	0.0183	43.0	35.8	A730049H05Rik	ENSMUST00000057977
10577508	1.201	0.0377	33.1	27.6	Ckap2	NM_001004140
10580829	-1.200	0.0027	187.2	224.6	Cngb1	BC045114
10477717	-1.201	0.0224	62.6	75.1	Procr	NM_011171
10530772	-1.201	0.0354	235.2	282.5	Nmu	NM_019515
10497935	-1.201	0.0215	9.1	10.9		---
10421934	-1.202	0.0230	278.5	334.7	Klhl1	NM_053105
10472034	-1.202	0.0170	238.2	286.3	Lypd6	NM_177139
10525923	-1.202	0.0159	263.5	316.7	Tmem132b	XM_915709
10427303	-1.204	0.0205	38.5	46.4	Hoxc4	NM_013553
10466344	-1.205	0.0214	55.9	67.3		---
10436750	-1.206	0.0226	59.8	72.1	EG546672	ENSMUST00000009191
10605113	-1.206	0.0311	869.9	1049.0	L1cam	NM_008478
10445758	-1.206	0.0271	46.2	55.8	Treml4	NM_001033922
10446312	-1.207	0.0151	207.2	250.1	Cntnap5c	NM_001081653
10602044	-1.207	0.0258	394.3	475.9	Frmpd3	NM_177750
10549388	-1.207	0.0269	68.0	82.1	Pthlh	NM_008970
10419854	-1.208	0.0033	387.6	468.1	Slc7a8	NM_016972
10368045	-1.208	0.0306	64.1	77.5	3110003A17Rik	NM_028440
10408146	-1.210	0.0411	27.9	33.7	V1rh9	NM_134218
10545212	-1.210	0.0443	18.3	22.2	ENSMUSG00000076563	ENSMUST00000103364
10485784	-1.211	0.0446	13.5	16.4	Olfr1297	NM_146888
10527963	-1.212	0.0273	27.4	33.2		---
10545886	-1.212	0.0155	119.6	145.0	1700019G17Rik	BC029200
10497613	-1.212	0.0363	30.4	36.8	EG545510	ENSMUST00000091270
10344897	-1.213	0.0048	389.8	473.0	Sulf1	NM_172294
10521759	-1.215	0.0241	713.9	867.3	Slit2	NM_178804

10537290	-1.216	0.0036	10.0	12.1		---
10576835	-1.220	0.0480	29.4	35.9	Cd209f	ENSMUST00000078702
10577349	-1.221	0.0261	31.9	38.9	Defb39	NM_183038
10436519	-1.223	0.0018	704.6	861.5	Robo1	NM_019413
10493867	-1.223	0.0491	38.1	46.6	Sprr2e	NM_011471
10467038	-1.224	0.0281	48.1	58.9	EG625995	BC096400
10537076	-1.226	0.0382	71.3	87.4		---
10484856	-1.226	0.0344	25.6	31.4	Olfr1259	NM_146341
10602688	-1.228	0.0226	279.9	343.7	LOC635253	ENSMUST00000095755
10578796	-1.228	0.0203	697.0	856.2	4930431L04Rik	BC111102
10377418	-1.232	0.0361	111.2	137.1	Tmem107	NM_028336
10498018	-1.233	0.0040	281.8	347.4	Pcdh18	NM_130448
10388234	-1.233	0.0084	18.8	23.1	Gsg2	NM_010353
10538658	-1.233	0.0084	944.7	1165.2	Herc3	NM_028705
10522827	-1.233	0.0064	19.6	24.1	Csn1s1	NM_007784
10597470	-1.234	0.0118	85.4	105.4	Cmtm8	NM_027294
10402394	-1.235	0.0098	35.2	43.5	Serpina1d	NM_009246
10568865	-1.236	0.0253	52.8	65.3	6430531B16Rik	BC145730
10392484	-1.237	0.0059	254.8	315.1	Abca8b	NM_013851
10462303	-1.237	0.0211	49.0	60.6	Kcnv2	NM_183179
10600988	-1.238	0.0001	46.9	58.1	Dgat2l3	NM_001081136
10438738	-1.239	0.0256	191.6	237.3	Bcl6	NM_009744
10496789	-1.239	0.0196	65.7	81.4	Lpar3	NM_022983
10378399	-1.239	0.0041	16.7	20.7	Olfr386	NM_207224
10453811	-1.240	0.0096	269.1	333.7	AK220484	NM_001083628
10485309	-1.240	0.0309	71.4	88.5	E530001K10Rik	ENSMUST00000099688
10440669	-1.241	0.0003	10.3	12.8	2310057N15Rik	BC104341
10499168	-1.242	0.0031	137.3	170.5	Kirrel	NM_130867
10523048	-1.242	0.0042	81.5	101.3	Npffr2	NM_133192
10351380	-1.243	0.0120	425.8	529.5	LOC100039795	ENSMUST00000111416
10470647	-1.246	0.0377	19.1	23.8		---
10355329	-1.246	0.0231	55.6	69.3	Bard1	NM_007525
10592289	-1.249	0.0178	73.9	92.2	Ccdc15	NM_001081429
10484701	-1.249	0.0135	11.4	14.3	Olfr1156	NM_146817
10427454	-1.251	0.0478	52.4	65.5	Card6	ENSMUST00000055038
10428453	-1.251	0.0197	651.7	815.1	Csmd3	NM_001081391
10511416	-1.253	0.0126	287.9	360.8	Tox	NM_145711

10548043	-1.253	0.0291	137.6	172.4	Kcna5	NM_145983
10409970	-1.253	0.0375	221.2	277.2	8430426H19Rik	NM_178875
10423230	-1.255	0.0097	174.3	218.7	Cdh9	NM_009869
10563728	-1.255	0.0018	28.9	36.3	EG435978	XM_884240
10469457	-1.256	0.0028	624.9	785.0	Plxdc2	NM_026162
10459671	-1.259	0.0121	531.8	669.6	Dcc	NM_007831
10420957	-1.259	0.0475	275.1	346.3	Ptk2b	NM_172498
10374704	-1.260	0.0020	19.0	23.9	1700030C12Rik	AK132720
10461840	-1.260	0.0103	21.5	27.1	Olfr1505	NM_001011850
10540359	-1.261	0.0218	367.8	463.9	Cntn4	NM_001109749
10464370	-1.265	0.0173	517.0	654.0	Slc18a2	NM_172523
10555894	-1.270	0.0342	20.6	26.1	Dub1	NM_007887
10423917	-1.271	0.0077	106.5	135.4		---
10439895	-1.282	0.0094	1126.6	1444.6	Alcam	NM_009655
10605616	-1.283	0.0019	316.8	406.6	Il1rapl1	BC119580
10505914	-1.290	0.0160	39.9	51.5	Zfp352	NM_153102
10358272	-1.291	0.0188	198.9	256.8	Lhx9	NM_001042577
10601927	-1.291	0.0324	110.9	143.2	Il1rapl2	NM_030688
10578794	-1.291	0.0231	396.5	512.0		---
10464905	-1.294	0.0333	143.4	185.6	Npas4	NM_153553
10452419	-1.301	0.0277	545.6	710.0	Efna5	NM_207654
10401002	-1.304	0.0026	32.9	42.9	Gphb5	NM_175644
10417517	-1.306	0.0098	53.2	69.5	ENSMUSG00000058570	ENSMUST00000081331
10553330	-1.307	0.0064	52.7	68.9	Mrgprb13	XM_884524
10501468	-1.317	0.0293	437.6	576.4	Ntng1	NM_030699
10499914	-1.319	0.0376	48.2	63.5	Lce1b	NM_026822
10406823	-1.321	0.0447	154.2	203.7		---
10401238	-1.325	0.0120	140.3	186.0	Zfp36l1	NM_007564
10559790	-1.327	0.0197	832.4	1104.3	Zim1	NM_011769
10407350	-1.341	0.0160	50.9	68.3	Fgf10	NM_008002
10473494	-1.342	0.0194	34.6	46.4	Olfr1034	NM_001011872
10537026	-1.348	0.0001	62.9	84.8	Cpa4	NM_027926
10596521	-1.351	0.0360	184.8	249.8	Grm2	BC115866
10603623	-1.359	0.0123	23.4	31.8		---
10578786	-1.364	0.0001	349.7	477.0	1700021K10Rik	AK006215
10569823	-1.365	0.0136	59.9	81.7	C330021F23Rik	BC089480
10444853	-1.378	0.0074	33.7	46.4	Pou5f1	NM_013633
10597592	-1.380	0.0174	50.6	69.8	Acaa1b	NM_146230
10518331	-1.381	0.0173	78.3	108.1		---
10486895	-1.398	0.0333	37.2	51.9	Mageb3	NM_008545
10440134	-1.399	0.0187	29.1	40.7	Olfr172	NM_147001

10419284	-1.401	0.0061	20.8	29.2	ENSMUSG00000061510	ENSMUST00000074862
10445022	-1.411	0.0457	22.3	31.4	H2-M10.5	NM_177637
10538519	-1.417	0.0013	196.9	279.0	Gsbs	NM_011153
10601993	-1.418	0.0028	167.6	237.6	D330045A20Rik	BC113128
10495878	-1.433	0.0035	446.7	640.3	Ndst4	NM_022565
10354644	-1.435	0.0095	293.0	420.3	EG627915	XM_892615
10415842	-1.455	0.0002	6.4	9.2		---
10367600	-1.580	0.0071	733.5	1158.9	Esr1	NM_007956
10371796	-1.598	0.0076	109.7	175.2	Slc17a8	NM_182959
10600892	-1.628	0.0009	227.3	370.1	Pgr15l	NM_001033361
10498965	-1.811	0.0069	405.0	733.5	Npy2r	NM_008731

Table 2.2 Gene Set Enrichment Analysis

Note: A value of 3 of the enrichment score corresponds to significant over expression (p-value <0.05)

Based on the genes with fold change >1.2x and p<0.05 using all samples

Function	GO ID	Enrichment Score	% genes in group present	# genes present	# genes in group
negative regulation of mitosis	45839	50.18	100	2	2
peptide YY receptor activity	1601	28.63	40	2	5
histidine decarboxylase activity	4398	26.01	100	1	1
spindle pole body	5816	26.01	100	1	1
negative regulation of mitotic metaphase/anaphase transition	45841	26.01	100	1	1
beta-galactoside alpha-2,3-sialyltransferase activity	3836	26.01	100	1	1
arsenite transmembrane transporter activity	15105	26.01	100	1	1
FasL biosynthetic process	45210	26.01	100	1	1
cellular monovalent inorganic anion homeostasis	30320	26.01	100	1	1
ectodermal cell fate commitment	1712	26.01	100	1	1
negative regulation of exocytosis	45920	26.01	100	1	1
negative regulation of calcium ion-dependent exocytosis	45955	26.01	100	1	1
flap endonuclease activity	48256	26.01	100	1	1
gluconokinase activity	46316	26.01	100	1	1
receptor signaling protein tyrosine phosphatase activity	4728	26.01	100	1	1
visceral mesoderm-endoderm interaction involved in midgut development	7495	26.01	100	1	1
positive regulation of urothelial cell proliferation	50677	26.01	100	1	1
gonad morphogenesis	35262	26.01	100	1	1
positive regulation of bone resorption	45780	26.01	100	1	1

positive regulation of bone remodeling	46852	26.01	100	1	1
negative regulation of mast cell cytokine production	32764	26.01	100	1	1
negative regulation of Rho protein signal transduction	35024	26.01	100	1	1
T-helper 2 type immune response	42092	26.01	100	1	1
regulation of memory T cell differentiation	43380	26.01	100	1	1
carbamoyl-phosphate synthase activity	4086	26.01	100	1	1
carbamoyl-phosphate synthase (ammonia) activity	4087	26.01	100	1	1
ornithine carbamoyltransferase activity	4585	26.01	100	1	1
ornithine carbamoyltransferase complex	9348	26.01	100	1	1
neuromedin U receptor binding	42922	26.01	100	1	1
homoiothermy	42309	26.01	100	1	1
stem cell factor receptor activity	5020	26.01	100	1	1
centrosome organization	51297	25.01	33.333	2	6
microtubule organizing center organization	31023	22.2	28.571	2	7
biogenic amine biosynthetic process	42401	21.52	17.647	3	17
neuropeptide receptor activity	8188	19.69	12.121	4	33
neuropeptide binding	42923	19.69	12.121	4	33
amino acid derivative biosynthetic process	42398	18.54	15	3	20
ovulation	30728	18.09	22.222	2	9
neuropeptide Y receptor activity	4983	18.09	22.222	2	9
urea cycle	50	18.09	22.222	2	9
urea metabolic process	19627	18.09	22.222	2	9
cellular amide metabolic process	43603	18.09	22.222	2	9
reproductive process in a multicellular organism	48609	17.99	9.0909	5	55

negative regulation of cell differentiation	45596	17.56	10.811	4	37
long-chain-alcohol O-fatty-acyltransferase activity	47196	17.53	50	1	2
progesterone receptor signaling pathway	50847	17.53	50	1	2
ovarian follicle rupture	1543	17.53	50	1	2
cholecystokinin receptor activity	4951	17.53	50	1	2
centrosome cycle	7098	17.53	50	1	2
orexin receptor activity	16499	17.53	50	1	2
ethanol catabolic process	6068	17.53	50	1	2
monohydric alcohol catabolic process	34310	17.53	50	1	2
tricarboxylic acid transport	6842	17.53	50	1	2
citrate transmembrane transporter activity	15137	17.53	50	1	2
tricarboxylic acid transmembrane transporter activity	15142	17.53	50	1	2
citrate transport	15746	17.53	50	1	2
C3a anaphylatoxin receptor activity	4943	17.53	50	1	2
fibril organization	43206	17.53	50	1	2
endodermal cell fate commitment	1711	17.53	50	1	2
germ-line stem cell maintenance	30718	17.53	50	1	2
spinal cord ventral commissure morphogenesis	21965	17.53	50	1	2
shikimate kinase activity	4765	17.53	50	1	2
growth hormone receptor binding	5131	17.53	50	1	2
chemoattractant activity	42056	17.53	50	1	2
regulation of receptor-mediated endocytosis	48259	17.53	50	1	2
positive regulation of receptor-mediated endocytosis	48260	17.53	50	1	2
ornithine decarboxylase activity	4586	17.53	50	1	2
follicle-stimulating hormone receptor activity	4963	17.53	50	1	2
regulation of mast cell cytokine	32763	17.53	50	1	2

production					
gamma-tubulin complex	930	17.53	50	1	2
beta-tubulin binding	48487	17.53	50	1	2
carboxyl- or carbamoyltransferase activity	16743	17.53	50	1	2
potassium channel regulator activity	15459	17.53	50	1	2
potassium channel inhibitor activity	19870	17.53	50	1	2
cell-cell adhesion mediated by integrin	33631	17.53	50	1	2
sialic acid binding	33691	17.53	50	1	2
interleukin-1, Type II, blocking receptor activity	4910	17.53	50	1	2
interleukin-1, Type II, blocking binding	19968	17.53	50	1	2
group II metabotropic glutamate receptor activity	1641	17.53	50	1	2
prolactin receptor binding	5148	17.53	50	1	2
positive regulation of JAK-STAT cascade	46427	17.53	50	1	2
signal complex assembly	7172	17.53	50	1	2
histidine catabolic process	6548	16.55	20	2	10
histidine family amino acid metabolic process	9075	16.55	20	2	10
histidine family amino acid catabolic process	9077	16.55	20	2	10
chemotaxis	6935	16.5	7.2289	6	83
taxis	42330	16.5	7.2289	6	83
catecholamine biosynthetic process	42423	15.24	18.182	2	11
heparan sulfate sulfotransferase activity	34483	15.24	18.182	2	11
axon guidance	7411	15.02	6.6667	6	90
detection of light stimulus involved in visual perception	50908	14.42	11.539	3	26
detection of light stimulus involved in sensory perception	50962	14.42	11.539	3	26
locomotion	40011	14.27	6.383	6	94
protein serine/threonine phosphatase inhibitor activity	4865	13.26	33.333	1	3
arylsulfatase activity	4065	13.26	33.333	1	3

N-acetylglucosamine-6-sulfatase activity	8449	13.26	33.333	1	3
pancreatic polypeptide receptor activity	1602	13.26	33.333	1	3
elastic fiber assembly	48251	13.26	33.333	1	3
fibroblast growth factor receptor binding	5104	13.26	33.333	1	3
prostaglandin-D synthase activity	4667	13.26	33.333	1	3
glycerol kinase activity	4370	13.26	33.333	1	3
negative regulation of cell-matrix adhesion	1953	13.26	33.333	1	3
negative regulation of T-helper 2 cell differentiation	45629	13.26	33.333	1	3
negative regulation of isotype switching to IgE isotypes	48294	13.26	33.333	1	3
negative regulation of astrocyte differentiation	48712	13.26	33.333	1	3
negative regulation of potassium ion transport	43267	13.26	33.333	1	3
leukocyte mediated immunity	2443	13.26	33.333	1	3
norepinephrine biosynthetic process	42421	13.26	33.333	1	3
maternal behavior	42711	13.26	33.333	1	3
positive regulation of vasoconstriction	45907	13.26	33.333	1	3
STAT protein nuclear translocation	7262	13.26	33.333	1	3
protein import into nucleus, translocation	60	12.27	14.286	2	14
response to amphetamine	1975	12.27	14.286	2	14
retinal ganglion cell axon guidance	31290	12.27	14.286	2	14
negative regulation of transport	51051	11.52	13.333	2	15
alveolus development	48286	11.52	13.333	2	15
lactation	7595	11.52	13.333	2	15
peptide binding	42277	11.29	7.1429	4	56
hormone receptor binding	51427	10.84	12.5	2	16
polypeptide N-acetylgalactosaminyltransferase activity	4653	10.84	12.5	2	16
auxiliary transport protein activity	15457	10.84	12.5	2	16

channel regulator activity	16247	10.84	12.5	2	16
presynaptic membrane	42734	10.84	12.5	2	16
histone deacetylase binding	42826	10.67	25	1	4
retinoic acid receptor binding	42974	10.67	25	1	4
retinoid X receptor binding	46965	10.67	25	1	4
mitotic cell cycle spindle assembly checkpoint	7094	10.67	25	1	4
spindle checkpoint	31577	10.67	25	1	4
axolemma	30673	10.67	25	1	4
alcohol dehydrogenase activity	4022	10.67	25	1	4
[heparan sulfate]-glucosamine N-sulfotransferase activity	15016	10.67	25	1	4
monovalent inorganic anion homeostasis	55083	10.67	25	1	4
endodeoxyribonuclease activity	4520	10.67	25	1	4
proteoglycan biosynthetic process	30166	10.67	25	1	4
positive regulation of vascular endothelial growth factor receptor signaling pathway	30949	10.67	25	1	4
polyamine biosynthetic process	6596	10.67	25	1	4
regulation of cytokine production during immune response	2718	10.67	25	1	4
regulation of T-helper 2 type immune response	2828	10.67	25	1	4
negative regulation of T-helper 2 type immune response	2829	10.67	25	1	4
negative regulation of cell-substrate adhesion	10812	10.67	25	1	4
surfactant homeostasis	43129	10.67	25	1	4
chemical homeostasis within a tissue	48875	10.67	25	1	4
chemorepellent activity	45499	10.67	25	1	4
cerebral cortex neuron differentiation	21895	10.67	25	1	4
arginine biosynthetic process	6526	10.67	25	1	4
cell adhesion mediated by integrin	33627	10.67	25	1	4

homotypic cell-cell adhesion	34109	10.67	25	1	4
negative regulation of synaptic transmission, glutamatergic	51967	10.67	25	1	4
dopamine beta-monooxygenase activity	4500	10.67	25	1	4
dopamine catabolic process	42420	10.67	25	1	4
catecholamine catabolic process	42424	10.67	25	1	4
behavioral response to ethanol	48149	10.67	25	1	4
myeloid progenitor cell differentiation	2318	10.67	25	1	4
germ cell programmed cell death	35234	10.67	25	1	4
axon part	33267	10.24	11.765	2	17
homophilic cell adhesion	7156	10.05	6.4516	4	62
intrinsic to membrane	31224	9.837	1.5023	78	5192
blood vessel remodeling	1974	9.69	11.111	2	18
response to organic nitrogen	10243	9.69	11.111	2	18
response to amine stimulus	14075	9.69	11.111	2	18
positive regulation of cell adhesion	45785	9.502	7.6923	3	39
axonogenesis	7409	9.349	6.0606	4	66
positive regulation of MAP kinase activity	43406	9.246	7.5	3	40
response to peptide hormone stimulus	43434	9.195	10.526	2	19
heterophilic cell adhesion	7157	9.195	10.526	2	19
fear response	42596	9.195	10.526	2	19
mitotic sister chromatid segregation	70	8.924	20	1	5
cell cycle checkpoint	75	8.924	20	1	5
sister chromatid segregation	819	8.924	20	1	5
anaphylatoxin receptor activity	4942	8.924	20	1	5
N-formyl peptide receptor activity	4982	8.924	20	1	5
collagen metabolic process	32963	8.924	20	1	5
mesodermal cell fate commitment	1710	8.924	20	1	5
positive regulation of axon extension	45773	8.924	20	1	5

[heparan sulfate]-glucosamine 3-sulfotransferase 1 activity	8467	8.924	20	1	5
nuclear-transcribed mRNA catabolic process, deadenylation-dependent decay	288	8.924	20	1	5
positive regulation of Wnt receptor signaling pathway	30177	8.924	20	1	5
regulation of vascular endothelial growth factor receptor signaling pathway	30947	8.924	20	1	5
bleb formation	32060	8.924	20	1	5
cerebellar Purkinje cell layer development	21680	8.924	20	1	5
germinal center formation	2467	8.924	20	1	5
regulation of T-helper 2 cell differentiation	45628	8.924	20	1	5
regulation of isotype switching to IgE isotypes	48293	8.924	20	1	5
negative regulation of chondrocyte differentiation	32331	8.924	20	1	5
regulation of astrocyte differentiation	48710	8.924	20	1	5
positive regulation of cell-cell adhesion	22409	8.924	20	1	5
oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, reduced ascorbate as one donor, and incorporation of one atom of oxygen	16715	8.924	20	1	5
developmental programmed cell death	10623	8.924	20	1	5
regulation of JAK-STAT cascade	46425	8.924	20	1	5
positive regulation of cellular process	48522	8.519	3.3457	9	269
membrane part	44425	8.384	1.4296	82	5736
protein tyrosine kinase activity	4713	8.352	4.1958	6	143
hormone activity	5179	8.236	4.6729	5	107

peptide receptor activity, G-protein coupled	8528	8.121	6.6667	3	45
integral to membrane	16021	8.069	1.4581	74	5075
neurite morphogenesis	48812	8.022	5.3333	4	75
cell surface	9986	8.022	5.3333	4	75
monosaccharide binding	48029	7.954	9.0909	2	22
response to hormone stimulus	9725	7.734	6.383	3	47
epithelial cell maturation	2070	7.669	16.667	1	6
glycoprotein biosynthetic process	9101	7.669	16.667	1	6
multicellular organismal macromolecule metabolic process	44259	7.669	16.667	1	6
L-glutamate transmembrane transporter activity	5313	7.669	16.667	1	6
L-glutamate transport	15813	7.669	16.667	1	6
MAP kinase kinase activity	4708	7.669	16.667	1	6
cellular carbohydrate biosynthetic process	34637	7.669	16.667	1	6
regulation of chemotaxis	50920	7.669	16.667	1	6
regulation of positive chemotaxis	50926	7.669	16.667	1	6
positive regulation of positive chemotaxis	50927	7.669	16.667	1	6
induction of positive chemotaxis	50930	7.669	16.667	1	6
female gonad development	8585	7.669	16.667	1	6
prostanoid metabolic process	6692	7.669	16.667	1	6
prostaglandin metabolic process	6693	7.669	16.667	1	6
calcium-independent cell-cell adhesion	16338	7.669	16.667	1	6
negative regulation of coagulation	50819	7.669	16.667	1	6
melanocortin receptor activity	4977	7.669	16.667	1	6
chromatin DNA binding	31490	7.669	16.667	1	6
regulation of T-helper cell differentiation	45622	7.669	16.667	1	6
negative regulation of Ras protein signal transduction	46580	7.669	16.667	1	6

negative regulation of small GTPase mediated signal transduction	51058	7.669	16.667	1	6
negative regulation of ion transport	43271	7.669	16.667	1	6
terminal button	43195	7.669	16.667	1	6
adenylate cyclase inhibiting metabotropic glutamate receptor activity	1640	7.669	16.667	1	6
regulation of synaptic transmission, glutamatergic	51966	7.669	16.667	1	6
reproductive behavior in a multicellular organism	33057	7.669	16.667	1	6
biogenic amine catabolic process	42402	7.669	16.667	1	6
response to estradiol stimulus	32355	7.669	16.667	1	6
phototransduction	7602	7.604	8.6957	2	23
L-amino acid transmembrane transporter activity	15179	7.604	8.6957	2	23
cell part morphogenesis	32990	7.293	4.9383	4	81
cell projection morphogenesis	48858	7.293	4.9383	4	81
cell motion	6928	7.205	3.0201	9	298
detection of stimulus involved in sensory perception	50906	7.044	5.8824	3	51
negative regulation of cell cycle	45786	6.759	4.6512	4	86
microtubule organizing center	5815	6.72	14.286	1	7
alcohol catabolic process	46164	6.72	14.286	1	7
excretion	7588	6.72	14.286	1	7
anion homeostasis	55081	6.72	14.286	1	7
fibrillar collagen	5583	6.72	14.286	1	7
negative regulation of secretion	51048	6.72	14.286	1	7
acidic amino acid transport	15800	6.72	14.286	1	7
regulation of mRNA stability	43488	6.72	14.286	1	7
glycerol-3-phosphate metabolic process	6072	6.72	14.286	1	7
response to insulin stimulus	32868	6.72	14.286	1	7
regulation of bone remodeling	46850	6.72	14.286	1	7
regulation of Rho GTPase activity	32319	6.72	14.286	1	7

positive regulation of cAMP biosynthetic process	30819	6.72	14.286	1	7
interleukin-1 binding	19966	6.72	14.286	1	7
lymphoid progenitor cell differentiation	2320	6.72	14.286	1	7
carboxy-lyase activity	16831	6.704	7.6923	2	26
extrinsic to membrane	19898	6.704	7.6923	2	26
cellular developmental process	48869	6.632	2.0492	20	976
regulation of protein kinase activity	45859	6.465	3.9063	5	128
nervous system development	7399	6.459	3.5088	6	171
carboxylic acid transmembrane transporter activity	46943	6.448	5.4546	3	55
pigmentation during development	48066	6.444	7.4074	2	27
organic acid transmembrane transporter activity	5342	6.311	5.3571	3	56
cell-cell adhesion	16337	6.292	3.4483	6	174
keratinocyte differentiation	30216	6.201	7.1429	2	28
cellular nitrogen compound metabolic process	34641	6.201	7.1429	2	28
regulation of kinase activity	43549	6.123	3.7594	5	133
detection of stimulus	51606	6.051	5.1724	3	58
adenylate kinase activity	4017	5.975	12.5	1	8
ovulation from ovarian follicle	1542	5.975	12.5	1	8
retinol metabolic process	42572	5.975	12.5	1	8
amine transmembrane transporter activity	5275	5.975	12.5	1	8
deoxyribonuclease activity	4536	5.975	12.5	1	8
calcium-dependent protein binding	48306	5.975	12.5	1	8
keratinocyte proliferation	43616	5.975	12.5	1	8
regulation of behavior	50795	5.975	12.5	1	8
prostaglandin biosynthetic process	1516	5.975	12.5	1	8
prostanoid biosynthetic process	46457	5.975	12.5	1	8
regulation of tissue remodeling	34103	5.975	12.5	1	8
regulation of immune effector process	2697	5.975	12.5	1	8

regulation of production of molecular mediator of immune response	2700	5.975	12.5	1	8
prefoldin complex	16272	5.975	12.5	1	8
osteoblast development	2076	5.975	12.5	1	8
protein deubiquitination	16579	5.975	12.5	1	8
regulation of smooth muscle contraction	6940	5.975	12.5	1	8
calcium channel regulator activity	5246	5.975	12.5	1	8
behavioral response to cocaine	48148	5.975	12.5	1	8
amino acid derivative catabolic process	42219	5.975	12.5	1	8
response to estrogen stimulus	43627	5.975	12.5	1	8
acetylgalactosaminyltransferase activity	8376	5.973	6.8966	2	29
regulation of transferase activity	51338	5.93	3.6765	5	136
voltage-gated potassium channel complex	8076	5.808	5	3	60
tissue remodeling	48771	5.76	6.6667	2	30
positive regulation of cell proliferation	8284	5.746	3.5971	5	139
germ cell development	7281	5.579	4.8387	3	62
cellular structure morphogenesis	32989	5.562	2.9412	7	238
mammary gland development	30879	5.559	6.4516	2	31
inorganic anion transmembrane transporter activity	15103	5.374	11.111	1	9
calcium-dependent cell-cell adhesion	16339	5.374	11.111	1	9
cellular component assembly	22607	5.374	11.111	1	9
protein serine/threonine/tyrosine kinase activity	4712	5.374	11.111	1	9
embryonic gut development	48566	5.374	11.111	1	9
smooth muscle cell differentiation	51145	5.374	11.111	1	9
glycogen biosynthetic process	5978	5.374	11.111	1	9
glucan biosynthetic process	9250	5.374	11.111	1	9
spermatid development	7286	5.369	6.25	2	32

response to organic substance	10033	5.364	4.6875	3	64
cytoplasmic vesicle	31410	5.266	3.0769	6	195
signal transducer activity	4871	5.204	1.5293	41	2681
molecular transducer activity	60089	5.204	1.5293	41	2681
regulation of Wnt receptor signaling pathway	30111	5.19	6.0606	2	33
glutamine family amino acid metabolic process	9064	5.19	6.0606	2	33
carboxylic acid transport	46942	5.161	4.5455	3	66
organic acid transport	15849	5.064	4.4776	3	67
locomotory behavior	7626	5.064	4.4776	3	67
membrane	16020	5.053	1.34	72	5373
receptor activity	4872	5.023	1.5441	38	2461
behavior	7610	4.979	3.7037	4	108
plasma membrane	5886	4.892	1.6278	30	1843
steroid hormone receptor signaling pathway	30518	4.879	10	1	10
photoreceptor cell maintenance	45494	4.879	10	1	10
retinol dehydrogenase activity	4745	4.879	10	1	10
melanin biosynthetic process	42438	4.879	10	1	10
cellular macromolecule biosynthetic process	34645	4.879	10	1	10
protein-hormone receptor activity	16500	4.879	10	1	10
erythrocyte development	48821	4.879	10	1	10
ubiquitin-specific protease activity	4843	4.879	10	1	10
nucleus organization	6997	4.879	10	1	10
regulation of pigmentation during development	48070	4.879	10	1	10
multicellular organismal response to stress	33555	4.86	5.7143	2	35
system development	48731	4.819	2.5478	8	314
negative regulation of multicellular organismal process	51241	4.708	5.5556	2	36
anatomical structure development	48856	4.631	1.8199	19	1044
potassium channel activity	5267	4.617	4.1667	3	72
regulation of cellular process	50794	4.615	1.3689	60	4383

heparin binding	8201	4.534	4.1096	3	73
L-amino acid transport	15807	4.463	9.0909	1	11
photoreceptor activity	9881	4.463	9.0909	1	11
protein-chromophore linkage	18298	4.463	9.0909	1	11
synaptic vesicle membrane	30672	4.463	9.0909	1	11
acrosome reaction	7340	4.463	9.0909	1	11
central nervous system projection neuron axonogenesis	21952	4.463	9.0909	1	11
negative regulation of multicellular organism growth	40015	4.463	9.0909	1	11
fibroblast growth factor receptor signaling pathway	8543	4.463	9.0909	1	11
lysosphingolipid and lysophosphatidic acid receptor activity	1619	4.463	9.0909	1	11
bioactive lipid receptor activity	45125	4.463	9.0909	1	11
cAMP-dependent protein kinase regulator activity	8603	4.463	9.0909	1	11
embryonic organ morphogenesis	48562	4.463	9.0909	1	11
regulation of cell-substrate adhesion	10810	4.463	9.0909	1	11
regulation of cAMP biosynthetic process	30817	4.463	9.0909	1	11
amino acid binding	16597	4.463	9.0909	1	11
anchored to plasma membrane	46658	4.463	9.0909	1	11
leukocyte adhesion	7159	4.463	9.0909	1	11
response to ethanol	45471	4.463	9.0909	1	11
hemopoietic progenitor cell differentiation	2244	4.463	9.0909	1	11
antigen binding	3823	4.463	9.0909	1	11
regulation of protein kinase cascade	10627	4.454	4.0541	3	74
regulation of epithelial cell proliferation	50678	4.425	5.2632	2	38
carbon-carbon lyase activity	16830	4.425	5.2632	2	38
non-membrane spanning protein tyrosine kinase activity	4715	4.425	5.2632	2	38

neuropeptide signaling pathway	7218	4.375	4	3	75
carbohydrate binding	30246	4.369	2.5641	7	273
anatomical structure homeostasis	60249	4.299	3.9474	3	76
regulation of neuron differentiation	45664	4.299	3.9474	3	76
anchored to membrane	31225	4.171	3.2787	4	122
steroid binding	5496	4.169	5	2	40
centrosome	5813	4.169	5	2	40
voltage-gated potassium channel activity	5249	4.152	3.8462	3	78
cell differentiation	30154	4.14	1.8797	15	798
lipid localization	10876	4.109	8.3333	1	12
lipid storage	19915	4.109	8.3333	1	12
sulfuric ester hydrolase activity	8484	4.109	8.3333	1	12
positive regulation of axonogenesis	50772	4.109	8.3333	1	12
bone mineralization	30282	4.109	8.3333	1	12
regulation of gliogenesis	14013	4.109	8.3333	1	12
regulation of glial cell differentiation	45685	4.109	8.3333	1	12
small conjugating protein-specific protease activity	19783	4.109	8.3333	1	12
response to cocaine	42220	4.109	8.3333	1	12
immune effector process	2252	4.109	8.3333	1	12
intracellular part	44424	4.009	0.8412	70	8321
receptor binding	5102	4.004	2.0561	11	535
amino acid transmembrane transporter activity	15171	3.936	4.7619	2	42
sulfotransferase activity	8146	3.936	4.7619	2	42
G-protein coupled receptor protein signaling pathway	7186	3.891	1.5825	26	1643
regulation of cell cycle	51726	3.87	2.7933	5	179
hydrolase activity	16787	3.826	0.5249	10	1905
neurotransmitter transport	6836	3.826	4.6512	2	43
positive regulation of protein kinase activity	45860	3.814	3.6145	3	83
nuclear hormone receptor binding	35257	3.802	7.6923	1	13
cellular amino acid and derivative metabolic process	6519	3.802	7.6923	1	13

NAD+ ADP-ribosyltransferase activity	3950	3.802	7.6923	1	13
collagen binding	5518	3.802	7.6923	1	13
coated vesicle membrane	30662	3.802	7.6923	1	13
clathrin coated vesicle membrane	30665	3.802	7.6923	1	13
motor axon guidance	8045	3.802	7.6923	1	13
regulation of isotype switching	45191	3.802	7.6923	1	13
glutamine metabolic process	6541	3.802	7.6923	1	13
endochondral ossification	1958	3.802	7.6923	1	13
regulation of cAMP metabolic process	30814	3.802	7.6923	1	13
neuroblast proliferation	7405	3.802	7.6923	1	13
regulation of calcium-mediated signaling	50848	3.802	7.6923	1	13
positive regulation of calcium-mediated signaling	50850	3.802	7.6923	1	13
mitotic chromosome condensation	7076	3.802	7.6923	1	13
response to stimulus	50896	3.723	1.5783	25	1584
transmembrane receptor activity	4888	3.691	1.5334	28	1826
pigmentation	43473	3.621	4.4444	2	45
response to chemical stimulus	42221	3.582	2.1028	9	428
visual perception	7601	3.571	3.4483	3	87
nucleotide kinase activity	19201	3.535	7.1429	1	14
interstitial matrix	5614	3.535	7.1429	1	14
retinoic acid metabolic process	42573	3.535	7.1429	1	14
protein amino acid ADP-ribosylation	6471	3.535	7.1429	1	14
trophectodermal cell differentiation	1829	3.535	7.1429	1	14
regulation of RNA stability	43487	3.535	7.1429	1	14
central nervous system neuron axonogenesis	21955	3.535	7.1429	1	14
positive regulation of neuron differentiation	45666	3.535	7.1429	1	14
induction of an organ	1759	3.535	7.1429	1	14
protein C-terminus binding	8022	3.535	7.1429	1	14
biomineral formation	31214	3.535	7.1429	1	14

glutamine family amino acid biosynthetic process	9084	3.535	7.1429	1	14
behavioral fear response	1662	3.535	7.1429	1	14
mannose binding	5537	3.535	7.1429	1	14
negative regulation of signal transduction	9968	3.525	4.3478	2	46
negative regulation of cell communication	10648	3.525	4.3478	2	46
G-protein coupled receptor activity	4930	3.523	1.5547	25	1608
sensory perception of light stimulus	50953	3.513	3.4091	3	88
regulation of catalytic activity	50790	3.459	2.2727	7	308
amino acid metabolic process	6520	3.446	2.8986	4	138
secretion	46903	3.433	4.2553	2	47
catalytic activity	3824	3.425	0.7517	34	4523
cell adhesion	7155	3.357	1.9724	10	507
biological adhesion	22610	3.357	1.9724	10	507
steroid hormone receptor activity	3707	3.344	4.1667	2	48
transferase activity, transferring sulfur-containing groups	16782	3.344	4.1667	2	48
epidermis development	8544	3.344	4.1667	2	48
multicellular organismal process	32501	3.336	1.5067	27	1792
regulation of cell proliferation	42127	3.328	2.8369	4	141
intracellular receptor-mediated signaling pathway	30522	3.299	6.6667	1	15
kinetochore	776	3.299	6.6667	1	15
neuropeptide hormone activity	5184	3.299	6.6667	1	15
pancreas development	31016	3.299	6.6667	1	15
temperature homeostasis	1659	3.299	6.6667	1	15
chromosome condensation	30261	3.299	6.6667	1	15
regulation of I-kappaB kinase/NF-kappaB cascade	43122	3.299	6.6667	1	15
response to steroid hormone stimulus	48545	3.299	6.6667	1	15
cellular polysaccharide biosynthetic process	33692	3.299	6.6667	1	15
developmental process	32502	3.262	1.4292	34	2379
ligand-dependent nuclear receptor activity	4879	3.258	4.0816	2	49

mitochondrion	5739	3.255	0.3398	3	883
regulation of signal transduction	9966	3.238	2.2013	7	318
regulation of cell communication	10646	3.217	2.1944	7	319
signal transduction	7165	3.194	1.3735	41	2985
melanocyte differentiation	30318	3.09	6.25	1	16
regulation of JUN kinase activity	43506	3.09	6.25	1	16
microtubule organizing center part	44450	3.09	6.25	1	16
regulation of nucleotide metabolic process	6140	3.09	6.25	1	16
regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	19219	3.09	6.25	1	16
regulation of cyclic nucleotide metabolic process	30799	3.09	6.25	1	16
regulation of muscle contraction	6937	3.09	6.25	1	16
associative learning	8306	3.09	6.25	1	16
leukocyte migration	50900	3.09	6.25	1	16
hydrolase activity, acting on acid anhydrides	16817	3.031	0.1835	1	545
hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	16818	3.018	0.1842	1	543

Table 2.3 Highest Expressed Transcripts

AFFY ID	Mean E2	Mean Oil	Gene Symbol
10593865	20185.1	20134.8	LOC236598
10400704	16194.3	15694.8	
10598025	15248.7	15673.1	
10445185	14860.6	15048.2	Pigt
10452415	14252.2	14201.9	100043560
10598069	12920.7	12986.3	CYTB
10598036	12852.8	13103.8	COX1
10353250	12687.3	12674.1	ENSMUSG00000073212
10411452	11329.5	11328.7	ENSMUSG00000070443
10426437	11127.9	11052.6	
10598067	10005.5	10181.8	ND5
10485357	9953.6	10004.1	
10466843	9752.1	9484.1	EG667806
10455780	9670.7	9625.9	EG433273
10498405	9561.4	9554.1	Gapdh
10595140	9525.6	9406.2	
10453451	9291.5	9487.4	Calm2
10422655	9267.7	9199.9	ENSMUSG00000072432
10587780	9121.8	9442.8	Tuba1b
10353630	9111.7	9075.1	COX2
10414313	8987.5	9082.0	Gm1821
10598043	8833.1	9210.1	ATP6
10584572	8669.3	8460.3	Hspa8
10409200	8305.8	8242.1	EG638833
10520390	8252.4	8233.0	ENSMUSG00000070408
10606538	7859.4	7992.9	Rpl30
10384493	7705.1	7405.1	
10553833	7670.5	7979.0	Ndn
10440467	7618.5	7621.2	EG545172
10598059	7540.8	7503.8	ND4L
10378848	7485.6	7606.9	Hsp90aa1
10546292	7472.6	7473.1	Rpl21
10451884	7457.6	7509.0	EG433125
10482507	7362.1	7338.7	Ppia
10398326	7327.5	7569.6	Meg3
10482432	7269.3	7195.6	LOC100044724
10363430	7203.1	7309.7	Psap
10567823	7170.7	7174.2	EG668319
10578545	7150.9	7167.2	ENSMUSG00000071102
10406417	7093.6	7212.6	Actg1
10463355	6996.4	7012.0	Scd2
10494662	6809.8	6545.6	Ywhah
10485654	6783.9	6594.7	Rpl10
10356778	6697.9	6855.8	
10569996	6696.1	6748.7	EG631359

10598055	6650.3	6453.7	ND3
10488415	6639.2	6750.1	Cst3
10397528	6620.4	6661.4	EG667287
10420986	6572.4	6401.5	EG668366
10531144	6569.2	6509.4	EG668319
10419469	6539.2	6408.6	
10360629	6512.1	6632.8	
10454039	6367.4	6341.9	Impact
10412665	6331.8	6423.7	EG382843
10415444	6299.4	6331.8	
10572146	6288.1	6195.0	Atp6v1b2
10554817	6287.6	6210.1	
10598626	6282.4	6813.6	Tspan7
10535381	6280.7	6094.1	Actb
10493891	6216.1	6261.5	Ywhaz
10601567	6179.6	6197.6	EG545741
10369210	6176.6	6088.6	Serinc1
10422161	6155.2	6193.3	Mycbp2
10425903	6148.0	6148.8	100043084
10477004	6096.2	6275.9	LOC100044416
10548246	5996.1	6075.6	EG667610
10465244	5981.5	5702.6	Malat1
10361710	5876.3	5822.4	EG382450
10531931	5824.0	5813.9	Sparcl1
10474239	5651.4	5543.2	A930018P22Rik
10414661	5589.5	5630.3	EG667348
10363699	5584.1	5624.5	Rps6
10578904	5547.8	5673.0	Cpe
10535577	5504.9	5843.0	Tmem130
10414431	5482.1	5446.8	EG624367
10451110	5464.6	5489.7	Hsp90ab1
10529873	5461.2	5384.5	Rab2a
10549653	5427.2	5432.5	Atp6v0c
10432404	5425.7	5627.2	Tuba1a
10461402	5396.8	5687.2	Fth1
10568050	5362.5	5420.8	Aldoa
10571815	5355.9	5713.7	Gpm6a
10554701	5315.9	5251.1	Hnrnpk
10463153	5275.9	5288.3	Morf4l1
10489049	5245.6	5512.2	Rpl9
10374453	5234.7	5032.3	Glul
10456974	5118.8	5094.8	Arf1
10493243	5104.7	5071.9	
10584122	5103.6	5027.8	666622
10511069	5094.4	5118.8	Gnb1
10363905	5056.8	5110.3	Zwint
10543317	5031.2	5088.8	
10410970	5029.2	4939.3	100042959

10392251	5011.4	4916.1	Ddx5
10448182	4911.7	5328.1	
10453373	4894.3	5165.5	Prepl
10381115	4872.0	4746.3	Eif1
10513737	4839.0	5050.8	Rpl17
10564159	4787.6	4731.2	
10456891	4782.7	4726.0	Atp5a1
10564183	4780.7	4742.7	
10511865	4778.7	4747.3	Ptges3
10379153	4774.4	4613.7	Aldoc
10419578	4762.2	4742.7	Ndrg2
10560624	4747.3	4815.9	Apoe
10386058	4720.4	4953.4	Sparc
10479996	4716.8	4618.8	Atp5c1
10465686	4707.4	4836.7	Rtn3
10440491	4638.0	4713.9	App
10537909	4637.7	4444.5	
10406939	4634.8	4625.8	OTTMUSG00000013242
10406499	4615.0	4687.8	EG667230
10605349	4614.0	4602.2	Ube2d3
10469772	4602.2	4630.0	OTTMUSG00000011467
10593490	4597.4	4543.5	EG629557
10522208	4593.6	4656.7	Uchl1
10595183	4569.4	4489.1	Eef1a1
10564161	4566.3	4644.5	Snord116
10537244	4524.1	4644.2	
10473240	4520.9	4176.0	Eno1
10559261	4463.3	4360.0	Cd81
10388042	4424.2	4631.0	6330403K07Rik
10389526	4398.2	4495.3	Cltc
10385599	4365.1	4405.8	Canx
10401695	4361.2	4292.2	ENSMUSG00000066443
10578539	4334.0	4099.4	Slc25a4
10598027	4333.1	4124.5	
10352457	4331.3	4413.2	EG433387
10548116	4321.2	4021.2	Ccnd2
10420988	4316.4	4416.8	Dpysl2
10533945	4281.8	4286.0	Ubc
10416187	4267.6	4264.0	
10362005	4258.7	4427.9	Ahi1
10564165	4243.4	4231.0	
10570000	4225.5	4197.7	
10375926	4224.9	4138.2	Ppp2ca
10434733	4127.9	3955.7	Eif4a2
10600390	4100.0	3995.9	Gdi1
10384150	4048.0	4094.0	Purb
10373498	4039.3	3856.6	Rps26
10584777	3979.9	3925.6	Ddx6

10383088	3968.3	4248.4	Gaa
10564169	3957.6	3976.8	
10365637	3954.3	3628.1	Arl1
10406278	3943.1	3928.1	Rps2
10547151	3938.1	4080.7	Rpl27a
10454805	3928.3	4037.6	Uba52
10601888	3925.6	3570.2	Plp1
10488020	3919.1	3939.8	Txndc13
10372324	3878.5	3906.3	Syt1
10499431	3868.6	3908.0	Syt11
10594320	3860.0	3811.4	ENSMUSG00000074250
10427241	3842.4	3908.0	Pcbp2
10531286	3841.6	3775.6	Vdac2
10410772	3835.5	3799.3	EG268676
10603649	3826.2	3674.7	
10549375	3811.7	3673.7	ENSMUSG00000059775
10447354	3808.0	3704.6	Txndc14
10563913	3787.9	3827.5	
10563917	3787.9	3827.5	
10563923	3787.9	3827.5	100040985
10505526	3786.9	3751.6	
10467842	3782.7	3490.0	Got1
10545041	3768.0	3457.5	Nap1l5
10540822	3739.9	3749.8	Slc6a11
10386388	3734.5	3758.7	1110031B06Rik
10411519	3732.4	3865.1	Mtap1b
10437080	3730.4	3750.6	Ttc3
10347917	3722.4	3733.5	Rpl19
10595046	3721.1	3630.9	100042107
10391963	3718.0	3851.2	Nsf
10447490	3682.6	3617.8	Pja2
10564001	3676.5	3614.3	AF357427
10564035	3676.5	3614.3	
10564059	3676.5	3614.3	
10564041	3673.7	3603.3	
10601834	3663.5	3631.9	Gprasp2
10439566	3653.1	3511.3	Atp6v1a
10469672	3636.7	3404.4	Gad2
10533483	3613.5	3594.1	Atp2a2
10564211	3568.2	3536.0	Snrpn
10564023	3565.0	3542.9	
10385572	3553.7	3595.3	Sqstm1
10345423	3532.8	3577.2	Plekhb2
10367106	3529.1	3458.4	Atp5b
10442155	3518.1	3690.0	Ppp2r1a
10347216	3504.3	3581.9	EG433319
10594248	3504.0	3570.7	Rplp1
10359689	3503.3	3519.6	Atp1b1

10516209	3496.3	3490.7	
10397752	3496.0	3443.4	Calm1
10400470	3493.6	3561.6	Cox6c
10560919	3488.3	3685.1	Atp1a3
10363178	3472.4	3336.7	Npm1
10603833	3459.4	3518.4	Usmg5
10466402	3457.7	3202.1	Eif4a1
10484318	3446.7	3386.3	Nckap1
10433445	3441.9	3267.8	Abat
10564137	3431.4	3395.5	
10526381	3412.7	3341.1	Mdh2
10384522	3409.4	3211.7	Actr2
10501661	3401.1	3286.4	Sfrs3
10578613	3396.4	3587.8	Rps16
10411393	3388.9	3331.6	Rps18
10454411	3384.7	3298.3	Hnrnpa1
10517682	3384.0	3113.5	2310028O11Rik
10564027	3383.7	3337.4	
10564089	3383.3	3332.1	
10585932	3380.7	3389.8	Pkm2
10402708	3373.4	3023.3	Ckb
10598359	3371.6	3627.3	Syp
10363415	3371.6	3446.7	Spock2
10506643	3371.6	3455.1	Tmem59
10592023	3363.4	3401.8	Aplp2
10559796	3362.5	3568.2	Peg3
10564033	3349.9	3306.3	
10515519	3320.1	3152.8	Atp6v0b
10547807	3294.9	3281.9	Eno2
10378739	3289.6	3335.3	Ywhae
10457929	3279.6	3328.6	Rit2
10454809	3272.3	3313.2	Matr3
10399407	3256.5	2990.1	Vsnl1
10529656	3256.3	3355.0	Nsg1
10385297	3255.4	3397.6	Gabra1
10354404	3245.4	3184.6	Dnajb6
10478424	3236.7	3487.1	Ywhab
10600886	3223.7	3553.7	Gpr165
10546054	3222.8	3364.6	Rpl3
10432492	3210.5	3323.1	Faim2
10490250	3201.9	3206.1	3100002L24Rik
10541089	3197.2	3137.9	EG640370
10408359	3194.1	3323.8	Nrsn1
10347036	3193.7	3165.2	Mtap2
10395737	3182.8	3124.5	EG665251
10371482	3174.9	3198.5	Hsp90b1
10471909	3164.4	2882.7	
10424413	3143.6	2982.9	EG432959

10564021	3140.5	3051.9	
10564025	3140.5	3051.9	
10563993	3140.5	3051.9	
10455238	3136.0	3123.6	Ndfip1
10427807	3135.5	3322.4	Sub1
10445239	3130.1	3108.9	EG546797
10577412	3121.4	3231.3	6820431F20Rik
10555055	3120.4	3134.7	Ndufc2
10579996	3120.2	3103.5	Gpsn2
10416057	3104.6	3236.7	Clu
10513818	3095.6	3351.5	Stmn1
10582888	3094.9	2851.7	
10598723	3091.1	3079.8	Ddx3x
10410625	3075.3	2958.4	Sdha
10521587	3061.5	3011.2	Dnaja1
10376245	3056.6	2917.1	Gria1
10347980	3051.1	2935.7	Itm2c
10392930	3014.3	3034.8	Atp5h
10600377	2976.3	2842.8	Atp6ap1
10605766	2967.4	2814.0	Maged1
10400926	2955.3	2998.9	Rtn1
10607302	2946.7	2972.2	Gnl3l
10364990	2945.7	2935.1	Eef2
10360544	2937.1	2859.8	Hnrnpu
10472378	2934.1	2792.8	Scn2a1
10384603	2929.2	2956.8	Mdh1
10538857	2926.8	2794.9	Serbp1
10426557	2926.0	3036.1	Pfkm
10564069	2923.3	2850.7	
10428020	2921.3	3074.8	6-Mar
10599855	2917.3	2853.1	Eif4e
10425757	2916.5	2804.4	1500032L24Rik
10502732	2909.0	2978.4	Prkacb
10563935	2906.0	2827.7	
10564135	2902.9	2866.2	
10564143	2901.5	2869.3	
10437992	2894.9	2803.5	Dnm1l
10360270	2888.9	2807.8	Atp1a2
10506488	2882.1	3056.8	Ppap2b
10569319	2881.1	3007.4	Ctsd
10374466	2876.1	2934.9	Rab1
10582658	2870.1	2977.1	Agt
10414093	2869.1	2895.3	Glud1
10567219	2864.8	2915.0	Arl6ip1
10381187	2860.4	3038.0	Atp6v0a1
10591747	2850.7	2739.5	Rpl15
10481711	2846.6	2938.4	Stxbp1
10345504	2844.8	2729.3	Cox5b

10564019	2843.6	2827.5	
10490818	2835.3	3127.9	Stmn2
10483604	2830.4	2772.7	Slc25a12
10356999	2830.4	2822.8	Prdx2
10599627	2827.3	2630.8	Hprt1
10421768	2825.1	2811.6	Akap11
10607391	2814.6	2920.7	Rps7
10490259	2803.7	2906.0	100043387
10490262	2803.7	2906.0	OTTMUSG00000016611
10434384	2801.1	2918.7	Ap2m1
10426751	2798.8	3101.0	Tegt
10590972	2792.0	2731.0	Mif
10564043	2783.3	2655.2	
10598678	2782.2	2859.8	Usp9x
10578916	2781.2	2588.7	Sc4mol
10560304	2773.3	2758.0	Calm3
10600593	2772.9	2568.8	Hnrnpa3
10561927	2771.8	2976.5	Aplp1
10395788	2771.4	2656.7	Srp54c
10388938	2770.8	2879.1	Wsb1
10584350	2767.6	2886.5	Tpt1
10382284	2759.7	2599.8	Prkar1a
10477630	2757.2	2725.7	Dynlrb1
10436783	2754.0	2723.6	Sod1
10458841	2754.0	2958.0	100042241
10375121	2752.8	2932.7	C530030P08Rik
10598029	2744.8	2643.1	ND1
10487629	2738.4	2602.7	Idh3b
10545417	2731.9	2779.1	Mat2a
10452639	2726.4	2739.1	Mylc2b

2.6 Figures

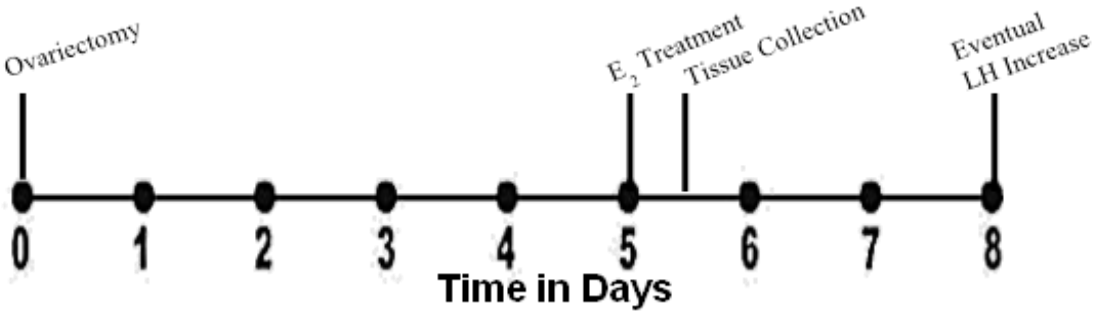


Figure 2.1 Experimental Design

Figure 2.2 AVPV Micropunch

Figure 2.2 300µm coronal section with AVPV excised. Dotted line indicates site of 1mm microdissection of AVPV.

2.7 References

Blutstein T, Devidze N, Choleris E, Jasnow AM, Pfaff DW, Mong JA (2006) Oestradiol up-regulates glutamine synthetase mRNA and protein expression in the hypothalamus and hippocampus: implications for a role of hormonally responsive glia in amino acid neurotransmission. Journal of neuroendocrinology 18:692-702.

Calizo LH, Flanagan-Cato LM (2003) Hormonal-neural integration in the female rat ventromedial hypothalamus: triple labeling for estrogen receptor-alpha, retrograde tract tracing from the periaqueductal gray, and mating-induced Fos expression. Endocrinology 144:5430-5440.

Chakraborty TR, Rajendren G, Gore AC (2005) Expression of estrogen receptor {alpha} in the anteroventral periventricular nucleus of hypogonadal mice. Experimental biology and medicine (Maywood, NJ 230:49-56.

Dalman MR, Deeter A, Nimishakavi G, Duan ZH (2012) Fold change and p-value cutoffs significantly alter microarray interpretations. BMC bioinformatics 13 Suppl 2:S11.

Davis EC, Shryne JE, Gorski RA (1996) Structural sexual dimorphisms in the anteroventral periventricular nucleus of the rat hypothalamus are sensitive to gonadal steroids perinatally, but develop peripubertally. Neuroendocrinology 63:142-148.

de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E (2003) Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. Proceedings of the National Academy of Sciences of the United States of America 100:10972-10976.

Dellovade TL, Merchenthaler I (2004) Estrogen regulation of neurokinin B gene expression in the mouse arcuate nucleus is mediated by estrogen receptor alpha. Endocrinology 145:736-742.

Felty Q, Singh KP, Roy D (2005) Estrogen-induced G1/S transition of G0-arrested estrogen-dependent breast cancer cells is regulated by mitochondrial oxidant signaling. Oncogene 24:4883-4893.

Flanagan-Cato LM, Calizo LH, Daniels D (2001) The synaptic organization of VMH neurons that mediate the effects of estrogen on sexual behavior. Hormones and behavior 40:178-182.

Gorski RA (1985) Sexual dimorphisms of the brain. Journal of animal science 61 Suppl 3:38-61.

Kalra SP (1993) Mandatory neuropeptide-steroid signaling for the preovulatory luteinizing hormone-releasing hormone discharge. Endocrine reviews 14:507-538.

Micevych PE, Kelly MJ (2012) Membrane estrogen receptor regulation of hypothalamic function. Neuroendocrinology 96:103-110.

Mitra SW, Hoskin E, Yudkovitz J, Pear L, Wilkinson HA, Hayashi S, Pfaff DW, Ogawa S, Rohrer SP, Schaeffer JM, McEwen BS, Alves SE (2003) Immunolocalization of estrogen receptor beta in the mouse brain: comparison with estrogen receptor alpha. Endocrinology 144:2055-2067.

Ottem EN, Godwin JG, Krishnan S, Petersen SL (2004) Dual-phenotype GABA/glutamate neurons in adult preoptic area: sexual dimorphism and function. J Neurosci 24:8097-8105.

Petersen SL, Ottem EN, Carpenter CD (2003) Direct and indirect regulation of gonadotropin-releasing hormone neurons by estradiol. Biology of reproduction 69:1771-1778.

Polston EK, Gu G, Simerly RB (2004) Neurons in the principal nucleus of the bed nuclei of the stria terminalis provide a sexually dimorphic GABAergic input to the anteroventral periventricular nucleus of the hypothalamus. Neuroscience 123:793-803.

Rosas-Arellano MP, Solano-Flores LP, Ciriello J (1999) Co-localization of estrogen and angiotensin receptors within subfornical organ neurons. Brain Res 837:254-262.

Sakakibara M, Uenoyama Y, Minabe S, Watanabe Y, Deura C, Nakamura S, Suzuki G, Maeda K, Tsukamura H (2013) Microarray analysis of perinatal-estrogen-induced changes in gene expression related to brain sexual differentiation in mice. PloS one 8:e79437.

Scordalakes EM, Shetty SJ, Rissman EF (2002) Roles of estrogen receptor alpha and androgen receptor in the regulation of neuronal nitric oxide synthase. The Journal of comparative neurology 453:336-344.

Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno JS, Jr., Shagoury JK, Bo-Abbas Y, Kuohung W, Schwinof KM, Hendrick AG, Zahn D, Dixon J, Kaiser UB, Slaugenhaupt SA, Gusella JF, O'Rahilly S, Carlton MB, Crowley WF, Jr., Aparicio SA, Colledge WH (2003) The GPR54 gene as a regulator of puberty. The New England journal of medicine 349:1614-1627.

Shughrue PJ, Bushnell CD, Dorsa DM (1992) Estrogen receptor messenger ribonucleic acid in female rat brain during the estrous cycle: a comparison with ovariectomized females and intact males. Endocrinology 131:381-388.

Simerly RB (1998) Organization and regulation of sexually dimorphic neuroendocrine pathways. Behavioural brain research 92:195-203.

Simerly RB, Carr AM, Zee MC, Lorang D (1996) Ovarian steroid regulation of estrogen and progesterone receptor messenger ribonucleic acid in the anteroventral periventricular nucleus of the rat. Journal of neuroendocrinology 8:45-56.

Simonian SX, Herbison AE (1997) Differential expression of estrogen receptor alpha and beta immunoreactivity by oxytocin neurons of rat paraventricular nucleus. Journal of neuroendocrinology 9:803-806.

Simonian SX, Spratt DP, Herbison AE (1999) Identification and characterization of estrogen receptor alpha-containing neurons projecting to the vicinity of the gonadotropin-releasing hormone perikarya in the rostral preoptic area of the rat. The Journal of comparative neurology 411:346-358.

Somponpun SJ, Johnson AK, Beltz T, Sladek CD (2004) Estrogen receptor-alpha expression in osmosensitive elements of the lamina terminalis: regulation by hypertonicity. American journal of physiology Regulatory, integrative and comparative physiology 287:R661-669.

Tsukahara S (2009) Sex differences and the roles of sex steroids in apoptosis of sexually dimorphic nuclei of the preoptic area in postnatal rats. Journal of neuroendocrinology 21:370-376.

Vida B, Deli L, Hrabovszky E, Kalamatianos T, Caraty A, Coen CW, Liposits Z, Kallo I (2010) Evidence for suprachiasmatic vasopressin neurones innervating kisspeptin neurones in the rostral periventricular area of the mouse brain: regulation by oestrogen. Journal of neuroendocrinology 22:1032-1039.

Watson RE, Jr., Langub MC, Jr., Engle MG, Maley BE (1995) Estrogen-receptive neurons in the anteroventral periventricular nucleus are synaptic targets of the suprachiasmatic nucleus and peri-suprachiasmatic region. Brain Res 689:254-264.

Wintermantel TM, Campbell RE, Porteous R, Bock D, Grone HJ, Todman MG, Korach KS, Greiner E, Perez CA, Schutz G, Herbison AE (2006) Definition of estrogen receptor pathway critical for estrogen positive feedback to gonadotropin-releasing hormone neurons and fertility. Neuron 52:271-280.

Xu Q, Hamada T, Kiyama R, Sakuma Y, Wada-Kiyama Y (2008) Site-specific regulation of gene expression by estrogen in the hypothalamus of adult female rats. Neuroscience letters 436:35-39.

CHAPTER 3

IN VIVO VALIDATION OF MICROARRAY-IDENTIFIED GENE TARGETS OF 17β-ESTRADIOL IN THE ANTEROVENTRAL PERIVENTRICULAR NUCLEUS OF THE FEMALE MOUSE

3.1 Introduction

The Affymetrix array uses multiple probes to identify a single gene. Unfortunately, after inputting several of their array probe sequences into the Basic Local Alignment Search Tool (BLAST) provided by the National Center for Biotechnology Information (NCBI), I discovered that some of their probes recognize conserved regions of superfamilies. Although it requires multiple hits for a gene to be designated as regulated, this can still lead to false-positives being identified on the array. Considering microarray data are routinely used to identify novel pathways based on the enrichment of genes that share common functions and known interactions (Curtis et al., 2005), false-positives represent a caveat of microarrays that should not be ignored (Pawitan et al., 2005, Cheng and Pounds, 2007). Conversely, pooling samples is sometimes considered favorable for equalizing variability (Allison et al., 2006). Still, before fully interpreting the microarray findings, it is first necessary to determine which differentially regulated transcripts are valid (Morey et al., 2006). To suss this out, I applied multiple levels of stringency to the data set provided by the Keck Institute and performed quantitative

reverse transcription polymerase chain reaction (QPCR) assays with primers specific to each transcript.

3.2 Materials and Methods

3.2.1 Animals

All protocols were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts and all animals were housed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Eight-week-old female C57Bl/6 mice (Jackson Labs; Bar Harbor, ME) were housed four to a cage in a temperature- and light-controlled room (12:12 light/dark cycle), with standard feed and water provided *ad libitum.* After a minimum of 48 h post-arrival, all mice were bilaterally ovariectomized under isofluorane anesthesia. Five days later, mice were injected subcutaneously with sesame oil vehicle or 0.05 μg/g b.w. E_2 dissolved in sesame oil. Twelve hours later, animals were anesthetized with CO_2, brains were collected, rapidly frozen on powdered dry ice, wrapped in Parafilm™ (Pechiney Plastic Packaging Company; Chicago, IL) and stored at -80°C in cryotubes.

3.2.2 Tissue Preparation and RNA Isolation

Brains were allowed to thaw slowly at -20°C, then coronally cryosectioned at 12 μm using a Leica CM3000 cryostat (Nussloch, Germany), until the early AVPV was reached. The early AVPV was determined by the appearance of the optic recess. I took a 300-μm coronal section and immediately excised the AVPV from it using a 1-mm circular Harris Uni-Core™ stainless steel tissue micropunch needle (Ted Pella Inc.; Redding, CA). I transferred the micropunched tissue to a 1.5-ml microcentrifuge tube, on

powdered dry ice. To obtain enough starting material, I pooled four AVPV micropunches to make one sample.

Total RNA was isolated from each pool using Trizol™ (Invitrogen; Carlsbad, CA) and Qiagen RNeasy Lipid kit (Qiagen; Valencia, CA). Sample concentration was determined via Nanodrop™ (Thermo Scientific; Wilmington, DE) and quality was verified using the Agilent 2100 Bioanalyzer® and RNA 6000 Nano LabChips (Agilent Technologies; Santa Clara, CA). Samples with 260/280 readings ≥1.4 and 260/230 readings ≥ 1.0 were deemed acceptable.

3.2.3 Gene Selection Criteria for Further Testing

The expression analysis performed by the Keck Institute identified 269 differentially regulated transcripts, comprised of transcripts having both a minimum fold-change ≥1.2 with a *p*-value ≤0.05 (see Chapter 2, Table 2.6.1). I increased the stringency of the fold-change lower limit to ≥1.4 for increased transcripts and ≤-1.5 for decreased transcripts (Morey et al., 2006). I only included transcripts having a mean raw fluorescence ≥75. Furthermore, I only included the two highest increased unannotated transcripts that remained within the list.

3.2.4 Quantitative Reverse-Transcription PCR (QPCR)

One μg total RNA was reverse transcribed into cDNA using QuantiTect Reverse Transcriptase Kit (Roche Diagnostics, Indianapolis, IN), following the manufacturer's protocol. I used Primer3™ software (http://bioinfo.ut.ee/primer3/) to design specific QPCR primers for: C1ql2, Creb3l1, Ensmusg00000022845, Ensmusg00000056615, Esr1, Ets2, Gadd45a, Hdc, Mad2l1, Npy2r, Nrip1, Pdzrn3, Pgr, Pgr15l1, Rasd1, Slc17a8 and

Slitrk6 (Table 3.5.1). I obtained the primers from Integrated DNA Technologies (Coralville, IA).

QPCR reactions were carried out in a Stratagene MX3000P™ thermocycler, utilizing MxPro™QPCR software (both Agilent Technologies; Santa Clara, CA). Reactions contained cDNA, diluted 1:10 with nuclease-free water, specific primers and SybrGreen™ QPCR Mastermix (Roche Diagnostics Corporation; Indianapolis, IN). Manufacturer's protocol was used with the following cycle settings: 95°C for 10 min, and 40 cycles of 95°C for 30 sec, 57°C for 30 sec and 72°C for 30 sec. Each sample was tested in duplicate. Primer specificity was verified via 2% agarose gel electrophoresis and confirmation of a single dissociative curve peak during each QPCR reaction.

3.2.5 Statistics

For QPCR, the duplicate raw cycle threshold (Ct) values were analyzed using the ΔΔCt method (Livak and Schmittgen, 2001), with β-actin employed as background control. Known E_2-induced transcripts within the AVPV, Esr1 and Kiss1, were used as positive treatment controls. QPCR reactions with nuclease-free water instead of cDNA were used as negative controls. I used Graphpad Prism™ to perform t-test with Welch's correction.

3.3 Results

3.3.1 QPCR Validation of Transcripts Increased by E_2 in the Female Mouse AVPV

By only including genes that met a higher fold-change cutoff and a minimum mean raw fluorescence ≥75, the gene list was reduced to 21 transcripts. This includes 17 increased transcripts, two of which are not yet annotated, and 4 decreased genes. Of the 17 E_2-induced transcripts selected, I was unable to either confirm or refute the changes in

Hcrtr1, Hs3st5, Mc4r or Pdzrn4, as the primers lacked specificity. Eleven of the remaining 13 transcripts were positively validated by QPCR (Table 3.5.2). The increase in *pgr* expression mirrors that of previous studies, and thus served as an internal positive treatment control (Simerly et al., 1996).

3.3.2 QPCR Validation of Transcripts Decreased by E_2 in the Female Mouse AVPV

All four of the E_2-dependent decreased transcripts positively validated via QPCR (Table 3.5.2). Three of these, Slc17a8, Pgr15l and Npy2r are novel E_2 targets within the AVPV.

3.4 Discussion

I was able to positively validate 88% of the transcripts tested. I attribute this level of validation to creating a careful and reproducible method for extracting the AVPV from surrounding brain nuclei. It has been noted that although there is greater variability in the capacity of a microarray to determine actual levels of expression, there is more confidence in their ability to determine ratios of expression (fold-change) (Beckman et al., 2004). Nevertheless, few of the transcripts validated fell in line with the fold-change values identified on the array (Table 3.5.2). This is supportive of the concept that microarrays are very useful in identifying robustly regulated transcripts; however further validation by more sensitive methods such as QPCR are warranted for more subtly regulated transcripts (Morey et al., 2006). In the case of the two transcripts that did not validate, there was a high amount of variability; therefore it may be necessary to increase the sample number in order to reach significance.

At this time, it is not impossible to draw conclusions about the expression of *pdzrn4*, *hcrtr1*, *hs3st5* and *mc4r*. Although multiple annealing temperatures were tested

during QPCR experiments, none provided a smooth and distinct dissociation curve, indicating a lack of primer specificity. This could mean that the primers were having off-target effects or there are perhaps yet unidentified splice variants of the transcripts, in either case, further testing is warranted. This is especially important considering my previous analysis using Ariadne™ Pathway software identified feeding and drinking behavior as a significantly regulated pathway (see Chapter 2), and both *hcrtr1* and *mc4r* fall within that gene set.

My QPCR validation of E_2-induced *mad2l1* (formerly referred to as *mad2*) expression, the most increased gene on the microarray and a critical spindle checkpoint protein (Wassmann et al., 2003), closely mirrors the level identified by the array (2.52-fold and 2.36-fold respectively). Besides the data herein, there are no other analyses of Mad2l1 mRNA levels in the rodent brain. The few studies that exist in humans positively correlate its high expression with grade IV gliomas (Bie et al., 2011). Similarly, there are high levels of *mad2l1* expression in ERα-positive breast cancer cells (Ghayad et al., 2009). Even though increased *mad2l1* expression causes cell cycle arrest at metaphase (Tunquist et al., 2003), it is quite unclear the role it may be playing in presumably non-dividing AVPV neurons (Sakuma, 2009). My data adds to the increasing body of work demonstrating the regulation of Mad2l1 by estrogens, while also being the first suggestion of a non-mitotic role for Mad2l1 in the brain, or in any tissue.

The decrease in *esr1* expression mirrored that of my microarray and previous studies, serving as an additional internal treatment control whereas the E_2-dependent decrease of Npy2r, Pgr15l and Slc17a8 within the AVPV are entirely novel. Npy2r was the most decreased transcript on the array and is notably involved in feeding and drinking

behavior (Kuo et al., 2007, Friedlander et al., 2010). Interestingly, Npy2r has recently been identified as a primary cilia marker in neurons, a discovery made based on its ciliary targeting sequence and its homology to Pgr15l (Loktev and Jackson, 2013), the second most decreased gene on the array. Although Pgr15l positively maps to the AVPV (http://www.brain-map.org/) little else is known about it; furthermore, this is the first indication that it is transcriptionally regulated by E_2.

As discussed in Chapter 2, the AVPV is almost entirely populated by dual-phenotypic GABA/glutamate neurons (Ottem et al., 2004) and several of the highest expressed genes were related to GABA and glutamate signaling, although not regulated by E_2. The exception is the validated marked decrease in Slc17a8 (vesicular glutamate transporter 3). Such a decrease would presumably limit the availability of glutamate release, making the GABA-mediated mechanisms more critical to understand.

The findings presented in this validation study confirm 11 increased transcripts and four decreased transcripts identified by the microarray, 13 of which are novel E_2 targets within the AVPV. This is not at all surprising, as this is the first comprehensive study investigating the E_2-induced transcriptome targeting the AVPV. As informative as this may be, this study was approached from a statistical standpoint and thus a high level of stringency was applied to both the fold-change and *p*-value cutoff. Consequently, there may be a wealth of physiologically relevant information within the data set that was filtered out. This will be addressed in Chapter 4.

3.5 Tables

Table 3.1 Primers Used in QPCR

NCBI Number	Transcript	Forward Primer	Reverse Primer	Amplicon
NM_207233	C1QL2	ATCCTGGGGAGGGAGAGGGA	TAGGGCCGCCTGTCTAGTCC	121 bp
NM_011957	Creb3l1	CCGACATGACCGTGCAGACA	CCACTCCTTGGGGTGGGAGA	115 bp
AK082585	ENSMUSG00000022845	AGCTGGGGTGATCGTGACCT	GGTCTGGTGTCCAGCAGGTT	118 bp
AK038867	ENSMUSG00000056615	ACCCCTCCTCAACTCCGTCC	CAGCAGACCAATCCGGAGCC	126 bp
NM_007956	Esr1	GTGCCAGGCTTTGGGGACTT	AGCAAACAGGAGCTTCCCCG	126 bp
NM_011809	ETS2	CCTTCAGTGGCTTCCAAAAG	ATTCACCAGGCTGAACTCGT	122 bp
NM_007836	GADD45A	CAGAGCAGAAGACCGAAAGG	GGGTCTACGTTGAGCAGCTT	127 bp
NM_008230	HDC	GCCCTGTGAATACCGTGAAT	GGTATCCAGGCTGCACATTT	128 bp
NM_019499	Mad2l1	AGAGAGGCAGGGAGGACAGC	CCTCGTTTCAGGCACCACCA	121 bp
NM_008731	NPY2R	ATTGCTCTGGACCGCCATCG	AGTGGACTTGCCAGCAGAGC	119 bp
NM_173440	NRip1	ACTTCCCGCTGCAGAAACTA	GCGTTTCCCAGAAGTCCATA	126 bp
NM_018884	PDZRN3	GTGGGCCTCTACAGGATGAA	CTTTGGCTGCAATGCTGTTA	124 bp
NM_008829	PGR	ACTGCCCAGCATGTCGTCTG	CGACTGGGGGAGAGCAACAC	125 bp
NM_001033361	Pgr15l	TATGGACCGGCACCGGGTAA	CGCATGAGGCAGAGCAAGGA	118 bp
NM_009026	Rasd1	TCGGCTCATCCAAAGTGGGC	CTTCGCCGCGGATCGAGTAA	123 bp
NM_182959	SLC17A8	GCCAGTAGCTTTTTGCAAGG	GGAGGTAAAAACCCCAGCTC	126 bp
NM_175499	Slitrk6	AGTCACCAATGCCCTCAGTC	TGGCACACTGATTTGGGATA	125 bp

Table 3.2 QPCR Validation of Microarray-Identified Transcripts

Gene	RefSeq	Keck Fold Change	Keck *p*-value	QPCR Fold Change	QPCR SEM	QPCR *p*-value
Mad2l1	NM_019499	2.36	0.001	2.52	±39.55	*
C1ql2	NM_207233	2.06	0.001	3.88	± 86.43	*
ENSMUSG00000022845	ENSMUST00000104915	2.02	0.000	1.48	±17.32	NS 0.107
Hdc	NM_008230	1.93	0.001	5.62	±70.04	***
Slitrk6	NM_175499	1.88	0.009	1.85	± 25.78	*
Pgr	NM_008829	1.81	0.000	1.69	± 21.57	*
ENSMUSG00000056615	ENSMUST00000070201	1.61	0.004	2.07	±16.43	**
Rasd1	NM_009026	1.58	0.012	1.66	± 14.84	*
Creb3l1	NM_011957	1.55	0.010	2.41	± 22.54	**
Nrip1	NM_173440	1.52	0.000	1.34	±11.96	NS 0.080
Pdzrn3	NM_018884	1.50	0.015	1.65	± 9.56	*
Ets2	NM_011809	1.43	0.002	1.51	±11.15	**
Gadd45a	NM_007836	1.41	0.001	2.27	± 27.88	**
Esr1	NM_007956	-1.58	0.007	-1.47	±6.81	**
Slc17a8	NM_182959	-1.60	0.008	-1.81	±1.79	**
Pgr15l	NM_001033361	-1.63	0.001	-1.55	±5.00	**
Npy2r	NM_008731	-1.81	0.007	-1.38	±1.54	**

3.6 References

Allison DB, Cui X, Page GP, Sabripour M (2006) Microarray data analysis: from disarray to consolidation and consensus. Nature reviews Genetics 7:55-65.

Beckman KB, Lee KY, Golden T, Melov S (2004) Gene expression profiling in mitochondrial disease: assessment of microarray accuracy by high-throughput Q-PCR. Mitochondrion 4:453-470.

Bie L, Zhao G, Cheng P, Rondeau G, Porwollik S, Ju Y, Xia XQ, McClelland M (2011) The accuracy of survival time prediction for patients with glioma is improved by measuring mitotic spindle checkpoint gene expression. PloS one 6:e25631.

Cheng C, Pounds S (2007) False discovery rate paradigms for statistical analyses of microarray gene expression data. Bioinformation 1:436-446.

Curtis RK, Oresic M, Vidal-Puig A (2005) Pathways to the analysis of microarray data. Trends in biotechnology 23:429-435.

Friedlander Y, Li G, Fornage M, Williams OD, Lewis CE, Schreiner P, Pletcher MJ, Enquobahrie D, Williams M, Siscovick DS (2010) Candidate molecular pathway genes related to appetite regulatory neural network, adipocyte homeostasis and obesity: results from the CARDIA Study. Annals of human genetics 74:387-398.

Ghayad SE, Vendrell JA, Bieche I, Spyratos F, Dumontet C, Treilleux I, Lidereau R, Cohen PA (2009) Identification of TACC1, NOV, and PTTG1 as new candidate genes associated with endocrine therapy resistance in breast cancer. Journal of molecular endocrinology 42:87-103.

Kuo LE, Kitlinska JB, Tilan JU, Li L, Baker SB, Johnson MD, Lee EW, Burnett MS, Fricke ST, Kvetnansky R, Herzog H, Zukowska Z (2007) Neuropeptide Y acts directly in the periphery on fat tissue and mediates stress-induced obesity and metabolic syndrome. Nature medicine 13:803-811.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.

Loktev AV, Jackson PK (2013) Neuropeptide Y family receptors traffic via the Bardet-Biedl syndrome pathway to signal in neuronal primary cilia. Cell reports 5:1316-1329.

Morey JS, Ryan JC, Van Dolah FM (2006) Microarray validation: factors influencing correlation between oligonucleotide microarrays and real-time PCR. Biological procedures online 8:175-193.

Ottem EN, Godwin JG, Krishnan S, Petersen SL (2004) Dual-phenotype GABA/glutamate neurons in adult preoptic area: sexual dimorphism and function. J Neurosci 24:8097-8105.

Pawitan Y, Michiels S, Koscielny S, Gusnanto A, Ploner A (2005) False discovery rate, sensitivity and sample size for microarray studies. Bioinformatics 21:3017-3024.

Sakuma Y (2009) Gonadal steroid action and brain sex differentiation in the rat. Journal of neuroendocrinology 21:410-414.

Simerly RB, Carr AM, Zee MC, Lorang D (1996) Ovarian steroid regulation of estrogen and progesterone receptor messenger ribonucleic acid in the anteroventral periventricular nucleus of the rat. Journal of neuroendocrinology 8:45-56.

Tunquist BJ, Eyers PA, Chen LG, Lewellyn AL, Maller JL (2003) Spindle checkpoint proteins Mad1 and Mad2 are required for cytostatic factor-mediated metaphase arrest. The Journal of cell biology 163:1231-1242.

Wassmann K, Niault T, Maro B (2003) Metaphase I arrest upon activation of the Mad2-dependent spindle checkpoint in mouse oocytes. Current biology : CB 13:1596-1608.

CHAPTER 4

17β-ESTRADIOL REGULATION OF PHYSIOLOGICALLY RELATED GENE GROUPS IN THE ANTEROVENTRAL PERIVENTRICULAR NUCLEUS OF THE FEMALE MOUSE

4.1 Introduction

The gene ontology analysis performed by the Keck Institute utilized all transcripts ≥1.2 fold-change with a *p*-value ≤ 0.05 within the array, however even after removal of non-validated transcripts, the minutiae of the GO IDs still did not provide much insight. As the overarching goal is to develop a global view of the functions of E_2 within the AVPV, it became apparent that a different approach was necessary. As mentioned in Chapter 3, false-positives represent a small, but potential problem with microarray analysis (Pawitan et al., 2005). However, there is also the problem of false-negatives: significantly regulated transcripts not detected due to the analysis method employed. While this is generally held to be a less common problem, it bears addressing.

Within the entirety of the microarray data set, there were many transcripts that met the requirement of ≥1.2 fold-change, though the calculated *p*-value was above 0.05. Recent publications have called into question the validity of such a stringent *p*-value in the context of microarray data (Morey et al., 2006, Dalman et al., 2012). Indeed, one of the most intensely studied transcriptional targets of E_2 within the AVPV, kiss1, failed to meet the *p*-value cutoff set by the Keck Institute and many others. The microarray

measured expression of kiss1, at 1.38-fold increase by E_2, with a *p*-value of 0.095, supports the more recent concept that is more appropriate to apply greater stringency to the fold-change cutoff, and greater leniency to the *p*-value (Zhang et al., 2013). Furthermore, as the Affymetrix mouse array is a dual channel array that measures ratios, the measured fold changes are considered more reliable than absolute expression values. This implies that even those transcripts with very low raw fluorescence, but high fold-change may be important.

To reap maximal useable information from the microarray, I took a more physiological approach to the data set. I did extensive literary searches to reclassify the transcripts beyond the limited functions of the Keck GO IDs, placing them into broader physiological groups. I also tested several transcripts outside of my previously high set stringency of ≥1.4-fold change, ≤0.05 and ≥75 raw mean fluorescence.

4.2 Materials and Methods

4.2.1 Animals

All protocols were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts and all animals were housed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Eight week old female C57Bl/6 mice (Jackson Labs; Bar Harbor, ME) were housed four to a cage in a temperature- and light-controlled room (12:12 light/dark cycle), with standard feed and water provided *ad libitum*. After a minimum of 48 h post-arrival, I bilaterally ovariectomized all female mice under inhaled isofluorane anesthesia. Five days later, mice were injected subcutaneously with sesame oil vehicle or 0.05µg/g b.w. E_2 dissolved in sesame oil. Twelve hours later, I anesthetized the animals with CO_2,

collected the brains, rapidly frozen them on powdered dry ice, wrapped them in Parafilm™ (Pechiney Plastic Packaging Company; Chicago, Illinois) and stored them at -80°C in cryotubes.

4.2.2 Tissue Preparation and RNA Isolation

Brains were allowed to thaw slowly at -20°C, then coronally cryosectioned at 12μm using a Leica CM3000 cryostat (Nussloch, Germany), until the early AVPV was reached. The early AVPV was determined by the appearance of the optic recess. I took a 300-μm coronal section and immediately excised the AVPV from it using a 1-mm circular Harris Uni-Core™ stainless steel tissue micropunch needle (Ted Pella Inc.; Redding, CA). I transferred the micropunched tissue to a 1.5-ml microcentrifuge tube, on powdered dry ice. To obtain enough starting material, I pooled three AVPV samples, 3 AVPVsconsidered to be n=1.

Total RNA was isolated from each pool using Trizol™ (Invitrogen; Carlsbad, CA) and Qiagen RNeasy Lipid kit (Qiagen; Valencia, CA). Sample concentration and quality were determined via Nanodrop™ (Thermo Scientific; Wilmington, DE).

4.2.3 Physiological Grouping of Validated E_2-Regulated Transcripts and Selection of Additional Genes for Validation

I clustered both validated and non-validated genes, based on extensive literature searches of published functions, into broad physiological groups. I included several transcripts between 1.4- and 1.2-fold-change, >0.05 *p*-value, and mean raw fluorescence <75 (see Chapter 3).

4.2.4 Quantitative Reverse-Transcription PCR (QPCR)

Oneμg total RNA was reverse transcribed into cDNA using QuantiTect Reverse Transcriptase Kit (Roche Diagnostics, Indianapolis, IN), following the manufacturer's protocol. I used Primer3™ software (http://bioinfo.ut.ee/primer3/) to design specific QPCR primers for: Crebl1, Esr1, Ets2, Gadd45a, Hdc, Kiss1, Mad2l1, Mmu-mir-21, Npy2r, Pdcd4, Phlda1, Pgr, Prl, Trp53 and trp53i11 mRNAs(Table 4.5.2). I obtained the primers from Integrated DNA Technologies (Coralville, IA).

QPCR reactions were carried out in a Stratagene MX3000P™ thermocycler, utilizing MxPro™QPCR software (both Agilent Technologies; Santa Clara, CA). Reactions contained cDNA, diluted 1:10 with nuclease-free water, specific primers and SybrGreen™ QPCR Mastermix (Roche Diagnostics Corporation; Indianapolis, IN). Manufacturer's protocol was used with the following cycle settings: 95°C for 10 min, and 40 cycles of 95°C for 30 sec, 57°C for 30 sec and 72°C for 30 sec. Each sample was tested in duplicate. Primer specificity was verified via 2% agarose gel electrophoresis and confirmation of a single dissociative curve peak during each QPCR reaction.

4.2.5 Statistics

For QPCR, the duplicate raw cycle threshold (Ct) values were analyzed using the ΔΔCt method (Livak and Schmittgen, 2001) with β-actin employed as background control. QPCR reactions with nuclease-free water instead of cDNA were used as negative controls. I used Student t-test with Welch's correction statistical analyses. Data is presented as mean ± SEM.

4.3 Results

4.3.1 Physiological Grouping of Validated E_2-Regulated Transcripts and Selection of Additional Genes for Validation

Based on the current literature, many of the previously validated genes (see Chapter 3) fell within 3 main physiological groups: cell death/tumor suppression, reproduction, and feeding/drinking behavior. I selected an additional 8 transcripts for further QPCR validation (Table 4.5.1).

Initially validated genes within the cell death/tumor suppressor group were the following: *creb3l1* (Mellor et al., 2013), *ets2* (Zhu et al., 2006, Kabbout et al., 2013), *gadd45a* (Maeda et al., 2002), *mad2l1* (Li and Murray, 1991, Cheslock et al., 2005). I further included *pdcd4* (Cmarik et al., 1999, Bitomsky et al., 2008), *phlda1* (Moad et al., 2013) and *trp53i11* (Liang et al., 2003, Wu et al., 2009), all of which were below the stringent 1.4-fold-change cutoff of the previous study. Considering that 3 of the genes in this functional grouping are within the Trp53 pathway, Pdcd4 being upstream (Wedeken et al., 2011) and both Gadd45a and Trp53i11 (Zhan et al., 1996) and (Zhu et al., 1999) being downstream, I also selected Trp53. Furthermore, Mmu-mir21 (microRNA21) was not tested for on the original microarray, however, it is associated with apoptosis (Carletti et al., 2010, Ruan et al., 2011), can be regulated by E_2 (Bhat-Nakshatri et al., 2009, Wickramasinghe et al., 2009) and Pdcd4 is one its primary targets (Asangani et al., 2008, Lu et al., 2008), hence it was also selected.

Initially validated genes within the reproduction group were *esr1* and *pgr*. I further included *prl*, not previously selected due to mean raw fluorescence <75, as there is significant Prl receptor expression in the AVPV (Kokay et al., 2011), and *kiss1*, not previously selected due to both a fold-change <1.4 and a *p*-value >0.05. It should be noted that reproduction-associated Nts (Dungan Lemko et al., 2010) was also identified by the microarray as significantly increased by E_2 (1.33-fold), but was not selected for

testing in this study as its regulation by E_2 in the female mouse AVPV is already well documented (Alexander et al., 1991, Dungan Lemko et al., 2010).

Initially validated genes within the feeding/drinking behavior group were *npy2r* (Friedlander et al., 2010, Loktev and Jackson, 2013) and *hdc* (Fulop et al., 2003, Jorgensen et al., 2006). I further included *cckar* (Bellissimo and Anderson, 2003, Li et al., 2011), not previously selected due to a fold-change <1.4.

4.3.2 QPCR Validation of Additional Physiologically Relevant Transcripts

The primers for Cckar nRNA failed to meet primer specificity quality control standards, consequently it was not testable. In the case of both Phlda1 and Prl, there existed a high amount of variability and, although there was an upward trend in their expression, both failed to reach significance and may require a higher number of samples. Mmu-mir21 was unchanged by E_2 treatment; however, there is no previous data for comparison, as it was not included on the microarray. Levels of Kiss1, Pdcd4 and Trp53i11, all increased with E_2 treatment, but Trp53 remained unchanged, all validating the microarray findings (Table 4.5.3).

4.4 Discussion

To better assess the physiological relevance of the E_2-induced transcriptome, I regrouped both validated and non-validated genes based on known biological functions. They fell into the following groups: cell death/tumor suppression, reproduction, and feeding/drinking behavior. Taking this broader view revealed that the most significant biological function regulated by E_2 in the AVPV is cell death/tumor suppression. This was a somewhat, but not entirely, shocking discovery.

As is the case with known tumor suppressor kiss1, these tumor suppressor genes have functions beyond that of apoptosis and tumor suppression. Indeed, a tumor suppressor gene network has been identified in the female rat hypothalamus and is critical during the onset of puberty (Roth et al., 2007). Although that network does not mirror the genes identified herein, it supports the notion that some tumor suppressors have critical roles regarding fundamental brain function, and likely in this particular nucleus, control of reproduction.

My data showing that Mmu-mir21 was not regulated by E_2 in the female AVPV and that its target gene *pdcd4* was increased are both novel findings. Likewise are the findings that the two downstream targets of Trp53, Gadd45a and Trp53i11, increase with E_2 treatment despite the lack of change in *trp53* expression. These are all in line with the microarray findings and support the idea that these are transcriptional targets of E_2. In addition, it is possible that the phosphorylation/acetylation status of Trp53 is being regulated by Pdcd4 (Kumar et al., 2013) to meet out these effects, and it therefore warrants further investigation. Further study of the presence of these genes and their regulation by E_2 in the AVPV is important, as many of these are primarily studied in the context of cancer or in mitotic cells, with little regard for their intrinsic function in non-mitotic brain neurons.

Both Prl and Kiss1 were tested as a part of the second largest physiological gene grouping, reproduction, and provided more insight into the methodology of analyzing microarray data than that of E_2 function within the AVPV. Although identified as significantly increased in the Keck Institute analysis, Prl was not originally selected for validation due to a mean raw fluorescence below 75 (see Chapter 3). In the QPCR

analysis, Prl mRNA failed to reach significance. It is likely that due to the very low level of expression, small fluctuations in expression carry a greater weight in the variability measurement and may require a higher sample number to distinguish changes amongst test groups. The significant increase detected in *kiss1* expression by QPCR mirrors previous studies (Smith et al., 2005), although it was not identified on the microarray due to a *p*-value >0.05. This supports the view that the fold-changes represented in microarray data are a more accurate predictor of true-positives as opposed to using stringent *p*-value cutoffs.

Feeding and drinking behavior was the only significantly regulated pathway identified in my previous Ariadne™ pathway analysis (see Chapter 2). Unfortunately, expression of newly selected Cckar, and the previously selected Hcrtr1 and Mc4r targets, remain unvalidated due to non-specific primers, which needs to be resolved. An increase in the sample number would also be advantageous, as Nrip1, a critical regulator of fat metabolism (Rosell et al., 2011) failed to reach significance in my previous validation study (see Chapter 3). It will be imperative to test these genes, as regulation of feeding and drinking behavior would represent a novel function for the AVPV, although not a surprising one. It has been alluded to previously, as the AVPV receives neuronal input from the ventral premammillary nucleus (Donato et al., 2011), which is implicated in mediating critical adiposity signals to the GnRH neurons (Amstalden et al., 2011). This will be discussed more fully in Chapter 7.

This more physiological approach to my previous microarray data adds to the number of novel genes that I have already validated, highlighting potential new roles for the AVPV. This particular study is the first to identify a broad network of E_2-regulated

tumor suppressors, and builds on previous indications regarding the mechanisms of feeding and drinking behavior. When taken together, this data set points to a broader and more integrative role for the AVPV in the neuroendocrine control of reproductive functions. Furthermore, this has provided additional insight into some of the caveats of microarray data analysis.

4.5 Tables

Table 4.1 Physiologically Related Gene Groups

Gene Symbol	Gene Name	Alias
Cell Death/Tumor Suppression		
Creb3l1	cAMP responsive element binding protein 3-like 1	old astrocyte specifically-induced substance
Ets2	E26 avian leukemia oncogene 2, 3' domain	oncogene homolog 2
Gadd45a	growth arrest and DNA-damage-inducible 45 alpha	DNA damage-inducible transcript
Kiss1*	KiSS-1 metastasis-suppressor	metastasis suppressor 1
Mad2l1	mitotic arrest deficient, homolog-like 1 (yeast)	mitotic spindle assembly checkpoint
mmu-mir21	mus musculus microRNA21	
Pdcd4	programmed cell death 4	nuclear antigen h731
Phlda1	pleckstrin homology-like domain, family A, member 1	T-cell death associated gene 51
Trp53	transformation related protein 53	cellular tumor antigen p53
Trp53i11	transformation related protein 53 inducible protein	pig11
Reproduction		
Esr1	estrogen receptor 1 (alpha)	nuclear receptor subfamily 3 group a
Kiss1*	kisspeptin	metastasis-suppressor
Pgr	progesterone receptor	nuclear receptor subfamily 3 group c member 3
Prl	prolactin	
Feeding/Drinking Behavior		
Cckar	cholecystokinin A receptor	
Hcrtr1	hypocretin receptor 1	orexin receptor
Hdc	histidine decarboxylase	
Mc4r	melanocortin 4 receptor	
Npy2r	neuropeptide Y receptor Y2	Y2 receptor
Nrip1	nuclear receptor interacting protein 1	receptor-interacting protein 140

Table 4.2 Primers Used in QPCR

NCBI Number	Transcript	Forward Primer	Reverse Primer	Amplicon
NM_007393	actinB	GGCTGTATTCCGCCTCCATCG	CCAGTTGGTAACAATGCCATG	154 bp
NM_178260	Kiss1	CTCGTAGGTCGTCGCCATGC	GACAGGTCCTTCTCCCGCTG	130 bp
NR_029738.1	mmu-mir21	GACATCGCATGGCTGTACCA	CCATGAGATTCAACAGTCAACATCA	Prevalidated (Carletti et al., 2010)
NM_011050	PDCD4	GTTGCTAGATAGGCGGTCCA	TCACATCCACCTCTTCCACA	122 bp
NM_009344	PHLDA1	CTGAAGGAAGGAGTGCTGGA	TGCTGCTGTTGTAGCTGCTT	122 bp
NM_011164	Prl	AAGAAGCCCCCGAATACATC	ATCCCATTTCCTTTGGCTTC	121 bp
NM_001127233	Trp53	GGGCTCACTCCAGCTACCTGAA	CTGAGTCAGGCCCCACTTTCTTG	185bp
NM_001025246	Tp53i11	TTTTTGATGGGGCTGAAGTC	AGAGTCCAGCGGATGATGAC	127 bp

Table 4.3 QPCR Validation of Physiologically Related Gene Groups

Gene	RefSeq	Keck Fold-Change	Keck *p*-value	QPCR (%) E_2 Mean	QPCR SEM	QPCR *p*-value
NM_178260	Kiss1	1.3816	0.0952	484.7785	100.7693	*
NR_029738.1	mmu-mir21	NA	NA	118.8763	11.5865	**0.1849**
NM_011050	PDCD4	1.3017	0.0074	148.6010	9.0826	**
NM_009344	PHLDA1	1.2931	0.0042	146.3610	20.2533	**0.1498**
NM_011164	Prl	1.4902	0.0433	187.8000	60.6166	**0.3025**
NM_001127233	Trp53	-1.0305	0.3363	126.7815	19.9726	**0.2286**
NM_001025246	Tp53i11	1.2755	0.0256	162.3300	21.3950	***

4.6 References

Alexander MJ, Kiraly ZJ, Leeman SE (1991) Sexually dimorphic distribution of neurotensin/neuromedin N mRNA in the rat preoptic area. The Journal of comparative neurology 311:84-96.

Amstalden M, Alves BR, Liu S, Cardoso RC, Williams GL (2011) Neuroendocrine pathways mediating nutritional acceleration of puberty: insights from ruminant models. Frontiers in endocrinology 2:109.

Asangani IA, Rasheed SA, Nikolova DA, Leupold JH, Colburn NH, Post S, Allgayer H (2008) MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. Oncogene 27:2128-2136.

Bellissimo N, Anderson GH (2003) Cholecystokinin-A receptors are involved in food intake suppression in rats after intake of all fats and carbohydrates tested. The Journal of nutrition 133:2319-2325.

Bhat-Nakshatri P, Wang G, Collins NR, Thomson MJ, Geistlinger TR, Carroll JS, Brown M, Hammond S, Srour EF, Liu Y, Nakshatri H (2009) Estradiol-regulated microRNAs control estradiol response in breast cancer cells. Nucleic acids research 37:4850-4861.

Bitomsky N, Wethkamp N, Marikkannu R, Klempnauer KH (2008) siRNA-mediated knockdown of Pdcd4 expression causes upregulation of p21(Waf1/Cip1) expression. Oncogene 27:4820-4829.

Carletti MZ, Fiedler SD, Christenson LK (2010) MicroRNA 21 blocks apoptosis in mouse periovulatory granulosa cells. Biology of reproduction 83:286-295.

Cheslock PS, Kemp BJ, Boumil RM, Dawson DS (2005) The roles of MAD1, MAD2 and MAD3 in meiotic progression and the segregation of nonexchange chromosomes. Nature genetics 37:756-760.

Cmarik JL, Min H, Hegamyer G, Zhan S, Kulesz-Martin M, Yoshinaga H, Matsuhashi S, Colburn NH (1999) Differentially expressed protein Pdcd4 inhibits tumor promoter-induced neoplastic transformation. Proceedings of the National Academy of Sciences of the United States of America 96:14037-14042.

Dalman MR, Deeter A, Nimishakavi G, Duan ZH (2012) Fold change and p-value cutoffs significantly alter microarray interpretations. BMC bioinformatics 13 Suppl 2:S11.

Donato J, Jr., Cravo RM, Frazao R, Gautron L, Scott MM, Lachey J, Castro IA, Margatho LO, Lee S, Lee C, Richardson JA, Friedman J, Chua S, Jr., Coppari R, Zigman JM, Elmquist JK, Elias CF (2011) Leptin's effect on puberty in mice is relayed by the ventral premammillary nucleus and does not require signaling in Kiss1 neurons. The Journal of clinical investigation 121:355-368.

Dungan Lemko HM, Naderi R, Adjan V, Jennes LH, Navarro VM, Clifton DK, Steiner RA (2010) Interactions between neurotensin and GnRH neurons in the positive feedback control of GnRH/LH secretion in the mouse. American journal of physiology Endocrinology and metabolism 298:E80-88.

Friedlander Y, Li G, Fornage M, Williams OD, Lewis CE, Schreiner P, Pletcher MJ, Enquobahrie D, Williams M, Siscovick DS (2010) Candidate molecular pathway genes related to appetite regulatory neural network, adipocyte homeostasis and obesity: results from the CARDIA Study. Annals of human genetics 74:387-398.

Fulop AK, Foldes A, Buzas E, Hegyi K, Miklos IH, Romics L, Kleiber M, Nagy A, Falus A, Kovacs KJ (2003) Hyperleptinemia, visceral adiposity, and decreased glucose tolerance in mice with a targeted disruption of the histidine decarboxylase gene. Endocrinology 144:4306-4314.

Jorgensen EA, Vogelsang TW, Knigge U, Watanabe T, Warberg J, Kjaer A (2006) Increased susceptibility to diet-induced obesity in histamine-deficient mice. Neuroendocrinology 83:289-294.

Kabbout M, Garcia MM, Fujimoto J, Liu DD, Woods D, Chow CW, Mendoza G, Momin AA, James BP, Solis L, Behrens C, Lee JJ, Wistuba, II, Kadara H (2013) ETS2 mediated tumor suppressive function and MET oncogene inhibition in human non-small cell lung cancer. Clinical cancer research : an official journal of the American Association for Cancer Research 19:3383-3395.

Kokay IC, Petersen SL, Grattan DR (2011) Identification of prolactin-sensitive GABA and kisspeptin neurons in regions of the rat hypothalamus involved in the control of fertility. Endocrinology 152:526-535.

Kumar N, Wethkamp N, Waters LC, Carr MD, Klempnauer KH (2013) Tumor suppressor protein Pdcd4 interacts with Daxx and modulates the stability of Daxx and the Hipk2-dependent phosphorylation of p53 at serine 46. Oncogenesis 2:e37.

Li R, Murray AW (1991) Feedback control of mitosis in budding yeast. Cell 66:519-531.

Li Y, Wu X, Zhou S, Owyang C (2011) Low-affinity CCK-A receptors are coexpressed with leptin receptors in rat nodose ganglia: implications for leptin as a regulator of short-term satiety. American journal of physiology Gastrointestinal and liver physiology 300:G217-227.

Liang XQ, Cao EH, Zhang Y, Qin JF (2003) P53-induced gene 11 (PIG11) involved in arsenic trioxide-induced apoptosis in human gastric cancer MGC-803 cells. Oncology reports 10:1265-1269.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.

Loktev AV, Jackson PK (2013) Neuropeptide Y family receptors traffic via the Bardet-Biedl syndrome pathway to signal in neuronal primary cilia. Cell reports 5:1316-1329.

Lu Z, Liu M, Stribinskis V, Klinge CM, Ramos KS, Colburn NH, Li Y (2008) MicroRNA-21 promotes cell transformation by targeting the programmed cell death 4 gene. Oncogene 27:4373-4379.

Maeda T, Hanna AN, Sim AB, Chua PP, Chong MT, Tron VA (2002) GADD45 regulates G2/M arrest, DNA repair, and cell death in keratinocytes following ultraviolet exposure. The Journal of investigative dermatology 119:22-26.

Mellor P, Deibert L, Calvert B, Bonham K, Carlsen SA, Anderson DH (2013) CREB3L1 is a metastasis suppressor that represses expression of genes regulating metastasis, invasion, and angiogenesis. Molecular and cellular biology 33:4985-4995.

Moad AI, Muhammad TS, Oon CE, Tan ML (2013) Rapamycin induces apoptosis when autophagy is inhibited in T-47D mammary cells and both processes are regulated by Phlda1. Cell biochemistry and biophysics 66:567-587.

Morey JS, Ryan JC, Van Dolah FM (2006) Microarray validation: factors influencing correlation between oligonucleotide microarrays and real-time PCR. Biological procedures online 8:175-193.

Pawitan Y, Michiels S, Koscielny S, Gusnanto A, Ploner A (2005) False discovery rate, sensitivity and sample size for microarray studies. Bioinformatics 21:3017-3024.

Rosell M, Jones MC, Parker MG (2011) Role of nuclear receptor corepressor RIP140 in metabolic syndrome. Biochimica et biophysica acta 1812:919-928.

Roth CL, Mastronardi C, Lomniczi A, Wright H, Cabrera R, Mungenast AE, Heger S, Jung H, Dubay C, Ojeda SR (2007) Expression of a tumor-related gene network increases in the mammalian hypothalamus at the time of female puberty. Endocrinology 148:5147-5161.

Ruan Q, Wang T, Kameswaran V, Wei Q, Johnson DS, Matschinsky F, Shi W, Chen YH (2011) The microRNA-21-PDCD4 axis prevents type 1 diabetes by blocking pancreatic beta cell death. Proceedings of the National Academy of Sciences of the United States of America 108:12030-12035.

Smith JT, Cunningham MJ, Rissman EF, Clifton DK, Steiner RA (2005) Regulation of Kiss1 gene expression in the brain of the female mouse. Endocrinology 146:3686-3692.

Wedeken L, Singh P, Klempnauer KH (2011) Tumor suppressor protein Pdcd4 inhibits translation of p53 mRNA. The Journal of biological chemistry 286:42855-42862.

Wickramasinghe NS, Manavalan TT, Dougherty SM, Riggs KA, Li Y, Klinge CM (2009) Estradiol downregulates miR-21 expression and increases miR-21 target gene expression in MCF-7 breast cancer cells. Nucleic acids research 37:2584-2595.

Wu Y, Liu XM, Wang XJ, Zhang Y, Liang XQ, Cao EH (2009) PIG11 is involved in hepatocellular carcinogenesis and its over-expression promotes Hepg2 cell apoptosis. Pathology oncology research : POR 15:411-416.

Zhan Q, Fan S, Smith ML, Bae I, Yu K, Alamo I, Jr., O'Connor PM, Fornace AJ, Jr. (1996) Abrogation of p53 function affects gadd gene responses to DNA base-damaging agents and starvation. DNA and cell biology 15:805-815.

Zhang L, Zhang J, Yang G, Wu D, Jiang L, Wen Z, Li M (2013) Investigating the concordance of Gene Ontology terms reveals the intra- and inter-platform reproducibility of enrichment analysis. BMC bioinformatics 14:143.

Zhu J, Jiang J, Zhou W, Zhu K, Chen X (1999) Differential regulation of cellular target genes by p53 devoid of the PXXP motifs with impaired apoptotic activity. Oncogene 18:2149-2155.

Zhu JD, Fei Q, Wang P, Lan F, Mao da Q, Zhang HY, Yao XB (2006) Transcription of the putative tumor suppressor gene HCCS1 requires binding of ETS-2 to its consensus near the transcription start site. Cell research 16:780-796.

CHAPTER 5

SEX DIFFERENCES IN TUMOR SUPPRESSOR GENES IN THE MOUSE AVPV

5.1 Introduction

The AVPV is a sexually dimorphic nucleus with a dense population of ERα-expressing neurons. The female AVPV is more than twice the size of the male AVPV, and has the main function of mediating the E_2 signal to the GnRH neurons to elicit the LH surge. However, there are subpopulations within the AVPV, for instance, the Kiss1-expressing cells and the Nts-expressing cells, while they do not colocalize with each other (Porteous et al., 2011), they both colocalize with ERα. This highlights the theme that E_2 has cell-specific functions in this nucleus. Considering the breadth of novel E_2-induced transcripts that I have identified thus far, it is possible that some are participatory in the ovulatory mechanisms, while others are supportive of more basic regulatory functions, independent of sex.

As shown in Chapters 3 and 4, there are a great many cell death/tumor suppressor genes regulated by E_2 in the AVPV. These are of high interest, particularly Ets2, a transcription factor, and Pdcd4 (Yang et al., 2003), a regulator of translation. These represent new avenues by which E_2 can exert global control over the proteome. To further characterize these findings, I used QPCR to investigate whether or not they are regulated by E_2 in a sex-specific manner.

5.2 Materials and Methods

5.2.1 Animals

All protocols were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts and all animals were housed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Eight-week-old male and female C57Bl/6 mice (Jackson Labs; Bar Harbor, ME) were housed four to a cage in a temperature- and light-controlled room (12:12 light/dark cycle), with standard feed and water provided *ad libitum*. After a minimum of 48 h post-arrival, I orchidectomized all male mice and bilaterally ovariectomized all female mice under inhaled isofluorane anesthesia. Five days later, mice were injected subcutaneously with sesame oil vehicle or 0.05 µg/g b.w. E_2 dissolved in sesame oil. Twelve hours later, I anesthetized the animals with CO_2, collected the brains, rapidly froze them on powdered dry ice, wrapped them in Parafilm™ (Pechiney Plastic Packaging Company; Chicago, Illinois) and stored them at -80°C in cryotubes.

5.2.2 Brain Tissue Preparation

Brains were allowed to thaw slowly at -20°C, then coronally cryosectioned at 12 µm using a Leica CM3000 cryostat (Nussloch, Germany), until the early AVPV was reached. The early AVPV was determined by the appearance of the optic recess. I took a 300-µm coronal section and immediately excised the AVPV from it using a 1-mm circular Harris Uni-Core™ stainless steel tissue micropunch needle (Ted Pella Inc.; Redding, CA). I transferred the micropunched tissue to a 1.5ml microcentrifuge tube, on powdered dry ice. To obtain enough starting material, I pooled three AVPV micropunches to make one sample.

5.2.3 RNA Isolation and QPCR

Total RNA was isolated from each pool of AVPV micropunches using Trizol™ (Invitrogen; Carlsbad, CA) and Qiagen RNeasy Lipid kit (Qiagen; Valencia, CA). Sample concentration and quality was determined via Nanodrop™ (Thermo Scientific; Wilmington, DE).

One μg total RNA was reverse transcribed into cDNA using QuantiTect Reverse Transcriptase Kit (Roche Diagnostics, Indianapolis, IN), following the manufacturer's protocol. I used Primer3™ software (http://bioinfo.ut.ee/primer3/) to design specific QPCR primers for: Esr1, Ets2, Kiss1, Pdcd4, Trp53 and Trp53i11 (Table 5.5.1). The sequence for primers for the primary transcript of mmu-mir21 was already published (Carletti et al., 2010). I obtained the primers from Integrated DNA Technologies (Coralville, IA).

QPCR reactions were carried out in a Stratagene MX3000P™ thermocycler, utilizing MxPro™QPCR software (both Agilent Technologies; Santa Clara, CA). Reactions contained cDNA, diluted 1:10 with nuclease-free water, specific primers and SybrGreen™ QPCR Mastermix (Roche Diagnostics Corporation; Indianapolis, IN). Manufacture's protocol was used with the following cycle settings: 95°C for 10 min, and 40 cycles of 95°C for 30 sec, 57°C for 30 sec and 72°C for 30 sec. Each sample was tested in duplicate. Primer specificity was verified via 2% agarose gel electrophoresis and confirmation of a single dissociative curve peak during each QPCR reaction.

5.2.4 Statistics

For QPCR, the duplicate raw cycle threshold (Ct) values were analyzed using the ΔΔCt method (Livak and Schmittgen, 2001) with β-actin employed as background

control. Known 17β-E_2-induced transcripts within the AVPV, esr1 and kiss1, were used as positive treatment controls. QPCR reactions with nuclease-free water instead of cDNA were used as negative controls. I used Graphpad Prism™ to perform t-test statistical analyses, with Welch's correction for variance. Data is presented as means ± SEM.

5.3 Results

5.3.1 Females Have Higher Expression of *esr1*, *kiss1* and *ets2* in AVPV Micropunches

Gonadectomized oil-treated males had 26% less *esr1* expression in their AVPV. E_2 treatment decreased Esr1 in both females and male, 50% and 40% respectively (Figure 5.6.1A). I found that males had 68% less *kiss1* expression in their AVPV and E_2 treatment increased kiss1 expression in females and males, to 480% and 369% respectively (Figure 5.6.1B). The changes in both Esr1 and Kiss1 are in accord with previous studies (Smith et al., 2005, Kauffman et al., 2007). Additionally, I found that males had 32% less *ets2* expression and that E_2 treatment increased Ets2 mRNA in both females and male, by 158% and 185% respectively (Figure 5.6.1C).

5.3.2 Males Have Higher Expression of Pdcd4 and Trp53 in AVPV Micropunches

Gonadectomized oil-treated males have 20% more *pdcd4* expression in their AVPV; however, E_2 treatment increased Pdcd4 mRNA to 165% in females and to 138% in males (Figure 5.6.2A). Males also have 24% more *trp53* expression, although it was not significantly regulated by E_2 in either sex (Figure 5.6.2B).

5.3.3 Males and Females Have the Same Expression Levels of mmu-mir21 and tp53i11 in AVPV Micropunches

Gonadectomized oil-treated males and females have identical levels of trp53i11 expression within the AVPV. However E_2 treatment increases the expression by 167% in females and to 138% in males (Figure 5.6.3A). There was no difference between the sexes in the expression of the primary transcript of microRNA mmu-mir-21, nor was there any change due to E_2 treatment in either sex (Figure 5.6.3B).

5.4 Discussion

The previous investigations of the function of E_2 in the AVPV have largely focused on already known gene targets, limiting the study of this nucleus to one function. Identification of multiple tumor suppressor genes within the AVPV was a novel finding (see Chapter 4), however, due to the very nature of the novel genes within the set, there is very little information regarding their function in non-tumorigenic cells, especially neurons. Further comparison of these genes revealed sex-specific expression of *ets2*, a transcription factor, and *pdcd4*, a translation inhibitor (Yang et al., 2003, Yang et al., 2004).

Even though gonadectomized females had higher *ets2* expression than males, E_2 treatment produced a greater effect in its expression. This indicates that there are multiple sex-specific mechanisms regulating Ets2, causing a more robust response in males, or perhaps the response is only occurring in a subpopulation of the Ets2-containing cells in females. It will therefore be important in the future to examine the colocalization of ERα with Ets2 in both males and females. Nevertheless, considering that Ets2 is a transcription factor, this is a very important finding. This offers a new mechanism by which E_2 to could indirectly regulate a host of targets, not only within the AVPV, but potentially wherever ERα is expressed. Bearing in mind that Ets2, like Esr1,

is heavily studied for its role in tumorigenesis, this has very broad implications. In fact, amongst trisomy 21 (Down's syndrome) patients, in which there is overexpression of *ets2* (Rahmani et al., 1989, Wolvetang et al., 2003), there is a lower incidence of all types of tumors, except leukemia and testicular (Yang et al., 2002). Recent work in mouse models of Down's syndrome has linked the higher amount of ets2 with the lower incidence of tumors (Reynolds et al., 2010). Therefore, further delineating this link between E_2 and the regulation of ets2 will be critical not only for better understanding the functions of the AVPV, but also regarding cancer research.

Though the AVPV is larger in females and they have 26% more ERα, Pdcd4 was higher in males, yet E_2 treatment had a greater effect on the expression in females. This could mean that there are multiple mechanisms regulating basal levels. One of the primary functions of Pdcd4 is to inhibit translation via binding to mRNAs that contain a structured 5' untranslated region (Wedeken et al., 2010). Importantly, known tumor suppressors Trp53 (Wedeken et al., 2011), Bcl-xl and Xiap (Liwak et al., 2012) are targets of Pdcd4-mediated translation inhibition. This is important in the context of the adult AVPV, as I found that males also have higher expression of Trp53; however with the abundance of Pdcd4, it is possible that not all of the Trp53 is actually translated. It will therefore be important to parallel these gene expression studies with protein studies. This finding, similar to that of Ets2, has wider implications beyond that of the AVPV. As a target of E_2, this represents another novel mechanism by which E_2 could indirectly exert widespread control over gene expression, but at the level of the proteome.

The fact that Trp53i11 was neither sex-specific in its expression nor its regulation by E_2 was quite interesting. As a downstream target of Trp53 (Zhu et al., 1999), I

expected Trp53i11 expression to mirror that of Trp53 and thus be higher in males; however as stated above, the expression level of Trp53 may not be a true indicator of its protein level or the activity of the protein. Importantly, the consistency of regulation of this gene between the sexes suggests a more basic function and regulation in this nucleus. Previous studies have shown that Trp53i11 is induced by reactive oxygen species (ROS) (Liang et al., 2004) and mediates apoptosis (Wu et al., 2009). This is important as E_2 can rapidly increase ROS, independently of nuclear ER (Felty et al., 2005a, Felty et al., 2005b), which would make the differences in AVPV size and ERα levels between the sexes irrelevant in the regulation of Trp53i11. However, the downstream result of increased expression of Trp53i11 in the brain is still unclear, as stated previously, there is no prior evidence of cyclical apoptosis in the female AVPV in adulthood.

Similar to Trp53i11, Mmu-mir21 was not sex-specific, but its expression remained unchanged following E_2 exposure in either sex. This suggests that the increase in *pdcd4* expression was not a downstream result of E_2-mediated Mmu-mir21 transcriptional suppression (Asangani et al., 2008). However this does not negate the possibility that basal levels of mmu-mir21 are involved in the regulation of Pdcd4.

The identification of novel sex-specific E_2 targets in the AVPV opens the door to a plethora of possible mechanisms regarding reproduction to be studied. These data have implications for not only other sexually dimorphic nuclei, but in all tissues with ERα expression. Most importantly, it represents two novel mechanisms by which E_2 may exert more global control over both the transcriptome and the proteome. In the future, it will be imperative to map the expression of these transcripts. It is likely that the differences in expression levels are indicative of differences in expression patterns,

especially if these are direct transcriptional targets of ERα. However, it is altogether possible that some of these transcripts are in identical subpopulations in males and females, with a difference in the robustness of their response.

5.5 Tables

Table 5.1 Primers Used in QPCR

NCBI Number	Transcript	Forward Primer	Reverse Primer	Amplicon
NM_007956	Esr1	GTGCCAGGCTTTGGGGACTT	AGCAAACAGGAGCTTCCCCG	126 bp
NM_011809	Ets2	CCTTCAGTGGCTTCCAAAAG	ATTCACCAGGCTGAACTCGT	122 bp
NM_178260	Kiss1	CTCGTAGGTCGTCGCCATGC	GACAGGTCCTTCTCCCGCTG	130 bp
NM_011050	Pdcd4	GTTGCTAGATAGGCGGTCCA	TCACATCCACCTCTTCCACA	122 bp
NR_029738.1	mmu-mir21	GACATCGCATGGCTGTACCA	CCATGAGATTCAACAGTCAACATCA	92bp
NM_001127233	Trp53	GGGCTCACTCCAGCTACCTGAA	CTGAGTCAGGCCCCACTTTCTTG	185bp
NM_001025246	Tp53i11	TTTTTGATGGGGCTGAAGTC	AGAGTCCAGCGGATGATGAC	127 bp

5.6 Figures

Figure 5.1 Females Have Higher Esr1, Kiss1 and Ets2 in AVPV Micropunches

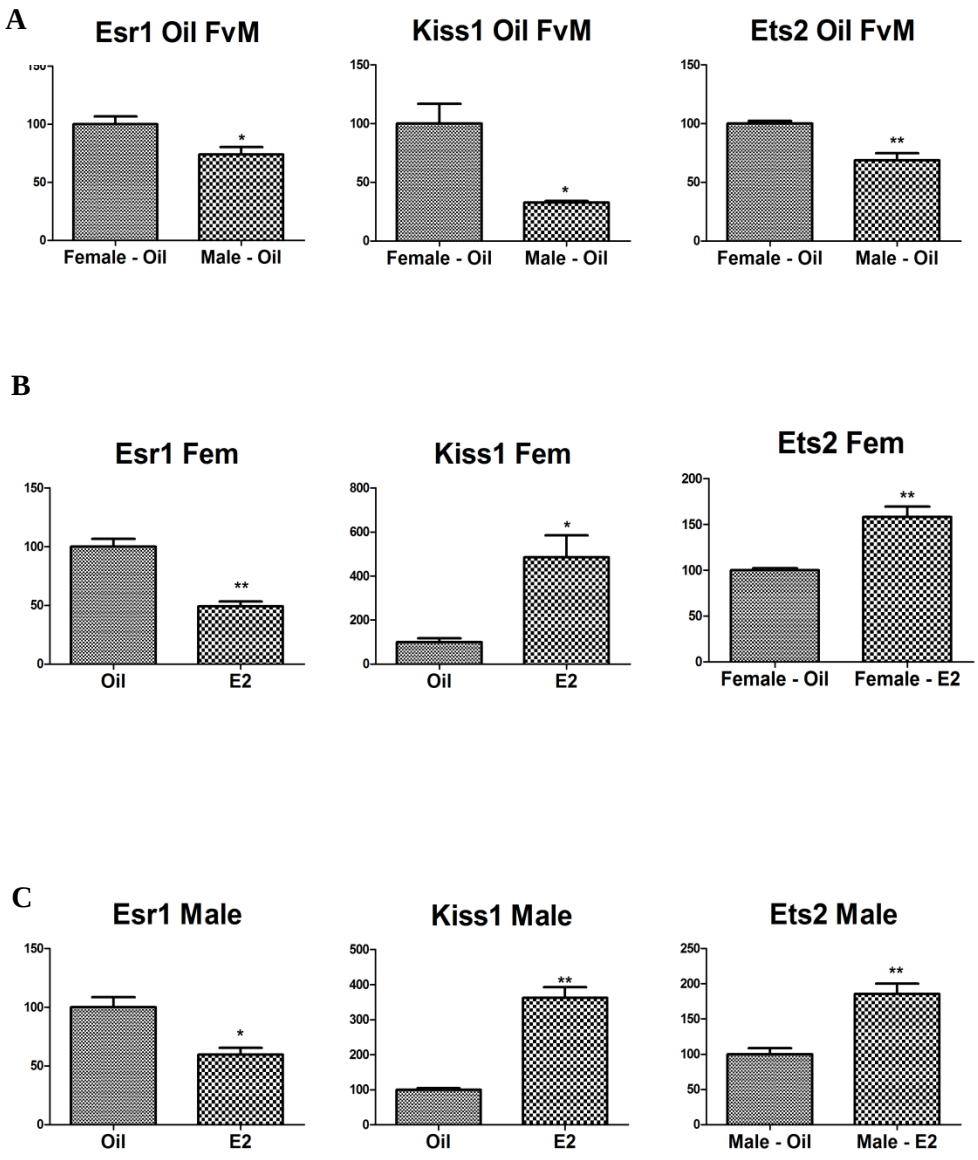


Figure 5.1 Levels of Esr1, Kiss1 and Ets2 in the AVPV of oil or E_2 treated mice. (A) Basal sex differences in gonadectomized mice. (B) Effects of E_2 in females. (C) Effects of E_2 in males. Bars = means ± SEM. Student t-test results: **p*-value <0.05; ***p*-value < 0.01.

Figure 5.2 Males Have Higher Expression of Pdcd4 and Trp53 in AVPV Micropunches

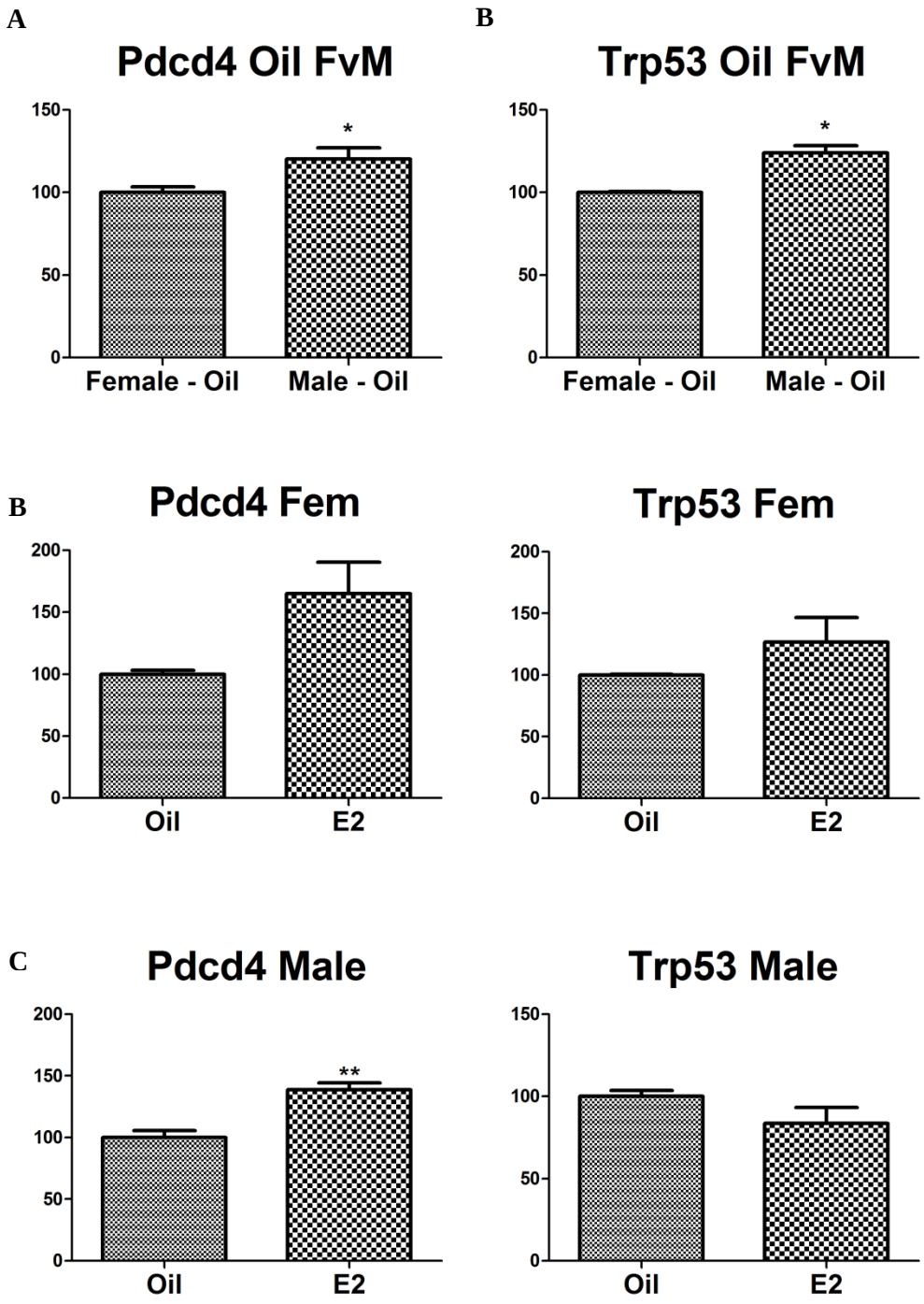


Figure 5.2 Levels of Pdcd4 and Trp53 in the AVPV of oil or E_2 treated mice. (A) Basal sex differences in gonadectomized mice. (B) Effects of E_2 in females. (C) Effects of E_2 in males. Bars = means ± SEM. Student t-test results: **p*-value <0.05; ***p*-value < 0.01.

Figure 5.3 Males and Females Have the Same Levels of Trp53i11 and mmu-mir21 in AVPV Micropunches

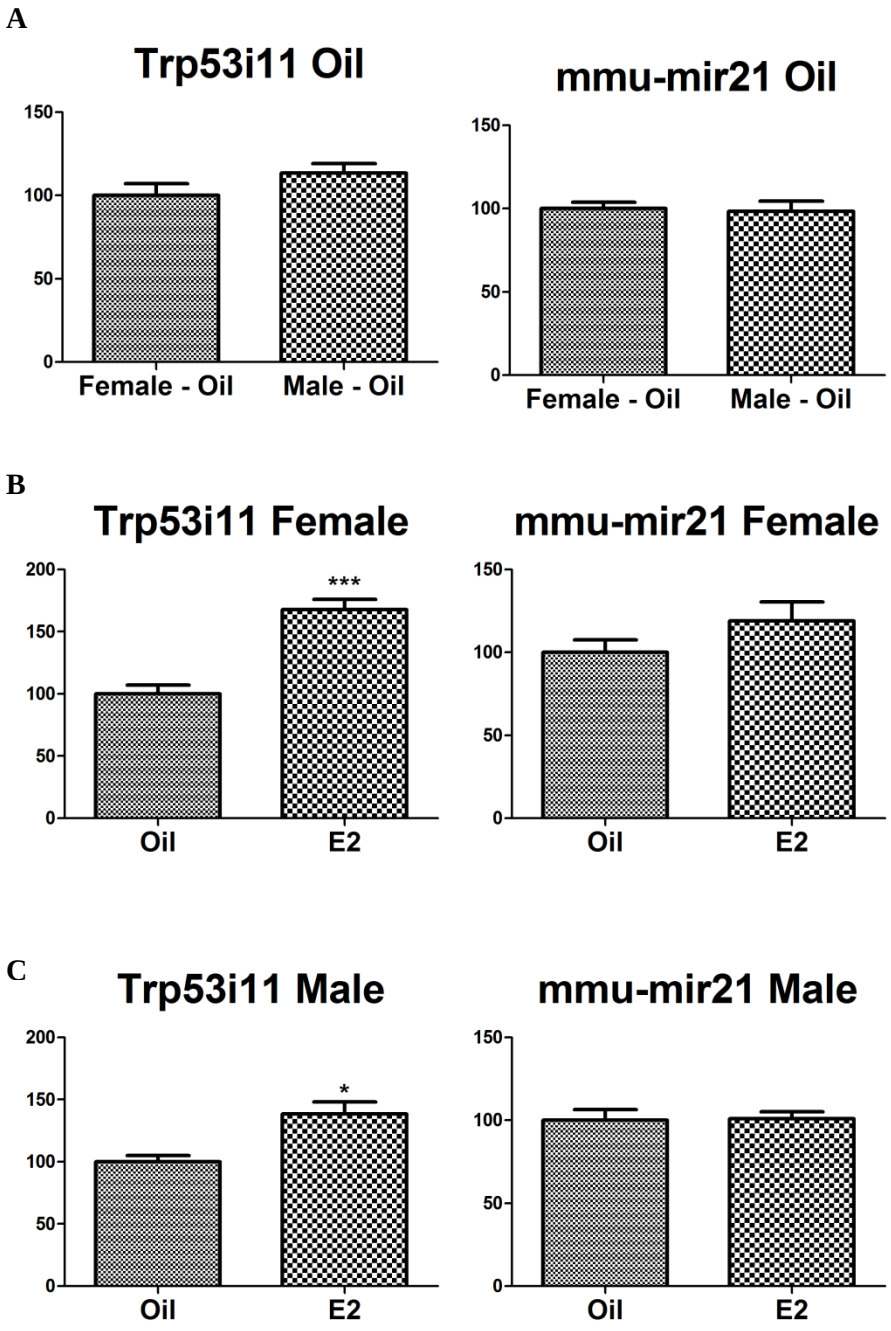


Figure 5.3 Levels of Trp53i11 and primary transcript of mmu-mir21 in the AVPV of oil or E_2 treated mice. (A) Basal sex differences in gonadectomized mice. (B) Effects of E_2 in females. (C) Effects of E_2 in males. Bars = means ± SEM. Student t-test results: **p*-value <0.05; ****p*-value < 0.001.

5.7 Bibliography

Asangani IA, Rasheed SA, Nikolova DA, Leupold JH, Colburn NH, Post S, Allgayer H (2008) MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. Oncogene 27:2128-2136.

Carletti MZ, Fiedler SD, Christenson LK (2010) MicroRNA 21 blocks apoptosis in mouse periovulatory granulosa cells. Biology of reproduction 83:286-295.

Felty Q, Singh KP, Roy D (2005a) Estrogen-induced G1/S transition of G0-arrested estrogen-dependent breast cancer cells is regulated by mitochondrial oxidant signaling. Oncogene 24:4883-4893.

Felty Q, Xiong WC, Sun D, Sarkar S, Singh KP, Parkash J, Roy D (2005b) Estrogen-induced mitochondrial reactive oxygen species as signal-transducing messengers. Biochemistry 44:6900-6909.

Kauffman AS, Gottsch ML, Roa J, Byquist AC, Crown A, Clifton DK, Hoffman GE, Steiner RA, Tena-Sempere M (2007) Sexual differentiation of Kiss1 gene expression in the brain of the rat. Endocrinology 148:1774-1783.

Liang XQ, Cao EH, Zhang Y, Qin JF (2004) A P53 target gene, PIG11, contributes to chemosensitivity of cells to arsenic trioxide. FEBS letters 569:94-98.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.

Liwak U, Thakor N, Jordan LE, Roy R, Lewis SM, Pardo OE, Seckl M, Holcik M (2012) Tumor suppressor PDCD4 represses internal ribosome entry site-mediated translation of antiapoptotic proteins and is regulated by S6 kinase 2. Molecular and cellular biology 32:1818-1829.

Porteous R, Petersen SL, Yeo SH, Bhattarai JP, Ciofi P, de Tassigny XD, Colledge WH, Caraty A, Herbison AE (2011) Kisspeptin neurons co-express met-enkephalin and galanin in the rostral periventricular region of the female mouse hypothalamus. The Journal of comparative neurology 519:3456-3469.

Rahmani Z, Blouin JL, Creau-Goldberg N, Watkins PC, Mattei JF, Poissonnier M, Prieur M, Chettouh Z, Nicole A, Aurias A, et al. (1989) Critical role of the D21S55 region on chromosome 21 in the pathogenesis of Down syndrome. Proceedings of the National Academy of Sciences of the United States of America 86:5958-5962.

Reynolds LE, Watson AR, Baker M, Jones TA, D'Amico G, Robinson SD, Joffre C, Garrido-Urbani S, Rodriguez-Manzaneque JC, Martino-Echarri E, Aurrand-Lions M, Sheer D, Dagna-Bricarelli F, Nizetic D, McCabe CJ, Turnell AS, Kermorgant S, Imhof BA, Adams R, Fisher EM, Tybulewicz VL, Hart IR, Hodivala-Dilke KM (2010) Tumour angiogenesis is reduced in the Tc1 mouse model of Down's syndrome. Nature 465:813-817.

Smith JT, Cunningham MJ, Rissman EF, Clifton DK, Steiner RA (2005) Regulation of Kiss1 gene expression in the brain of the female mouse. Endocrinology 146:3686-3692.

Wedeken L, Ohnheiser J, Hirschi B, Wethkamp N, Klempnauer KH (2010) Association of Tumor Suppressor Protein Pdcd4 With Ribosomes Is Mediated by Protein-Protein and Protein-RNA Interactions. Genes & cancer 1:293-301.

Wedeken L, Singh P, Klempnauer KH (2011) Tumor suppressor protein Pdcd4 inhibits translation of p53 mRNA. The Journal of biological chemistry 286:42855-42862.

Wolvetang EJ, Wilson TJ, Sanij E, Busciglio J, Hatzistavrou T, Seth A, Hertzog PJ, Kola I (2003) ETS2 overexpression in transgenic models and in Down syndrome predisposes to apoptosis via the p53 pathway. Human molecular genetics 12:247-255.

Wu Y, Liu XM, Wang XJ, Zhang Y, Liang XQ, Cao EH (2009) PIG11 is involved in hepatocellular carcinogenesis and its over-expression promotes Hepg2 cell apoptosis. Pathology oncology research : POR 15:411-416.

Yang HS, Cho MH, Zakowicz H, Hegamyer G, Sonenberg N, Colburn NH (2004) A novel function of the MA-3 domains in transformation and translation suppressor Pdcd4 is essential for its binding to eukaryotic translation initiation factor 4A. Molecular and cellular biology 24:3894-3906.

Yang HS, Jansen AP, Komar AA, Zheng X, Merrick WC, Costes S, Lockett SJ, Sonenberg N, Colburn NH (2003) The transformation suppressor Pdcd4 is a novel eukaryotic translation initiation factor 4A binding protein that inhibits translation. Molecular and cellular biology 23:26-37.

Yang Q, Rasmussen SA, Friedman JM (2002) Mortality associated with Down's syndrome in the USA from 1983 to 1997: a population-based study. Lancet 359:1019-1025.

Zhu J, Jiang J, Zhou W, Zhu K, Chen X (1999) Differential regulation of cellular target genes by p53 devoid of the PXXP motifs with impaired apoptotic activity. Oncogene 18:2149-2155.

CHAPTER 6

ETS2 IS BOTH A TRANSCRIPTIONAL TARGET OF 17β-ESTRADIOL AND A POTENTIAL MEDIATOR OF 17β-ESTRADIOL-RESPONSIVE GENES

6.1 Introduction

The identification of Ets2, a novel and sex-specific target of E_2 in the AVPV, has revealed a new possible mechanism by which E_2 may exert secondary, expansive control over the transcriptome, and ultimately the proteome. Putative binding sites for Ets family transcription factors appear almost ubiquitously in promoters; however they are not always functional (FitzGerald et al., 2004). On the other hand, research indicates a very high positive correlation between the clustering of multiple Ets, Sp1 and Ap-1 binding sites with actual Ets regulatory function (Hollenhorst et al., 2011). Furthermore, there is a strong negative correlation between functional Ets promoter regions and the presence of a functional TATA box (FitzGerald et al., 2004). It is therefore possible to screen for promoters that have a high probability of being regulated by Ets family transcription factors.

Ets2, the highest expressed member of the ETS superfamily in the brain (Hollenhorst et al., 2004), was increased 1.5-fold by E_2 in the AVPV (see Chapter 5). This is especially interesting for two reasons. Firstly, Ets2 is linked to early neural development as well as post-mitotic neurons, and it is postulated that it may be necessary to maintain proper neuronal function (Maroulakou et al., 1994). Secondly, ERα interacts

with Sp1 to synergistically mediate transcription (Krishnan et al., 1994, Porter et al., 1997), and Ets2 functions in this manner as well (Shirasaki et al., 1999, Jinnin et al., 2006, Sun et al., 2006). It is therefore plausible that Ets2 increases the stability between Sp1 and its response elements. This could particularly impact the E_2-target genes that rely heavily upon Sp1 tethering for promoter activation, such as cathepsin D (Krishnan et al., 1994) and Kiss1 (Li et al., 2007).

In both humans and rodents, Kiss1 is the critical mediator of the E_2 signal to the GnRH neurons, being obligatory in the LH surge mechanism, pubertal onset and the maintenance of fertility (de Roux et al., 2003, Seminara et al., 2003). Through *in vitro* assays, it is known that the human KISS1 (KISS1) promoter is driven by ERα cooperativity with Sp1 (Li et al., 2007). Even though the human and mouse Kiss1 proximal promoters share no sequence homology, they both exhibit a similar clustering of Sp1 and ERα response element (ERE) half sites, indicating that their regulatory mechanisms may be conserved, perhaps beyond that of an Sp1 site (Table 6).

Considering that Sp1 and Ap-1 binding sites are important for the functioning of both Ets2 and ERα, it stands to reason that some of the E_2-responsive genes could be independently regulated by Ets2. Of particular concern is Pdcd4, as it was expressed in a sex-specific manner, regulated by E_2 (see Chapter 5), and represents a new mechanism by which E_2 may exert general control over the proteome via translation inhibition (Wedeken et al., 2010).

To address these new questions and gain a broader perspective into how E_2 might be mediating some its effects, I employed *in silico* promoter analysis to map putative binding sites for Sp1, ERα, Ap-1 and Ets2. This group comprised 15 proximal

promoters. Further, I utilized N43 cells, a neuronal hypolthalamic mouse cell line to test the effects of Ets2 overexpression and knockdown on the mRNA levels of Esr1, Kiss1 and Pdcd4.

6.2 Materials and Methods

6.2.1 *In silico* Promoter Analysis

I analyzed the proximal promoters of 14 genes for potential response elements for *ap-1*, *sp1*, *esr1* and ets2. Considering 1kb - 1.5kb immediately upstream of the transcription start site, I utilized the Transcriptional Element Search System (TESS), formerly provided by the Computational Biology and Informatics Laboratory (University of Pennsylvania; Philadelphia, PA). The program annotated the nucleotide sequences with potential transcription factor binding sites, based on published response element sequences. I manually color coded the annotations and added additional annotations for possible weak ets2 binding sites, using the core binding sequence GGAA/T.

6.2.2 N43 Cell Culture

For these experiments, I used N43 immortalized embryonic hypothalamic neuronal cells (Cellutions Biosystems, Inc.; Burlington, Ontario, Canada). Cells were maintained in 6-well plates at 37° C and 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) Hyclone fetal bovine serum (FBS; Thermo Fisher Scientific, Rockford, IL), 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (PS-Gln; GIBCO-BRL; Gaithersburg, MA). Once seeded, cells were grown until they reached approximately 70% confluence. They were then rinsed with phosphate buffered saline (PBS) and media was replaced with phenol red-free DMEM with 10% charcoal-stripped FBS and PS-Gln. Cells were treated either with 10 μM E_2, transfected

with Ets2 overexpression vector with and without E_2, or transfected with siEts2 with and without E_2 treatment. Individual assays are described below.

6.2.3 E_2 Treatment of N43 Cells

After 12 h in phenol red-free media, cells were treated with vehicle or 10 μM E_2, diluted from a stock solution of 100 mM E_2 dissolved in 100% ethanol (EtOH), in phenol red-free media for 12 h. Cells were rinsed three times with phosphate buffered saline, then harvested in 1 mL of TRIzol ™ (Invitrogen; Carlsbad, CA) and stored in 1.5 mL microcentrifuge tube at -80°C until mRNA isolation.

6.2.4 Ets2 Overexpression in N43 Cells

After 8 h in phenol red-free media, cells were transiently transfected with either pCMV6 empty vector control (PS100001) or mEts2-kanamycin/neomycin expression plasmid MC200894), both from OriGene Technologies (Rockville, Md), using NeuroMag Magnetofection™ (OzBiosciences; San Diego, CA), in a ratio of 1:2, per maufacturers' protocols. Eight hours post transfection, I treated the cells with either or 10 μM E_2 or vehicle for 12 h, and harvested as written above.

6.2.5 N43 siEts2

After 8 h in phenol red-free media, cells were transiently transfected with either control siRNA-A (sc-37007) or Ets-2 siRNA (m) (sc-37856), both from Santa Cruz Biotechnology, Inc. (Dallas, TX), using NeuroMag Magnetofection™ (NM51000 from OzBiosciences; San Diego, CA), in a ratio of 1:2, per maufacturers' protocols. Eight hours post transfection, I treated the cells with either 10 μM E_2 or vehicle for 12 h, and harvested as written above.

6.2.6 RNA Isolation and QPCR

One μg total RNA was reverse transcribed into cDNA using MMLV-RT (Roche Diagnostics, Indianapolis, IN), following the manufacturer's protocol. I used Primer3™ software (http://bioinfo.ut.ee/primer3/) to design specific QPCR primers for: Esr1, Kiss1 and Pdcd4 (Table 6.6.2). I obtained the primers from Integrated DNA Technologies (Coralville, IA).

QPCR reactions were carried out in a Stratagene MX3000P™ thermocycler, utilizing MxPro™QPCR software (both Agilent Technologies; Santa Clara, CA). Reactions contained cDNA, diluted 1:10 with nuclease-free water, specific primers and SybrGreen™ QPCR Mastermix (Roche Diagnostics Corporation; Indianapolis, IN). Manufacturer's protocol was used with the following cycle settings: 95°C for 10 min, and 40 cycles of 95°C for 30 sec, 57°C for 30 sec and 72°C for 30 sec. Each sample was tested in duplicate. Primer specificity was verified via 2% agarose gel electrophoresis and confirmation of a single dissociative curve peak during each QPCR reaction.

6.2.7 Statistics

For QPCR, the duplicate raw cycle threshold (Ct) values were analyzed using the ΔΔCt (Livak and Schmittgen, 2001) method with β-actin employed as background control. Known E_2-induced transcripts within the AVPV, Esr1 and Kiss1, were used as positive treatment controls. QPCR reactions with nuclease-free water instead of cDNA were used as negative controls. I used Graphpad Prism™ to perform perform student t-test with Welch's correction for the E_2 only treatments, and two-way ANOVA statistical analyses with Bonferroni post-hoc analyses for Ets2 overexpression and siEts2 with and without E_2. Data is presented as mean ± SEM.

6.3 Results

6.3.1 *In silico* Analysis of the Proximal Promoters of 17β-Estradiol Target Genes Reveals Potential ets2 Response Elements

In silico promoter analysis of 14 E_2-responsive genes revealed multiple strong and weak ets2 transcription factor binding sites, clustered with half-sites for ER and multiple response elements for sp1 and ap-1. Only three promoters, for slitrk6, rasd1 and nts, possess putative TATA boxes (Table 6.6.1)

6.3.2 E_2 Increases Expression of esr1, but Decreases pdcd4 in N43 Cells

In N43 cells, 10 nM E_2 treatment increased expression of *esr1* to 165%, but decreased expression of *pdcd4* to 57%. There were no significant decreases in Kiss1 or Ets2 mRNA levels (Figure 6.7.1).

6.3.3 Ets2 Overexpression in N43 Cells Increases Expression of Esr1 and Pdcd4, and Increases Kiss1 in the Presence of E_2

In N43 cells, overexpression of *ets2* increased levels of Esr1 mRNA by 238% and Pdcd4 by 128%, with no further increase in either transcript observed using *ets2* overexpression with E_2 co-treatment. *Ets2* overexpression did not significantly increase Kiss1 mRNA, however *ets2* overexpression with E_2 co-treatment increased Kiss1 to 183%. *Ets2* overexpression increased Ets2 44,000% without E_2 and 42,000% with E_2, which was not significantly different (Figure 6.7.2).

6.3.4 Ets2 Knockdown Decreases esr1 Expression

In N43 cells, siEts2 decreased *esr1* expression to 77% and the decrease was maintained with E_2 co-treatment. Knockdown of Ets2 did not significantly decrease Kiss1 or Pdcd4 and E_2 co-treatment had no additional effects (Figure 6.7.3). Use of siEts2 decreased Ets2 to 34%, both with and without E_2.

6.4 Discussion

My *in silico* promoter analysis revealed many putative ets2 binding sites clustered with sp1 and ap-1 sites within 1.5kb upstream of the start sites of the 15 transcripts, with only three having putative TATA sequences. This type of clustering is also important to the mechanisms of ERα function, as it often operates via an sp1 site, as is the case with regulation of the kiss1 promoter (Li et al., 2007). In fact, ERα binding to Sp1 is so central to the basic mechanism by which functions, that inhibition of Sp1 is sufficient to decrease basal and E_2-induced levels of E_2 transcriptional targets (Abdelrahim et al., 2002). It therefore was conceivable that some of the clustering of Sp1/Ets2 binding sequences observed within the proximal promoters of the identified E_2-responsive genes represented regions that may also be responsive to Ets2. Granting that 11 of the *in silico* mapped genes represented plausible transcriptional targets of Ets2, I chose to further investigate *esr1*, *kiss1* and *pdcd4*, as all three differ in expression between the sexes in the AVPV and are regulated by E_2 (see Chapter 5).

Although the *in vitro* data presented herein regarding the regulation of esr1, ets2, kiss1 and pdcd4 by E_2 alone do not mirror my prior *in vivo* experiments, it is very possible that it is a consequence of the embryonic neurons not yet being fully differentiated, or perhaps there are insufficient cofactors. Interestingly, the overexpression of Ets2 in these cells increases *esr1* expression, mimicking the *in vitro* E_2 effect, whereas loss of Ets2 resulted in a concomitant loss of Esr1 that could not be compensated for by co-treatment with E_2. These data point toward a mechanism whereby E_2, presumably via ERα, regulates Ets2 expression, which in turn regulates Esr1 expression.

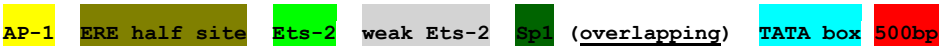
E_2 treatment alone was insufficient to regulate Kiss1 in either direction, but co-treatment with Ets2 resulted in a significant increase in Kiss1. This actually mimics the *in vivo* data, wherein E_2 treatment increased Kiss1 in the presence of an Ets2 increase. Although the loss of Ets2 did not significantly reduce levels of Kiss1, it could be a timing issue that depends on half-life of Kiss1. Nevertheless, this implicates Ets2 as necessary, at least in N43 cells, for E_2-mediated *kiss1* transcription. This is an important and novel finding, especially because both the mouse and human Kiss1 promoter have strong and weak Ets2 binding sites clustered within the first 300bp upstream of the transcription start site (Table 6.6.2).

While E_2 decreased *pdcd4* expression, the overexpression of ets2 alone was sufficient to increase *pdcd4* expression, mimicking the *in vivo* effect of E_2. However, knockdown of Ets2 failed to significantly decrease Pdcd4 mRNA levels, although this could be a timing issue. This suggests that in the N43 cells, basal levels of Kiss1 and Pdcd4 can be maintained by other mechanisms, but that Ets2 can act as an independent inducer, and is required for E_2-dependent increases.

When considered with the data from Chapters 3 and 5, it is apparent that Ets2 is not only a target of E_2, but can also regulate E_2-responsive genes, two of which, *esr1* and *kiss1*, are critical in the neuroendocrine control of ovulation (Petersen et al., 2003, Gore et al., 2011) and (Li et al., 2007, Teles et al., 2008). This may prove significant not only for the onset of puberty, but also for mechanisms involving reproductive senescence, when a decrease Kiss1 expression occurs (Lederman et al., 2010) and thus it bears further investigation.

6.5 Tables

Table 6.1 *In silico* Promoter Analysis



1.5kb upstream C1ql2:

```
   1 gactgtatct gcaaggaag ggaagagaac tcacaaagca accacacagc cgttcttagc
  61 aggaatattg tggagacctg tgctgtggtg ggaccagcaa gtggccagcg gtgggagcag
 121 aagcaaccac agtgaaaagt gagactccac tgagcatggc atctgataaa caaccactct
 181 ctttagagca caagccccgc cccggaaggt ggcccagaaa ccagaggtgt gggggtcactc
 241 acttgtgtag gtgatagaca gcaccaggac atccagccta cccagaccag gggctcttcc
 301 cctctgaggt aggtgccttc ccagctcccc gggggagcag gggccagtg tgtgtggctc
 361 tgtttcctct ttagtagcag tagaaggaca tagatgattc tcctttaatt gtgcccagc
 421 tggaggaggg gtgcaaatgc atttggatgc agggagaggg agctggggtg ttcctggaga
 481 ggaactcact caccagcctg gagcagggct ccctgtgcct tcagtgtgaa tggtgaaggt
 541 aagggaggag aatttactaa aacacgttgt tttaaaatgc tataatgaca tctaatattt
 601 tgtatgatgt ttacgaaaga aactaaattt aaagaagaaa ataagtaaca acaggaaggg
 661 ggaagagatt gagaccctga ggaggaggag gaggaggaga aggaatctgc ctttaagaaa
 721 ttagagttga ggcaggggag agagggcttg agttaagagt actggctgct cttcctagag
 781 gacctagatg caattctcag cacccacata gcagctcaca aatgtctgaa actccaattc
 841 ttgggaatct gacacgatca cacatgcagg caaaatacca atgtacatga attaaaaaaa
 901 aaaaaaacaa cctttaaaag aaacaagggt tcagtaccac tactgacatc ttgtttcccc
 961 agaggcctta ctttaattat ttattgtttc cacttagttg ctcaattaat taatttagag
1021 gtttttttct tccttttctt ttctttttc tttctctctt ttttttcttc ttaagacagg
1081 gtttctctgt gtagctcagg ctatcctgga actcactctg tagaccaggc tggccttgta
1141 ctcaaagatc tgcctgcctc tgcctcccca gtgctgggat taaagacatg caccatcact
1201 gccctgcttt cctcttttta ttttgaaaat tgttcatcaa cagttactaa acgtgttcga
1261 attccaagag ctgactagac atataagacc attcagcctt ctgaataaga tgtaggtgtg
1321 tccctcctct tactcctcta tttggaagtt ggttactttc tgtatgtagt atgcgaatcc
1381 ccctctgcca ccccgctttc tgttttaaaa cagaaaaggc tgcaacatac agtgtgtggt
1441 tctgttcttg aactggaagc ttaggctgtc ctggacttgg gttgagacct gggctcatcc
1501 a
```

1.5kb upstream from Creb3l1:

```
   1 tggctgaatc gctcttccac caggggtccc gcagccacat ttcgggaacc ctcgggccag
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1.5kb upstream from Ets-2:

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1.5kb upstream from Gadd45a:

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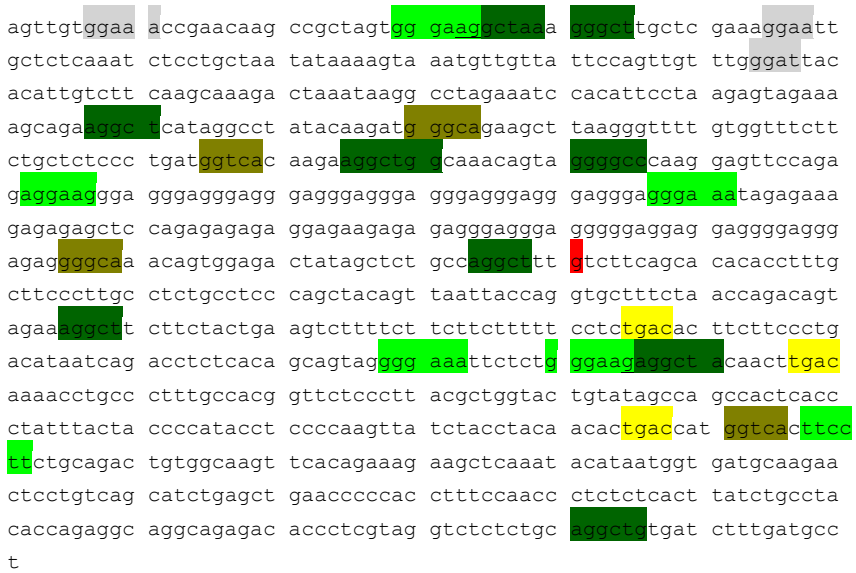
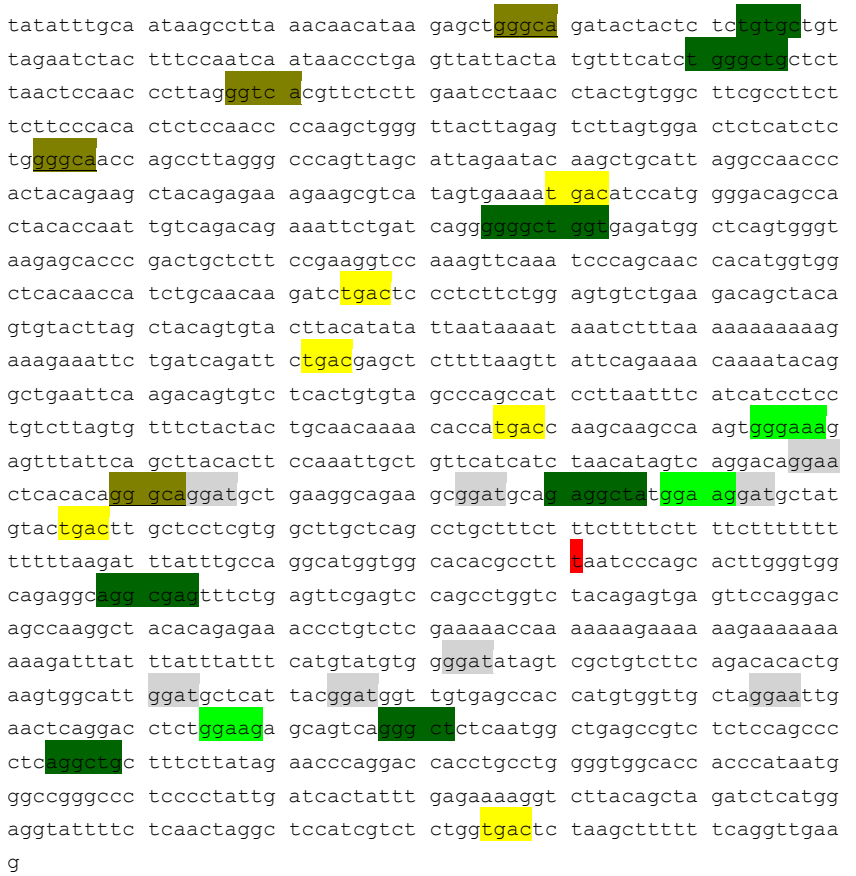
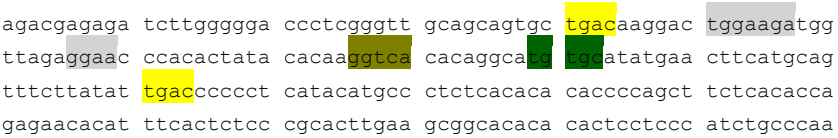


1.5kb upstream from HDC



1kb upstream from KISS1



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361 gctaggagaa cagcatgctt aatagaatag gtcttgagtt gatgattgcc ctcctagagg
421 tacagagaca caccccagag tgtttaggaa taaaaagccc ttctaaatat caacatcatg
481 ctgtcatgat gcgtgatgat gtgagctcac agcagtatcg ataatttggg agatcctgga
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721 ttttcatcct agacgtgtga gtataggggt acccctgggg taagtagtgc tgtgttttct
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901 tttcttgatc tgcaactggc tgactcagat ggaaaccctg ccatggcaga gaggcatgac
961 aggtcagtgg ggaaacacagg ctctggtatc aggagaccta t
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(-190) 2.5 fold E2 induction; (-534) 4 fold; (-1kb) 8 fold

1 kb upstream of Kiss1

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```

1.5kb upstream of Nts

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 481 ttctaagtta tccttttctt agtctcaata ttctgtcttt aaatataaat tctctactca
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 661 cttcatttat ttacttctcc taatttatca gcagatgttt ttgatttttg tttgtttttt
 721 acattttctt ctatatatct tattttgaaa taacctgtag ttttttaaaa aaataaacag
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```
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1501 a
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1.5kb upstream from Pdzrn3:

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1.5kb upstream from **human PGR**

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1501 t
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1.5kb upstream from Pgr:

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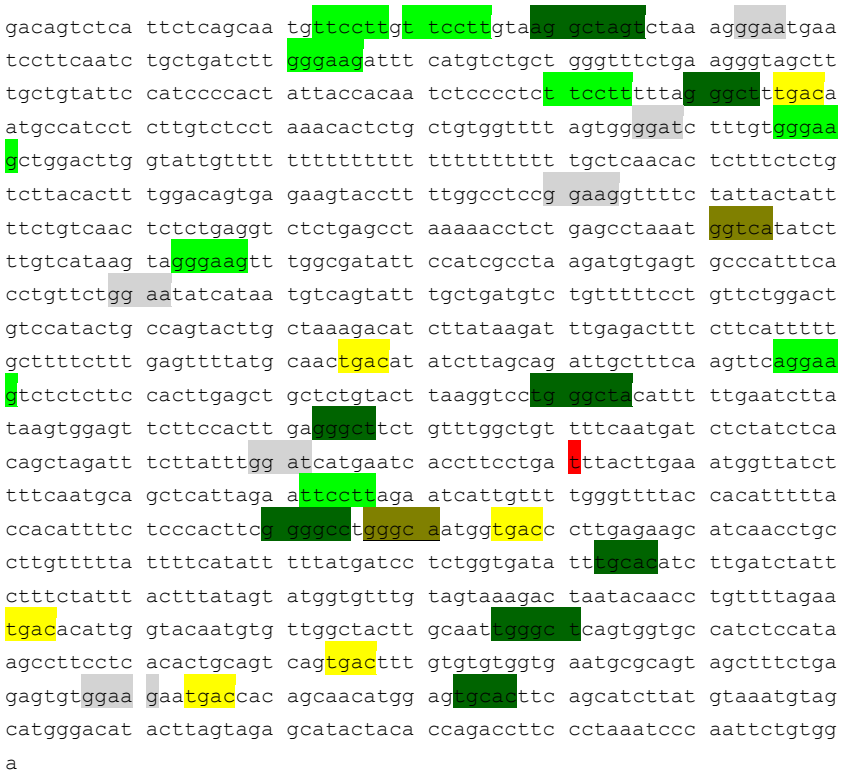
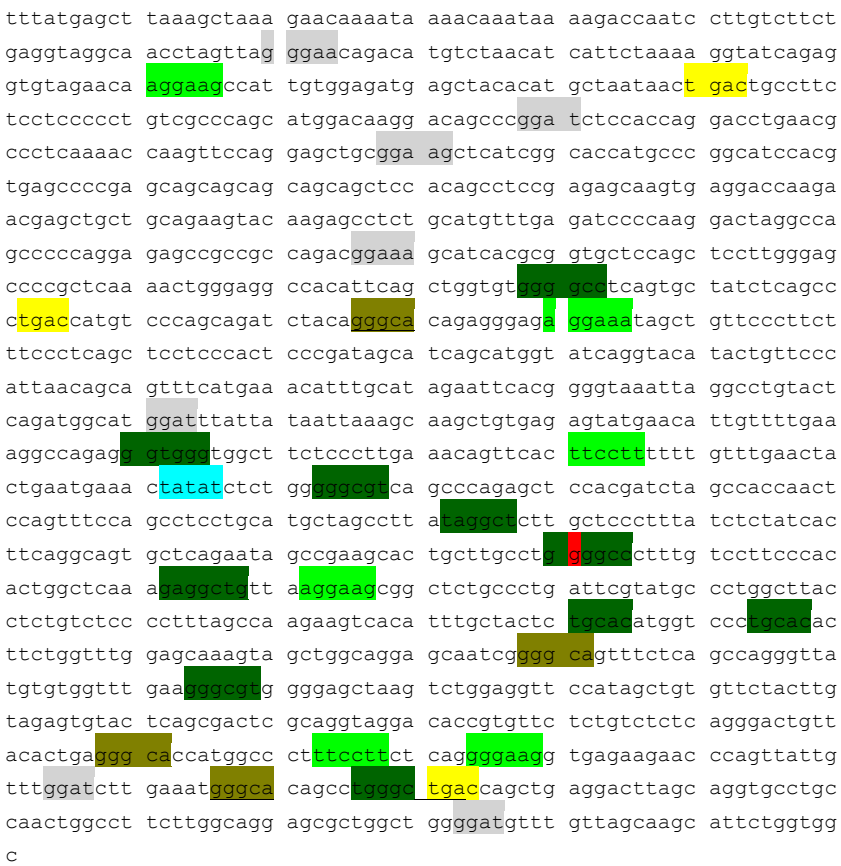


1.5kb upstream from Rasd1:



1.5kb upstream from Slitrk6:

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 721 aaggcctcga tgtcttaacg tcacgaggac ttgcatgtta tttactggct actgtgactt
 781 ttctgagaaa tcttcctata acttacatat catagagtaa gccagaatta aaaatactgt
 841 tgcgaaaata ttatactact gtcaatacat tacagaaatt taattttgta cataactaaa
 901 gatatacagc gatacacaat gaaaacaaga tcatttctac ttggaacaag aaagtaacca
 961 tatataatta aggtgtgttc aggtaaccac ctcatatggc aaagatgaaa gtactgtaat
1021 aagaattgga gcactgggtt actctgttag ctcaggggcc aagtgacttt gtaaacaagt
1081 aatgaaatag ctctcatttt gtttaataaa aaaggtatat agaacaaaag taaataatca
1141 aagacttaaa ttaaaaatgt ttgacagatc ataccttatt tgaaatacaa gccaacaatt
1201 aataattagt aggaaaatac atggcaaggg aatatctact gagaaataaa tattcagtta
1261 cctaaaatat tggtacacct tggaagctat aatatttttc acatccttta aagttttact
1321 catattttat tgcttttaat tttgtggata actgtaactt gaagaatata cttacatggt
1381 ttcataaata actttgtttc tcttgataat aagttgaaaa attaagacta ttaatttcag
1441 caatgaaaaa aaatccatgg agatataaaa tgttactgca ctgaaaagag aaggatagtc
1501 c
```

1.5kb upstream from Rasd1:

```
   1 tttatgagct taaagctaaa gaacaaaata aaacaaataa aagaccaatc cttgtcttct
  61 gaggtaggca acctagttag ggaacagaca tgtctaacat cattctaaaa ggtatcagag
 121 gtgtagaaca aggaagccat tgtggagatg agctacacat gctaataact gactgccttc
 181 tcctcccccc gtcgcccagc atggacaagg acagcccgga tctccaccag gacctgaacg
 241 ccctcaaaac caagttccag gagctgcgga agctcatcgg caccatgccc ggcatccacg
 301 tgagccccga gcagcagcag cagcagctcc acagcctccg agagcaagtg aggaccaaga
 361 acgagctgct gcagaagtac aagagcctct gcatgtttga gatccccaag gactaggcca
 421 gcccccagga gagccgccgc cagacggaaa gcatcacgcg gtgctccagc tccttgggag
 481 ccccgctcaa aactgggagg ccacattcag ctggtgtggg gcctcagtgc tatctcagcc
 541 ctgaccatgt cccagcagat ctacagggca cagagggaga ggaaatagct gttcccttct
 601 ttccctcagc tcctcccact cccgatagca tcagcatggt atcaggtaca tactgttccc
 661 attaacagca gtttcatgaa acatttgcat agaattcacg gggtaaatta ggcctgtact
 721 cagatggcat ggatttatta taattaaagc aagctgtgag agtatgaaca ttgttttgaa
 781 aggccagagg gtgggtggct tctcccttga aacagttcac ttccttcttt gtttgaacta
 841 ctgaatgaaa ctatatctct gggggcgtca gcccagagct ccacgatcta gccaccaact
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 961 ttcaggcagt gctcagaata gccgaagcac tgcttgcctg gggccctttg tccttcccac
1021 actggctcaa agaggctgtt aaggaagcgg ctctgccctg attcgtatgc cctggcttac
1081 ctctgtctcc cctttagcca agaagtcaca tttgctactc tgcacatggt ccctgcagac
1141 ttctggtttg gagcaaagta gctggcagga gcaatcgggg cagtttctca gccagggtta
1201 tgtgtggttt gaagggcgtg gggagctaag tctggaggtt ccatagctgt gttctacttg
```

```
1261 tagagtgtac tcagcgactc gcaggtagga caccgtgttc tctgtctctc agggactgtt
1321 acactgaggg caccatggcc ctttccttct caggggaagg tgagaagaac ccagttattg
1381 tttggatctt gaaatgggca cagcctgggc tgaccagctg aggacttagc aggtgcctgc
1441 caactggcct tcttggcagg agcgctggct ggggatgttt gttagcaagc attctggtgg
1501 c
```

1.5kb upstream from Trp53i11:

```
   1 cacatacttg cacatactcc ctacctctag ggcacagagg tgttgccttt cctgcggagg
  61 gagcccagga caggacagat ggattagcag ctgcagggtc ttcgtaggag taaccctcac
 121 cggttggctg ctccctactt ctaaggcttt cggtagaaca ttttagcaca ttcggaatcg
 181 agcttctaca ggaacaatag gaatctgagt ttccacttgg caaacctttt taagcagtcg
 241 ccttactggc ttctcgaaag ctccaaggag cagtgataac tgcatgtcca tttccttccc
 301 agatgcaaca gagggtcact aggacagggg gtttggagag gcctttacca tcacagttca
 361 cgtcggctaa ctatgcgtgt tgcttcatgt tactatgtgc ctgaccaggt aagctcgcct
 421 gcagccgatg aaaggatgag gtaacttaga aacatgtttg aacaaaggac tcagcacata
 481 ttttctctta ctctgccagg cacctctggg aaggatgcat gcccccatgc tgtctccatt
 541 tattggttga agaaatacaa ggactaaatg gtggccttaa gaatgatctt aaacaaatgc
 601 agagttttgg agaatgtatg cacagaggtg ggcacactag gggagtggat ggggagatgg
 661 ttggatagtc tcttgtccct cagtactgtt ttttccaatt agctgtagtg agcgctctgt
 721 gtctatcagc atgcaagcag aagccagaga gaggggtgag gtttaccaga gggctctgta
 781 cttggcattg ccgtcgtgga tatcctctga gctctgtgtt ctttcagtct cccagcctgt
 841 cagccttgat tgtgagcagt ttcccttctg ttagagatca tgggtacata tctgtcctcc
 901 caacaggtg ggagtcaggg atgccttttct gtcaactcca aggccccagg actcagcact
 961 ttacctagca cacatctgga tagctatacc ttctgtgatg gtttgtatat gcttggccta
1021 gggagtggca ctattaggag gtgtgacctt gttggaatag gtgtgttcct gttgatgtgg
1081 cttaaggccc tcaccctagc tgcctggaag ccagtattct gctagcagcc ttcagatgaa
1141 gatgtagaac tctcagctcc tcctgcatca tgcctgcctg gatgctgcca ttttcccacc
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1321 tatggggctc tcacttcctg ataagacagg catggaatct ccagaacaca gatgcccaga
1381 gcttatagct ctgtctacac cgagcatcac tgaaagtgta agtcctaaca ctggtgaccc
1441 tgtttggttc cacttgcttg ctgctaatga aagtgagatc attacttcta acatagcctc
1501 t
```

Table 6.2 Primers Used in QPCR

NM_007956	Esr1	GTGCCAGGCTTTGGGGACTT	AGCAAACAGGAGCTTCCCCG	126 bp
NM_011809	Ets2	CCTTCAGTGGCTTCCAAAAG	ATTCACCAGGCTGAACTCGT	122 bp
NM_178260	Kiss1	CTCGTAGGTCGTCGCCATGC	GACAGGTCCTTCTCCCGCTG	130 bp
NM_011050	Pdcd4	GTTGCTAGATAGGCGGTCCA	TCACATCCACCTCTTCCACA	122 bp

6.6 Figures

Figure 6.1 E_2 Treatment of N43 Cells

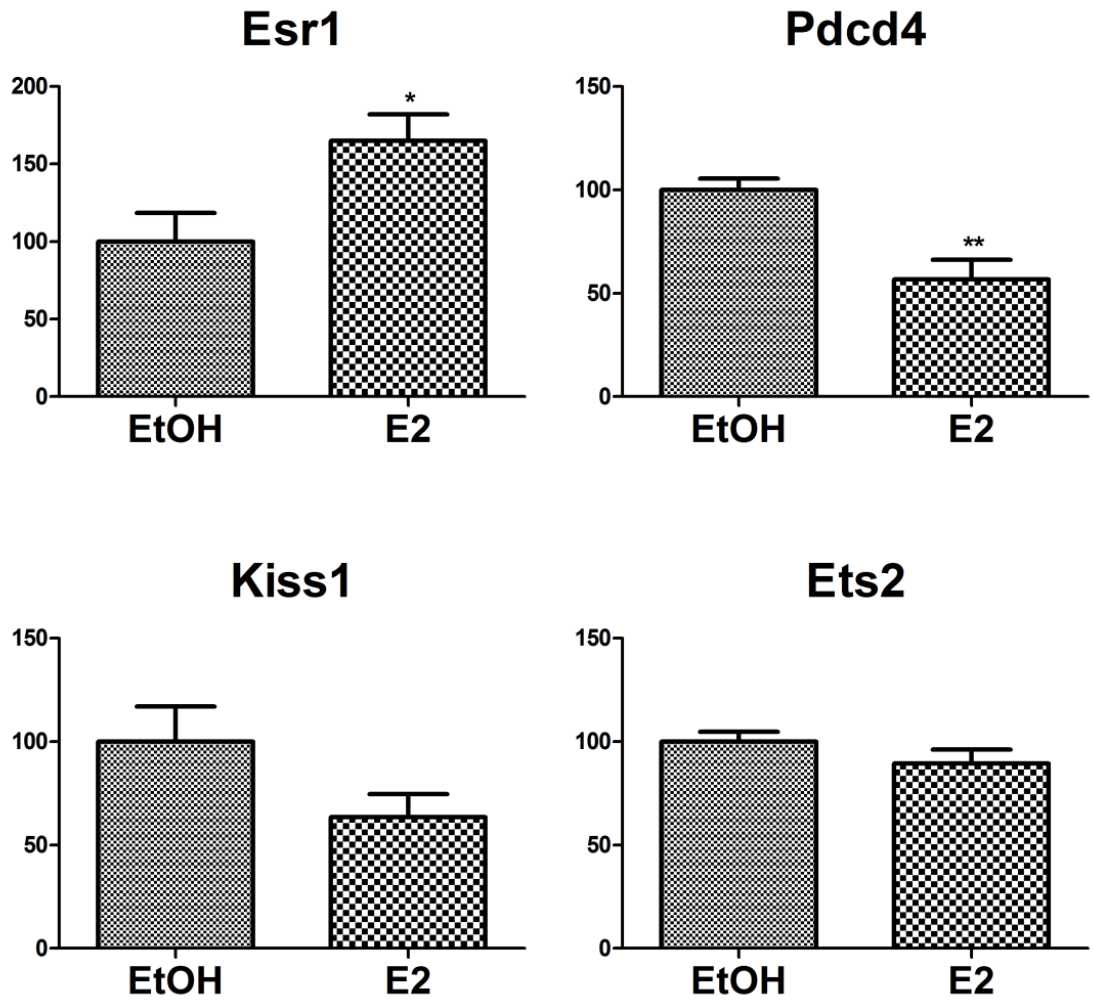

Figure 6.1 Levels of Esr1, Pdcd4, Kiss1 and Ets2 in N43 neuronal cells following 12 h E_2 treatment. Bars = means ± SEM. Student t-test results: *p-value < 0.05; ** p-value < 0.001.

Figure 6.2 Ets2 Overexpression in N43 Cells, With and Without E_2

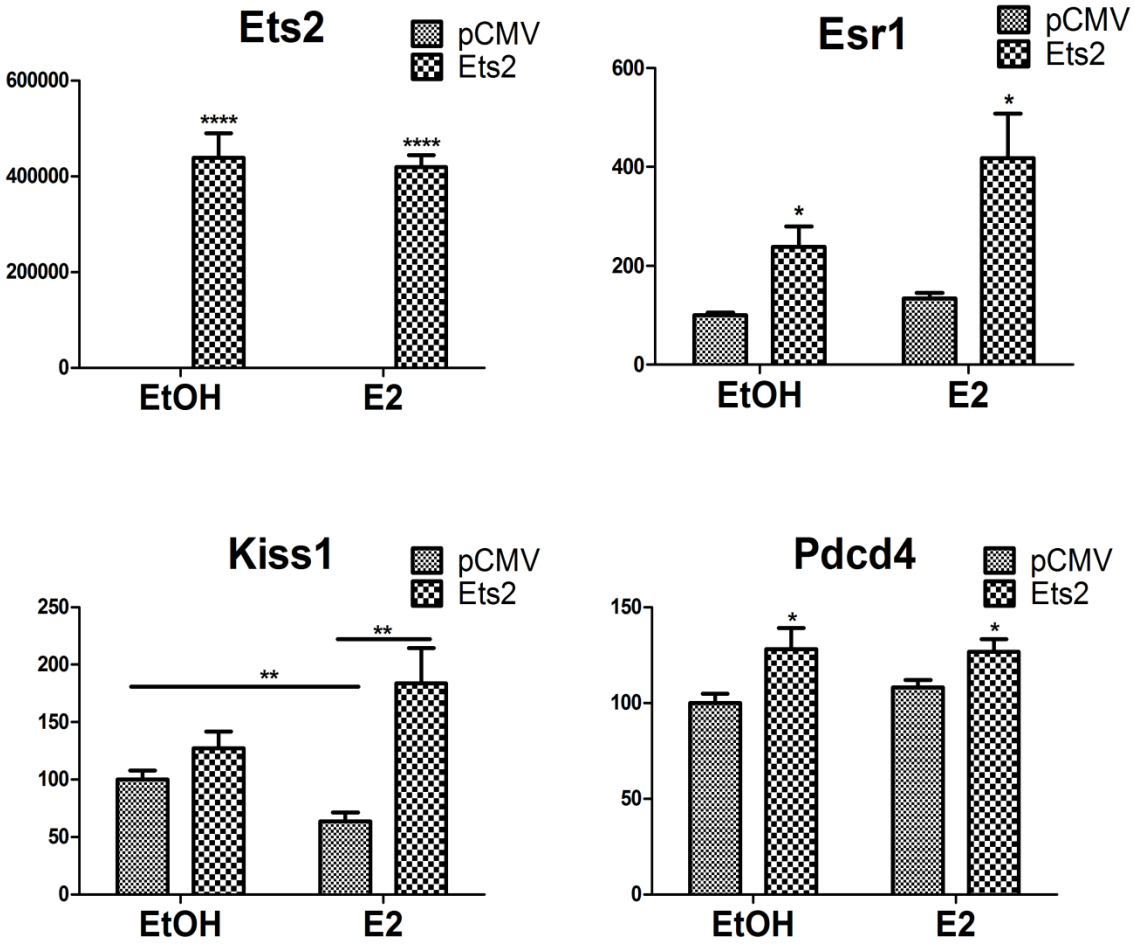

Figure 6.2 Levels of Ets2, Esr1, Kiss1 and Pdcd4 following Ets2 overexpression, with and without E_2 cotreatment.

Figure 6.3 Ets2 Knockdown in N43 Cells, With and Without E_2

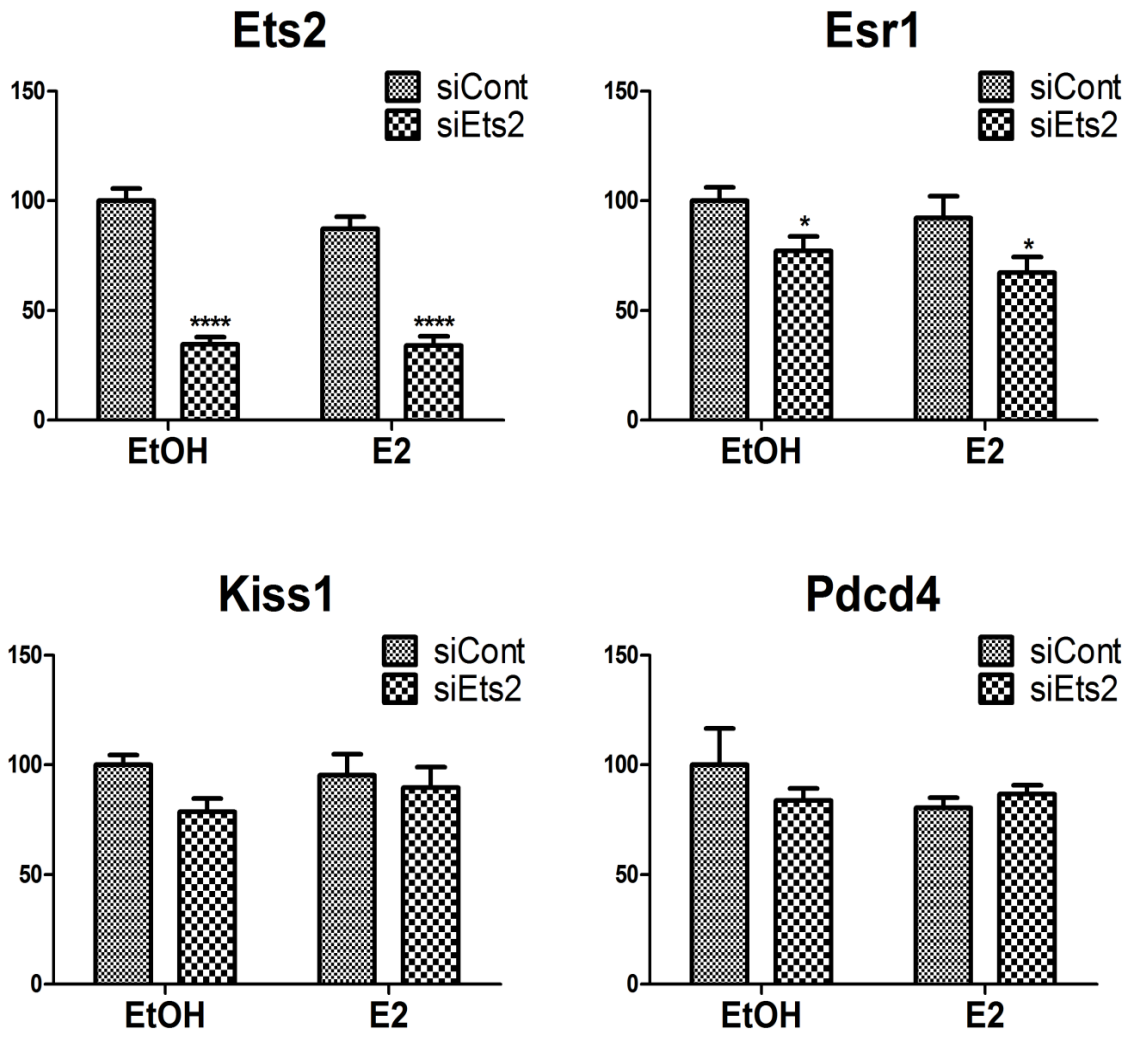

Figure 6.3 Levels of Ets2, Esr1, Kiss1 and Pdcd4 following Ets2 knockdown, with and without E_2 cotreatment.

6.7 Bibliography

Abdelrahim M, Samudio I, Smith R, 3rd, Burghardt R, Safe S (2002) Small inhibitory RNA duplexes for Sp1 mRNA block basal and estrogen-induced gene expression and cell cycle progression in MCF-7 breast cancer cells. The Journal of biological chemistry 277:28815-28822.

de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E (2003) Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. Proceedings of the National Academy of Sciences of the United States of America 100:10972-10976.

FitzGerald PC, Shlyakhtenko A, Mir AA, Vinson C (2004) Clustering of DNA sequences in human promoters. Genome research 14:1562-1574.

Gore AC, Walker DM, Zama AM, Armenti AE, Uzumcu M (2011) Early life exposure to endocrine-disrupting chemicals causes lifelong molecular reprogramming of the hypothalamus and premature reproductive aging. Molecular endocrinology (Baltimore, Md 25:2157-2168.

Hollenhorst PC, Jones DA, Graves BJ (2004) Expression profiles frame the promoter specificity dilemma of the ETS family of transcription factors. Nucleic acids research 32:5693-5702.

Hollenhorst PC, McIntosh LP, Graves BJ (2011) Genomic and biochemical insights into the specificity of ETS transcription factors. Annual review of biochemistry 80:437-471.

Jinnin M, Ihn H, Asano Y, Yamane K, Trojanowska M, Tamaki K (2006) Platelet derived growth factor induced tenascin-C transcription is phosphoinositide 3-kinase/Akt-dependent and mediated by Ets family transcription factors. Journal of cellular physiology 206:718-727.

Krishnan V, Wang X, Safe S (1994) Estrogen receptor-Sp1 complexes mediate estrogen-induced cathepsin D gene expression in MCF-7 human breast cancer cells. The Journal of biological chemistry 269:15912-15917.

Lederman MA, Lebesgue D, Gonzalez VV, Shu J, Merhi ZO, Etgen AM, Neal-Perry G (2010) Age-related LH surge dysfunction correlates with reduced responsiveness of hypothalamic anteroventral periventricular nucleus kisspeptin neurons to estradiol positive feedback in middle-aged rats. Neuropharmacology 58:314-320.

Li D, Mitchell D, Luo J, Yi Z, Cho SG, Guo J, Li X, Ning G, Wu X, Liu M (2007) Estrogen regulates KiSS1 gene expression through estrogen receptor alpha and SP protein complexes. Endocrinology 148:4821-4828.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.

Maroulakou IG, Papas TS, Green JE (1994) Differential expression of ets-1 and ets-2 proto-oncogenes during murine embryogenesis. Oncogene 9:1551-1565.

Petersen SL, Ottem EN, Carpenter CD (2003) Direct and indirect regulation of gonadotropin-releasing hormone neurons by estradiol. Biology of reproduction 69:1771-1778.

Porter W, Saville B, Hoivik D, Safe S (1997) Functional synergy between the transcription factor Sp1 and the estrogen receptor. Molecular endocrinology (Baltimore, Md 11:1569-1580.

Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno JS, Jr., Shagoury JK, Bo-Abbas Y, Kuohung W, Schwinof KM, Hendrick AG, Zahn D, Dixon J, Kaiser UB, Slaugenhaupt SA, Gusella JF, O'Rahilly S, Carlton MB, Crowley WF, Jr., Aparicio SA, Colledge WH (2003) The GPR54 gene as a regulator of puberty. The New England journal of medicine 349:1614-1627.

Shirasaki F, Makhluf HA, LeRoy C, Watson DK, Trojanowska M (1999) Ets transcription factors cooperate with Sp1 to activate the human tenascin-C promoter. Oncogene 18:7755-7764.

Sun HJ, Xu X, Wang XL, Wei L, Li F, Lu J, Huang BQ (2006) Transcription factors Ets2 and Sp1 act synergistically with histone acetyltransferase p300 in activating human interleukin-12 p40 promoter. Acta biochimica et biophysica Sinica 38:194-200.

Teles MG, Bianco SD, Brito VN, Trarbach EB, Kuohung W, Xu S, Seminara SB, Mendonca BB, Kaiser UB, Latronico AC (2008) A GPR54-activating mutation in a patient with central precocious puberty. The New England journal of medicine 358:709-715.

Wedeken L, Ohnheiser J, Hirschi B, Wethkamp N, Klempnauer KH (2010) Association of Tumor Suppressor Protein Pdcd4 With Ribosomes Is Mediated by Protein-Protein and Protein-RNA Interactions. Genes & cancer 1:293-301.

CHAPTER 7

DISCUSSION

7.1 General Discussion

The adult female AVPV is obligatory in mediating the positive effects that E_2 has on the GnRH neurons to elicit the LH surge. Nearly all of these neurons are ERα-expressing GABAergic/glutamatergic cells, yet within there exist subpopulations. This dictates the way in which the effects of E_2 are conveyed through this nucleus, and is indicative of a complex intersection of functions necessary for both pubertal onset and reproductive maintenance. The novel genes identified within this transcriptome study further support that.

7.2 E_2 Regulation of Feeding and Drinking Genes

My initial microarray findings establish a new foundation of exploration into the functions of E_2 within the AVPV. This targeted, yet global approach reveals new prospective players in the neuroendocrine control of female reproduction, for which this nucleus is most widely known. With the identification of feeding and drinking behavior via the Ariadne ™ pathway analysis in Chapter 2, this further supports the growing body of literature indicating a broader role for the AVPV, concerning integration of energy balance, body weight management and pubertal onset.

Leptin, produced by body fat, is the primary adiposity signal to initiate puberty. In both *ex vivo* and *in vivo* studies, it was shown that targeted loss of leptin receptors (LR) in the GABAergic, but not the glutamatergic, neurons resulted in impaired pubertal

maturation (Vong et al., 2011, Martin et al., 2014). Though Kiss1 colocalizes with both GABAergic cells (Petersen et al., 2012) and LR-expressing cells, there is likely a redundant mechanism of signaling to the GnRH neurons regarding adiposity, as Kiss1 cells do not gain LR until after puberty (Cravo et al., 2013).

Importantly, the AVPV receives inputs from the ventral premammillary nucleus (PMv), a region dense with LR and important for mediating the adiposity signal. These neurons also colocalize with glutamate, and as stated above, loss of LR in glutamatergic cells did not impair fertility. This is intriguing because lesion of this nucleus prevents pubertal onset (Donato et al., 2011). This again indicates that there are secondary mechanisms, besides that of LR, working through these nuclei that participate in the control of puberty and ovulation. Indeed, the premammillary nucleus is a production site for neuropeptide Y, α-melanocortin stimulating hormone and cholecystokinin (Lantos et al., 1995), receptors for which, Npy2r, Mc4r and Cckar, were all identified as regulated by E_2 in the AVPV on my microarray (see Chapter 2).

One of these receptors, npy2r, was the most significantly decreased transcript on the microarray. Based on its protein sequence homology to the second most decreased gene on the array pgr15l, it was recently identified as a marker of primary cilia (non-motile cilia) in neurons (Loktev and Jackson, 2013). This is important because mild cilopathies, such as Bardet-Biedl syndrome (BBS), manifest with hyperphagia and truncal obesity (Sheffield, 2010). Furthermore, *tubby* mice, characterized by delayed onset obesity and similar to BBS (Noben-Trauth et al., 1996), lack npy2r in their hypothalamic cilia, which has been implicated in the pathophysiology of their obese

phenotype (Loktev and Jackson, 2013). Importantly, the E_2-induced decreases in both Npy2r and Pgr15l validated by QPCR.

Taken together, the role for the AVPV as an integrative center for feeding behavior, energy balance and the onset of puberty is growing. In the future, it will be valuable to map Cckar, Mc4r, Npy2r, and Pgr15l within the AVPV, as well as perform colocalization studies with LR, ERα and gad (a marker of GABAergic cells).

7.3 E_2 Regulation of Novel Tumor Suppressor Genes, *trp53i11* and *pdcd4* in the AVPV

Identification of numerous tumor suppressors was intriguing, nevertheless it does not mean that their function in this nucleus is limited to tumor suppression, nor does the fact that this was identified within the AVPV necessarily mean that they are participatory in the LH surge mechanism. The data presented herein suggests they represent basic mechanism by which E_2 functions, both directly and indirectly. In the case of Trp53i11, there was no sex-specific differences in expression or regulation by E_2, indicating that it may be occurring independently of ERα. However, as Trp53i11 induction is generally a precursor to apoptosis, E_2-mediated cell death in the adult female AVPV bears closer investigation.

Regulation of Pdcd4 is of particular interest because it supports a far-reaching influence of E_2. Higher in males, Pdcd4 is capable of interacting directly with the structured 5'-UTR of a transcript, or indirectly by binding to elongation initiation factor 4a1 (eif4a1), both methods preventing binding to the ribosome and thus translation. One of the primary targets of Pdcd4 is Trp53 (Wedeken et al., 2011). Inhibition of Trp53 is

associated with cell survival and thus would provide a new method by which E_2 exerts some of its neuroprotective effects.

Considering this may represent a generalized mechanism of E_2 function, a more comprehensive characterization is warranted. As the sex differences in the size of this nucleus occurs early in development, it may be informative to determine the expression of Pdcd4 both before and during that differentiation process. Likewise, it is imperative to characterize the distribution of Pdcd4 in the AVPV in both sexes, but may also be informative to do so in the SDN and ARH as well.

7.4 Ets2 as Both a Target of E_2 and a Potential Mediator of E_2-Responsive Genes

The male AVPV has 26% less esr1 expression than females, but it was not inhibited as much in males by E_2 treatment. Interestingly, the sex difference in basal *ets2* expression was very similar to that of esr1; however the males yielded a more robust response. Conversely, females exhibited a more robust E_2-response in both Kiss1 and Pdcd4 levels. The differences in the strength of the E_2 responses between male and females is suggestive of differences in their subpopulations, especially as some of these may not be primary responses, but secondary. When considered with the *in vitro* data, it could be a function of which cells have ERα/Ets2 colocalization.

7.5 Conclusion

The methods and effects of E_2 action in the AVPV are complex and not limited to pubertal onset and ovulation. As a target of E_2 and a regulator of Esr1, Ets2 could be pivotal in the sexual differentiation of the AVPV as well as in the onset of puberty. Furthermore, it may represent a basic mechanism by which E_2 acts, which has numerous

implications outside of reproduction, importantly, mechanisms of endocrine disruption and cancer progression/treatment.

7.6 Bibliography

Cravo RM, Frazao R, Perello M, Osborne-Lawrence S, Williams KW, Zigman JM, Vianna C, Elias CF (2013) Leptin signaling in Kiss1 neurons arises after pubertal development. PloS one 8:e58698.

Donato J, Jr., Cravo RM, Frazao R, Gautron L, Scott MM, Lachey J, Castro IA, Margatho LO, Lee S, Lee C, Richardson JA, Friedman J, Chua S, Jr., Coppari R, Zigman JM, Elmquist JK, Elias CF (2011) Leptin's effect on puberty in mice is relayed by the ventral premammillary nucleus and does not require signaling in Kiss1 neurons. The Journal of clinical investigation 121:355-368.

Lantos TA, Gorcs TJ, Palkovits M (1995) Immunohistochemical mapping of neuropeptides in the premamillary region of the hypothalamus in rats. Brain research Brain research reviews 20:209-249.

Loktev AV, Jackson PK (2013) Neuropeptide Y family receptors traffic via the Bardet-Biedl syndrome pathway to signal in neuronal primary cilia. Cell reports 5:1316-1329.

Martin C, Navarro VM, Simavli S, Vong L, Carroll RS, Lowell BB, Kaiser UB (2014) Leptin-responsive GABAergic neurons regulate fertility through pathways that result in reduced kisspeptinergic tone. J Neurosci 34:6047-6056.

Noben-Trauth K, Naggert JK, North MA, Nishina PM (1996) A candidate gene for the mouse mutation tubby. Nature 380:534-538.

Petersen SL, Krishnan S, Aggison LK, Intlekofer KA, Moura PJ (2012) Sexual differentiation of the gonadotropin surge release mechanism: a new role for the canonical NfkappaB signaling pathway. Frontiers in neuroendocrinology 33:36-44.

Sheffield VC (2010) The blind leading the obese: the molecular pathophysiology of a human obesity syndrome. Transactions of the American Clinical and Climatological Association 121:172-181; discussion 181-172.

Vong L, Ye C, Yang Z, Choi B, Chua S, Jr., Lowell BB (2011) Leptin action on GABAergic neurons prevents obesity and reduces inhibitory tone to POMC neurons. Neuron 71:142-154.

Wedeken L, Singh P, Klempnauer KH (2011) Tumor suppressor protein Pdcd4 inhibits translation of p53 mRNA. The Journal of biological chemistry 286:42855-42862.

BIBLIOGRAPHY

Abdelrahim M, Samudio I, Smith R, 3rd, Burghardt R, Safe S (2002) Small inhibitory RNA duplexes for Sp1 mRNA block basal and estrogen-induced gene expression and cell cycle progression in MCF-7 breast cancer cells. The Journal of biological chemistry 277:28815-28822.

Alexander MJ, Kiraly ZJ, Leeman SE (1991) Sexually dimorphic distribution of neurotensin/neuromedin N mRNA in the rat preoptic area. The Journal of comparative neurology 311:84-96.

Allison DB, Cui X, Page GP, Sabripour M (2006) Microarray data analysis: from disarray to consolidation and consensus. Nature reviews Genetics 7:55-65.

Amstalden M, Alves BR, Liu S, Cardoso RC, Williams GL (2011) Neuroendocrine pathways mediating nutritional acceleration of puberty: insights from ruminant models. Frontiers in endocrinology 2:109.

Asangani IA, Rasheed SA, Nikolova DA, Leupold JH, Colburn NH, Post S, Allgayer H (2008) MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. Oncogene 27:2128-2136.

Axelson JF, Shannon W, Van Leeuwen FW (1992) Immunocytochemical localization of estrogen receptors within neurotensin cells in the rostral preoptic area of the rat hypothalamus. Neuroscience letters 136:5-9.

Beckman KB, Lee KY, Golden T, Melov S (2004) Gene expression profiling in mitochondrial disease: assessment of microarray accuracy by high-throughput Q-PCR. Mitochondrion 4:453-470.

Bellissimo N, Anderson GH (2003) Cholecystokinin-A receptors are involved in food intake suppression in rats after intake of all fats and carbohydrates tested. The Journal of nutrition 133:2319-2325.

Bhat-Nakshatri P, Wang G, Collins NR, Thomson MJ, Geistlinger TR, Carroll JS, Brown M, Hammond S, Srour EF, Liu Y, Nakshatri H (2009) Estradiol-regulated microRNAs control estradiol response in breast cancer cells. Nucleic acids research 37:4850-4861.

Bie L, Zhao G, Cheng P, Rondeau G, Porwollik S, Ju Y, Xia XQ, McClelland M (2011) The accuracy of survival time prediction for patients with glioma is improved by measuring mitotic spindle checkpoint gene expression. PloS one 6:e25631.

Bitomsky N, Wethkamp N, Marikkannu R, Klempnauer KH (2008) siRNA-mediated knockdown of Pdcd4 expression causes upregulation of p21(Waf1/Cip1) expression. Oncogene 27:4820-4829.

Blutstein T, Devidze N, Choleris E, Jasnow AM, Pfaff DW, Mong JA (2006) Oestradiol up-regulates glutamine synthetase mRNA and protein expression in the hypothalamus and hippocampus: implications for a role of hormonally responsive glia in amino acid neurotransmission. Journal of neuroendocrinology 18:692-702.

Calizo LH, Flanagan-Cato LM (2003) Hormonal-neural integration in the female rat ventromedial hypothalamus: triple labeling for estrogen receptor-alpha, retrograde tract tracing from the periaqueductal gray, and mating-induced Fos expression. Endocrinology 144:5430-5440.

Carletti MZ, Fiedler SD, Christenson LK (2010) MicroRNA 21 blocks apoptosis in mouse periovulatory granulosa cells. Biology of reproduction 83:286-295.

Chakraborty TR, Rajendren G, Gore AC (2005) Expression of estrogen receptor {alpha} in the anteroventral periventricular nucleus of hypogonadal mice. Experimental biology and medicine (Maywood, NJ 230:49-56.

Chappell PE, Levine JE (2000) Stimulation of gonadotropin-releasing hormone surges by estrogen. I. Role of hypothalamic progesterone receptors. Endocrinology 141:1477-1485.

Cheng C, Pounds S (2007) False discovery rate paradigms for statistical analyses of microarray gene expression data. Bioinformation 1:436-446.

Cheslock PS, Kemp BJ, Boumil RM, Dawson DS (2005) The roles of MAD1, MAD2 and MAD3 in meiotic progression and the segregation of nonexchange chromosomes. Nature genetics 37:756-760.

Cmarik JL, Min H, Hegamyer G, Zhan S, Kulesz-Martin M, Yoshinaga H, Matsuhashi S, Colburn NH (1999) Differentially expressed protein Pdcd4 inhibits tumor promoter-induced neoplastic transformation. Proceedings of the National Academy of Sciences of the United States of America 96:14037-14042.

Colledge WH (2009) Transgenic mouse models to study Gpr54/kisspeptin physiology. Peptides 30:34-41.

Cravo RM, Frazao R, Perello M, Osborne-Lawrence S, Williams KW, Zigman JM, Vianna C, Elias CF (2013) Leptin signaling in Kiss1 neurons arises after pubertal development. PloS one 8:e58698.

Curtis RK, Oresic M, Vidal-Puig A (2005) Pathways to the analysis of microarray data. Trends in biotechnology 23:429-435.

Dalman MR, Deeter A, Nimishakavi G, Duan ZH (2012) Fold change and p-value cutoffs significantly alter microarray interpretations. BMC bioinformatics 13 Suppl 2:S11.

Davis EC, Shryne JE, Gorski RA (1996) Structural sexual dimorphisms in the anteroventral periventricular nucleus of the rat hypothalamus are sensitive to gonadal steroids perinatally, but develop peripubertally. Neuroendocrinology 63:142-148.

de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E (2003) Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. Proceedings of the National Academy of Sciences of the United States of America 100:10972-10976.

Dellovade TL, Merchenthaler I (2004) Estrogen regulation of neurokinin B gene expression in the mouse arcuate nucleus is mediated by estrogen receptor alpha. Endocrinology 145:736-742.

Donato J, Jr., Cravo RM, Frazao R, Gautron L, Scott MM, Lachey J, Castro IA, Margatho LO, Lee S, Lee C, Richardson JA, Friedman J, Chua S, Jr., Coppari R, Zigman JM, Elmquist JK, Elias CF (2011) Leptin's effect on puberty in mice is relayed by the ventral premammillary nucleus and does not require signaling in Kiss1 neurons. The Journal of clinical investigation 121:355-368.

Dungan Lemko HM, Naderi R, Adjan V, Jennes LH, Navarro VM, Clifton DK, Steiner RA (2010) Interactions between neurotensin and GnRH neurons in the positive feedback control of GnRH/LH secretion in the mouse. American journal of physiology Endocrinology and metabolism 298:E80-88.

Everett JW, Radford HM (1961) Irritative deposits from stainless steel electrodes in the preoptic rat brain causing release of pituitary gonadotropin. Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine 108:604-609.

Felty Q, Singh KP, Roy D (2005a) Estrogen-induced G1/S transition of G0-arrested estrogen-dependent breast cancer cells is regulated by mitochondrial oxidant signaling. Oncogene 24:4883-4893.

Felty Q, Xiong WC, Sun D, Sarkar S, Singh KP, Parkash J, Roy D (2005b) Estrogen-induced mitochondrial reactive oxygen species as signal-transducing messengers. Biochemistry 44:6900-6909.

FitzGerald PC, Shlyakhtenko A, Mir AA, Vinson C (2004) Clustering of DNA sequences in human promoters. Genome research 14:1562-1574.

Flanagan-Cato LM, Calizo LH, Daniels D (2001) The synaptic organization of VMH neurons that mediate the effects of estrogen on sexual behavior. Hormones and behavior 40:178-182.

Friedlander Y, Li G, Fornage M, Williams OD, Lewis CE, Schreiner P, Pletcher MJ, Enquobahrie D, Williams M, Siscovick DS (2010) Candidate molecular pathway genes related to appetite regulatory neural network, adipocyte homeostasis and obesity: results from the CARDIA Study. Annals of human genetics 74:387-398.

Fulop AK, Foldes A, Buzas E, Hegyi K, Miklos IH, Romics L, Kleiber M, Nagy A, Falus A, Kovacs KJ (2003) Hyperleptinemia, visceral adiposity, and decreased glucose tolerance in mice with a targeted disruption of the histidine decarboxylase gene. Endocrinology 144:4306-4314.

Ghayad SE, Vendrell JA, Bieche I, Spyratos F, Dumontet C, Treilleux I, Lidereau R, Cohen PA (2009) Identification of TACC1, NOV, and PTTG1 as new candidate genes associated with endocrine therapy resistance in breast cancer. Journal of molecular endocrinology 42:87-103.

Goodman RL (1978) The site of the positive feedback action of estradiol in the rat. Endocrinology 102:151-159.

Gore AC, Walker DM, Zama AM, Armenti AE, Uzumcu M (2011) Early life exposure to endocrine-disrupting chemicals causes lifelong molecular reprogramming of the hypothalamus and premature reproductive aging. Molecular endocrinology (Baltimore, Md 25:2157-2168.

Gorski RA (1985) Sexual dimorphisms of the brain. Journal of animal science 61 Suppl 3:38-61.

Gu GB, Simerly RB (1997) Projections of the sexually dimorphic anteroventral periventricular nucleus in the female rat. The Journal of comparative neurology 384:142-164.

Guan XM, Hess JF, Yu H, Hey PJ, van der Ploeg LH (1997) Differential expression of mRNA for leptin receptor isoforms in the rat brain. Molecular and cellular endocrinology 133:1-7.

Hollenhorst PC, Jones DA, Graves BJ (2004) Expression profiles frame the promoter specificity dilemma of the ETS family of transcription factors. Nucleic acids research 32:5693-5702.

Hollenhorst PC, McIntosh LP, Graves BJ (2011) Genomic and biochemical insights into the specificity of ETS transcription factors. Annual review of biochemistry 80:437-471.

Jinnin M, Ihn H, Asano Y, Yamane K, Trojanowska M, Tamaki K (2006) Platelet derived growth factor induced tenascin-C transcription is phosphoinositide 3-kinase/Akt-dependent and mediated by Ets family transcription factors. Journal of cellular physiology 206:718-727.

Jorgensen EA, Vogelsang TW, Knigge U, Watanabe T, Warberg J, Kjaer A (2006) Increased susceptibility to diet-induced obesity in histamine-deficient mice. Neuroendocrinology 83:289-294.

Kabbout M, Garcia MM, Fujimoto J, Liu DD, Woods D, Chow CW, Mendoza G, Momin AA, James BP, Solis L, Behrens C, Lee JJ, Wistuba, II, Kadara H (2013) ETS2 mediated tumor suppressive function and MET oncogene inhibition in human non-small cell lung cancer. Clinical cancer research : an official journal of the American Association for Cancer Research 19:3383-3395.

Kalra SP (1993) Mandatory neuropeptide-steroid signaling for the preovulatory luteinizing hormone-releasing hormone discharge. Endocrine reviews 14:507-538.

Kauffman AS, Gottsch ML, Roa J, Byquist AC, Crown A, Clifton DK, Hoffman GE, Steiner RA, Tena-Sempere M (2007) Sexual differentiation of Kiss1 gene expression in the brain of the rat. Endocrinology 148:1774-1783.

Kokay IC, Petersen SL, Grattan DR (2011) Identification of prolactin-sensitive GABA and kisspeptin neurons in regions of the rat hypothalamus involved in the control of fertility. Endocrinology 152:526-535.

Krishnan V, Wang X, Safe S (1994) Estrogen receptor-Sp1 complexes mediate estrogen-induced cathepsin D gene expression in MCF-7 human breast cancer cells. The Journal of biological chemistry 269:15912-15917.

Kumar N, Wethkamp N, Waters LC, Carr MD, Klempnauer KH (2013) Tumor suppressor protein Pdcd4 interacts with Daxx and modulates the stability of Daxx and the Hipk2-dependent phosphorylation of p53 at serine 46. Oncogenesis 2:e37.

Kuo LE, Kitlinska JB, Tilan JU, Li L, Baker SB, Johnson MD, Lee EW, Burnett MS, Fricke ST, Kvetnansky R, Herzog H, Zukowska Z (2007) Neuropeptide Y acts directly in the periphery on fat tissue and mediates stress-induced obesity and metabolic syndrome. Nature medicine 13:803-811.

Lantos TA, Gorcs TJ, Palkovits M (1995) Immunohistochemical mapping of neuropeptides in the premamillary region of the hypothalamus in rats. Brain research Brain research reviews 20:209-249.

Lederman MA, Lebesgue D, Gonzalez VV, Shu J, Merhi ZO, Etgen AM, Neal-Perry G (2010) Age-related LH surge dysfunction correlates with reduced responsiveness of hypothalamic anteroventral periventricular nucleus kisspeptin neurons to estradiol positive feedback in middle-aged rats. Neuropharmacology 58:314-320.

Li D, Mitchell D, Luo J, Yi Z, Cho SG, Guo J, Li X, Ning G, Wu X, Liu M (2007) Estrogen regulates KiSS1 gene expression through estrogen receptor alpha and SP protein complexes. Endocrinology 148:4821-4828.

Li R, Murray AW (1991) Feedback control of mitosis in budding yeast. Cell 66:519-531.

Li Y, Wu X, Zhou S, Owyang C (2011) Low-affinity CCK-A receptors are coexpressed with leptin receptors in rat nodose ganglia: implications for leptin as a regulator of short-term satiety. American journal of physiology Gastrointestinal and liver physiology 300:G217-227.

Liang XQ, Cao EH, Zhang Y, Qin JF (2003) P53-induced gene 11 (PIG11) involved in arsenic trioxide-induced apoptosis in human gastric cancer MGC-803 cells. Oncology reports 10:1265-1269.

Liang XQ, Cao EH, Zhang Y, Qin JF (2004) A P53 target gene, PIG11, contributes to chemosensitivity of cells to arsenic trioxide. FEBS letters 569:94-98.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.

Liwak U, Thakor N, Jordan LE, Roy R, Lewis SM, Pardo OE, Seckl M, Holcik M (2012) Tumor suppressor PDCD4 represses internal ribosome entry site-mediated translation of antiapoptotic proteins and is regulated by S6 kinase 2. Molecular and cellular biology 32:1818-1829.

Loktev AV, Jackson PK (2013) Neuropeptide Y family receptors traffic via the Bardet-Biedl syndrome pathway to signal in neuronal primary cilia. Cell reports 5:1316-1329.

Lu Z, Liu M, Stribinskis V, Klinge CM, Ramos KS, Colburn NH, Li Y (2008) MicroRNA-21 promotes cell transformation by targeting the programmed cell death 4 gene. Oncogene 27:4373-4379.

Maeda T, Hanna AN, Sim AB, Chua PP, Chong MT, Tron VA (2002) GADD45 regulates G2/M arrest, DNA repair, and cell death in keratinocytes following ultraviolet exposure. The Journal of investigative dermatology 119:22-26.

Maroulakou IG, Papas TS, Green JE (1994) Differential expression of ets-1 and ets-2 proto-oncogenes during murine embryogenesis. Oncogene 9:1551-1565.

Martin C, Navarro VM, Simavli S, Vong L, Carroll RS, Lowell BB, Kaiser UB (2014) Leptin-responsive GABAergic neurons regulate fertility through pathways that result in reduced kisspeptinergic tone. J Neurosci 34:6047-6056.

Mayer C, Acosta-Martinez M, Dubois SL, Wolfe A, Radovick S, Boehm U, Levine JE (2010) Timing and completion of puberty in female mice depend on estrogen receptor alpha-signaling in kisspeptin neurons. Proceedings of the National Academy of Sciences of the United States of America 107:22693-22698.

Mellor P, Deibert L, Calvert B, Bonham K, Carlsen SA, Anderson DH (2013) CREB3L1 is a metastasis suppressor that represses expression of genes regulating metastasis, invasion, and angiogenesis. Molecular and cellular biology 33:4985-4995.

Micevych PE, Kelly MJ (2012) Membrane estrogen receptor regulation of hypothalamic function. Neuroendocrinology 96:103-110.

Mitra SW, Hoskin E, Yudkovitz J, Pear L, Wilkinson HA, Hayashi S, Pfaff DW, Ogawa S, Rohrer SP, Schaeffer JM, McEwen BS, Alves SE (2003) Immunolocalization of estrogen receptor beta in the mouse brain: comparison with estrogen receptor alpha. Endocrinology 144:2055-2067.

Moad AI, Muhammad TS, Oon CE, Tan ML (2013) Rapamycin induces apoptosis when autophagy is inhibited in T-47D mammary cells and both processes are regulated by Phlda1. Cell biochemistry and biophysics 66:567-587.

Morey JS, Ryan JC, Van Dolah FM (2006) Microarray validation: factors influencing correlation between oligonucleotide microarrays and real-time PCR. Biological procedures online 8:175-193.

Noben-Trauth K, Naggert JK, North MA, Nishina PM (1996) A candidate gene for the mouse mutation tubby. Nature 380:534-538.

Oakley AE, Clifton DK, Steiner RA (2009) Kisspeptin signaling in the brain. Endocrine reviews 30:713-743.

Ottem EN, Godwin JG, Krishnan S, Petersen SL (2004) Dual-phenotype GABA/glutamate neurons in adult preoptic area: sexual dimorphism and function. J Neurosci 24:8097-8105.

Pawitan Y, Michiels S, Koscielny S, Gusnanto A, Ploner A (2005) False discovery rate, sensitivity and sample size for microarray studies. Bioinformatics 21:3017-3024.

Petersen SL, Barraclough CA (1989) Suppression of spontaneous LH surges in estrogen-treated ovariectomized rats by microimplants of antiestrogens into the preoptic brain. Brain Res 484:279-289.

Petersen SL, Krishnan S, Aggison LK, Intlekofer KA, Moura PJ (2012) Sexual differentiation of the gonadotropin surge release mechanism: a new role for the canonical NfkappaB signaling pathway. Frontiers in neuroendocrinology 33:36-44.

Petersen SL, Ottem EN, Carpenter CD (2003) Direct and indirect regulation of gonadotropin-releasing hormone neurons by estradiol. Biology of reproduction 69:1771-1778.

Polston EK, Gu G, Simerly RB (2004) Neurons in the principal nucleus of the bed nuclei of the stria terminalis provide a sexually dimorphic GABAergic input to the anteroventral periventricular nucleus of the hypothalamus. Neuroscience 123:793-803.

Porteous R, Petersen SL, Yeo SH, Bhattarai JP, Ciofi P, de Tassigny XD, Colledge WH, Caraty A, Herbison AE (2011) Kisspeptin neurons co-express met-enkephalin and galanin in the rostral periventricular region of the female mouse hypothalamus. The Journal of comparative neurology 519:3456-3469.

Porter W, Saville B, Hoivik D, Safe S (1997) Functional synergy between the transcription factor Sp1 and the estrogen receptor. Molecular endocrinology (Baltimore, Md 11:1569-1580.

Rahmani Z, Blouin JL, Creau-Goldberg N, Watkins PC, Mattei JF, Poissonnier M, Prieur M, Chettouh Z, Nicole A, Aurias A, et al. (1989) Critical role of the D21S55 region on chromosome 21 in the pathogenesis of Down syndrome. Proceedings of the National Academy of Sciences of the United States of America 86:5958-5962.

Reynolds LE, Watson AR, Baker M, Jones TA, D'Amico G, Robinson SD, Joffre C, Garrido-Urbani S, Rodriguez-Manzaneque JC, Martino-Echarri E, Aurrand-Lions M, Sheer D, Dagna-Bricarelli F, Nizetic D, McCabe CJ, Turnell AS, Kermorgant S, Imhof BA, Adams R, Fisher EM, Tybulewicz VL, Hart IR, Hodivala-Dilke KM (2010) Tumour angiogenesis is reduced in the Tc1 mouse model of Down's syndrome. Nature 465:813-817.

Ronnekleiv OK, Kelly MJ (1986) Luteinizing hormone-releasing hormone neuronal system during the estrous cycle of the female rat: effects of surgically induced persistent estrus. Neuroendocrinology 43:564-576.

Rosas-Arellano MP, Solano-Flores LP, Ciriello J (1999) Co-localization of estrogen and angiotensin receptors within subfornical organ neurons. Brain Res 837:254-262.

Rosell M, Jones MC, Parker MG (2011) Role of nuclear receptor corepressor RIP140 in metabolic syndrome. Biochimica et biophysica acta 1812:919-928.

Roth CL, Mastronardi C, Lomniczi A, Wright H, Cabrera R, Mungenast AE, Heger S, Jung H, Dubay C, Ojeda SR (2007) Expression of a tumor-related gene network increases in the mammalian hypothalamus at the time of female puberty. Endocrinology 148:5147-5161.

Ruan Q, Wang T, Kameswaran V, Wei Q, Johnson DS, Matschinsky F, Shi W, Chen YH (2011) The microRNA-21-PDCD4 axis prevents type 1 diabetes by blocking pancreatic beta cell death. Proceedings of the National Academy of Sciences of the United States of America 108:12030-12035.

Sakakibara M, Uenoyama Y, Minabe S, Watanabe Y, Deura C, Nakamura S, Suzuki G, Maeda K, Tsukamura H (2013) Microarray analysis of perinatal-estrogen-induced changes in gene expression related to brain sexual differentiation in mice. PloS one 8:e79437.

Sakuma Y (2009) Gonadal steroid action and brain sex differentiation in the rat. Journal of neuroendocrinology 21:410-414.

Scordalakes EM, Shetty SJ, Rissman EF (2002) Roles of estrogen receptor alpha and androgen receptor in the regulation of neuronal nitric oxide synthase. The Journal of comparative neurology 453:336-344.

Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno JS, Jr., Shagoury JK, Bo-Abbas Y, Kuohung W, Schwinof KM, Hendrick AG, Zahn D, Dixon J, Kaiser UB, Slaugenhaupt SA, Gusella JF, O'Rahilly S, Carlton MB, Crowley WF, Jr., Aparicio SA, Colledge WH (2003) The GPR54 gene as a regulator of puberty. The New England journal of medicine 349:1614-1627.

Sheffield VC (2010) The blind leading the obese: the molecular pathophysiology of a human obesity syndrome. Transactions of the American Clinical and Climatological Association 121:172-181; discussion 181-172.

Shirasaki F, Makhluf HA, LeRoy C, Watson DK, Trojanowska M (1999) Ets transcription factors cooperate with Sp1 to activate the human tenascin-C promoter. Oncogene 18:7755-7764.

Shughrue PJ, Bushnell CD, Dorsa DM (1992) Estrogen receptor messenger ribonucleic acid in female rat brain during the estrous cycle: a comparison with ovariectomized females and intact males. Endocrinology 131:381-388.

Shughrue PJ, Lane MV, Merchenthaler I (1997) Regulation of progesterone receptor messenger ribonucleic acid in the rat medial preoptic nucleus by estrogenic and antiestrogenic compounds: an in situ hybridization study. Endocrinology 138:5476-5484.

Simerly RB (1998) Organization and regulation of sexually dimorphic neuroendocrine pathways. Behavioural brain research 92:195-203.

Simerly RB, Carr AM, Zee MC, Lorang D (1996) Ovarian steroid regulation of estrogen and progesterone receptor messenger ribonucleic acid in the anteroventral periventricular nucleus of the rat. Journal of neuroendocrinology 8:45-56.

Simonian SX, Herbison AE (1997) Differential expression of estrogen receptor alpha and beta immunoreactivity by oxytocin neurons of rat paraventricular nucleus. Journal of neuroendocrinology 9:803-806.

Simonian SX, Spratt DP, Herbison AE (1999) Identification and characterization of estrogen receptor alpha-containing neurons projecting to the vicinity of the gonadotropin-releasing hormone perikarya in the rostral preoptic area of the rat. The Journal of comparative neurology 411:346-358.

Smith JT, Cunningham MJ, Rissman EF, Clifton DK, Steiner RA (2005) Regulation of Kiss1 gene expression in the brain of the female mouse. Endocrinology 146:3686-3692.

Somponpun SJ, Johnson AK, Beltz T, Sladek CD (2004) Estrogen receptor-alpha expression in osmosensitive elements of the lamina terminalis: regulation by hypertonicity. American journal of physiology Regulatory, integrative and comparative physiology 287:R661-669.

Sun HJ, Xu X, Wang XL, Wei L, Li F, Lu J, Huang BQ (2006) Transcription factors Ets2 and Sp1 act synergistically with histone acetyltransferase p300 in activating human interleukin-12 p40 promoter. Acta biochimica et biophysica Sinica 38:194-200.

Teles MG, Bianco SD, Brito VN, Trarbach EB, Kuohung W, Xu S, Seminara SB, Mendonca BB, Kaiser UB, Latronico AC (2008) A GPR54-activating mutation in a patient with central precocious puberty. The New England journal of medicine 358:709-715.

Tsukahara S (2009) Sex differences and the roles of sex steroids in apoptosis of sexually dimorphic nuclei of the preoptic area in postnatal rats. Journal of neuroendocrinology 21:370-376.

Tunquist BJ, Eyers PA, Chen LG, Lewellyn AL, Maller JL (2003) Spindle checkpoint proteins Mad1 and Mad2 are required for cytostatic factor-mediated metaphase arrest. The Journal of cell biology 163:1231-1242.

Vida B, Deli L, Hrabovszky E, Kalamatianos T, Caraty A, Coen CW, Liposits Z, Kallo I (2010) Evidence for suprachiasmatic vasopressin neurones innervating kisspeptin neurones in the rostral periventricular area of the mouse brain: regulation by oestrogen. Journal of neuroendocrinology 22:1032-1039.

Vong L, Ye C, Yang Z, Choi B, Chua S, Jr., Lowell BB (2011) Leptin action on GABAergic neurons prevents obesity and reduces inhibitory tone to POMC neurons. Neuron 71:142-154.

Wassmann K, Niault T, Maro B (2003) Metaphase I arrest upon activation of the Mad2-dependent spindle checkpoint in mouse oocytes. Current biology : CB 13:1596-1608.

Watson RE, Jr., Langub MC, Jr., Engle MG, Maley BE (1995) Estrogen-receptive neurons in the anteroventral periventricular nucleus are synaptic targets of the suprachiasmatic nucleus and peri-suprachiasmatic region. Brain Res 689:254-264.

Wedeken L, Ohnheiser J, Hirschi B, Wethkamp N, Klempnauer KH (2010) Association of Tumor Suppressor Protein Pdcd4 With Ribosomes Is Mediated by Protein-Protein and Protein-RNA Interactions. Genes & cancer 1:293-301.

Wedeken L, Singh P, Klempnauer KH (2011) Tumor suppressor protein Pdcd4 inhibits translation of p53 mRNA. The Journal of biological chemistry 286:42855-42862.

Wickramasinghe NS, Manavalan TT, Dougherty SM, Riggs KA, Li Y, Klinge CM (2009) Estradiol downregulates miR-21 expression and increases miR-21 target gene expression in MCF-7 breast cancer cells. Nucleic acids research 37:2584-2595.

Wintermantel TM, Campbell RE, Porteous R, Bock D, Grone HJ, Todman MG, Korach KS, Greiner E, Perez CA, Schutz G, Herbison AE (2006) Definition of estrogen receptor pathway critical for estrogen positive feedback to gonadotropin-releasing hormone neurons and fertility. Neuron 52:271-280.

Wolvetang EJ, Wilson TJ, Sanij E, Busciglio J, Hatzistavrou T, Seth A, Hertzog PJ, Kola I (2003) ETS2 overexpression in transgenic models and in Down syndrome predisposes to apoptosis via the p53 pathway. Human molecular genetics 12:247-255.

Wu Y, Liu XM, Wang XJ, Zhang Y, Liang XQ, Cao EH (2009) PIG11 is involved in hepatocellular carcinogenesis and its over-expression promotes Hepg2 cell apoptosis. Pathology oncology research : POR 15:411-416.

Xu Q, Hamada T, Kiyama R, Sakuma Y, Wada-Kiyama Y (2008) Site-specific regulation of gene expression by estrogen in the hypothalamus of adult female rats. Neuroscience letters 436:35-39.

Yang HS, Cho MH, Zakowicz H, Hegamyer G, Sonenberg N, Colburn NH (2004) A novel function of the MA-3 domains in transformation and translation suppressor Pdcd4 is essential for its binding to eukaryotic translation initiation factor 4A. Molecular and cellular biology 24:3894-3906.

Yang HS, Jansen AP, Komar AA, Zheng X, Merrick WC, Costes S, Lockett SJ, Sonenberg N, Colburn NH (2003) The transformation suppressor Pdcd4 is a novel eukaryotic translation initiation factor 4A binding protein that inhibits translation. Molecular and cellular biology 23:26-37.

Yang Q, Rasmussen SA, Friedman JM (2002) Mortality associated with Down's syndrome in the USA from 1983 to 1997: a population-based study. Lancet 359:1019-1025.

Yeo SH, Herbison AE (2014) Estrogen-Negative Feedback and Estrous Cyclicity Are Critically Dependent Upon Estrogen Receptor-alpha Expression in the Arcuate Nucleus of Adult Female Mice. Endocrinology 155:2986-2995.

Zhan Q, Fan S, Smith ML, Bae I, Yu K, Alamo I, Jr., O'Connor PM, Fornace AJ, Jr. (1996) Abrogation of p53 function affects gadd gene responses to DNA base-damaging agents and starvation. DNA and cell biology 15:805-815.

Zhang L, Zhang J, Yang G, Wu D, Jiang L, Wen Z, Li M (2013) Investigating the concordance of Gene Ontology terms reveals the intra- and inter-platform reproducibility of enrichment analysis. BMC bioinformatics 14:143.

Zhang S, Zeitzer JM, Yoshida Y, Wisor JP, Nishino S, Edgar DM, Mignot E (2004) Lesions of the suprachiasmatic nucleus eliminate the daily rhythm of hypocretin-1 release. Sleep 27:619-627.

Zhu J, Jiang J, Zhou W, Zhu K, Chen X (1999) Differential regulation of cellular target genes by p53 devoid of the PXXP motifs with impaired apoptotic activity. Oncogene 18:2149-2155.

Zhu JD, Fei Q, Wang P, Lan F, Mao da Q, Zhang HY, Yao XB (2006) Transcription of the putative tumor suppressor gene HCCS1 requires binding of ETS-2 to its consensus near the transcription start site. Cell research 16:780-796.