Generation of Kisspeptin Cell Lines to Investigate the Sex Steroid-Mediated Initiation of Puberty

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Abstract

Mammalian female reproduction requires preovulatory surges of gonadotropin-releasing hormone (GnRH) from neurons in the preoptic area (POA) of the hypothalamus, initiated by elevated estradiol (E$_2$). Rising E$_2$ activates a subset of sexually dimorphic Kisspeptin neurons in the female, located in the anteroventral periventricular nuclei (AVPV). Conversely, E$_2$ negative feedback is mediated by aneuroanatomically separate population of Kisspeptin neurons in the arcuate (Arc) nuclei. Kisspeptin is a regulator of GnRH neuronal activity in vivo, and the development of the Kisspeptin system is critical for the initiation of puberty. To elucidate how phenotypically similar neuronal populations react in a dramatically different fashion to the same E$_2$ exposure, we have begun generating two kisspeptin-expressing cell lines from kiss1-GFP post-pubertal female mice. AVPV and Arc explants were removed from 9-wk old females using a vibratome, and dissociated enzymatically, with neurite process extension occurring within four to five days after harvest. Cells were re-suspended, sorted for GFP fluorescence, and re-plated in DMEM with 20% FBS. We found that AVPV neurons retain differential expression patterns when exposed to E$_2$, but interestingly we observed that E$_2$-stimulated increased in GFP occurred within 20 minutes of exposure, suggesting a rapid time course of kiss1 stimulation. In contrast, Arc-derived primary neurons exhibited a slightly higher baseline level of GFP fluorescence, which diminished within 4h of E$_2$ exposure, recapitulating decreases in kiss1 expression observed previously. We are using a third generation lentiviral packaging system to infect both AVPV and Arc kisspeptin neurons with SV40-large T antigen, to immortalize both populations via suppression of tumor-suppressing gene p53. Four weeks post-infection, AVPV neurons began proliferating, retaining their unique expression pattern when treated with E$_2$. These cell lines will
be useful tools to probe the molecular mechanisms underlying this differential regulation of \textit{kiss1} expression in response to sex steroids.

\textbf{Introduction}

Mammalian reproductive function hinges on successful transduction of signals through the hypothalamic-pituitary-gonadal (HPG) axis. General characteristics of this central axis and the neuronal modulators of its physiology are relatively conserved among mammalian species. The HPG axis is strictly regulated by neighboring populations of neuronal and non-neuronal glial and astrocytic cell types (Sharif et al., 2013). The complex intercommunication among hypothalamic cells allows for rigorous control of neuropeptide release within the preoptic area (POA) of the hypothalamus. Axonal terminals extending from cell bodies within the hypothalamus terminate at the median eminence located at the base of the brain to release signals to the hypothalamic-hypophyseal portal vein system connecting the hypothalamus to the hypophysis (Sharif et al., 2013). This portal vein system allows direct communication between the hypothalamus and hypophysis, allowing regulated secretions of key POA neurons to modulate adenohypophysial hormone response efficiently. In a reproductive context, the adenohypophysis is stimulated to secrete specific gonadotropins to the circulatory system that facilitate gonadal development and reproductive physiology (Gojska et al., 2014). These sex steroids, at high levels, suppress hypothalamic secretions to the hypophysis to auto regulate the HPG axis (Oakley et al., 2009). Sex steroids act to ultimately influence specific hypothalamic neuronal cell types in the POA: gonadotropin-releasing hormone (GnRH) neurons that terminate in the hypothalamic-hypophyseal portal system through the median eminence (Oakley et al., 2009).

\textit{GnRH neurons and HPG regulation}
GnRH neurons are paramount regulators of HPG axis function, as GnRH release stimulates the adenohypophysis to secrete gonadotropins: luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Oakley et al., 2009). In males, LH stimulates Leydig cells in the testes to secrete testosterone and acts synergistically with FSH to allow spermatocytes to undergo meiosis to generate viable gametes (Clarkson et al., 2008). In females, LH induces estrogen secretion from follicular granulosa cells, while FSH recruits immature ovarian follicles within the ovary (Clarkson et al., 2008). Typically, elevated blood concentration of testosterone or estrogen provides negative feedback to GnRH neurons (Clarkson et al., 2008). In females, GnRH neurons express estrogen receptor beta (ERβ) that responds to rising estrogen levels by down regulating GnRH secretions, preventing further adenohypophysial gonadotropin-releasing activity (Smith et al., 2005). However, GnRH neuron activity requires complex regulation to elicit specific secretion profiles to direct reproductive behavior. This is regulation is attained by neighboring neuronal populations within the hypothalamus.

**Kisspeptin-releasing neurons and GnRH secretion modulation**

GnRH neuronal perikarya are influenced by two neuroanatomically distinct kisspeptin-releasing nuclei: the anteroventral periventricular (AVPV) and arcuate (Arc) nuclei (Smith et al., 2005). These nuclei provide regulatory signals to GnRH neurons by kisspeptin, a neuropeptide encoded by the kiss1 gene expressed highly within the AVPV and arcuate nuclei to modulate reproduction (Smith et al., 2005). Kiss1 is transcribed and translated to kisspeptin in the perikaryon of the neuron and released into extracellular space by vesicular axon transport. Kisspeptin binds to GnRH neuron soma receptors and axon terminals using a kisspeptin receptor, KISS1R (Clarkson et al., 2008). GnRH neurons increase secretion activity upon kisspeptin
binding (Clarkson et al., 2008). Much like GnRH neurons, AVPV and arcuate neurons are sensitive to rising estrogen levels in the blood (Smith et al., 2005). Estrogen effects the transcription activity of kiss1 in AVPV/Arc neuronal populations in females, while male rodents lack a sizable AVPV population as they are incapable of generating this surge in gonadotropins. Estrogen receptor alpha (ERα) is expressed in the soma of these kisspeptin-expressing neurons, but between the two populations of AVPV and Arc kisspeptin-expressing neurons, the transcript level modification of kiss1 is completely different in several female mammalian species (Smith et al., 2005).

Exposure to elevated estrogen causes an increase in kiss1 expression in the AVPV, whereas estrogen causes a decrease in kiss1 expression in arcuate nuclei (Smith et al., 2005). This differential expression response is only observed in female rodents; male rodents do not express appreciable levels of kisspeptin in the sexually dimorphic AVPV. However, arcuate kiss1-expressing neurons in male rodents exhibit negative feedback from estrogen acting on arcuate neurons, also seen in female rodents (Oakley et al., 2009). The sexually dimorphic nature of both physiology and anatomy of AVPV kisspeptin neurons is of considerable interest, considering female rodents require GnRH/LH surges to elicit ovulation and AVPV kisspeptin neurons provide positive feedback to GnRH neurons (Oakley et al., 2009).

Arcuate kisspeptin neurons colocalize neurokinin B (NKB) and dynorphin neurotransmitters, therefore mammalian arcuate cells have been termed KNDy neurons (Navarro et al., 2009). This unique colocalization of three neuropeptides to a single neuronal population is restricted to mammalian species, as there are no orthologous neuropeptides expressed by kiss2 populations in zebrafish (Navarro et al., 2009). The characteristic kisspeptin system in mammals is absolutely required for proper reproductive function in adults and pre-pubertal mammalian
species. An increase in kisspeptin drives the onset of puberty, though the mechanism responsible for this initiation is still unknown (Clarkson et al., 2008).

**Kisspeptin and puberty**

Pubertal onset is initiated by pulses of GnRH secretion from the hypothalamus to direct the pituitary to secrete LH/FSH to begin the development of secondary sexual characteristics (Clarkson et al., 2008). However, in pre-pubertal rodents this pulsatile secretion of GnRH is quiescent until sexual maturation. A complete mechanism that explains this pulse generation has yet to be developed, but it is clear that kisspeptin plays a pivotal role. Pre-pubertal rodent models that lack a functional kiss1/kiss1r system either by mutated *kiss1* or KISS1R antagonists, fail to initiate puberty as characterized by insufficient blood gonadotropin concentrations (Clarkson et al., 2008). Sexually mature rodents that lack kisspeptin or its receptor on GnRH neurons do not have an appropriately regulated HPG axis. Therefore, in the context of pubertal onset and GnRH/LH secretions, it is clear that kisspeptin is critical for reproduction.

**In vivomodel limitations and need for in vitro approach**

The prevailing method of investigating kisspeptin expression and regulation utilizes tissue fixation and in situ hybridization that makes cellular manipulation *in vivo* difficult. An *in vitro* model would yield rigorous research opportunities to investigate *kiss1* mRNA expression under specific physiological conditions, thereby illustrating the neuroanatomically distinct peptidyl releases that have not yet been elucidated between AVPV/Arc nuclei. Engineering an *in vitro* model system derived from differentiated quiescent neurons renders typical immortalization
methods difficult. Post-mitotic cells present a particular challenge when attempting to introduce exogenous nucleic acid constructs. The challenge associated with stably transfecting neurons is compounded by extreme sensitivity to physical stress and low efficiencies in terms of plasmid uptake. Considering these limitations, a stable transfection approach cannot be reliably used to generate AVPV/Arc nucleus cell lines, instead a virus-based transfection method will be used.

The utility of viral vectors is evidenced in the efficiency of introducing transgenes to non-dividing cells in vivo and in vitro. This approach addresses the limitation of efficiency and reduces physical stress experienced by the neurons, though there are drawbacks with which viral-vector technique is most appropriate as well as cautious handling and enhanced biosafety facilities. Highly efficient adenoviruses (ADVs) cannot be used as the introduced transgene is only expressed transiently, because ADVs do not integrate into the host genome. This would not be useful in a system model if these AVPV/Arc nucleus cell lines are to be used long term, which requires constitutive expression. Adeno-associated viruses (AAVs) are optimized for gene delivery to neurons, but the associated capsid limits the insert size to less than 5 kb. Lentiviruses, derived from the HIV-1 genome are capable of infecting non-dividing cell with high efficiency and integrating into the host genome, allowing stable expression of the transgene over a long period of time. Similar to AAVs, lentiviral genetic content is encapsulated, but unlike AAVs this capsid is enveloped, thereby affording a greater insertion size. Much like other virus-based transfection methods, the lentiviral system is replication incompetent and relatively safe to use. Lentiviruses have the capacity to transduce a wide range of cells, including non-dividing neurons and integrate into the genome to allow sustained gene expression (Mochizuki et al., 1998). Biosafety is enhanced if a third generation lentiviral system is utilized, which requires four separate plasmids to transfect an intermediary cell type to generate a complete virus. The
components include a transgene-carrying plasmid, a responsive element and several structural polyproteins. The individual components are: (1) a transfer plasmid with an associated transgene, (2) a packaging plasmid that encodes a responsive element, Rev, that acts post-transcriptionally, (2) a packaging plasmid coding for polyproteins Gag/Pol that ultimately yield viral enzymes reverse transcriptase, protease, and integrase, and (4) a plasmid that encodes the G-protein of the vesicular stomatitis virus (VSV-G) envelope gene. These plasmids are transfected into a viral particle-assembling cell type that ultimately generates a replication incompetent virus to be used to infect the target cell. While this third generation lentiviral packaging system is cumbersome and complicated, it is the most viable and efficient approach to infect quiescent neurons with a transgene that will generate immortalized cultures of AVPV/Arc nuclei.

The feasibility of immortalizing specific differentiated neurons in vivo by targeting tumorigenesis in transgenic mice has previously been demonstrated by Mellon et. al. (1990). Constitutive expression of Simian virus 40 (SV40) large T-antigen (TAg) driven by GnRH promoters infected into embryonic mice develop proliferative GnRH-expressing tumors (Mellon et al., 1990). GT1-7 cells that serve as a model system for GnRH expression were derived from these tumors, which are now ubiquitously used in neuroendocrinology research. Applying this method of immortalization in the context of generating separate AVPV and arcuate nucleus model systems is void; the utility of approaching immortalization by targeting tumorigenesis in vivo using transgenic mice is not valid if the aim is to distinguish between two hypothalamic regions in culture. Expression of kiss1 cannot be used to drive the expression of SV40 TAg as this gene is expressed in both populations of interest, therefore an approach that exploits expression of a unique gene among a population would generate a heterogeneous mixture of both
AVPV and arcuate nuclei. HPG modulation by AVPV and arcuate nucleus peptidyl release need to be explored separately.

**Objectives**

The goal of this project is to design a reliable method to isolate AVPV and arcuate kisspeptin-releasing neurons from a pre-existing mouse model. These neurons will be used to generate two unique immortalized kiss1-expressing cell lines using an adult female mouse model and a third generation lentiviral packaging system. Generated neuropeptide secretion profiles between the two kiss1 subpopulations and their response to sex steroids will be invaluable in determining the kiss1-expressing AVPV/Arc role in the reproductive axis.

**Materials and Methods**

**Animals**

Experiments were performed using 9-13 week old female Kiss1-GFP (Kiss1-hrGFP) mice. Female transgenic mice were generated by Dr. Carol Elias’ lab at the University of Michigan using homologous recombination techniques, derived from Kiss1-hrGFP mice. Female Kiss1-GFP positive mice were generated from breeding pairs, with all mice housed at the Oregon State University Laboratory Animal Research Center (LARC). All mice were maintained on a 12:12 h lighting schedule, with lights on at 6:30 AM in groups of three to five to a cage. Access to standard rodent chow and water was *ad libitum*. For sacrifice, mice were briefly anesthetized using an isofluorane chamber followed by cervical dislocation. Care was in accordance with the
Institutional Animal Care and Use Committee guidelines and approved by Oregon State University.

**Vibratome Preparation**

A Microslicer Zero 1 vibratome purchased from Ted Pella was utilized to isolate AVPV and arcuate nucleus explants from adult female *Kiss1*-GFP mice. Coronal sectioning was designed based on *in situ* hybridization data provided by the Allen Brain Atlas of an adult male mouse. The vibratomespecimen mount was placed in a cold slurry of ice and water before mouse anesthesia and this slurry was maintained throughout the procedure. Two stacked agar blocks were anchored with Loctite to the specimen plate to support the brain. Sectioning protocol was followed as described by the vibratome manual.

**Tissue Extraction and Explant Collection**

Complete brain extraction was performed post isofluorane anesthesia followed by standard pinch test and cervical dislocation. The brain was removed from the skull quickly under aseptic conditions. A small portion of the cerebellum was truncated using a sterile razor blade to allow a flat surface for anchorage on the vibratome specimen plate. The brain was fixed to the center of the specimen plate caudally with the rostral olfactory bulb oriented upwards with Loctite placed in front of the stacked agar blocks. The agar blocks and specimen were submerged in chilled artificial cerebral spinal fluid with calcium chloride, described fully by Sue Moenter at the University of Michigan, once Loctite solidified. Two 2500μm rostral-caudal cuts were made,
followed by a 500μm cut containing the AVPV nucleus of the hypothalamus. Sagittal and transverse trims were made on the AVPV nucleus explant and the explant was placed in a covered 1.5mL Eppendorf tube containing 1 mL serum free Dulbecco’s Modified Eagle Medium (DMEM) with collagenase. Another 1000μm cut was made to expose the arcuate nucleus. A final 1000μm cut was made to isolate the arcuate, followed by sagittal and transverse trimming and placed in a covered 1.5mL Eppendorf tube containing 1mL serum free DMEM with collagenase. AVPV and arcuate nucleus explants were enzymatically digested for 45 minutes on a shaker plate at 37°C before plating.

*Media, Incubation and Estrogen Treatment*

Matrigel was diluted 1:3 in serum free DMEM with 100μL added to each well in a 24-well plate and incubated at 5% CO₂ and 37°C for 30 minutes. Enzymatic activity of collagenase was deactivated by addition of fetal bovine serum (FBS) to each AVPV nucleus and arcuate sample to yield 20% FBS DMEM. In a 24-well plate, approximately 100μL of AVPV or Arcuate digest was added to six individual wells for a single explant. The final volume was brought up to 1mL with 20% FBS DMEM. Cultured neurons were treated with penicillin, streptomycin, and gentamicin for the duration of the experiment. Plates were incubated at 5% CO₂ and 37°C. Post-infection, AVPV and arcuate nucleus neurons were treated with 100nM estrogen for 20 minutes in 1mL of 1X PBS. Cells were imaged using fluorescent microscopy. Media change occurred every 48 hours after initial plating. Two weeks after plating, neurons were distinguished from other biological material and isolated by fluorescence-activated cell sorting (FACS).

*Fluorescence-Activated Cell Sorting (FACS) and Plating*
AVPV nucleus and arcuate neurons were washed with 1X PBS before re-suspension for FACS. Samples were re-suspended in 20% FBS DMEM after a 10 minute 100µL treatment of 1X TrypLE in 5% CO₂ at 37°C. AVPV nucleus and arcuate wells were pooled together in separate 15mL conicals and centrifuged for two minutes at 1000 rpm. Supernatants from both samples were aspirated and the cell pellet was re-suspended in 1X PBS. Samples were filtered using a nylon mesh of pore size equal to 47 microns. FACS was performed with a Beckman Coulter (Brea, California) MoFlo XDP using a solid state blue laser with excitation at 488 nm. Cells were sorted based on positive fluorescence signal in the green channel (530 nm PMT detector with a 40 nm bandpass filter) gated on a scatter to exclude cell debris, in a bivariant histogram. Cells within the gate were collected in 1mL of 20% FBS DMEM. Cells were re-plated in a 24-well plate with four wells being designated as AVPV neurons and four wells being designated as arcuate neurons.

Plasmid Isolation

All plasmids were delivered in a bacterial stab from Addgene and stored at 4°C for one week before each individual plasmid was cultured and purified. Agar plates were inoculated with ampicillin resistant bacteria and cultured over night at 37°C, followed by a liquid culture. Maxiprep yielded purified plasmids as confirmed by restriction digest. Four separate plasmids were isolated: (1) pLenti CMV/TO SV40 small and larger T (w612-1) was a gift from Eric Campeau (Addgene plasmid #22298), (2) pMDLg/pRRE was a gift from Didier Trono (Addgene plasmid #12251), (3) pRSV-Rev was a gift from Didier Trono (Addgene plasmid #12253), and (4) pMD2.G was a gift from Didier Trono (Addgene plasmid #12259).
Immortalization

Human embryonic kidney (HEK) 293T cells (gifted from Nichol Miller of David Schlaepfer’s lab at the Moores Cancer Center at University of California San Diego) were grown to 60-70% confluent cell density overnight in 37°C and 5% CO₂ incubation in a T25 flask in 10% FBS DMEM with penicillin, streptomycin, and gentamicin. Media change occurred once, one hour before transfection with lentiviral plasmids. Transfection of HEK 293T cells was performed in adherence to the lipofectamine 3000 (Life Technologies) protocol with RPMI replacing Opti-MEM medium to dilute 7.5µL lipofectamine 3000 and plasmid DNA for a 6-well volume. 2µg of plasmid DNA in a 4:2:2:1 ratio was added, with four parts SV40 TAg plasmid, two parts pMDLg/pRRE plasmid, two parts PMD2.G plasmid, and one part pRSV-Rev. The DNA-lipid complex was incubated at room temperature for 5 minutes after a gentle mix and added to HEK 293T cells. HEK 293T cells were incubated with lentiviral DNA for 12 hours before collection. The complete virus was filtered by a 45 um syringe filter into a 15mL conical tube. 1mL of complete virus was aliquoted to two 1.5mL microfuge tube, one for the AVPV nucleus and one for the arcuate nucleus. From a stock of 4µg/µL, 2µL of polybrene was added to the 1mL aliquots of complete virus. Three weeks post primary culture harvest, AVPV and arcuate nucleus T25 flasks were aspirated and 1mL of virus was added, followed by 4mL of 20% FBS DMEM. Approximately 16 hours after initial infection, the virus was aspirated and samples were resuspended in 20% FBS DMEM.

Results

Cell Plating and Viability
AVPV nucleus and arcuate explants were stable in artificial cerebral spinal fluid (ACSF) with calcium chloride. Explants were viable in SF DMEM with collagenase for the duration of enzymatic digestion at 37°C before plating. Neurite process extension typically occurs four to five days after initial harvest regardless of supplemental Matrigel coating. AVPV (Figure 1a) and arcuate nucleus neuron (Figure 1b) samples were stable in 20% FBS DMEM for three to five weeks with penicillin and streptomycin and gentamicin, though extensive glial cell populations were observed in arcuate samples (Figure 1b). AVPV nucleus samples co-express GFP with kisspeptin (Figure 2a) as well as in the arcuate (Figure 2b), as observed using UV-stimulated fluorescence. AVPV and arcuate neuron samples retained viability after treatment with 1X TrypLE for 10 minutes in 37°C and 5% CO₂ incubator to prepare for FACS. Cells centrifuged at 1000 rpm for two minutes and re-suspended in 1X PBS are still viable after flow cytometry. Post-sort, neurite process extension resumes after four to five days with reduced glial cells in arcuate samples.

Figure 1a (Left): Brightfield image of AVPV nucleus neuron sample five days post primary harvest with vibratome. Figure 1b (Right): Brightfield image of Arcuate nucleus neuron sample five days post primary harvest with vibratome.
Flow Cytometry Data

Neurons filtered through nylon mesh into 1X PBS from AVPV and arcuate samples before being sorted are sheared and fragmented (data not shown). Neurons suspended in 1X PBS that have not been filtered are not sheared and remain intact throughout the sorting process. Both AVPV neurons (Figure 3a) and arcuate neurons (Figure 3b) samples exhibit a unique bimodal data spread. Neurite process extension occurs four to five days after sorting and retain their viability in AVPV (Figure 4a) and Arc (Figure 4b) samples as well as had reduced glial population (Figure 4b).
Figure 3a (Left): Bi-variant flow cytometry results for kiss1-GFP expressing neurons from AVPV nucleus composite samples. Total event count = $1.3 \times 10^6$. Events within black boundary were collected in GFP positive 1.5 mL Eppendorf tube for re-plating. Figure 3b (Right): Bi-variant flow cytometry results for kiss1-GFP expressing neurons from arcuate composite samples. Total event count = $5.3 \times 10^5$. Just as with AVPV nucleus samples, events within boundary were collected for re-plating.

Figure 4a (Left). AVPV nucleus samples post fluorescence activated cell sorting, with magnified image (bordered inlet). Figure 4b (Right): Arcuate samples post fluorescence activated cell sorting, with magnified image (bordered inlet).

**Immortalization and Estrogen Treatments**
AVPV and arcuate nucleus samples began dividing after four weeks post initial infection (Figure 5a-b). AVPV and arcuate nucleus samples retain expression of kisspeptin, as observed with UV fluorescent microscopy (Figure 6a-d). Arcuate neurons demonstrated increased basal expression of *kiss1* compared to AVPV nucleus neurons (Figure 6c). Unique expression patterns of AVPV and arcuate nucleus neurons under estrogen exposure are retained. After 20 minute exposure with a 100 pM estrogen solution in 1X PBS, AVPV nucleus neurons increased expression of *kiss1* (Figure 6b). Arcuate neurons showed a decreased expression of *kiss1* with the same treatment (Figure 6d) after four hours.

Figure 5a (Left): Brightfield AVPV neuron image after lentiviral infection. Figure 5b (Right): Brightfield arcuate neurons after lentiviral infection.
Figure 6a (Top left): AVPV neurons co-expressing *kiss1* and GFP before 100 pM estrogen (E$_2$) treatment with magnified image (bordered inlet) under UV exposure. Figure 6b (Bottom left): AVPV neurons demonstrating an increase in *kiss1* expression after 20 minute incubation.
with 100pM E₂. Figure 6c (Top right): Arcuate neurons co-expresing kiss1 and GFP before 100 pM E₂ treatment with magnified image (bordered inlet) under UV exposure. Figure 6d (Bottom right): Arcuate neurons demonstrating a decrease in kiss1 expression after four hour incubation with 100 pM E₂.

Conclusions

AVPV and arcuate neurons are successfully excised using a vibratome from an adult female mouse brain. These explants are stable in SF DMEM with collagenase to allow sufficient breakdown of collagen to promote neuron culture. AVPV and Arc samples are stable in 20% FBS DMEM for six weeks with common antibiotics and antifungals: penicillin, streptomycin, and gentamicin. Extensive glial and non-kiss1-expressing neurons populate are present in arcuate samples. Specific kiss1-expressing neurons are purified using FACS, removing non-essential cell bodies from the cultures.

Assembled virus from HEK 293T cells infect AVPV and arcuate kisspeptin-releasing neurons after four weeks post-infection. AVPV and arcuate kisspeptin neurons retain differential expression patterns under estrogen exposure, with kiss1 up-regulation being observed after 20 minutes, and kiss1 down-regulation being observed after four hours for AVPV and arcuate samples respectively. Arcuate neurons also demonstrate an increase basal level of kiss1-expression compared to AVPV samples.

Discussion

Preliminary results are promising, suggesting we are using a viable method of isolating kisspeptin-expressing AVPV and arcuate neurons. Aliquots of both populations adhered and began neurite extension within four to five days following the initial harvest. Common growth media containing 20% FBS DMEM with penicillin, streptomycin, and gentamicin stabilize these
cells for approximately six weeks before degradation. Extensive glial populations populate arcuate neuron samples, but these are removed after FACS resulting in a purified arcuate kisspeptin neurons sample. AVPV nucleus samples have few glial cells. The proportion of excess glial cells to kisspeptin-releasing cells is not surprising between AVPV and arcuate neuron explants, considering that the arcuate explant is 1 mm thick compared to AVPV nucleus 0.5 mm thickness. Both neuron populations are stable in 20% FBS DMEM for approximately six weeks after initial harvest and retain stability after FACS, a large time window is afforded for further experiments and manipulation on these purified AVPV nucleus and arcuate neuron samples. Four weeks after initial harvest, infected AVPV and arcuate neurons begin proliferating after 12 hours incubation with complete lentiviral package. Unsuccessful TAg confirmation in HEK 293T cells by stable transfection was performed after RNA harvest (data not shown).

Retention of the differential expression pattern between AVPV and arcuate nucleus kisspeptin neurons when exposed to estrogen in these cell lines is also promising. This suggests that the correct immortalized cell type was successfully targeted in this project. Increased baseline arcuate nucleus kiss1-GFP expression in menopausal female mice relative to the AVPV nucleus suggests higher kiss1 transcription activity in the arcuate occurs late in life. The fact that increased expression of kiss1 as a function of estrogen exposure occurs after 20 minutes in AVPV kisspeptin-releasing neurons suggests a much more rapid transcription mechanism than previously thought. Unique peptidyl releases between AVPV and arcuate nuclei post infection suggest accurate infection of the correct target neuron, but more robust investigations regarding genetic profiles must take place to complete verification.

**Future Direction**
Neuron characterization post immortalization will follow to access AVPV nucleus and arcuate neuron genetic content to confirm immortalized targets. Arcuate neurons have been characterized as expressing genes for neurokinin B (Tac2) and its receptor (NK3R), dynorphin (Dyn), and kisspeptin (kiss1). However, arcuate neurons do not express the receptor for kisspeptin (kiss1R). Therefore, once immortalized arcuate neurons are stable, we can characterize appropriate gene expression with Tac2, NK3R, Dyn, kiss1, and kiss1R primers. Presence and absence of particular mRNA will justify the identity of the arcuate cell line.

The complex interplay between hypothalamic Kiss1-expressing populations and GnRH neurons is integral to elucidate regulation of the HPG axis. Intercommunication between AVPV nucleus and arcuate neurons may provide insight in regards to their differential expression pattern when exposed to rising levels of estrogen. This in vitro model will allow further experimentation to explore these regulatory mechanisms. These immortalized cell lines will provide an invaluable tool in elucidating the regulation of the kisspeptin system and how it modulates GnRH neuron activity. These in vitro models will also be useful in determining the sensitivity to endocrine disruptors like perfluorinated compounds (PFCs) and how they influence the HPG axis at the kisspeptin neuron level, a cellular mechanism that has not yet been explored.

PFCs comprise a large part of synthetic chemicals used frequently in industry, typically focusing on domestic products. Products designed to resist staining, resist water, and provide non-stick coating (Teflon) all utilize PFCs as they reduce friction based on their unique physical and chemical properties (Kjeldsen et al. 2013). These exploitable properties allow extensive application of these chemicals, though the molecular stability promote environmental persistence as they are resistant to several forms of degradation (Giesy and Kannan 2002). Human exposure is typically attributed to ingestion from contaminated food from PFC-coated packaging or from
tainted water as well as from plant or animal foods that have accumulated sufficient levels (Giesy and Kannan 2002). To a lesser extent, dermal exposure and inhalation pose viable means to be exposed to these chemicals (Anderson et al. 2014). Specific subclasses of PFCs, like perfluoroalkyl acids (PFAAs) are of particular interest as they act as endocrine disruptors.

PFAAs have been reported as endocrine disruptors by mimicking endogenous hormone function and causing deleterious effects. Binding to an estrogen receptor elicits an estrogen response; considering there are two subclasses of estrogen receptors in the mammalian hypothalamus (ERα and ERβ), PFAAs can alter several cell types inappropriately. Moiety and chain length significantly alter binding capacity to estrogen receptors (Gao et al., 2012). It can be concluded that several classes of PFCs are of concern regarding neurotoxicity, but the specific neuronal cell type acted upon by PFCs is not well understood.

Considering both GnRH and kisspeptin-releasing neurons in the AVPV and arcuate nucleus express estrogen receptors, it would appear the PFCs act specifically on these hypothalamic regions to influence the reproductive axis. However, an investigation regarding various PFC effects on kisspeptin release have not been possible. With AVPV and arcuate kisspeptin cell lines, this cellular mechanism can be explored in great detail. Once the mechanism that alters AVPV and arcuate nucleus kisspeptin-release upon PFC estrogen receptor binding, the complete toxicodynamics for these endocrine disruptors can be constructed.
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