The Ever-Changing Female-in Toxicology

Explaining variability, highlighting the role of the CNS and future challenges

Birth Defects Research and Prevention Meeting

Ralph L. Cooper, Ph.D.

Quality Scientific Solutions

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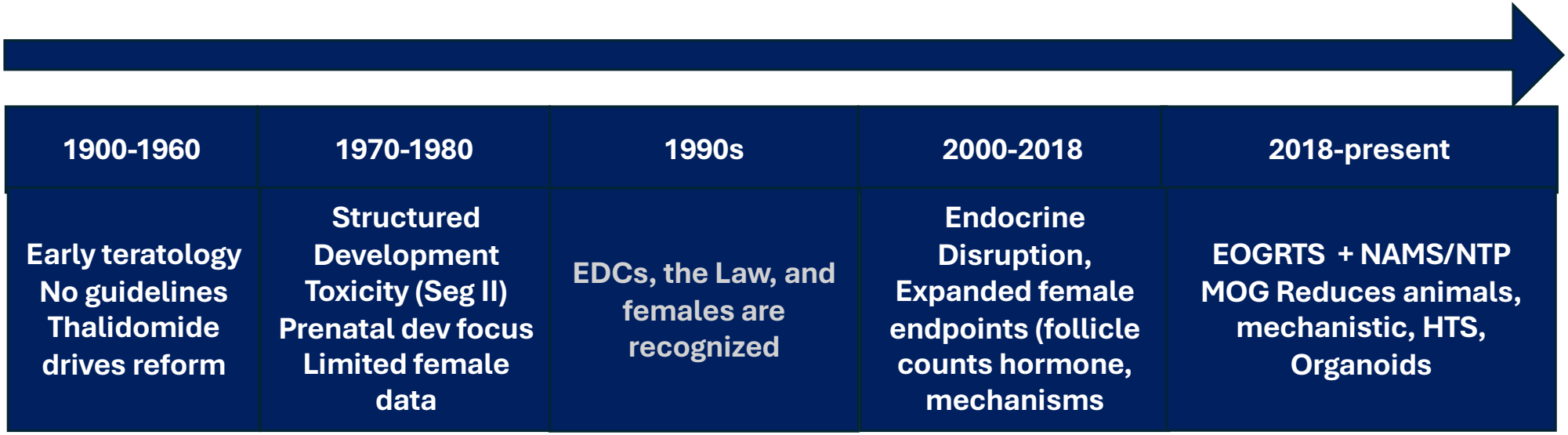
The Century-Long Evolution of Female Reproductive Toxicology

From Descriptive Pathology to Mechanistic, Endocrine-Aware Science

Female reproductive toxicology has advanced only through crisis, scientific pressure, and regulatory recognition of complexity.



The Century-Long Evolution of Female Reproductive Toxicology

From Descriptive Pathology to Mechanistic, Endocrine-Aware Science
Female reproductive toxicology has advanced only through crisis, scientific pressure, and regulatory recognition of complexity.

- **Early testing (1900s–1960s) treated female biology as "noise.**
- **Thalidomide forced global recognition that reproductive and developmental hazards require structured, predictive testing. (1960) Teratology Society formed (Warkany was a prime mover)**
- **Emphasis was placed on mating and fertility, limited evaluation of female (no cycles, no female developmental endpoints) tissue weights, maybe pituitary and mammary tumors.**
- **Ignoring cyclicity, follicle dynamics, and neuroendocrine control demonstrated by Long and Evans, 1922, Holweg and Junkman, 1923, 1927, and John Everett. 1940.**
- **Cultural/Regulatory Rationale,** Male-centric toxicology culture, female too complex, Regulatory inertia.

1900-1960	1970-1980	1990s	2000-2011	2018-present
Early teratology No guidelines Thalidomide drives reform	Structured Development Toxicity (Seg II) Prenatal dev focus Limited female data	EPA 1996 Repro Toxicity Guidelines, First unified US female repro focus	Endocrine Disruption, Expanded female endpoints (follicle counts hormone, mechanisms	EOGRTS + NAMS/NTP MOG Reduces animals, mechanistic, HTS, Organoids

Early literature did provide very useful advice about certain measures of the female's reproductive physiology as the era of reproductive toxicology began. However, the understanding of the estrous cycle was well under way. For example: Joseph A. Long, J. A.and Evans, H. M.1922 The oestrous cycle in the rat and its associated phenomena. Univ. of California Press.

There is no need to explain the profound importance of an infallible method for recognizing what is thus the periodic function of the ovary. The embryologists, moreover, is interested in the precise time of ovulation inasmuch as knowledge of the sequence of events in the earliest development of mammals depends on such information.

Citing the work of Stockard and Papanicolaou "By the fortunate discovery that in the guinea pig these mucosal transformations are accompanied by the deliverance of epithelial cells so that at times the lumen of the vagina has a characteristic cell content it has been possible for Stockard and Papanicolaou to show that **we may discover with ease in the living animal the exact occurrence and progress of these cycles**." When it has been proved, as Stockard and Papanicolaou have done for the guinea pig, and as we have been able to do with exactitude for the rat, that these cycles are correlated with the rhythmic discharge of ova from the ovary, it will be seen **that we now have in our hands for the first time an accurate method** for the detection of ovarian function in experimental animals. This fact promises important consequences, for it enables us to investigate disturbances of ovarian function which may be experimentally produced

The Regulatory Framework Takes Shape

Structured Developmental Toxicity → EPA 1996 → Global Harmonization

1970s–1980s:

- **Segment II studies standardized developmental toxicity. Adult female endpoints still under evaluated.**
- **Basic research on female reproductive neuroendocrinology, molecular biology, (releasing hormones, neurotransmitters, and circadian functions) developed rapidly.**
  - **Established critical time linkages with ovarian steroids and CNS circadian activity. Rodent hormone and CNS timing established.**
  - **The brain can be a target for EDCs**
  - **Ignored by most toxicologists.**
  - **1977 Roger Guillemin and Andrew V. Schally shared the 1977 Nobel Prize in Physiology or Medicine** *"for their discoveries concerning the peptide hormone production of the brain."*

1900-1960	**1970-1980**	**1990s**	2000-2018	2018-present
Early teratology No guidelines. Thalidomide drives reform	**Structured Development Toxicity (Seg II) Prenatal dev focus Limited female data**	**EPA 1996 Repro Toxicity Guidelines, expanded US female repro focus**	Endocrine Disruption, Expanded female endpoints (follicle counts hormone, mechanisms	EOGRTS + NAMS/NTP MOG Reduces animals, mechanistic, HTS, Organoids

Mechanistic Expansion and the Endocrine Disruption Era

Female Biology Moves to the Center of Hazard Identification

Endocrine disruption science forced regulators to confront the mechanistic complexity and susceptibility of the female reproductive system.

- **1991-1996 three wingspread conferences highlight Endocrine Disrupting Chemicals.**
  - **Cataloged evidence of environmental chemicals as hormonally active**
  - **Wildlife sex-reversals, malformations, developmental issues, human populations, timing of dose etc.**
- **1996 Food Quality Protection Act (FQPA)**
- **1996 Safe Drinking Water Act Amendments (SDWA amendments)**
  - **Mandated that EPA create a program to screen chemicals for endocrine disruption.**
  - **Tier 1 assays: TG 450-456 adopted in 2007 (female relevant ER assays, steroidogenesis aromatase, uterotropic and pubertal.)**
  - **Tier 2 tests** Extended One Generation Reproductive Study, EOGRTS 2011)
- **1998 OPPTS 870.3800 endpoints for non-pregnant females (3 Week pre-mating vaginal smear)**

1900-1960	1970-1980	**1990s**	**2000-2018**	2018-present
Early teratology No guidelines Thalidomide drives reform	Structured Development Toxicity (Seg II) Prenatal dev focus Limited female data	**EDCs, the Law, and females are recognized**	**Endocrine Disruption, Expanded female endpoints (follicle counts hormone, mechanisms**	EOGRTS + NAMS/NTP MOG Reduces animals, mechanistic, HTS, Organoids

The Extended One-Generation Era, NAMs, and the New Challenge
OECD 443, NTP MOG, HTS, and the emphasis on limiting the number of animals
Modern testing integrates mechanistic insight and reduced-animal designs, but female biological variability remains one unresolved barrier.

- **2001** **OECD 416 added the estrous cycle before mating**
- **2003** **Kisspeptin recognized as a reproductive hormone**
- **2008** **OECD 407 updated to include estrous cycle (last 2 weeks)**
- **Tier 1 guideline studies adopted**
- 2014 **EPA formalized New Alternative Methods**
- **2011** **OECD 443 (EOGRTS) included estrous cycles, VO, AGD, Ovarian counts histo2014**

1900-1960	1970-1980	1990s	**2000-2011**	**2016-present**
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2016 **Lautenberg TSCA amendments legally require EPA to: Reduce vertebrate testing; Use NAMs whenever Maintain a formal list of NAMs**

2018 **OECD 408 (90 day added vaginal cycle if triggered by other findings**

2018–2020, EPA shifts toward NAMs (New Approach Methodologies), Increasing reliance on HTS, in silico, and non-animal methods.

2021–present EDSP effectively transitions into a NAM-driven program. ToxCast/Tox21 data used for: Prioritization, screening, and WoE evaluation

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The ever-changing female
Variability in the ovarian cycle and the HPO axis.

Knowledge of ovarian cycle by monitoring vaginal cytology can be used to explain variability and can be diagnostic for pathology

Summary:

Since the 1990s, significant advances have been made for evaluating potential female reproductive toxicants. The progressive addition of new endpoints have brought about a significant improvement in the evaluation of the female test animal.

Combining the new *in vitro*, high-throughput assays with the enhanced guidelines offers significant insight into the potential risk of a test chemical on female reproduction.

Question: With emphasis on *in vitro* and alternate test, can the important timing, integration or sequence of change (variability) which dominate the intact female be addressed in vitro?

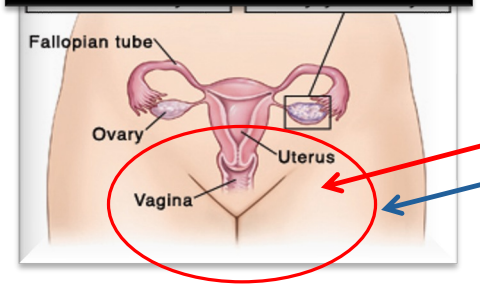
In vivo: By identifying the sources of this variability and building a deeper understanding of the rhythmical changes in the underlying physiology that occur over the ovarian cycle, much of the concern about day-to-day or hour-to-hour changes in many of the female endpoints has been addressed. This has strengthened confidence in the results of guideline studies.

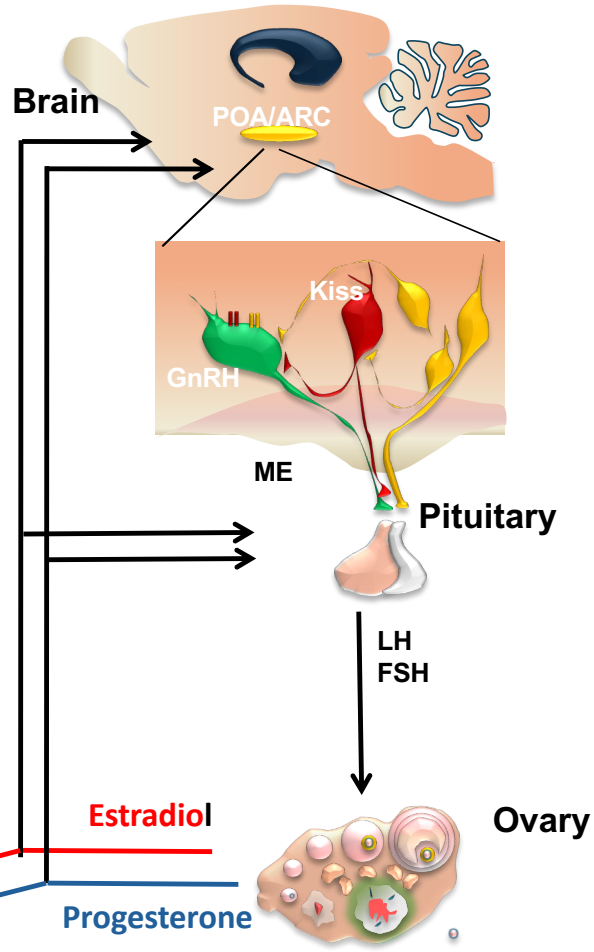
Example: Knowledge of the ovarian cycle by monitoring vaginal cytology had a major impact in defining the role of the brain in driving pituitary-ovarian function, and removes variability.

Guideline	VO (F1) Estrogen driven	Estrous cycle (P0/F1)	AGD (F1) Androgen driven	Follicle counts	Ovarian pathology
OPPTS 870.3800 (2-gen, EPA) **August 1998** (EPA 712-C-98-208)	**Not required**	**F1** yes (estrous cycle evaluation)	**Not required**	**Triggered**	**Yes** – ovary histopathology in P0/F1
OECD TG 416 (2-gen) (original) / **2001** (major revision)	**Not required**	**P0 & F1:** yes (estrous cycle)	**Not required**	**Not required (classic TG 416)**	**Yes** – ovary histo-pathology in P0/F1
OECD TG 443 (EOGRTS) **2011** (adopted)	**Yes** – VO in F1 Cohort 1	**P0 & F1:** yes (estrous cycle in selected females)	**Yes** – AGD in F1 (and often F2 if triggered)	**Yes** – follicle counts/oocyte maturation in females (triggered or default, depending on design)	**Yes** – detailed ovarian histopathology, integrity, follicle staging
OECD TG 421 (screening) **1995** (original) / **2015** (last major revision)	**Yes** – VO in F1 if offspring examined (design-dependent)	**P0:** yes – estrous cycle before and during mating; F1 usually not (screening)	**Yes** – AGD in F1 pups (standard in modern use)	**In general, no formal follicle counts** (screening level)	**Yes** – ovary histopathology in P0 (screening detail, but less extensive than 416/443)
OECD TG 422 (combined repeat-dose + repro screen) **1996** (original) / **2016** (last major revision)	**Yes** – VO in F1 if offspring examined (design-dependent)	**P0:** yes – estrous cycle before mating; F1 usually not (screening)	**Yes** – AGD in F1 pups (standard)	**No routine follicle counts** (screening)	**Yes** – ovary histopathology in P0 (screening-level detail)

Summary of endpoints measured in selected regulatory test. The vaginal smear is primary for diagnosing the underlying changes in most all other endpoints

Target Tissues
The fallopian tubes uterus, vaginal and mammary glands brain and pituitary are primary targets for the ovarian hormones, containing receptors for both hormones.



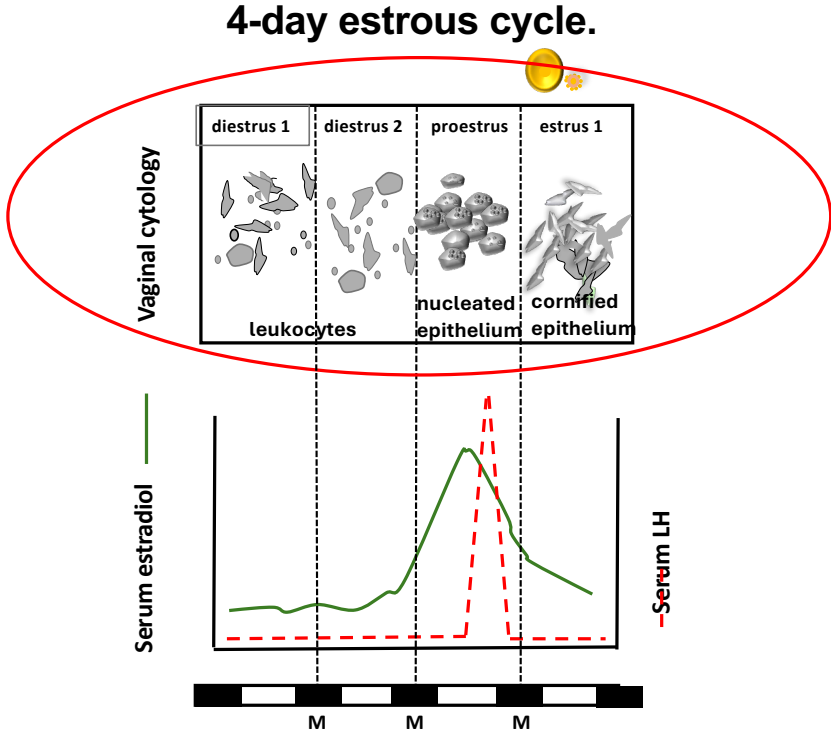


Endpoints	Pub	28 day	90 day	421 PND13	422 PND13	416 2 gen	443
Vaginal opening	•					•	•
Vaginal smear at kill	•	•	•	•	•	•	•
Estrous cyclicity	√+ •		?	√+ •	√+ •	√+ •	√+ •
Sexual behavior				plug	plug	plug	plug
Mating index				•	•	•	•
Pituitary Wt	•	op	•			•	•
Pituitary histo		•	•				•
Ovarian Wt	•	•	•	•	•	•	•
Ovarian histo	•	•	•	•	•	•	•
Follicular counts						•	•
Uterine wt	•	•	•	•	•	•	•
Uterus histo	•		•	?	?	•	•
Mammary glands histo		•	•				•
Thyroid Wt	•	op	•	•	•	•	•
Thyroid Histo	•	•	•	•	•	•	•
Adrenal Wt	•		•				•
Adrenal Histo		•	•				•
Fertility				•	•	•	•
Cervix			•	•	•	•	•
Vagina		•	•				
Gestational length				•	•	•	•
Implantations				•	•	•	•
implantation loss				•	•	•	•
Pup sex ratio				•	•	•	•
Pup AGD				•	•	•	•
Thyroxine (T4) TSH T3	T4 TSH	T4 TSH T3	T4 TSH T3	T4 TSH T3	T4 TSH T3		T4 TSH T3
Gonadotropin E2, P4			E				

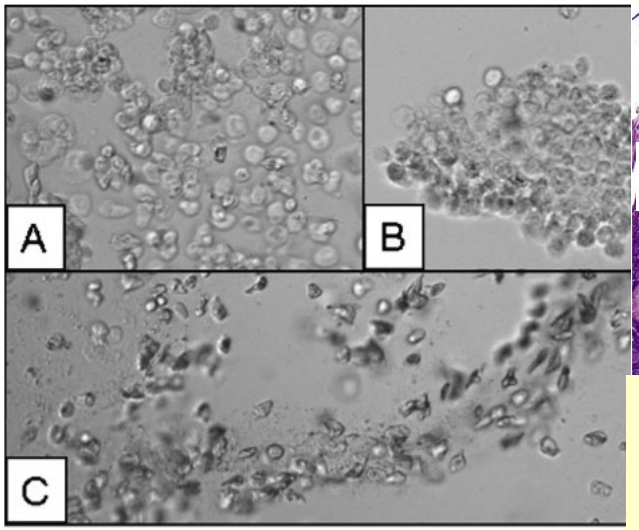
Ovarian Staging: Using vaginal cytology

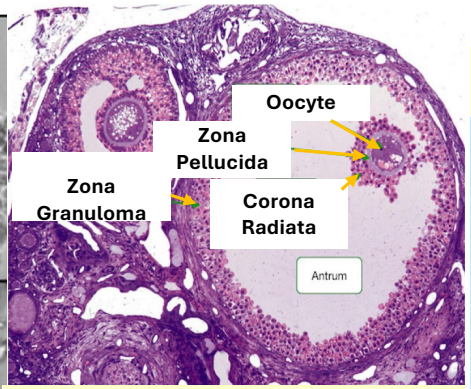
- What does the vaginal cytology tell us about the underlying neuroendocrine control of ovarian function?
- Should be examined for a period long enough to discern a pattern, (e.g., 2 weeks).
  - A single smear is not really that informative.
  - 3 weeks prior to mating is required in some mulitgens.
  - Chronic studies would be enhanced by intermittent sampling (e.g., 2 weeks on, 2 weeks off) to identify PoF.
- Vaginal smears identify the functional status of the ovary
  - Is the ovary functioning properly (i.e., cycling "regular")?
  - If not: is the cycling irregularly, persistent estrus, constant estrus, persistent diestrus (PSP), constant diestrus (anestrus)?
  - Each of the above is diagnostic for a unique follicular type.
- Useful in interpreting necropsy findings (what to expect for reproductive tract tissues
  - Uterine, pituitary & ovarian weight (reduces/explains variance)
- Identifying sampling times for hormone measures
  - LH just before lights out on proestrus
  - Prolactin (diurnal and nocturnal surges)
  - Estradiol (proestrus rise)
  - Progesterone Evening of proestrus and estrus
- Identify dose times

Use of vaginal cycle to characterize Knowledge of ovarian cycle by monitoring vaginal cytology can be used to explain variability and can be diagnostic for pathology

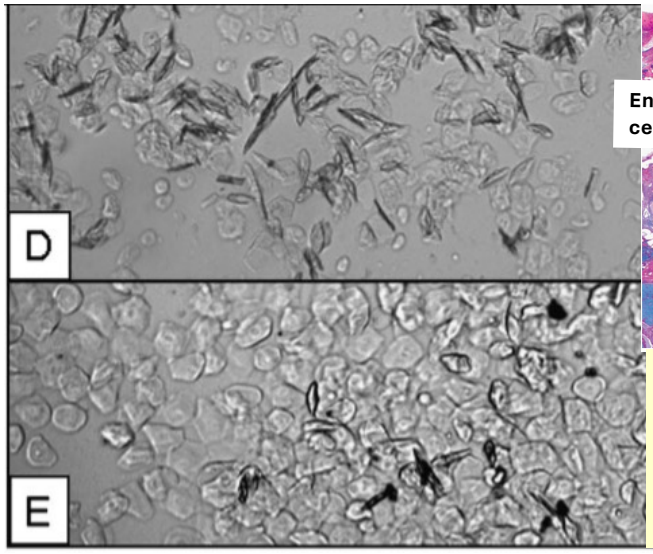


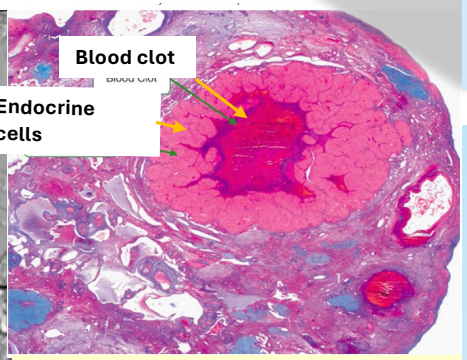
Cooper et al., JAGS 1986, 34:735-751





- **Ovulatory surge of LH**
- **Female is sexually active after lights out**
- **Uterus large, fluid filled**





- **Ovulation occurs ~ 0100 h**
- **Female receptive**
- **Oocytes present in ampulla. CL forms.**
- **Uterus shrinks**

Proestrus

Early Proestrus, the dominant follicle shows rapid granulosa cell proliferation, Aromatase surges and estradiol rises steeply. FSH induces LHR expression in granulosa cells. Theca cells increase androgen production. Follicle enlarges, antrum expands, cumulus still compact. Late Proestrus Estradiol peaks, triggering the GnRH/LH surge.
Progesterone from granulosa peak just before LH surge. Meiosis resumes, GVBD starts, cumulus expansion, follicular wall remodeling, increased vascular permeability. Proestrus follicles transform from estrogen-producing growth units into ovulation-ready, progesterone-primed structures.

Estrus

Estrus: The "ovulatory" follicle. Estrus begins around the time of **ovulation** in the rat. Which **~12–14 hours after the LH surge. The follicle rupture (localized** proteolysis at the stigma). Theca externa contraction. Release of the **cumulus–oocyte complex (COC)** into the oviduct.
Oocyte state: Completed **GVBD**, extruded the **first polar body**, Arrested at **metaphase II**.
Post-ovulatory follicle Granulosa and theca cells collapse inward. Basement membrane breaks down. Blood vessels invade the granulosa layer. Estrus is the **rupture and release** phase **Follicle transitions to a luteal gland**
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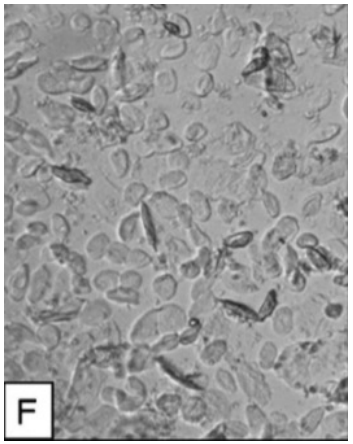
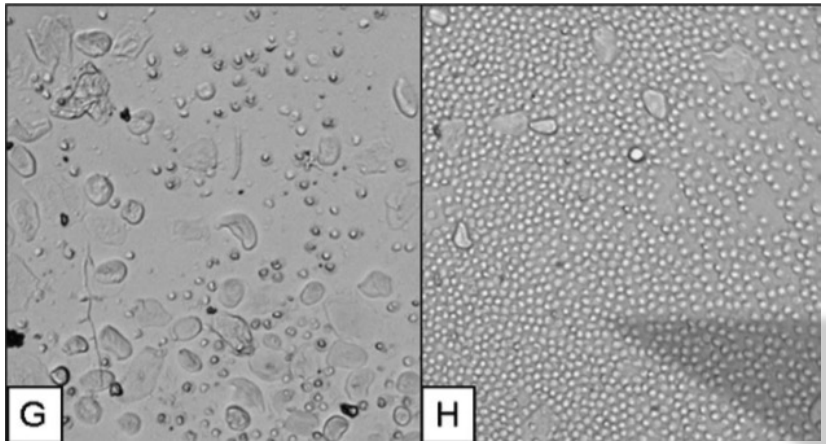


- **If not pregnant, CL regress**
- **Not sexually receptive.**
- **Luteal phase is short or absent.**



Uterus small, avascular, slit-like lumen

Metestrus - diestrus 1

The "late antral, estrogen-quiet" follicle

Follicular state

Several **medium-sized antral follicles** are present; one or a few begin to show **dominance**.

Granulosa cells:

Low aromatase expression
Low LH receptor expression
Primarily FSH-responsive

Theca cells:

Produce androgens at a modest rate
LH-responsive but not yet strongly steroidogenic

Endocrine environment

High progesterone from the previous CL suppresses GnRH/LH pulses.

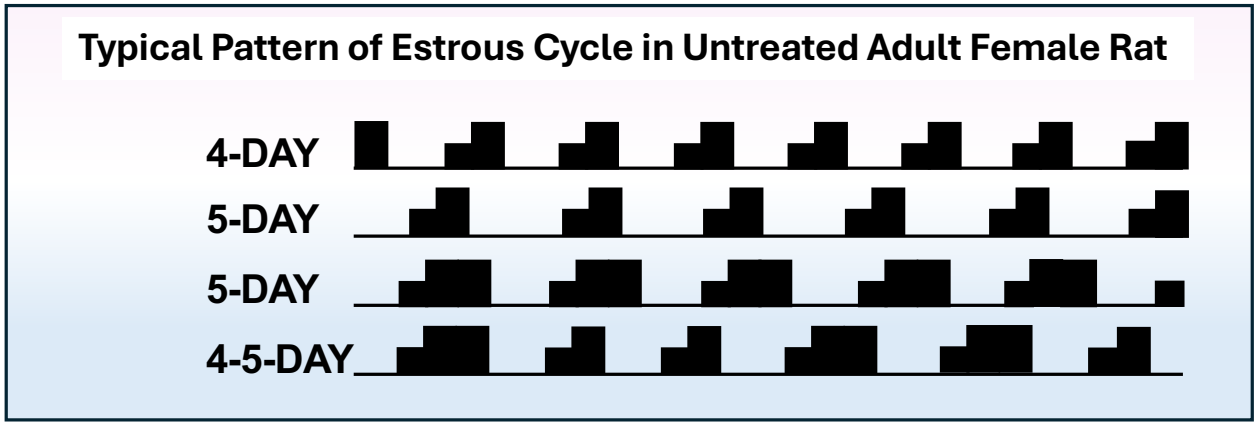
Estradiol is **low**, rising only slowly as the dominant follicle grows.

Morphology

Follicles are **antral**, but the antrum is not yet massively expanded.

Cumulus–oocyte complex (COC) is compact; oocyte is in **GV arrest**.

Key point: Diestrus follicles are **growing but steroidogenesis "switched on."** They are preparing for the estrogenic surge of proestrus.



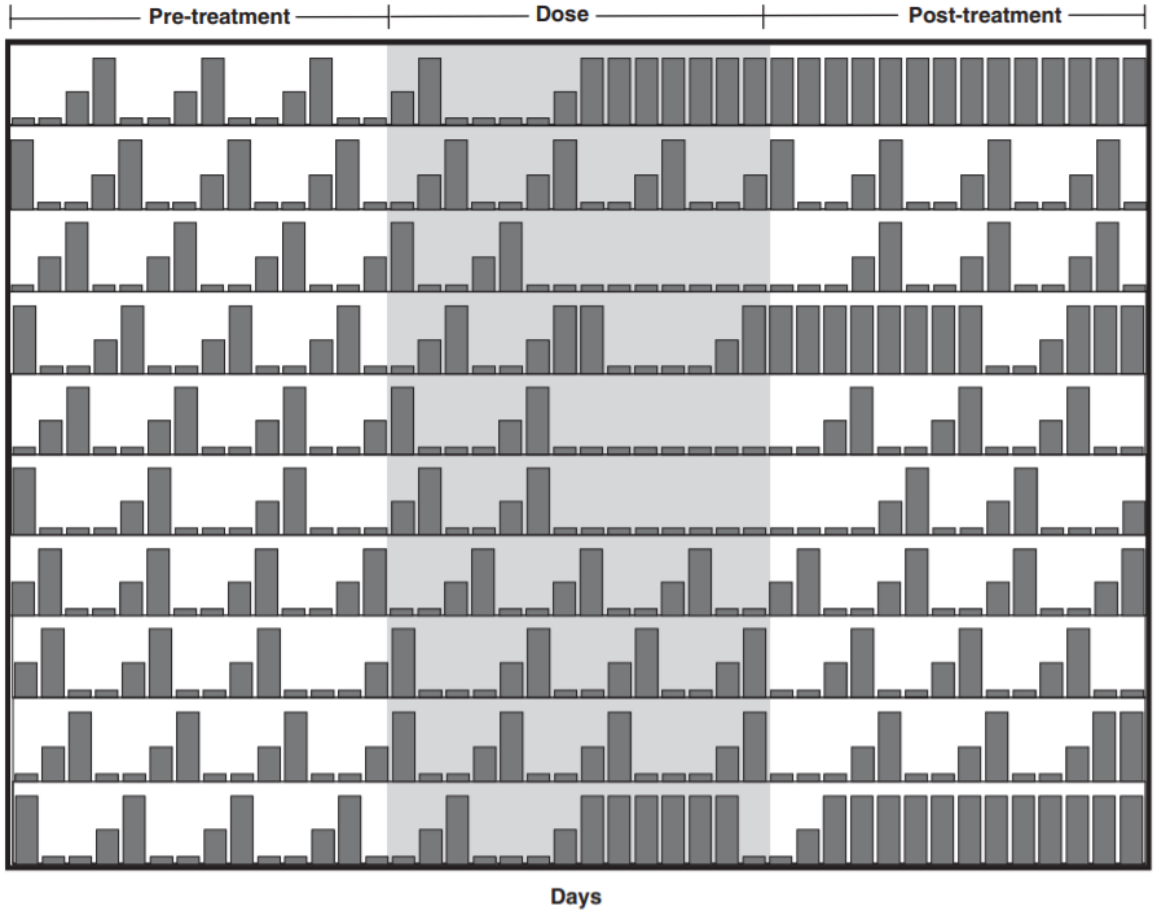
Full height columns depict the day of estrus, 1/2 height columns the day of proestrus, and ½ height columns the day of diestrus. (Cooper and Goldman (1999) In: *An Evaluation and Interpretation of Reproductive Endpoints for Human Health Risk Assessment* ILSI press.

- **Under normal conditions (14:10 or 12:12 light:dark cycle) the majority of untreated females (>85 %) display regular 4-5 ovarian cycles.**
- **Importantly, the ovarian cycle requires an intact CNS (i.e., hypothalamus). Ovarian hormones feedback to discrete hypothalamic areas and in coordination with circadian signals regulate the cycle via pituitary LH and FSH.**
- **Information on the ovarian cycle can be used to inform when to sample for most endpoints**
  - **Knowing when to sample, will reduce variance in data (uterine, pituitary weight, endocrine measurements, ovulation (eggs) etc.**
  - **Changes in the pattern will can be diagnostic for underlying ovarian histopathology, uterine, pituitary weights, endocrine milieu, LUF formation and other disorders.**

Characterizing estrous cycle in the female rat.

Representative depiction of estrous cycles in xenobiotic-exposed females over 14 days of treatment (shaded area) bracketed between 2-week pre-treatment and 2-week post-treatment periods. Both episodes of persistent estrus and persistent diestrus (likely indicative of a pseudopregnancy) are present.

- **PE ovaries were polyfollicular, no CL, steady E, no P, have reduced food intake & are typically sexually active.**
- **PSP ovaries have CL, maintain P4, have increased food intake & are not sexually receptive** *From Cooper et al., Toxicol Sci. 2000 Feb;53(2):297-307*



Relationship Between Cycle Length and Ovulation					
Cycle length			Oocyte retrieval performed		
4	4/5	5	Cornification day 1	Cornification day 2	# Oocytes
X			X		15
X			X		14
X			X		18
	X		X		0
	X		X		0
	X		X		0
		X	X		0
		X	X		0
	X			X	14
	X			X	0
	X			X	12
		X		X	16
		X		X	13
		X		X	9
		X		X	16

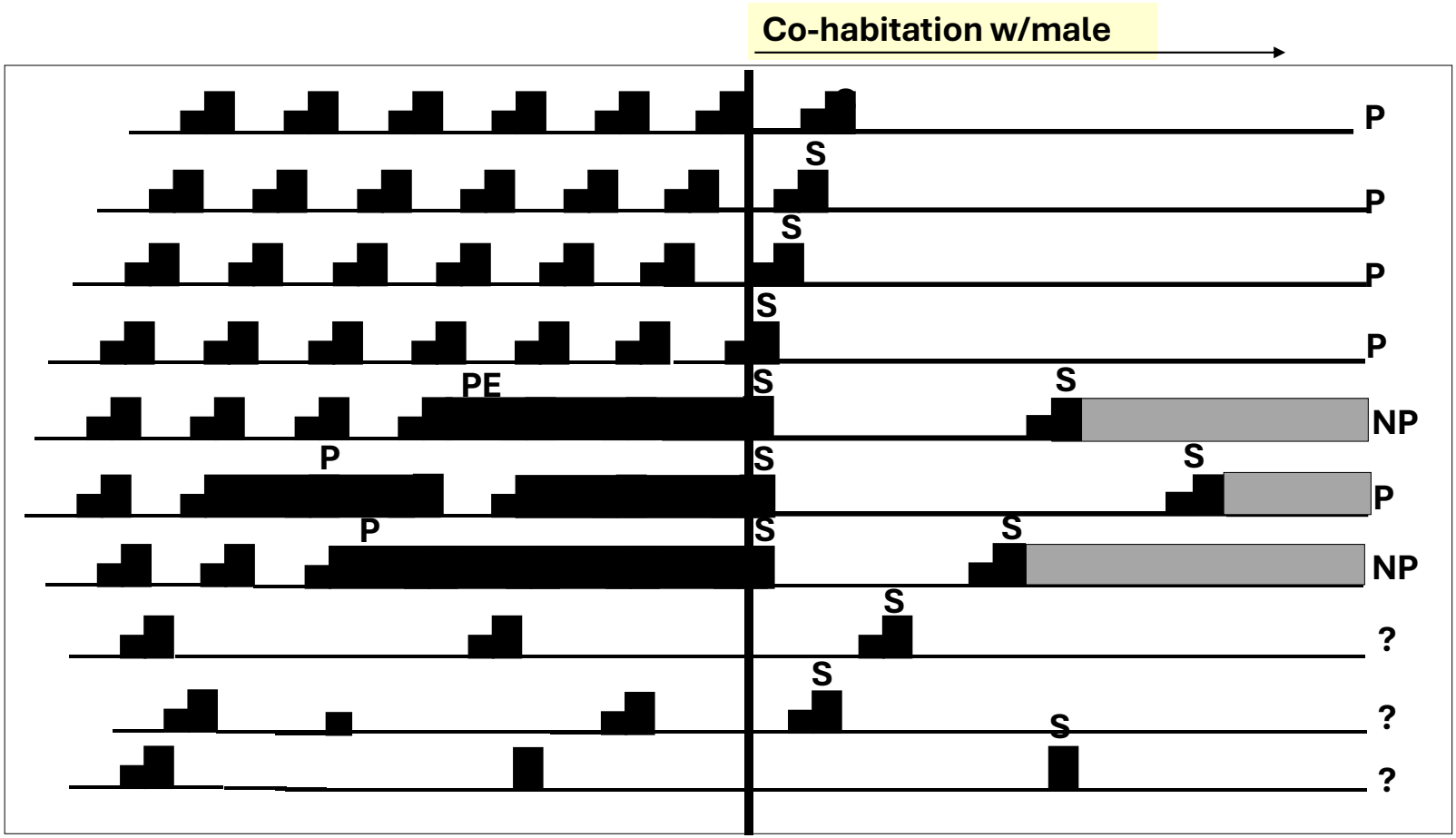
Cycles were characterized over a two-week period to determine their length and were classified as regular 4-day or 5-day (2 days of vaginal cell cornification) cycles, or a combination of each. The cycle length and the day of oocyte retrieval for each rat are indicated by "X." Oocyte numbers represent the totals obtained from oviducts of both left and right ovaries. *Based on Goldman, Murr & Cooper 2007*

Using smear data to reduce variability

Knowledge of ovarian cycle and when to look for eggs:

- **4 Day Cycle: ddPE Females ovulate around 0100 h on first day of E. Oviducts are flushed around noon and examined for eggs.**
- **5 Day Cycle: DDPEE Females ovulate on the 2nd day of E. Actual LH surge appears on 1st day of E.**
- **4-5 day females: difficult to interpret. If ballooned uterus (indicative of an endocrine proestrus) will invariably be empty, may be spontaneous shift. and it is important in evaluating**

Relationship between ovarian cycle to mating and pregnancy outcome



Usually strain typical letter size,

Female usually receptive, some pregnant (few implants/rarely are pups delivered

Receptivity/sperm + not pregnant
Not LUFs-LUFs are shorter di periods

S = sperm positive smears
P = pregnant NP = not pregnant

Hardy, D.F., (1972) Sexual Behavior in the continuously cycling rats. Behaviour, 4:288-297

"In brief, the female is in control"
"But HPO is in control of the female's behavior," and in this case, the O controls the B.

Behavior, Female is "in charge" . In essence the female's endocrine status determines if the male can mount, intromit etc. (Hardy1972)

$$\text{Lordosis Quotient (LQ)} = \frac{\text{Number of Lordosis Responses}}{\text{number of Mounts by the Male}}$$

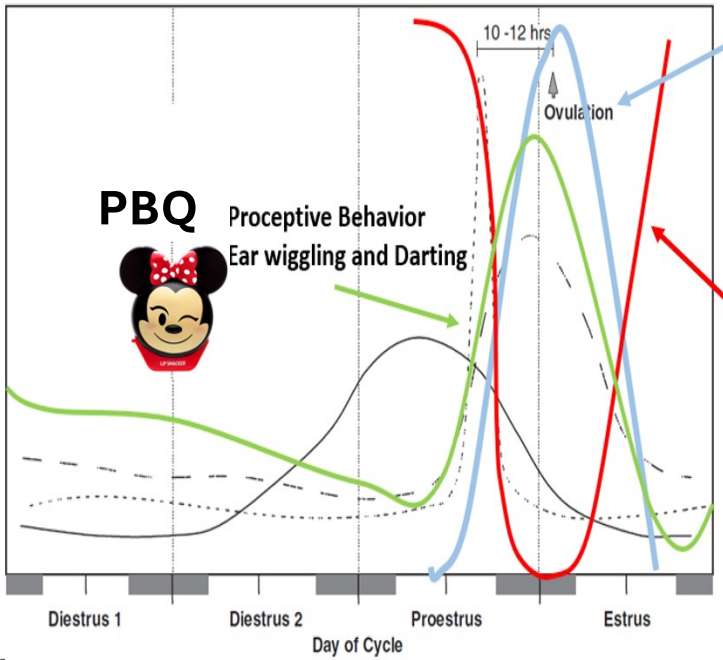
$$\text{Rejection Quotient (RQ)} = \frac{\text{Number of Rejections of the Male}}{\text{Number of Mounts or Mount Attempts}}$$

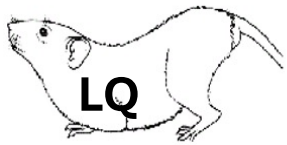
$$\text{Proceptive Behavior (PBQ)} = \frac{\text{Ear Wiggling Hops \& Darting}}{\text{Total observation time (min)}}$$

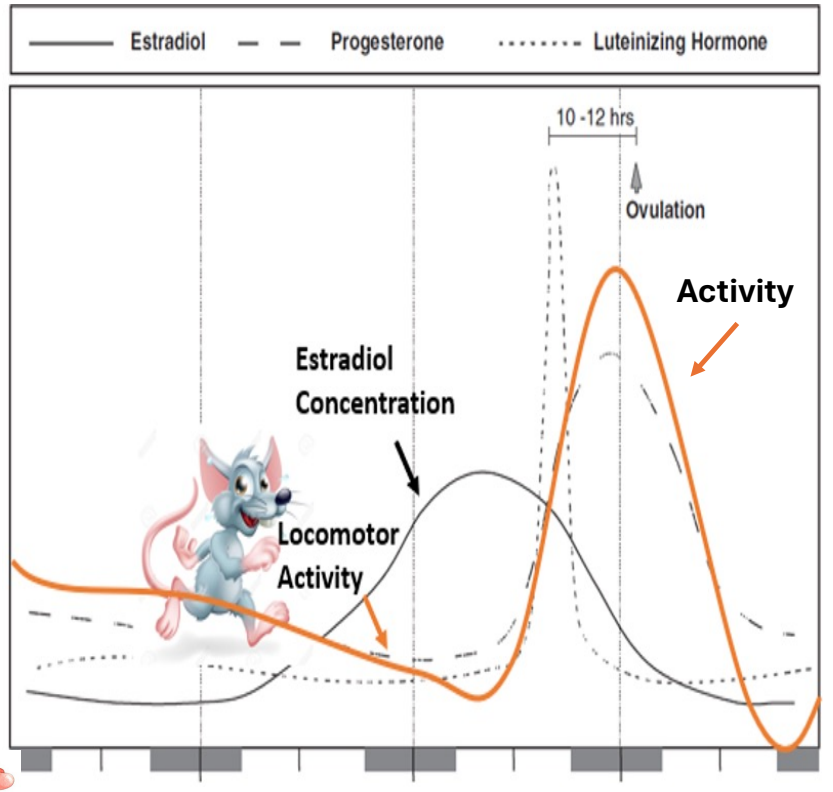
or

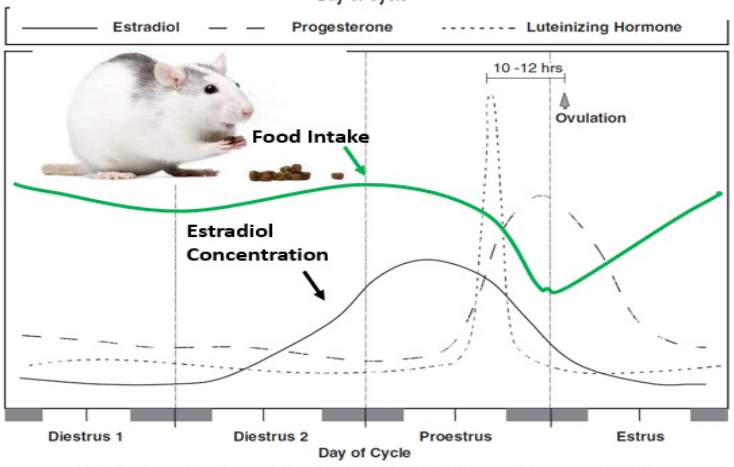
$$= \frac{\text{Ear Wiggling Hops \& Darting}}{\text{Total observation time + Lordosis}}$$

Proceptive behaviors mainly depend upon the secretion of progesterone while lordosis primarily relies on estrogen production.









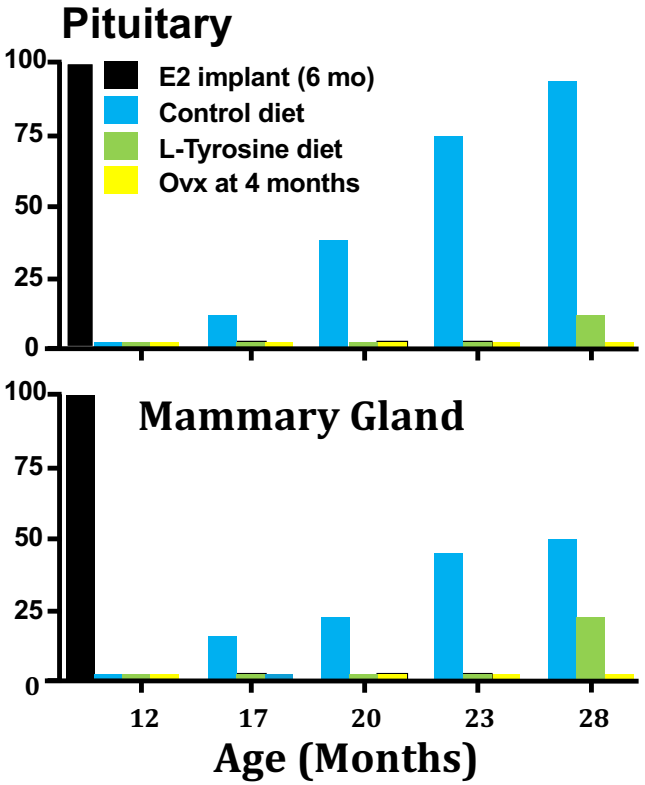
Behavioral Changes Associated with the Rat Ovarian Cycle

Behavioral Changes Associated with the Rat Ovarian Cycle. The underlying hormonal milieu predicts sexual activity, food intake, activity, and body temperature. Day to Day & Hour to Hour.

Age versus Ovarian Status in LE female rats

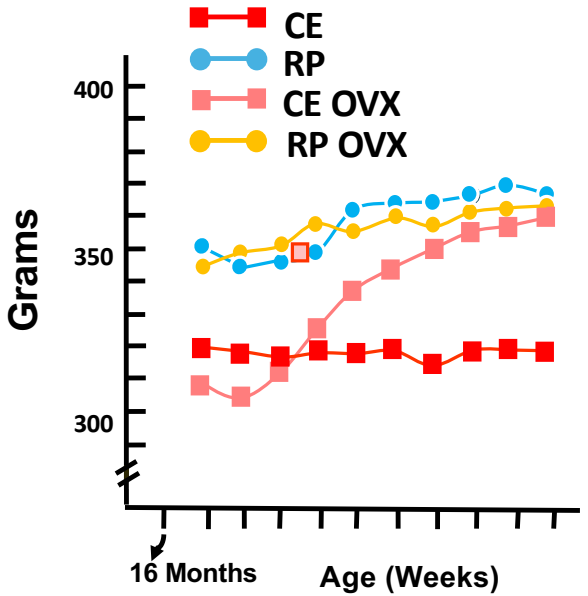
The hormonal status of the aged animal drives the response

Tumor Incidence Develop as a result of Reproductive senescence ↑ E2, Prl & no P4.



From Cooper, Experimental and Clinical Interventions in Aging 1983

Ovarian Hormones Impact Body Weight



From: Cooper and Linnoila, Behav Biol. 1976 Dec;18(4):551-61

THE EFFECTS OF FEMALE REPRODUCTIVE HORMONES ARE PERVASIVE AND UNDER THE CONTROL OF THE CNS

Note also that the effects of the ovarian cycle are pervasive, influencing immune, cardiovascular, liver (metabolism), bone, renal fluid balance, GI microbiome, and adipose tissue, etc.

The previous slides demonstrate that much of the variability present in many of the endpoints included in female reproduction assessment can be accounted for by using the vaginal smear to determine the best day of the cycle and the best time of day to examine the endpoint.

- **There is no stated requirement for determining the ovarian cycle before termination in the multigenerational studies.**
- **Care should be taken to control the stage of the cycle (and even the time of the day) that the measurements of most endpoints are taken.**

Importantly, all of the changes in females' reproductive organs are controlled by the brain (hypothalamus) through the neuronal regulation of LH, FSH, and prolactin. Within the hypothalamus, different populations of neurons control the circadian rhythm. The circadian rhythm synchronizes with the 4-5 day ovarian hormone cycle to generate the ovulatory surge of LH, CL formation, and support pregnancy. Ne

Tox in the 21st Century,
Is there hope for the female?

- The current emphasis in reproductive toxicology to transition from relying *on in vivo* data to high-throughput **screening (HTS) and New Approach Methodologies (NAMs) have improved reproductive-toxicant identification by making screening *faster*, *more mechanistic*, *more human-relevant*, and *far more scalable* than traditional in vivo studies.**

- **But**:, this transition presents new challenges for the identification of a chemical effect in more complex biological processes, such as the neuroendocrine network controlling pituitary-ovarian function. Consider the following:
  - The literature detailing the neuroendocrine/neuropeptide changes that control gonadotropin secretion and the ovarian cycle (folliculogenesis, ovulation, and corpora lutea formation is extensive.
  - **Although there *are in vitro* efforts to capture the effects of xenobiotics on *pieces* of this axis, none integrate them into a functional LH-regulating circuit, and none address the complex upstream neural network(s).**

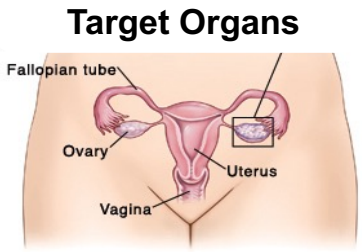
- **Is it feasible to build a human *in vitro* system that accurately models the neuroendocrine network regulating pituitary ovarian function?**
  - A system that would model the full **multi-node architecture** of reproductive function, amenable to the study of the critical temporal aspects, hormonal rhythms, and the dynamic changes associated with the reproductive cycle. Measurements that, to date, have been restricted to whole animal studies.
  - If successful, this model would enable mechanistic studies to be done in vitro, maybe improve how chemicals are prioritized and perhaps limit the need for *in vivo* confirmation.

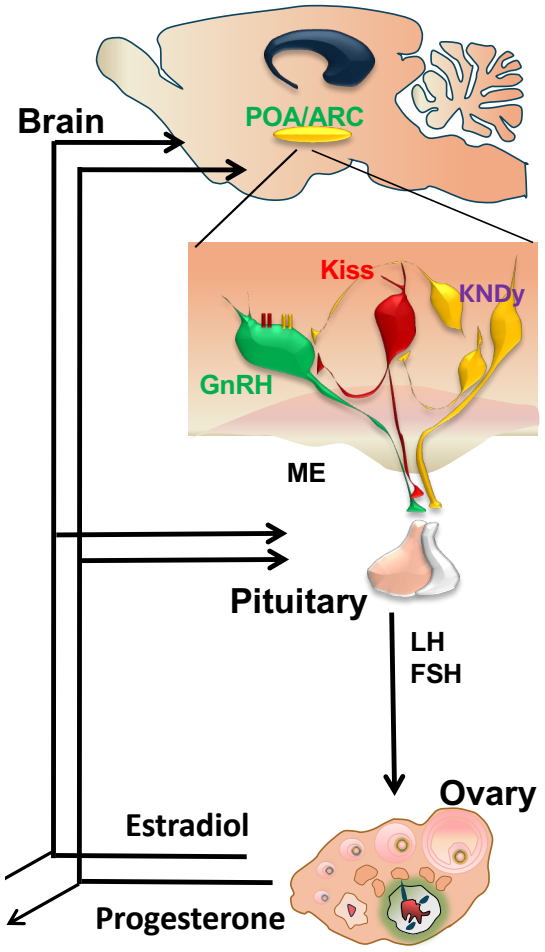
Setting the Stage Hypothalamic Control of the Female Reproductive Axis

The hypothalamus and pituitary have a major role in the regulation of reproductive development, adult reproduction function and reproductive senescence. Is it possible to model part or all of this complex system.

GnRH the final common signal from the brain: A key event in the HPO axis is the release of Gonadotropin-releasing hormone (GnRH) which is produced by hypothalamic neurons and released into the circulation. This in turn acts on the pituitary gonadotrophs to release of two gonadotropins [follicle-stimulating hormone (FSH) and luteinizing hormone (LH)], which then stimulate folliculogenesis and ovulation respectively.

Without GnRH, the individual is sterile.... and anosmic!
Without LH there is no ovulation & there are no offspring.
Alterations in LH secretion can delay ovulation leading to polyploidy and aneuploidy.
Without proper hormone feedback and/or and proper hypothalamic activity, there would be no GnRH release.
Without the proper environmental cues (mainly light:dark cycle) there would be no GnRH or LH.



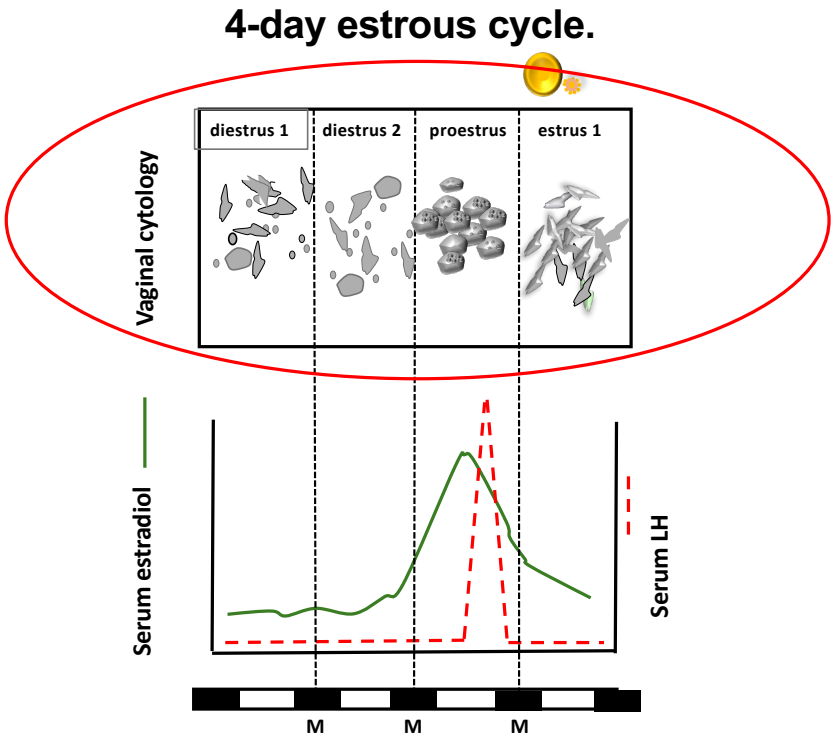


The ovulatory surge of LH: A key event in the ovarian cycle
Two GnRH-LH regulatory mechanisms.

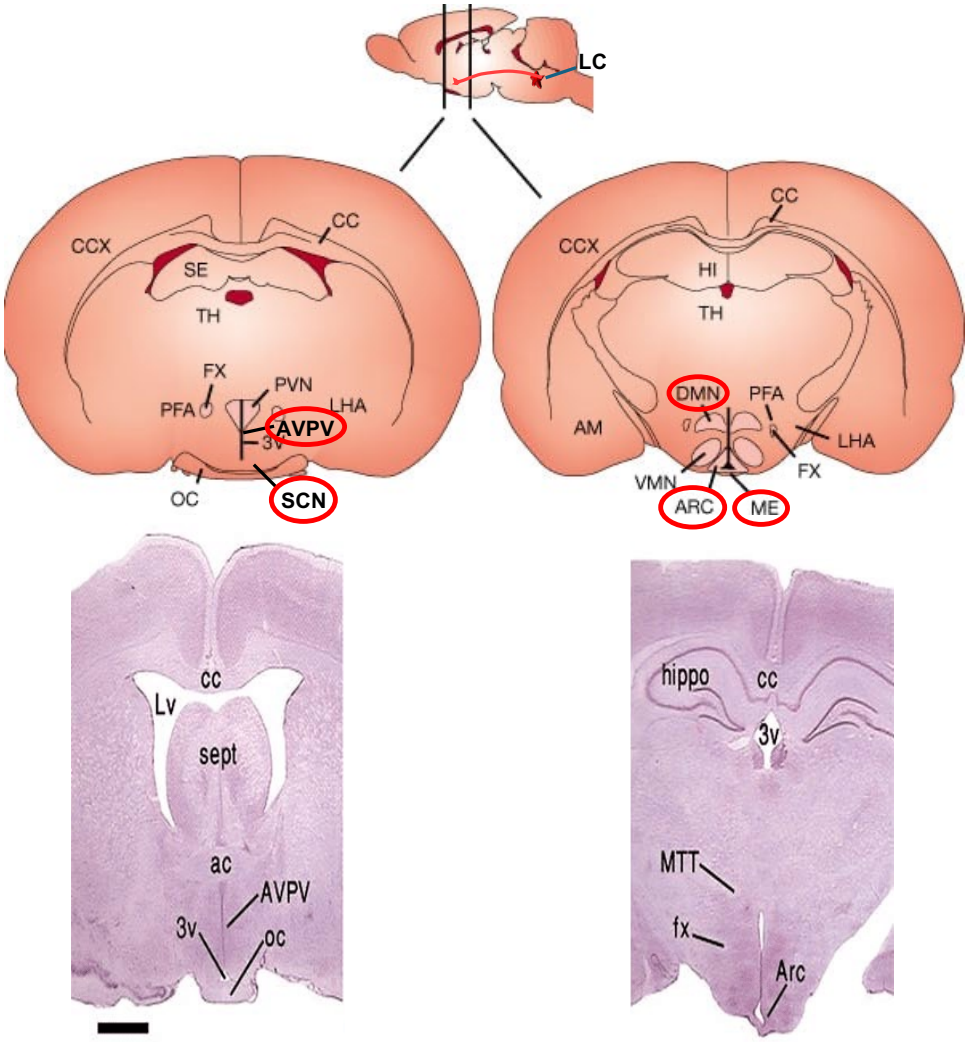
- GnRH neurons are **under CNS, ER-PR Control**
  - **Two areas of the hypothalamus regulate GnRH secretion. For most of the 4-day cycle GnRH is pulsed into the portal system at a frequency of about one burst per hour in humans (40-60 min in rat; 20-40 min in mouse). At this time the Kisspeptin/Neurokinin B/Dynorphin KNDy neurons in the arcuate nucleus control GnRH activity. The resulting pulsatile secretion of GnRH favors FSH secretion, which stimulate the follicular growth culminating a set of graafian.**

- **On the day of vaginal proestrus, in response to rising blood E2, the GnRH neurons are driven by AVPV kisspeptin neurons responding to a synchronous increase in activity from the SCN (AVP & VIP) activity and locus coeruleus (NE). This is the neural trigger for the ovulatory surge of LH in the blood on the afternoon of vaginal proestrus.**

- **Two different endocrine patterns, one CNS releasing factor.**
  - **Can this be recapitulated in vitro?**



Cooper et al., JAGS 1986, 34:735-751

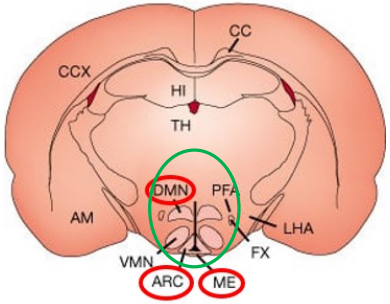


Anatomical locations of primary structures controlling GnRH-LH release in the rat brain

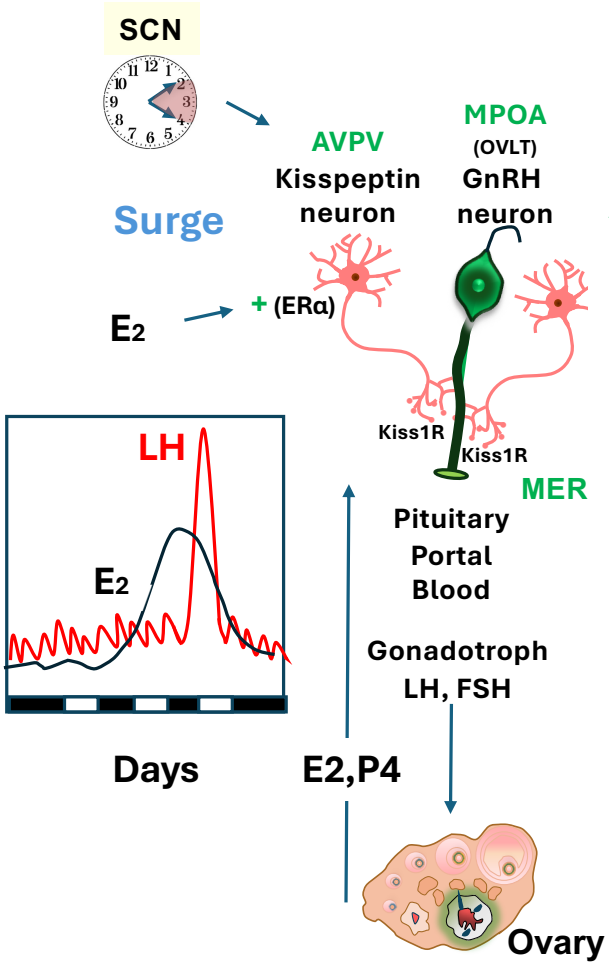
The parts are well defined. Are the amenable to study in vitro? Diagrams of rat brain, showing major hypothalamic regions implicated GnRH control of LH secretion. top is a longitudinal view of a rat brain, Cross-sections of the brain at two levels (indicated by vertical lines) are shown at the left and right**. Human and Rodent nuclei do vary, but differences are not major**.

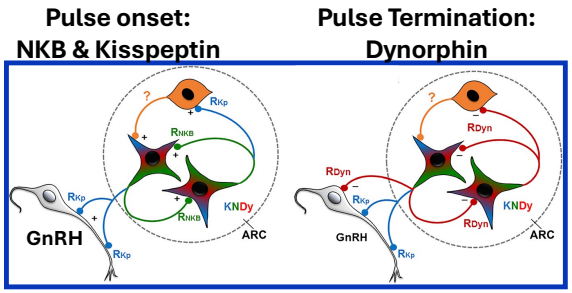
AVPV (kisspeptin cell bodies), SCN (circadian clock) ARC (KNDy cell bodies). ME (Kisspeptin, NKDy, GnRH terminals), DMN (GnIF cell bodies), VMN (ventromedial nucleus), PFX perifornical area, LHA (lateral hypothalamus), LC (locus coeruleus) LV (lateral ventricle), 3V (third ventricle), LC (locus coeruleus NE neurons cell bodies). OC optic chiasm, sept (septum) FX (fornix), TH (thalamus), HI (hippocampus), CC (corpus callosum), AM (amygdala).

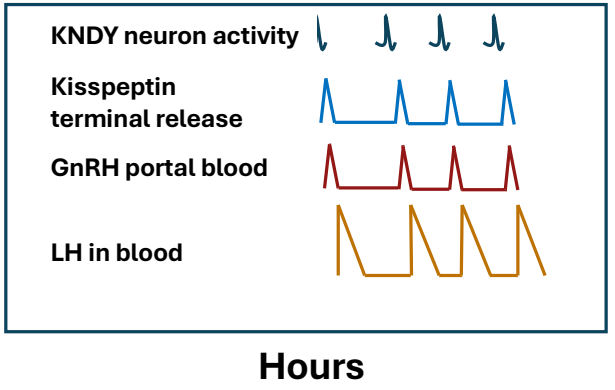
NEUROENDOCRINE CONTROL OF ADULT FEMALE GnRH-LH SECRETION



- Surge v Pulse generators controlling GnRH are anatomically distinct and have a different role in controlling the ovarian cycle.
  - In response to low E the surge mechanism is absent and the pulse mechanism maintains baseline levels of LH and FSH maintain folliculogenesis.
  - As serum E2 peaks on the afternoon of vaginal proestrus, the surge generator synchronizes with circadian activity (SCN) to produce the ovulatory surges of LH.
- The ovulatory surge of Luteinizing hormone represents a highly integrated neuroendocrine signal influenced by light:dark cycle, ovarian hormone (E2 and P4), and the proper sequence of neurotransmitter (NE etc.) and neuropeptide signals.



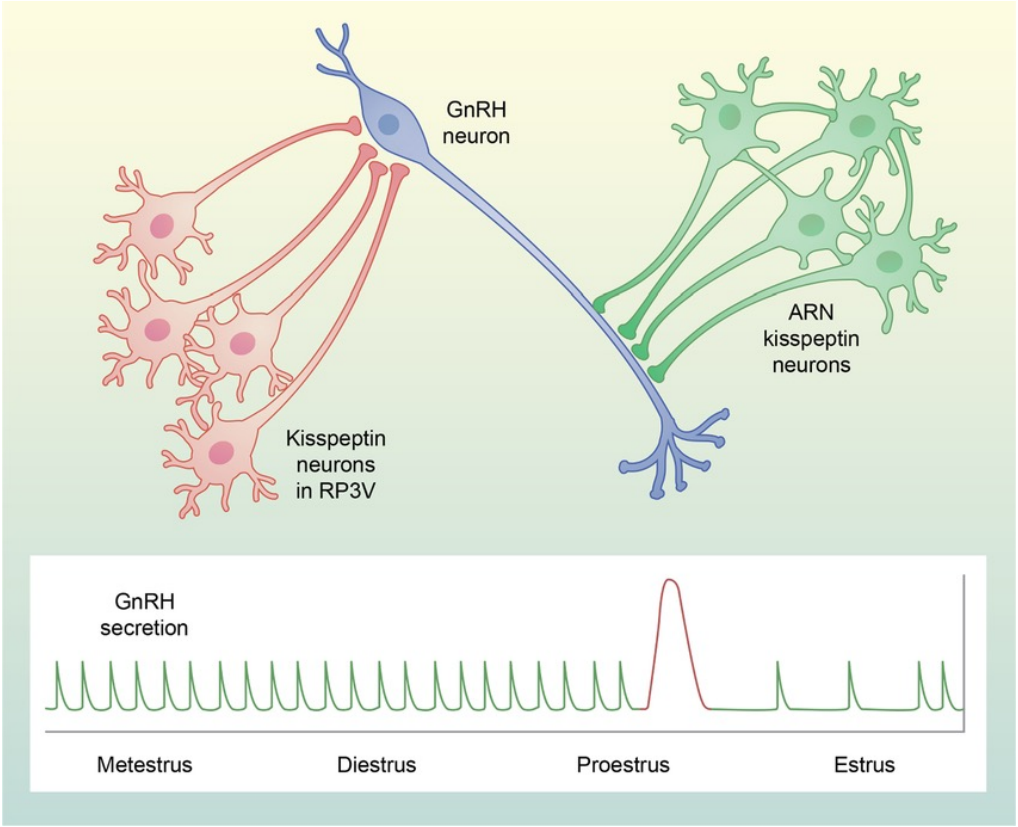




Neuroendocrine Axis controlling Adult Female Reproduction.

- **The ovulatory surge of Luteinizing hormone represents a highly integrated neuroendocrine signal influenced by light:dark cycle, ovarian hormone (E2 and P4), neurotransmitter (NE etc.) and neuropeptide signals.**
  - **Surge v Pulse generators controlling GnRH are anatomically distinct and have a different role in controlling the ovarian cycle.**
    - **In response to low E the surge mechanism is absent and the pulse mechanism maintains baseline levels of LH and FSH maintain folliculogenesis.**
    - **As serum E2 peaks on the afternoon of vaginal proestrus, the surge generator synchronizes with circadian activity (SCN) to produce the ovulatory surges of LH.**

Simplified version of dual control of the GnRH pulses.

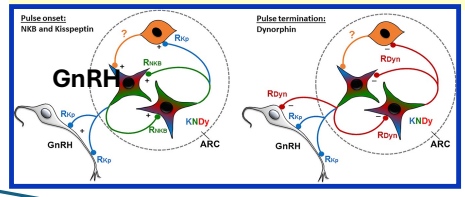


From: Goodman et al., 2022 Neuroendocrinology

CNS Regulation of the LH surge A SIMPLIFIED MODEL

PF-670462 → *CK1δ/ε inhibition ↓ $PER2$ → SCN arrhythmicity → loss of VIP/AVP timing signal → RP3V kisspeptin not activated → no GnRH surge → no LH surge → no ovulation*

μ- κ & δ-**opioid receptor** agonist
Naloxone & Naltrexone disrupts timing
early advance late delay.
NK3R (TACR3) antagonists delay surge.

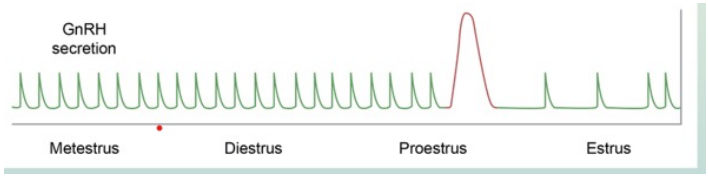


(AVP)
(V1a)
(VIP)
(VPAC2)
DMN
GnIF
GPR147
ARC
KNDy
Erα-
GPR147
(pulse)
ER, PR
SCN
(AVP)
(VIP)
RP3V
(surge)
POA
(OVLT}
MER
Kiss terminals
Gonadotroph
KISSR
(VPAC2)
(V1a)
(NEα1)
Kisspeptin
(ERα) +
GnRH
GPR147
GPR147
Slow FSH Basal
Fast LH Surge
Mult synaptic pathway, to synchronize NE with SCN input to Kisspeptin
NE
LC
A1,A2 & A6 (the locus coeruleus) being the dominant contributor.
NE synthesis or blockers
Dithiocarbamates
Chlordimeform
Prazozin
ER, PR
ER blockers
E Synthesis inhibitors
Coplanar PCBs (e.g., PCB-126)
AHR activation suppresses estradiol positive feedback
And estrogen negative feedback in KNDy neurons

Ovary
Follicle
Follicular wall rupture
Luteinization
Steroid switch

Oocyte
Maturation
Cumulus expansion

Can we produce this in vitro



CNS Regulation of the LH surge A SIMPLIFIED MODEL

- **CURRENT STATUS:**

- **Important structures and molecular pathways are mapped *in vivo* & species difference are recognized (**e.g., AVPV in rats RP3V/POA/periventricular Per in humans. Arcuate in rats v infundibulum in humans)I
- **Anatomy of ERα, Erβ & GPER identified, other neurotransmitter and neuropeptide inputs defined.**
- Certain Kisspeptin, KNDy, NE, and GnIH pathways, have been described in **receptor-centric GPCR cell lines** (e.g., KISS1R, TACR3, OPRK1, ADRA1/2, GPR147) are available for HTS. This can be used to guide model development and testing.
- Effort to establish embryonic human stem cells EHSC) or induced human pluripotent stem cells (iHPSCs) are in progress. GnRH is promising (Poliandri et al, 2017; Keen et al. 2022). Similarly, a mouse kNDY mESC ARC KNDy cell line has been published but (Miyaki et al., 2026).
  - Several studies with GT7 (mouse GnRH neurons). But, several issues.
- **Ex vivo methods such as tissue slices provide information on interaction of some neuronal pathways. This information is needed for verifying fully *in vitro* methods.**

- **Limitations:**
  - Cell availability (see above). Is it feasible to evaluate them within an *assembloid* (different culture demands, growth rates etc.)
  - **Culture media (any special requirements for different cell populations).**
  - **Chamber/device configuration and scalability.**

CNS Regulation of the LH surge A SIMPLIFIED MODEL

- **CURRENT STATUS (continued)**

- **Limitations:**
  - Cell availability (see above). Is it feasible to evaluate them within an *assembloid* (different culture demands, growth rates etc.)
  - **Culture media (any special requirements for different cell populations).**
  - **Chamber/device configuration and scalability.**
- **Being optimistic:**
  - **A step-by-step approach: first pairing certain duos or trios of neurons (e.g., kiss and GnRH and measure LH from a gonadotroph or SCN cells and Kisspeptin activity, etc.**
  - **An integrated model permitting individual components, temporal changes and system dynamics would be of considerable value for screening potential reproductive toxicants.**
  - **The basic literature in this area is growing.**
  - **Similarly, there is a considerable amount of work in progress evaluating other components of the female reproductive system that can inform a brain model.**
  - **So, it's just a matter of time?**

Thank you

Ralph L Cooper,
Quality Scientific Solutions
8612 Millers Bend,
Bahama, North Carolina
rlcooper14@hotmail.com
919-452-2763