PATHOLOGY ENDPOINTS: EVALUATION OF THYROTOXICANTS IN ANIMAL STUDIES

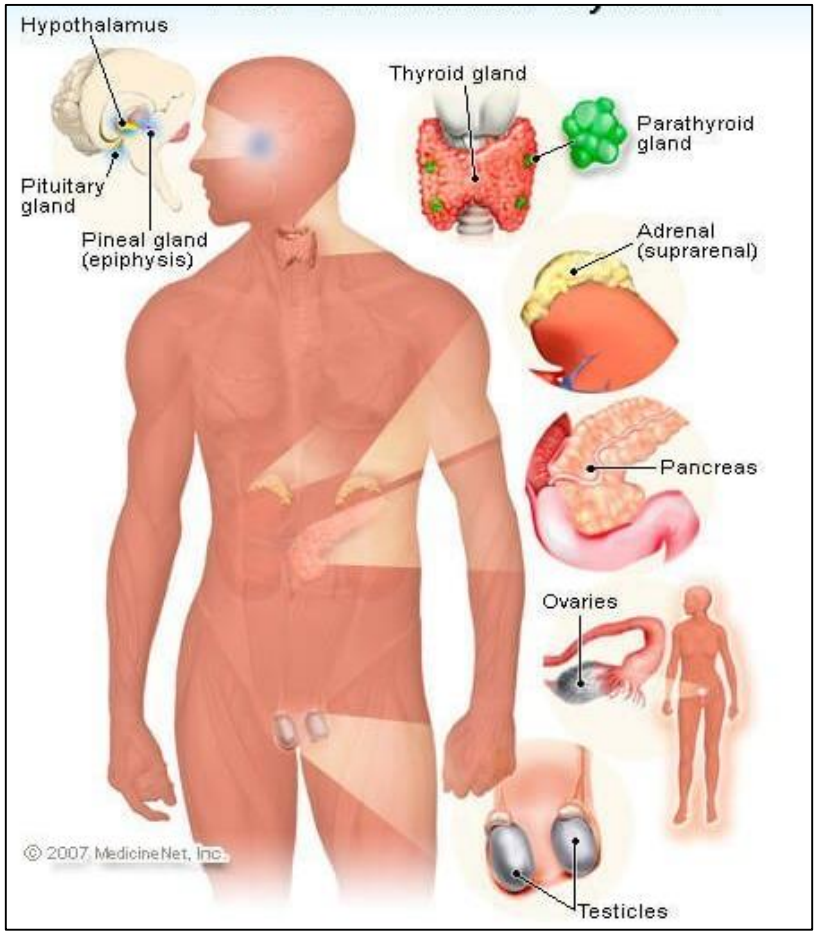
HESI Thyroid Workshop. May 9-10, 2019. Washington, DC

ENDOCRINE SYSTEM

Organs with endocrine function

- Hypothalamus
- Pituitary gland
- Thyroid gland
- Adrenal glands
- Pancreas
- Pineal gland
- Parathyroid glands
- Gonads

- Heart
- Thymus
- Adipose tissue
- Digestive tract
- Kidneys



https://www.emedicinehealth.com/anatomy_of_the_endocrine_system/article_em.htm#hypothalamus

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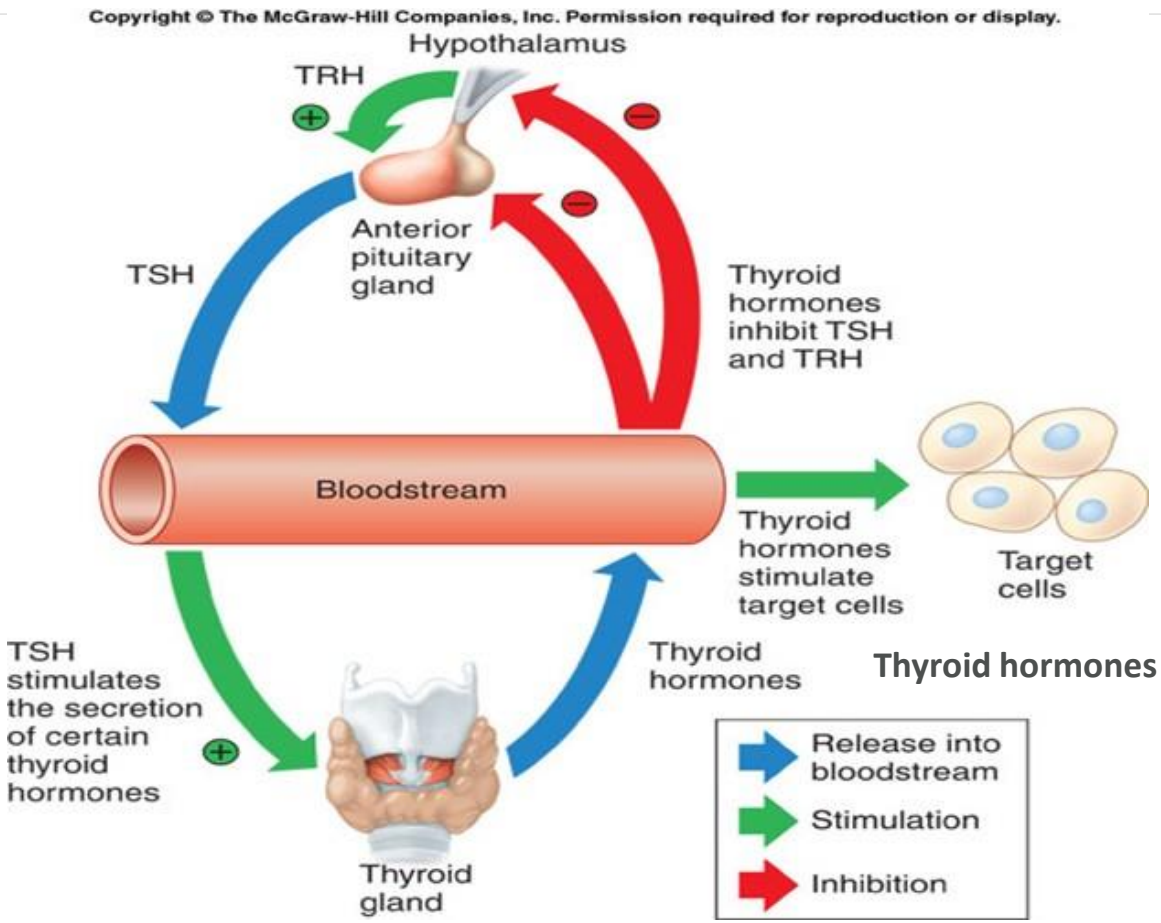
HYPOTHALAMIC-PITUITARY-THYROID (HPT) AXIS



Thyroid hormones = T_4 and T_3

https://slideplayer.com/slide/9234885/

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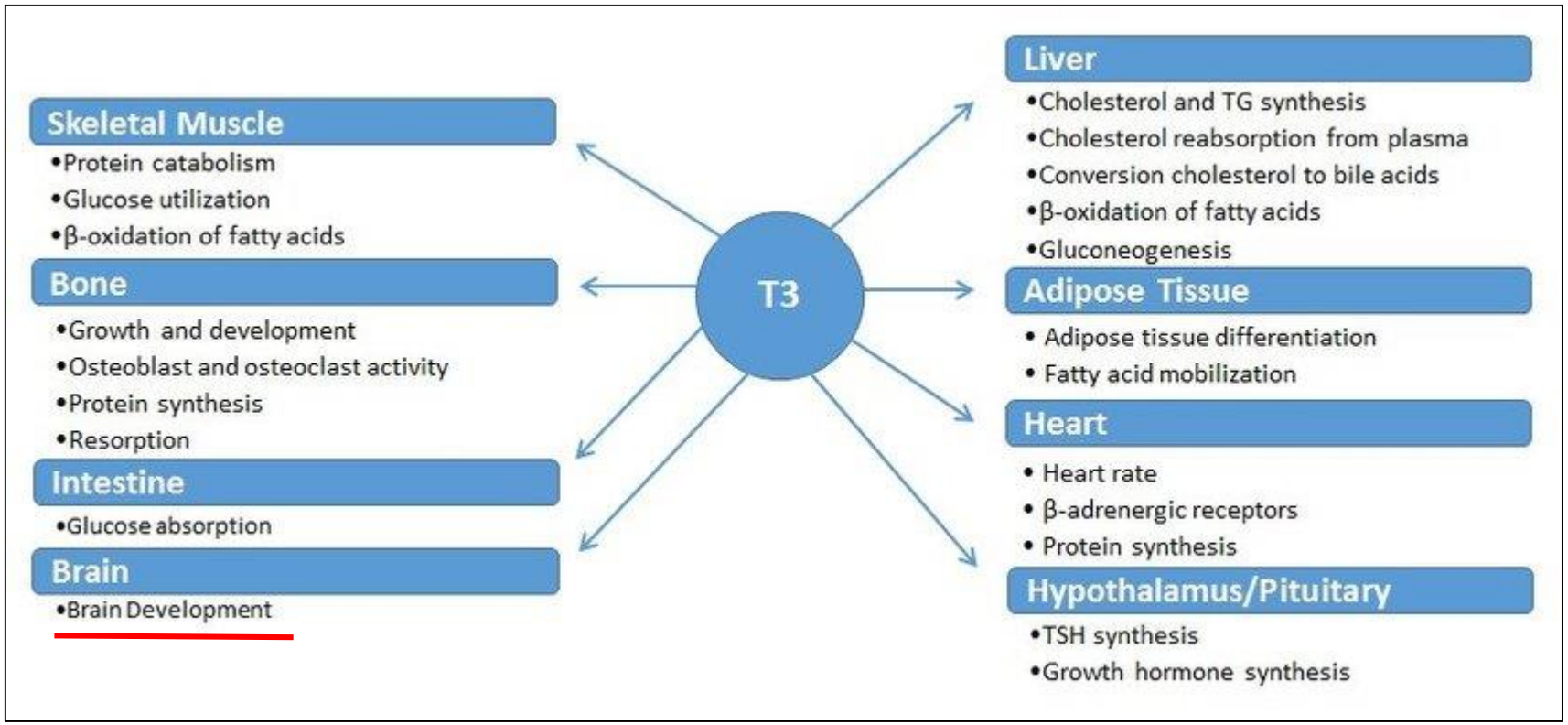
THYROID HORMONE FUNCTION



https://www.researchgate.net/figure/Specific-functions-of-thyroid-hormones-on-target-organs_fig3_330902320

↑ CHO utilization

↑ Protein catabolism

↑ Nitrogen excretion

↑ Fat oxidation

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THYROID HORMONE METABOLISM

TABLE 17.4 Thyroxine (T_4) binding to serum proteins in selected vertebrate species[a,b]

Species	T_4-binding globulin	Postalbumin	Albumin	Prealbumin
Human	++	–	++	+
Monkey	++	–	++	+
Dog	+	–	++	–
Mouse	–	++	++	–
Rat	–	+	++	+
Chicken	–	–	++	–

[a]Modified from Döhler et al. (1979) Pharmacol Ther 5:305–318

[b]Degree of T_4 binding to serum proteins: + or + + . Absence of binding of T_4 to serum protein: –

TABLE 17.5 Triiodothyronine (T_3) binding to serum proteins in selected vertebrate species[a,b]

Species	T_3-binding globulin	Postalbumin	Albumin	Prealbumin
Human	+	–	+	–
Monkey	+	–	+	–
Dog	+	–	+	–
Mouse	–	+	+	–
Rat	–	–	+	–
Chicken	–	–	+	–

[a]Modified from Döhler et al. (1979) Pharmacol Ther 5:305–318

[b]Degree of T_3 binding to serum proteins: + or + + . Absence of binding of T_3 to serum protein: –

488 CHOKSI ET AL. (2003)

Table 2
Selected Parameters of Thyroid System in Humans and Rats (Adapted From U.S. EPA, 1998)

Parameter	Human	Rat
Half-life of T4	5–9 days	0.5–1 day
Half-life of T3	1 day	0.25 day
Thyroxine-binding globulin levels	High	Very low
Amount of T4 required in absence of functional thyroid gland	2.2 µg/kg bw/day	20 µg/kg bw/day
T4 production (rate/kg bw)	1×	10×
Sex difference in serum TSH levels	No difference	Adult males have higher levels than adult females
Follicular cell morphology	Low cuboidal Follicular height is equal in males and females.	Cuboidal Follicular height in males is greater than in females

- Half-life of T_4 in rats is 0.5-1 day
- Rat maintains relatively high TSH levels which gives the thyroid gland a slightly more cellular appearance

(HISTO)PATHOLOGIC ENDPOINTS OF THYROTOXICITY IN ADULTS

Metabolic
- ↓ Basal metabolic rate
  - ↑ Body weight w/o increased appetite
  - ↓lipid metabolism

Cardiovascular
- ↓ Heart rate
- Atherosclerosis

Internal organs
- Liver: lipid deposition and hepatomegaly
- Kidney: lipid accumulation in glomeruli

Reproduction
- ↓ libido
- ↓ sperm count
- Abnormal/absence of estrous cycles

Elevated blood cholesterol, decreased cholesterol excretion (clinical pathology)

Thyroid gland (rats)
- Decreased follicular size
- Decreased colloid
- Increased epithelial cell height
- Degeneration/vacuolation of epithelium

di-(2-ethylhexyl) phthalate (DEHP) in male Wistar rats (Howarth et al 2001)

di-(2-ethylhexyl) phthalate (DEHP) in SD rats (Poon et al 1996)

Dichlorodiphenyltrichloroethane (DDT) in Wistar Rats (Yaglova and Yaglov, 2017)

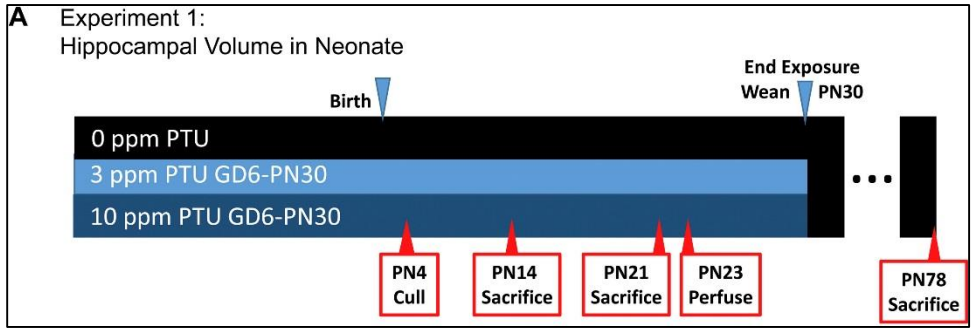
Polybrominated diphenyl ether in SD rats (Lee et al., 2010)

HISTOPATHOLOGIC ENDPOINTS

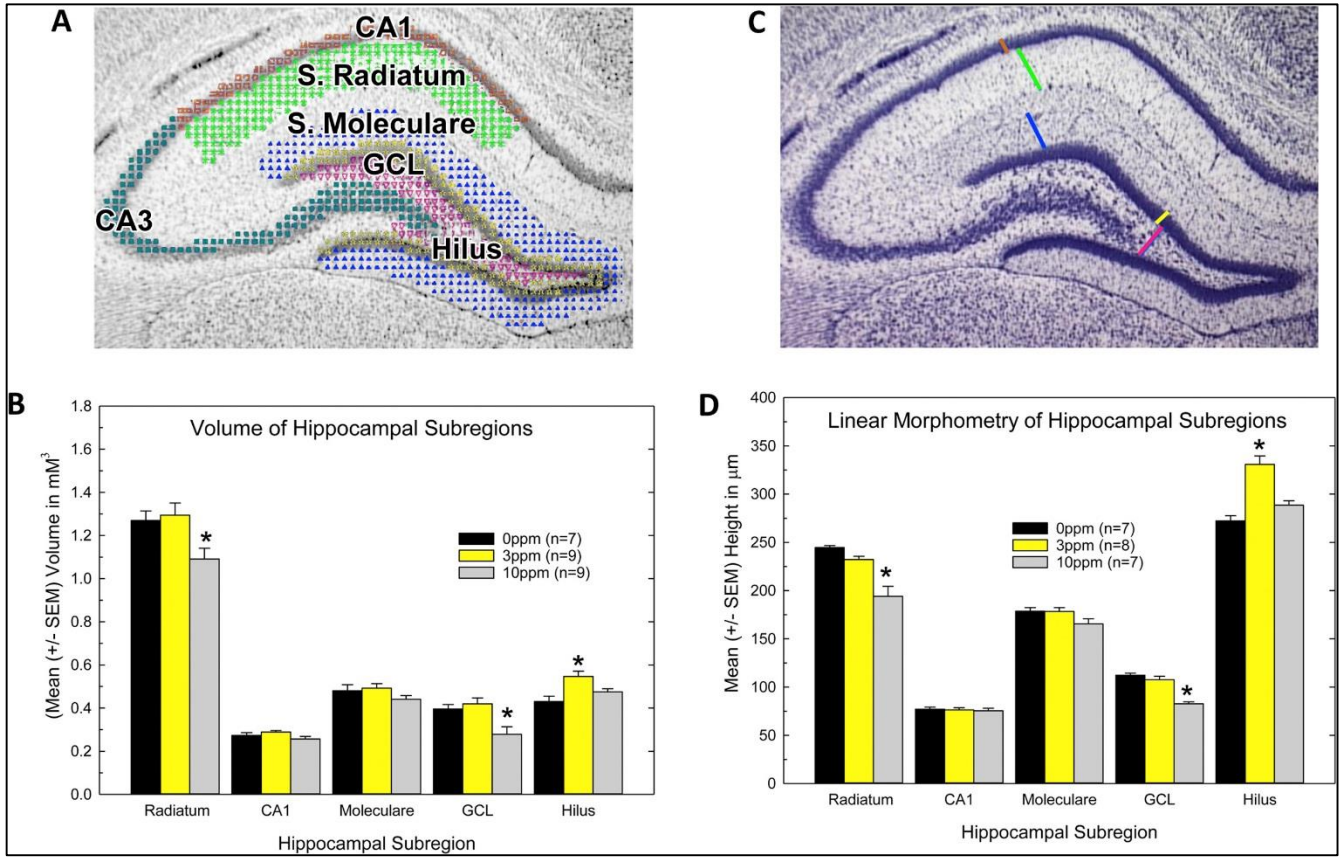
Gilbert et al., 2017

Adult hippocampal neurogenesis is impaired by transient and moderate developmental thyroid hormone disruption☆

M.E. Gilbert[a,*], J.H. Goodman[b,c], J. Gomez[b], A.F.M. Johnstone[a], R.L. Ramos[d]



- Brain and hippocampus weights ↓ in 10 ppm group as well
- Study demonstrated a method of quantitative assessment of neuronal development in rats



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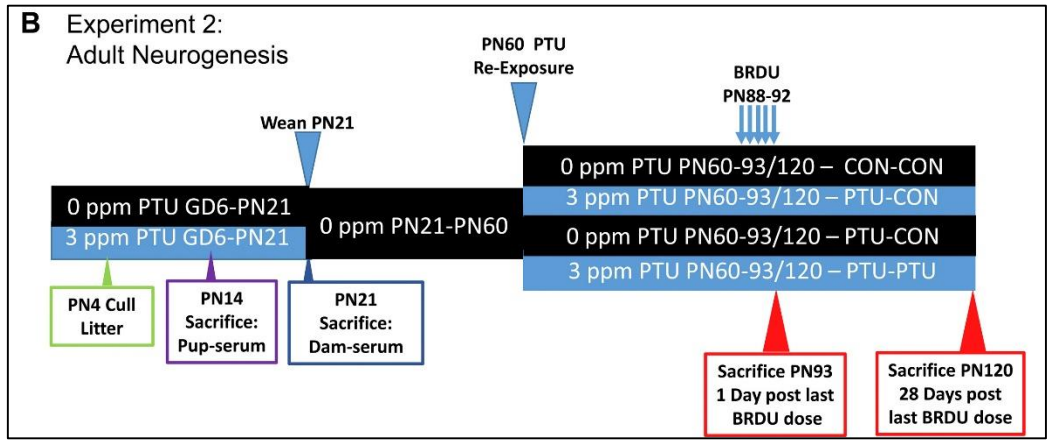
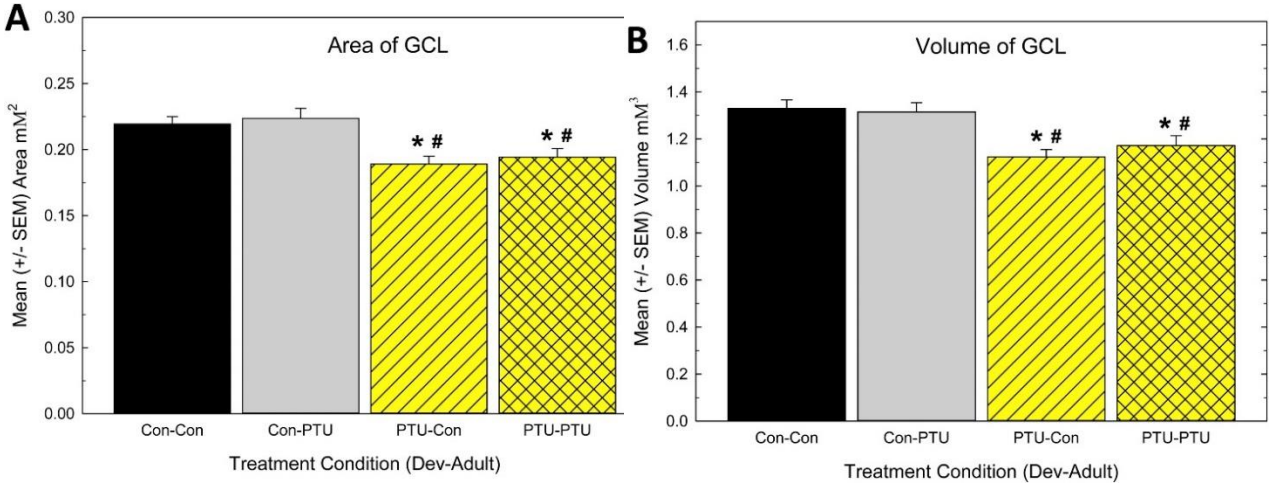
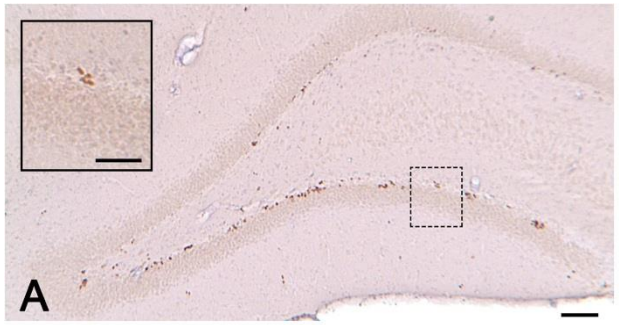
HISTOPATHOLOGIC ENDPOINTS

Gilbert et al., 2017

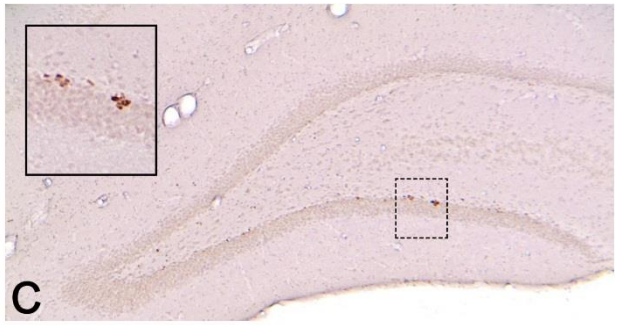
- Neurogenesis in dentate gyrus continues throughout life
- Severe hypothyroidism in adults ↓ new neuron survival



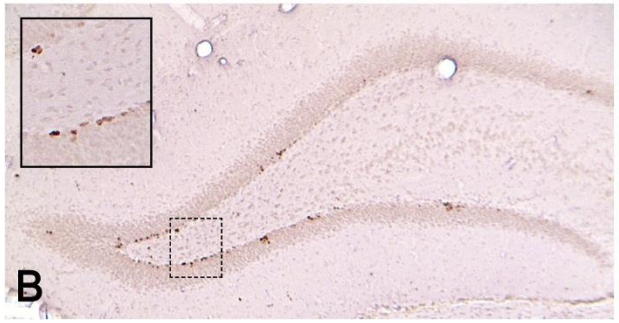




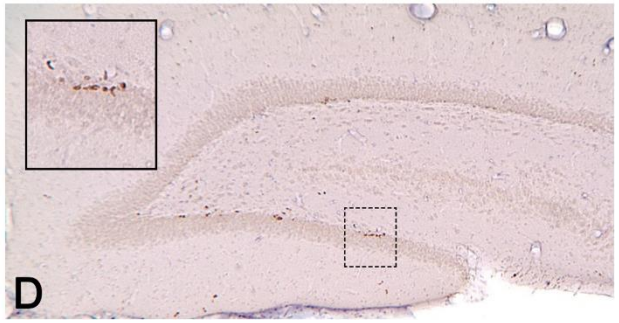
Untreated (normal) rat



Rat: ↓ T_4 during fetal-early PND



Rat with adult onset ↓ T_4



Rat: ↓ T_4 during fetal-early PND + adult

BrdU labeling for cell proliferation

- Delay in neurologic lesions in adults after mild hypothyroidism in late gestational/early neonatal rats

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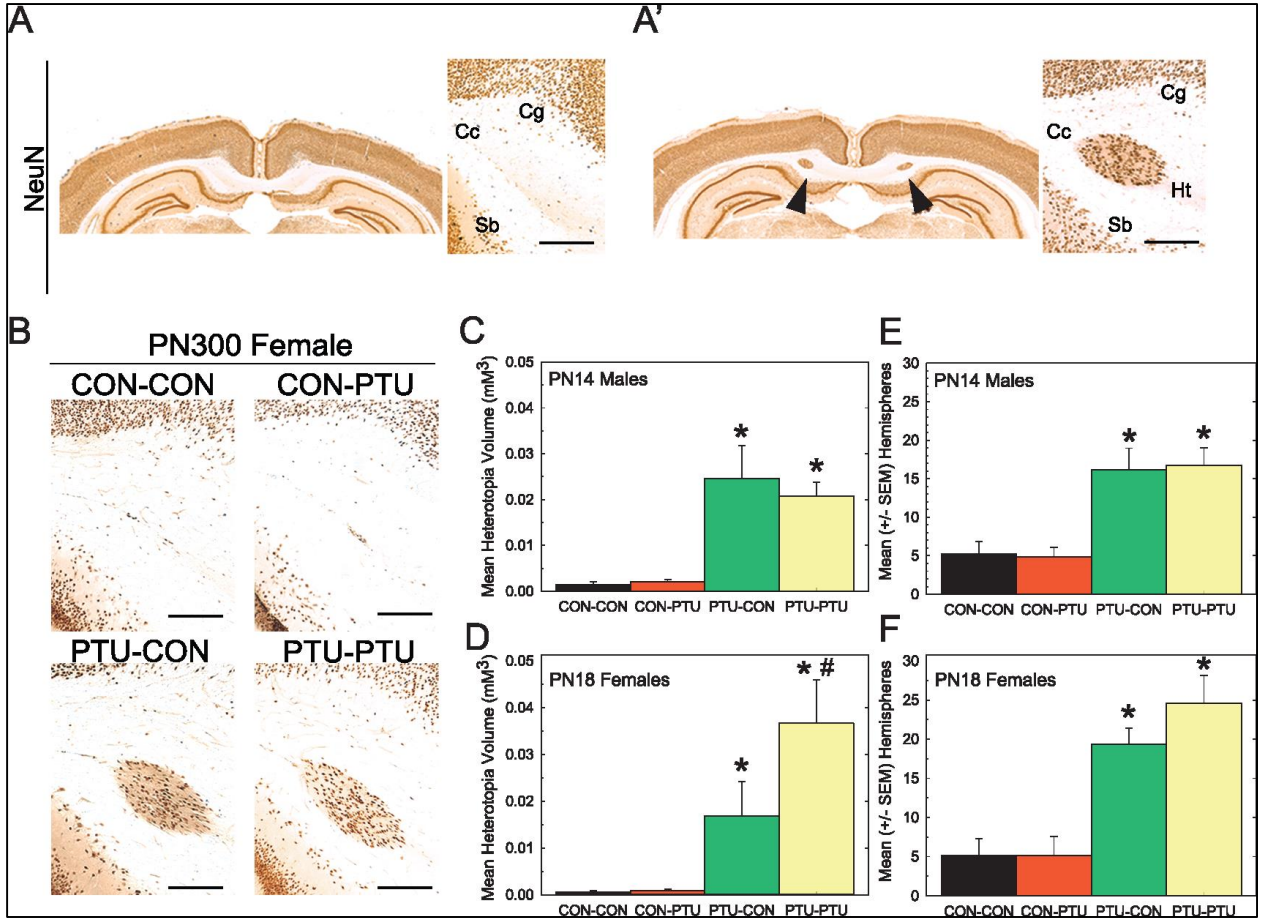
HISTOPATHOLOGIC ENDPOINTS

Developmental Thyroid Hormone Insufficiency Induces a Cortical Brain Malformation and Learning Impairments: A Cross-Fostering Study

Katherine L. O'Shaughnessy,*,† Patricia A. Kosian,‡ Jermaine L. Ford,§ Wendy M. Oshiro, Sigmund J. Degitz,‡ and Mary E. Gilbert*,1

- PTU exposure resulted in permanent cortical heterotopia in rats
- Lesion is bilateral to the corpus callosum
- Composed of glutamatergic and GABAergic neurons
- Heterotopic neurons connected structurally and functionally to cortical neurons
- Rats also exhibit increased seizure sensitivity along with learning and memory deficits



Toxicological Sciences, Volume 163, Issue 1, May 2018, Pages 101–115, https://doi.org/10.1093/toxsci/kfy016

HISTOPATHOLOGIC ENDPOINTS

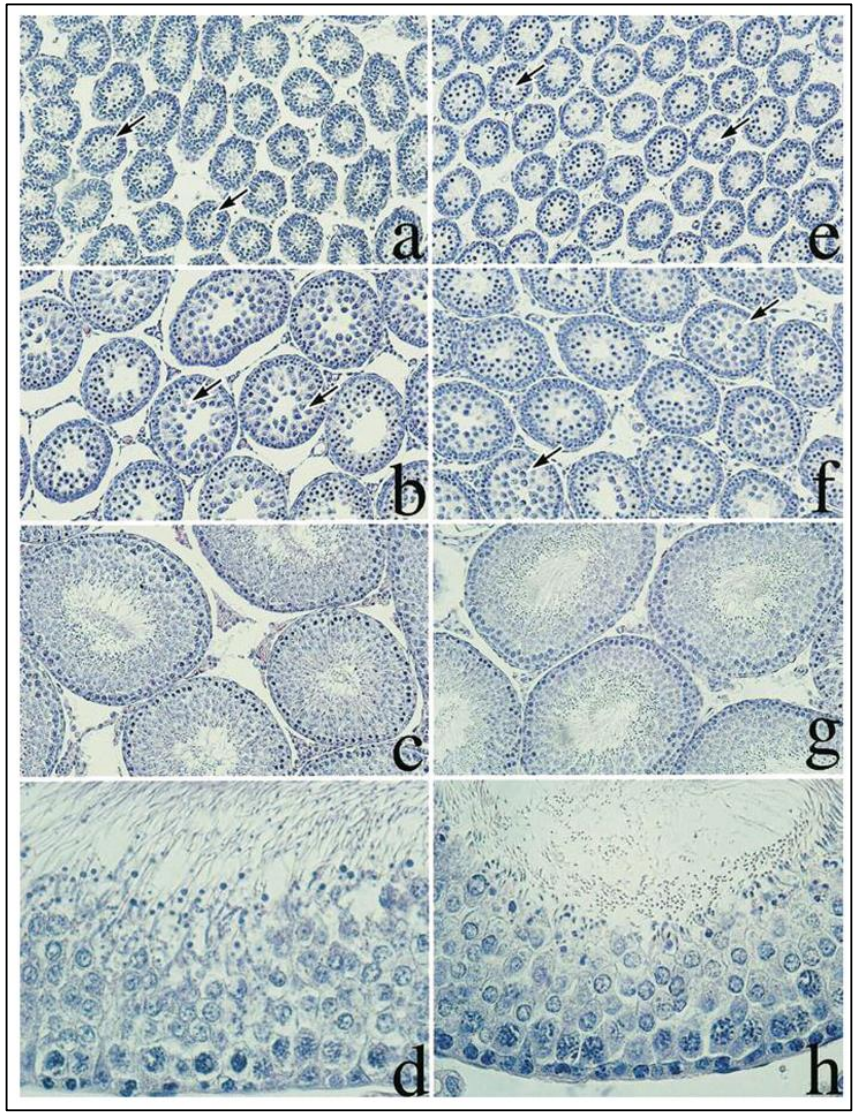
Develop. Growth Differ. (2004) **46**, 327–334

Developmental delay and unstable state of the testes in the *rdw* rat with congenital hypothyroidism

Yasuhiro Sakai,[1]* Shohei Yamashina[1] and Sen-ichi Furudate[2]

[1]*Department of Anatomy and* [2]*Department of Laboratory Animal Science, Kitasato University School of Medicine, Sagamihara 228-8555, Japan*

- RDW rat: decreased serum T_4
- Delay in full development of the normal structures
- After full development, degeneration of testes began
- Partially reversed by T_4 treatment
- These findings, along with other studies, suggests thyroid hormones have a role in both development and maintenance of testicular function



A-D: control rat at 2, 4, and 8 weeks
E-H: rdw (hypothyroid) rat at 4, 8, 22 weeks

Wagner et al., 2008

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(HISTO)PATHOLOGIC ENDPOINTS OF THYROTOXICITY

Upregulation of CYP enzymes in the liver

- Xenobiotic administration
  - Phenobarbital
  - Rifampicin
- Pesticide exposure
- Increased expression of sulfo- and glucuronyl-transferases in liver

Increased metabolism of T_4 (and T_3)

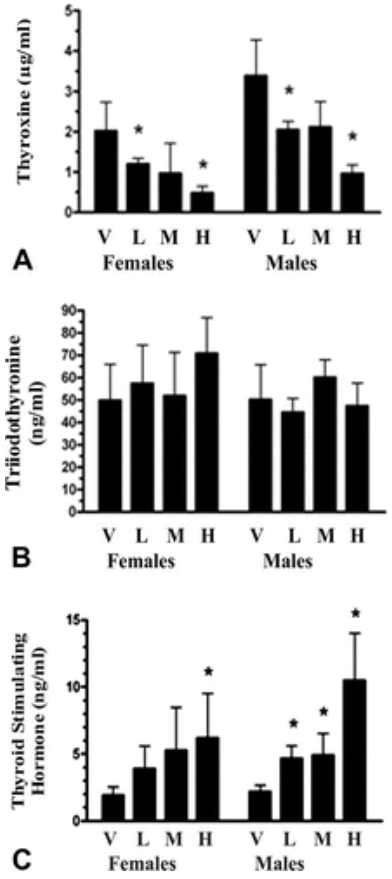
Decreased T4 levels stimulates TSH production/release

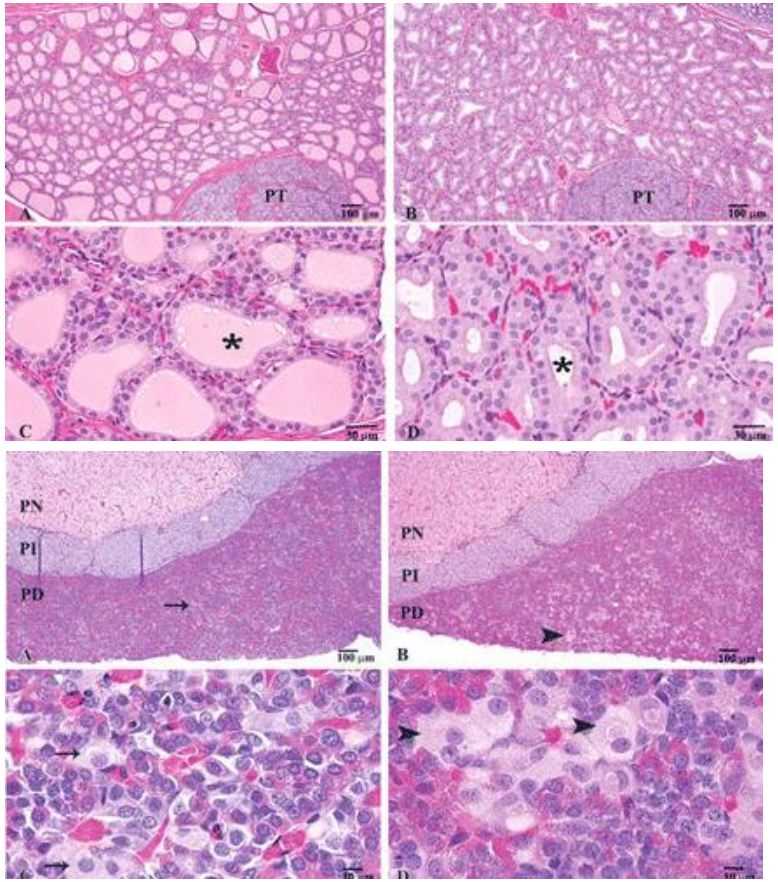
Consequences

- Thyroid gland hypertrophy + ↑ thyroid weight (+/-)
  - Follicular hyperplasia (early)
  - Follicular neoplasia (late)
- Thyroid hyperplasia/neoplasia
- Liver hypertrophy
- Pituitary hypertrophy

Characterization of Xenobiotic-Induced Hepatocellular Enzyme Induction in Rats: Anticipated Thyroid Effects and Unique Pituitary Gland Findings

Tanja S. Zabka,[1] Mark R. Fielden,[2] Rosario Garrido,[1] Jianhua Tao,[3] Adrian J. Fretland,[1] Jennifer L. Fretland,[1] Mudher A. Albassam,[1] Thomas Singer,[1] and Kyle L. Kolaja[1]





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HISTOPATHOLOGIC LESIONS IN THYROTOXICOSIS

Neurologic

- Altered neurologic development
  - Decreased cell numbers
  - Decreased dendritic arborization
  - Decreased axonal myelination
  - Decreased synaptogenesis
- Decreased hippocampal neurogenesis in adult
  - Decreased granule cell layer area
  - Decreased granule cell layer volume
- Increased apoptosis of cerebellar granular neurons

Reproductive

- Altered spermatogenesis

Inhibit growth of the kidney, liver, and diaphragm*

Inhibit development of the hypothalamo-pituitary-adrenal axis in the rat*

Thyroidectomy in utero on sheep fetus

- Smaller type II muscle fibers
- Delayed epiphyseal closure
- Delayed bone maturation
- Thin skin, abnormal or no wool (follicle) development
- Fewer type II pneumocytes
- Abnormal neonatal cardiovascular adaptations

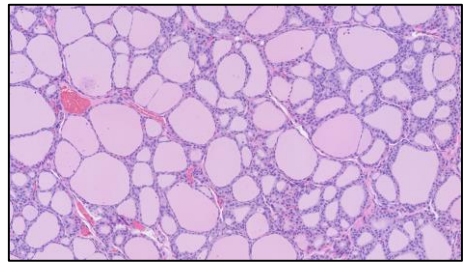
Forhead and Fowden, 2014

THYROID GLAND/HORMONE DEVELOPMENT

Developmental stage	Rat (days)	Human (weeks)
Gestational age at term	21	40
Colloid production	17	13-14
Follicular cell organogenesis	18 days-3 weeks postnatally	>14
Synthesis/secretion hormones	17.5	16-18
Rise in plasma T_3	Birth to 3 weeks postnatally	30 weeks to birth
H-T-P maturation	Postnatally	Last trimester+postnatally

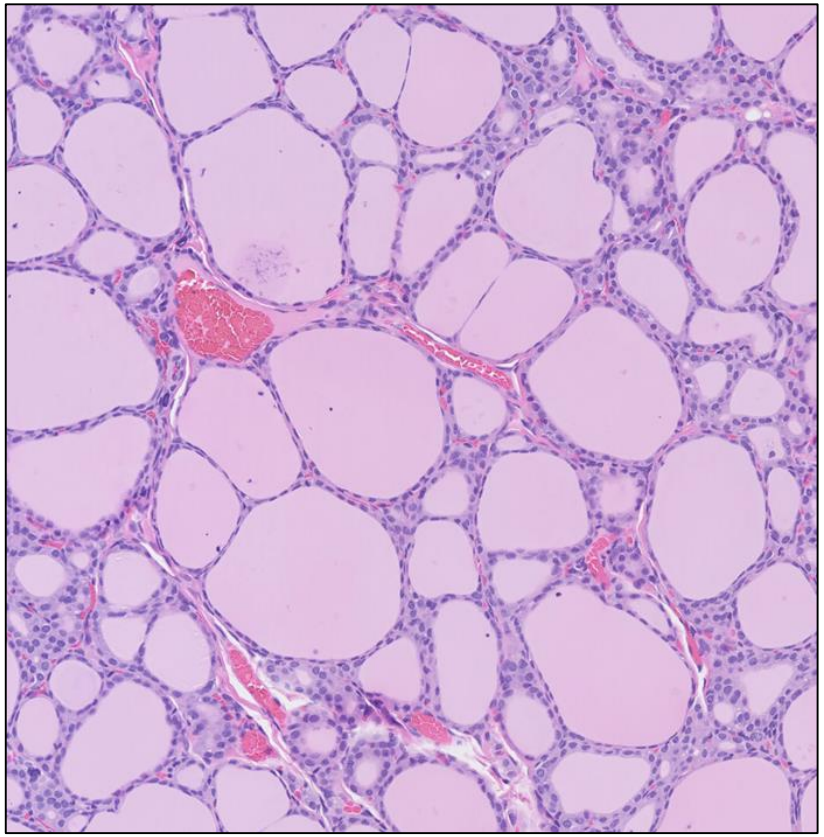
Modified from Forhead and Fowden, 2014

- Thyroid gland is histologically mature by PND 21 in the rat
- Thyroid gland and HTP axis not fully developed until PND 28 in the rat

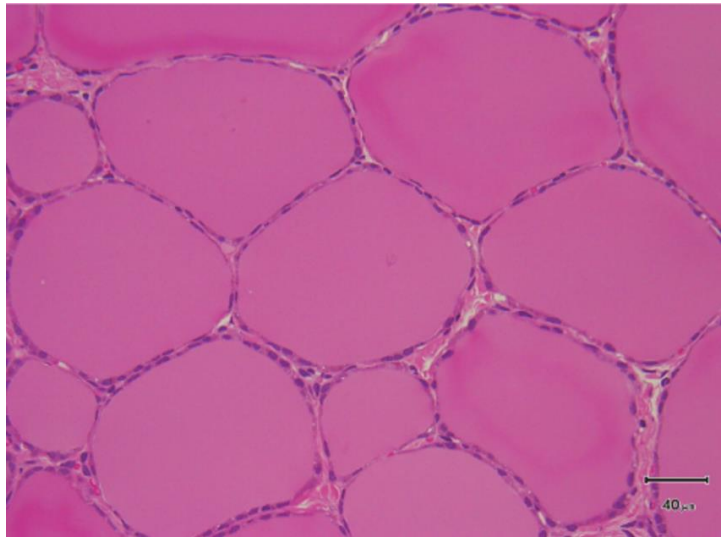


THYROID GLAND HISTOLOGY

- Follicular epithelial cells
- Follicles-colloid
- C-cells
- Vasculature
- Stroma
- Capsule
- Parathyroid (when present)
- Ectopic tissue



Photomicrograph courtesy of Cathy Picut



Hatakeyama et al 2011

THYROID-RELATED END POINTS OF THE EPA PUBERTAL ASSAYS

Guidance for Thyroid Assays in Pregnant Animals, Fetuses and Postnatal Animals, and Adult Animals (2005)

- Maternal, fetal (GD 20), and F1 offspring (PNS 4 and 21) thyroids should be collected…for pathologic analyses
- The thyroids/parathyroids, attached to a section of the trachea, of all maternal, fetal, and offspring samples should be excised and immersion fixed immediately after collection in 10% neutral buffered formalin or other appropriate fixative. Following fixation, the thyroid/parathyroid tissue samples should be carefully trimmed and weighed.
- Hormonal analyses should be conducted on GD 20 fetuses and dams, PND 4 pups, and PND 21 pups and dams.

Thyroid gland weights

- 2-4 week exposure required to see weight changes
- Male assay (4~4.5 weeks) more sensitive than female assay
- Technical considerations when collecting weighing thyroid gland
  - Need entire gland/remove extraneous tissues
  - Post fixation weighing recommended

Hormone evaluation (T4, T3, TSH)

- T4 measurements complicated by circadian rhythms, short half-life, inter-animal variability, body weight
- Advances in assay technology allow for quantifying T3 and T4 in GD20 and PND 4 rats

THYROID-RELATED END POINTS OF THE EPA PUBERTAL ASSAYS

Histologic evaluation

- Most reliable indicator of thyrotoxicosis
- Interpretation is subjective
- Follicular epithelial cell height males > females
- Variation in cell height and colloid area in different regions
- ↓ follicular colloid can occur within days

EPA Endocrine Disruptor Screening Program Test Guidelines:

Examination of colloid area and follicular cell height

Colloid content

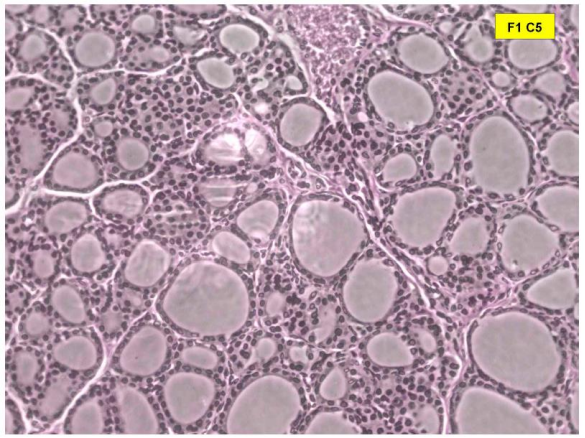
- C1 to C5 (5 point scale) with C5=normal*

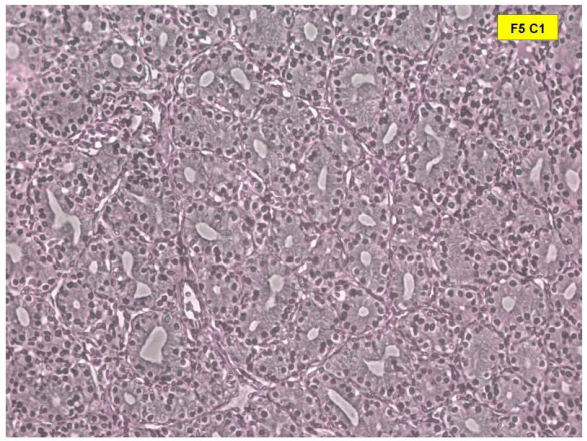
Follicular epithelial cell height

- F1 to F5 (5 point scale) with F1=normal*

Generally colloid content decreases while follicular cell height increases

- Follows a pattern
  - F1C5 (normal) → F2C4 → F3C3 → F4C2 → F5C1
  - Not set in stone





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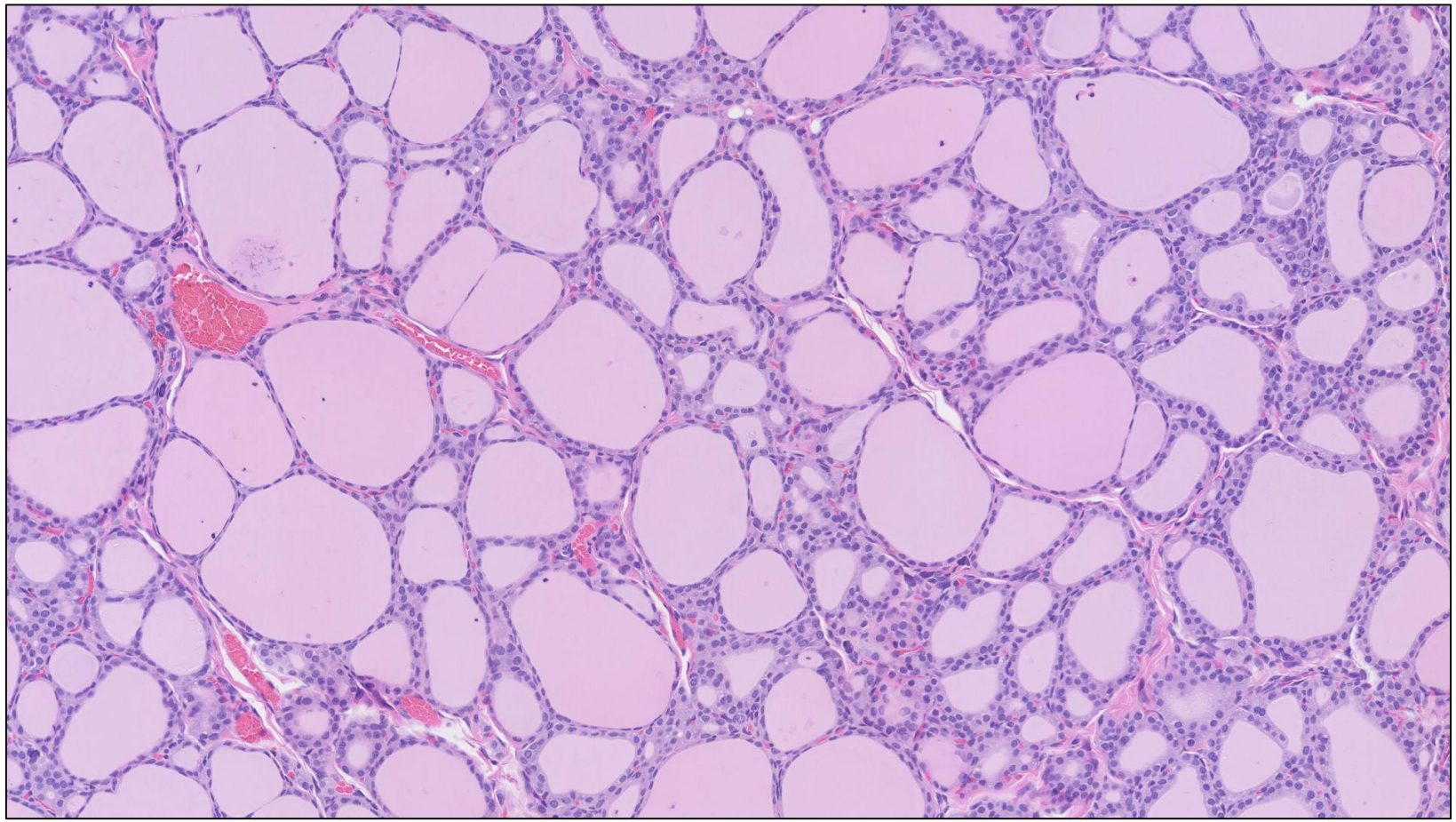
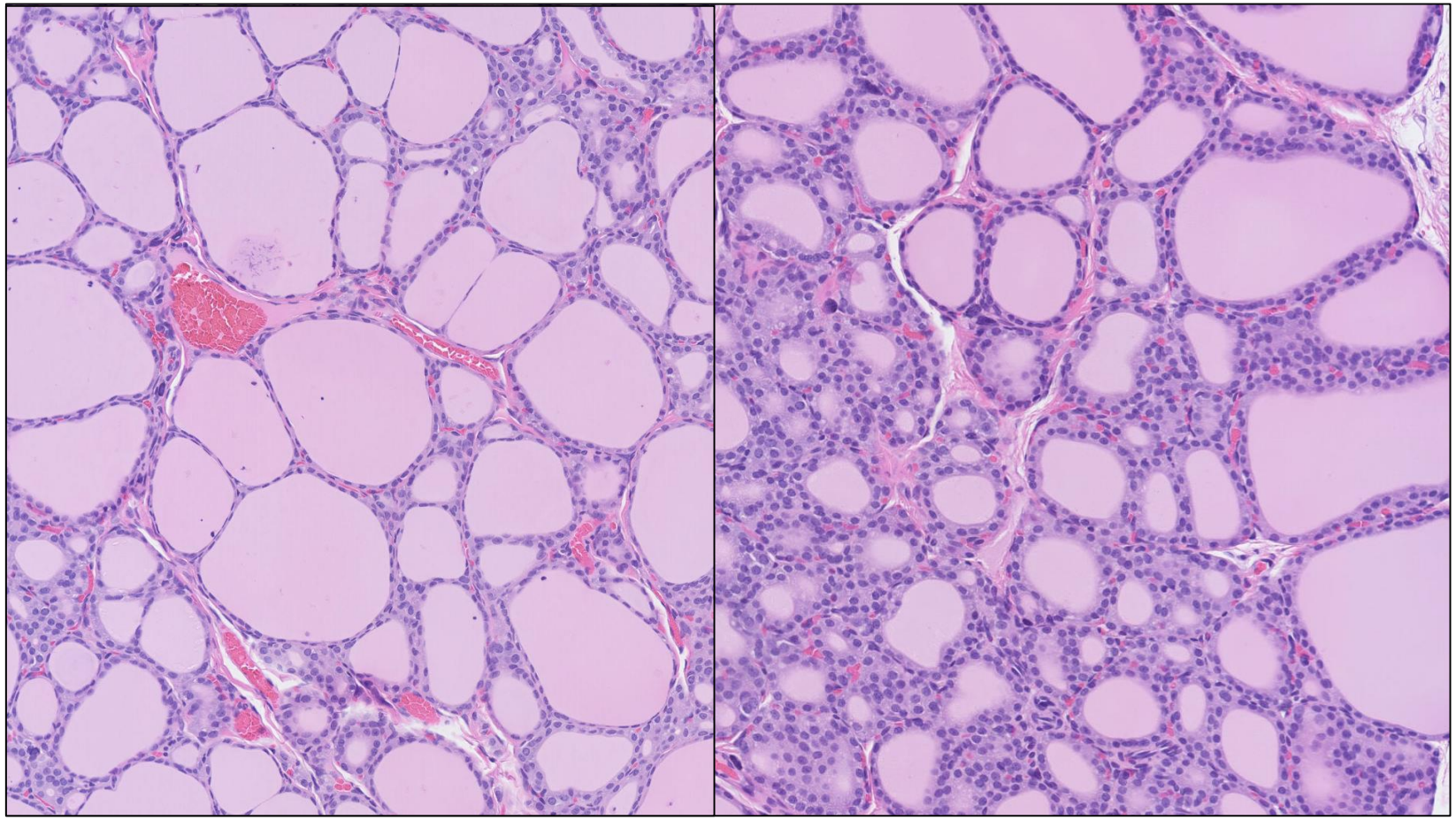
Control adult rat: Grade 1F 5C



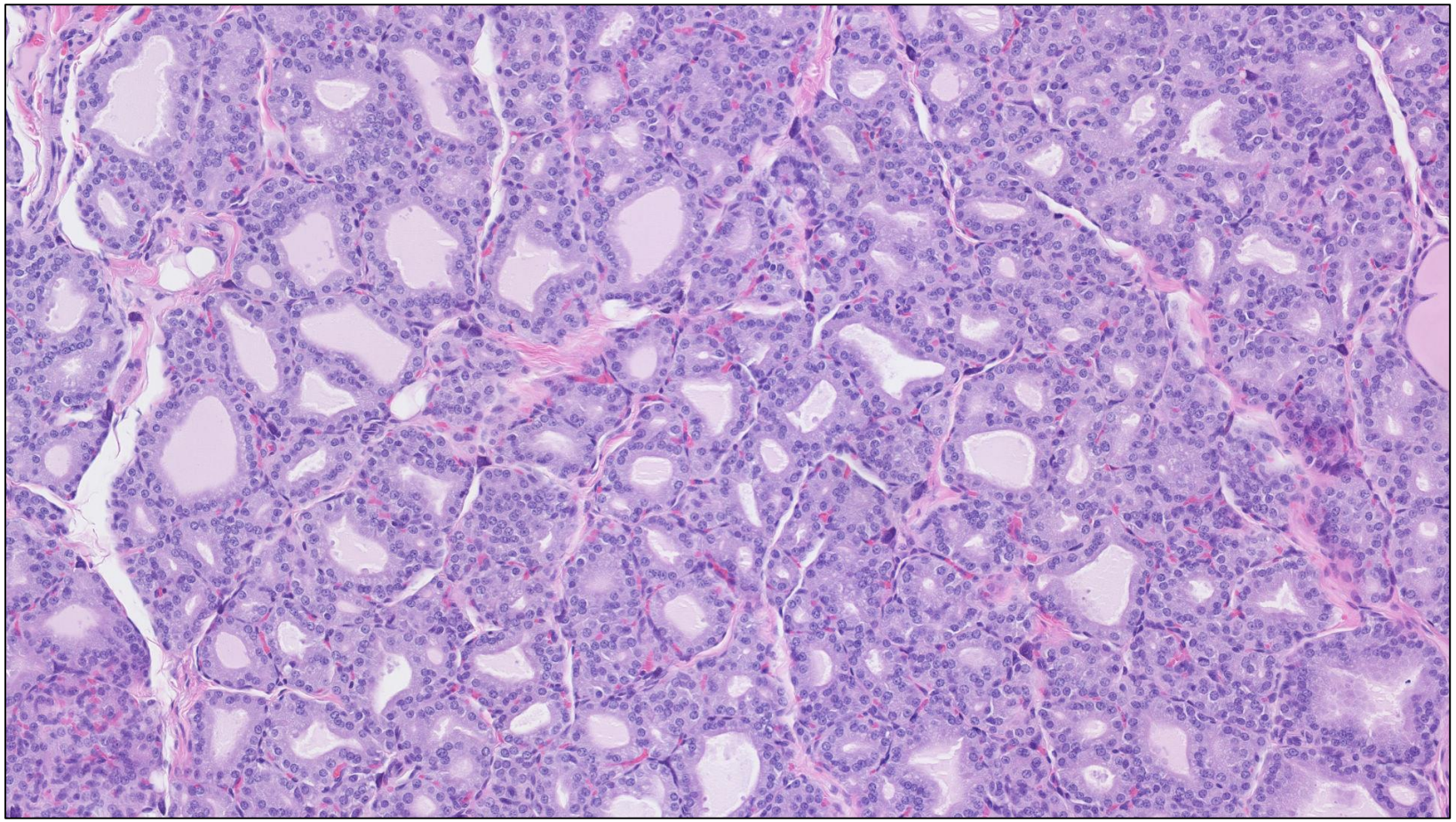
Photomicrograph courtesy of Cathy Picut

Control adult rat: Grade 2F 4C



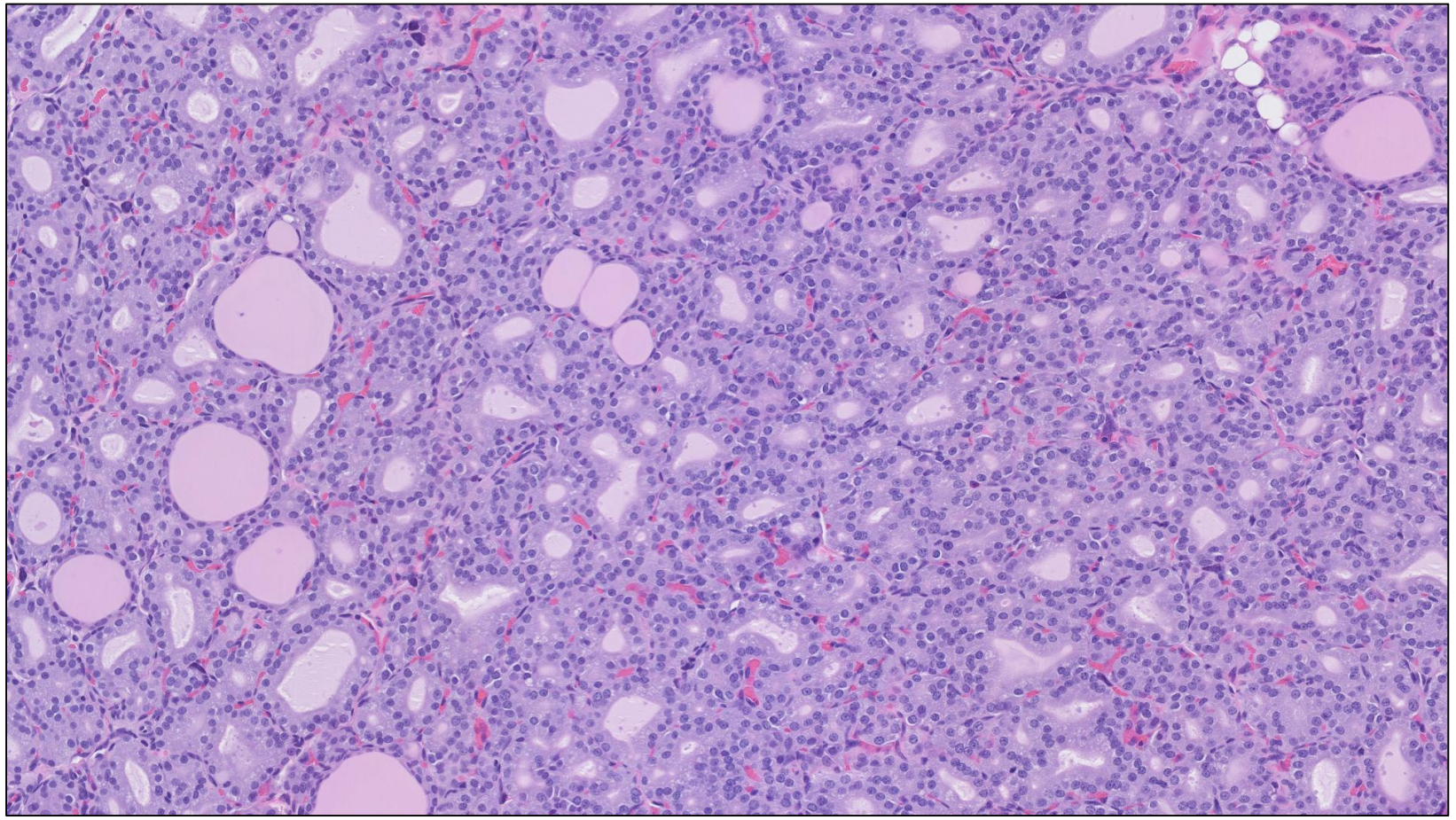
Photomicrograph courtesy of Cathy Picut

PTU treated adult rat: Grade 3F 3C



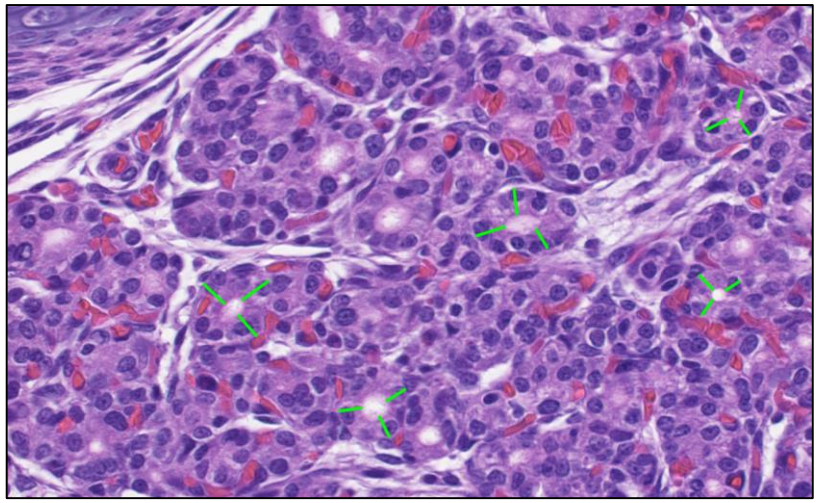
Photomicrograph courtesy of Cathy Picut

PTU treated adult rat: Grade 4F 2C

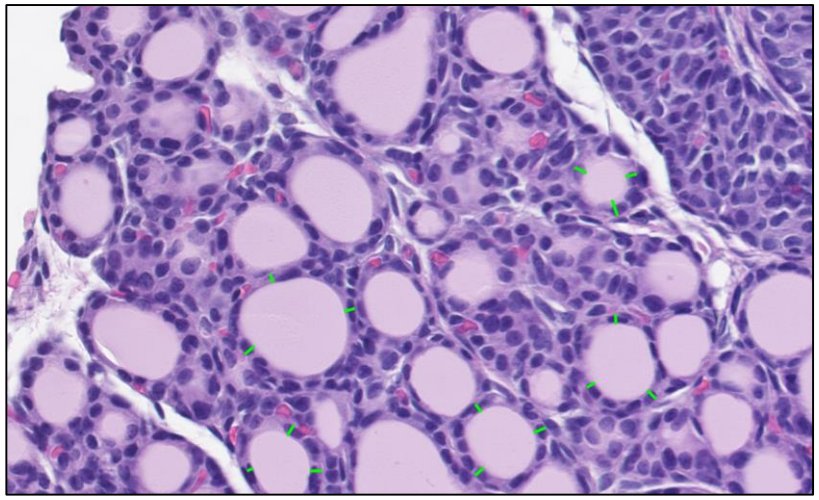


Photomicrograph courtesy of Cathy Picut

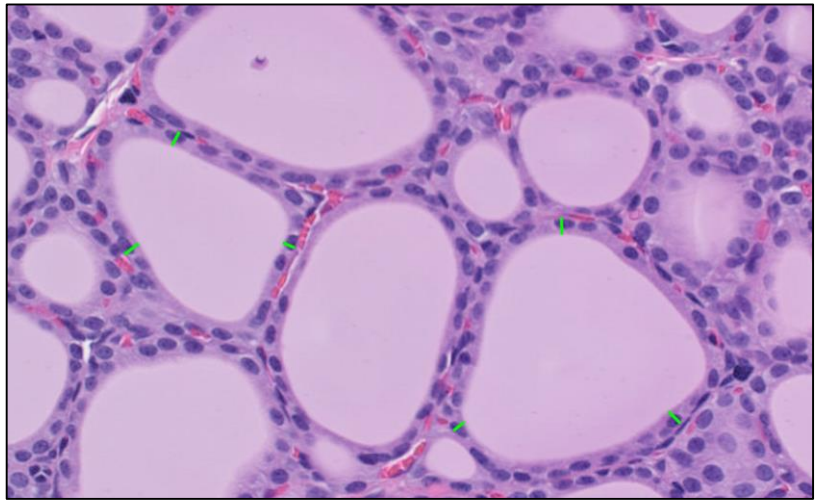
PRENATAL AND EARLY POSTNATAL HISTOLOGY OF THE THYROID GLAND IN THE RAT



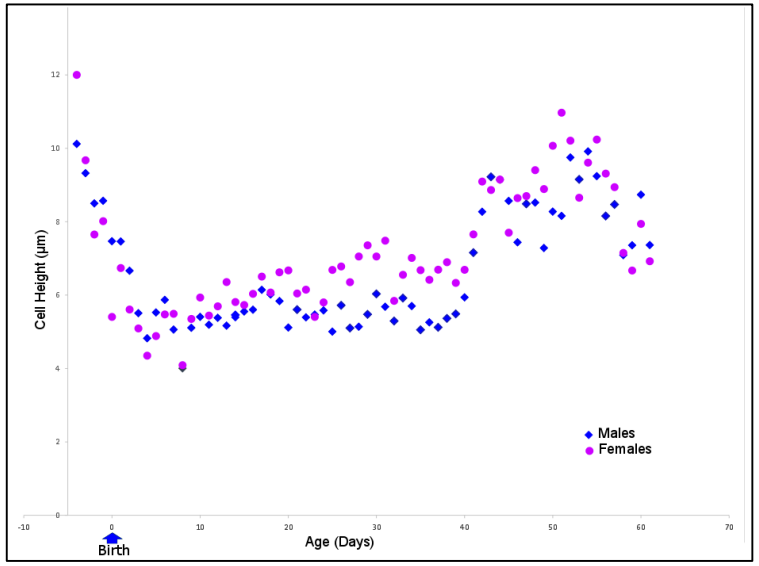
GD 20



PND 3-4



PND 30



Picut et al., 2017

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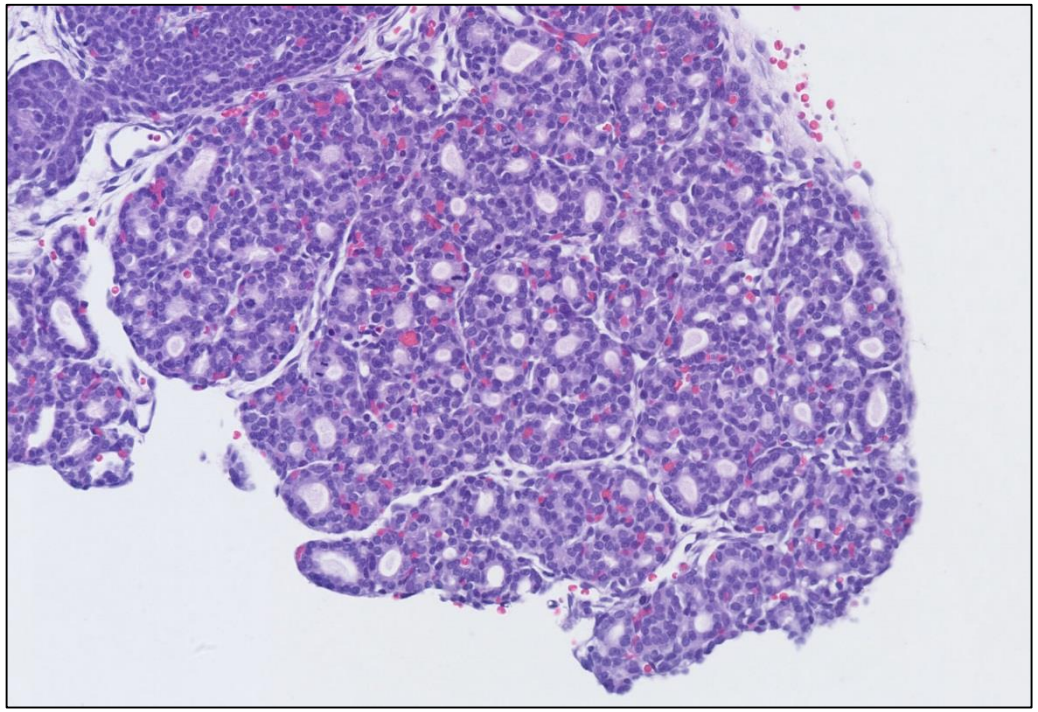
APPLICATION OF EPA GUIDELINES TO A KNOWN THYROTOXICANT

Female Sprague Dawley rats administered propylthiouracil (6-PTU) in the diet at a concentration of 0 and 3 parts per million (ppm) daily from Gestation Day (GD) 6 through Postnatal Day (PND) 21

Microscopic examination of the thyroid gland was performed on GD 20 fetuses, PND4 pups, PND 21 pups, and adult females

Thyroid hormone levels and thyroid weights also measured

GD 20 MALE FETUS



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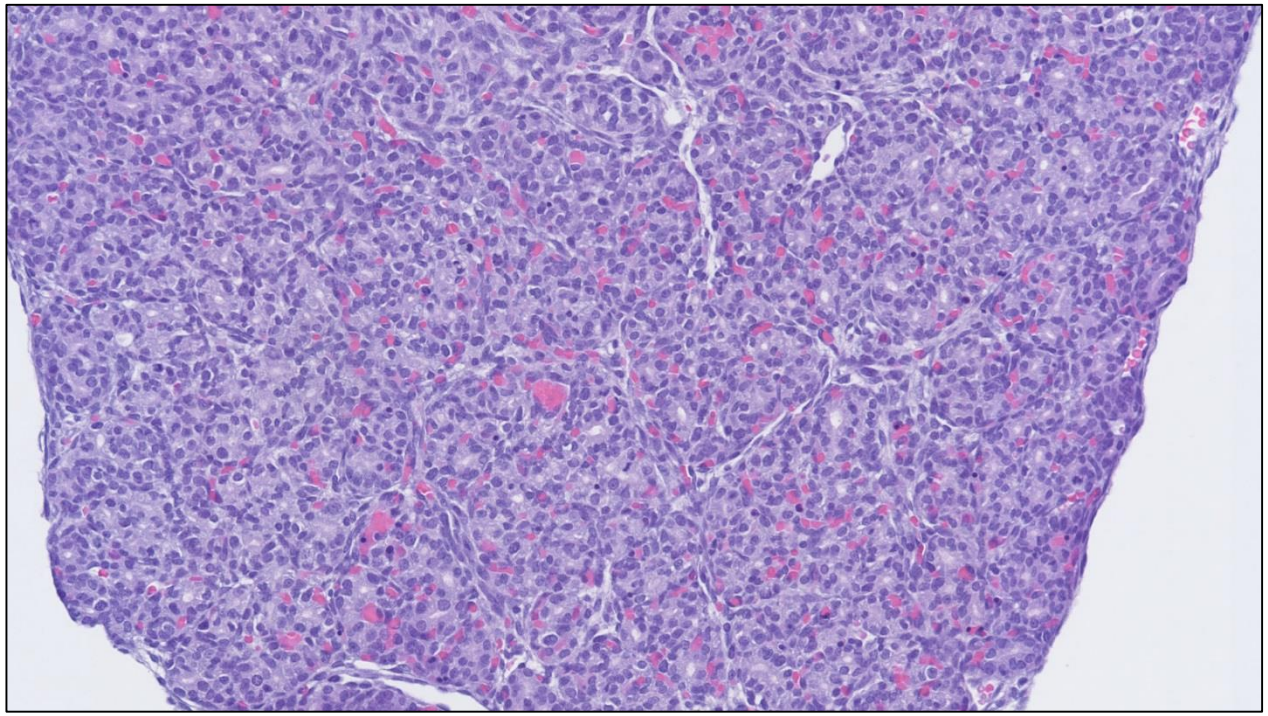
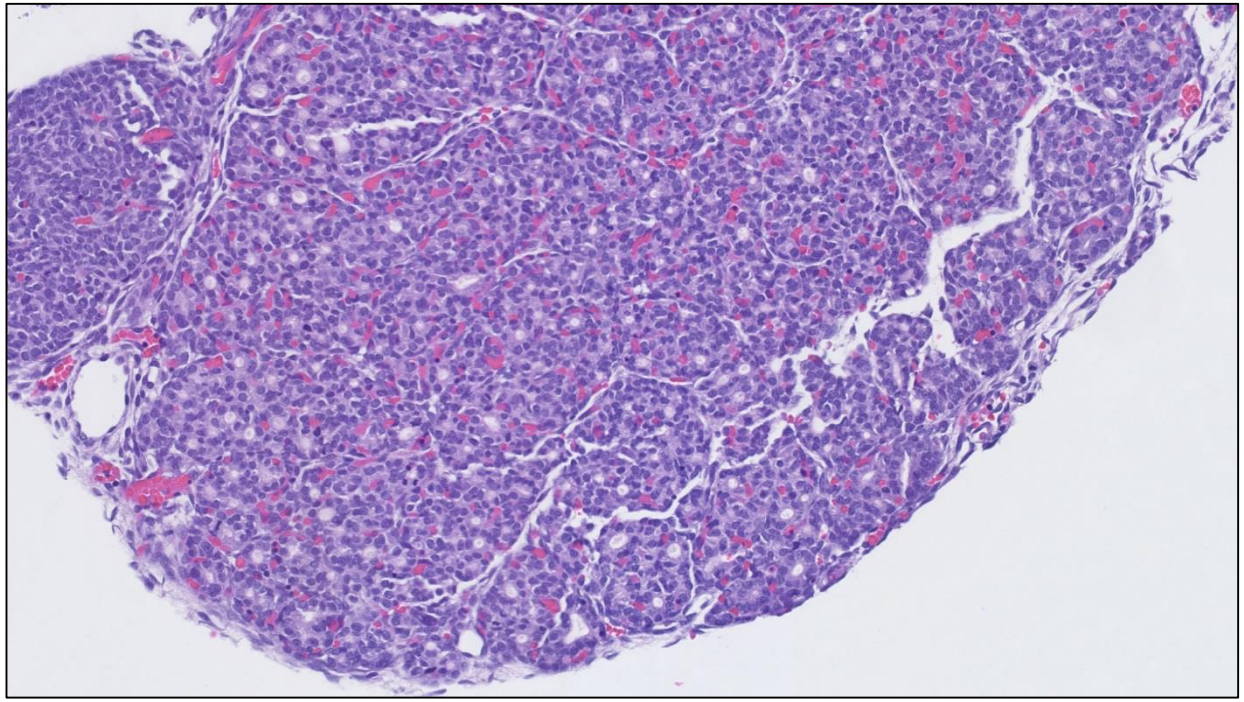


PTU

GD 20 MALE FETUS



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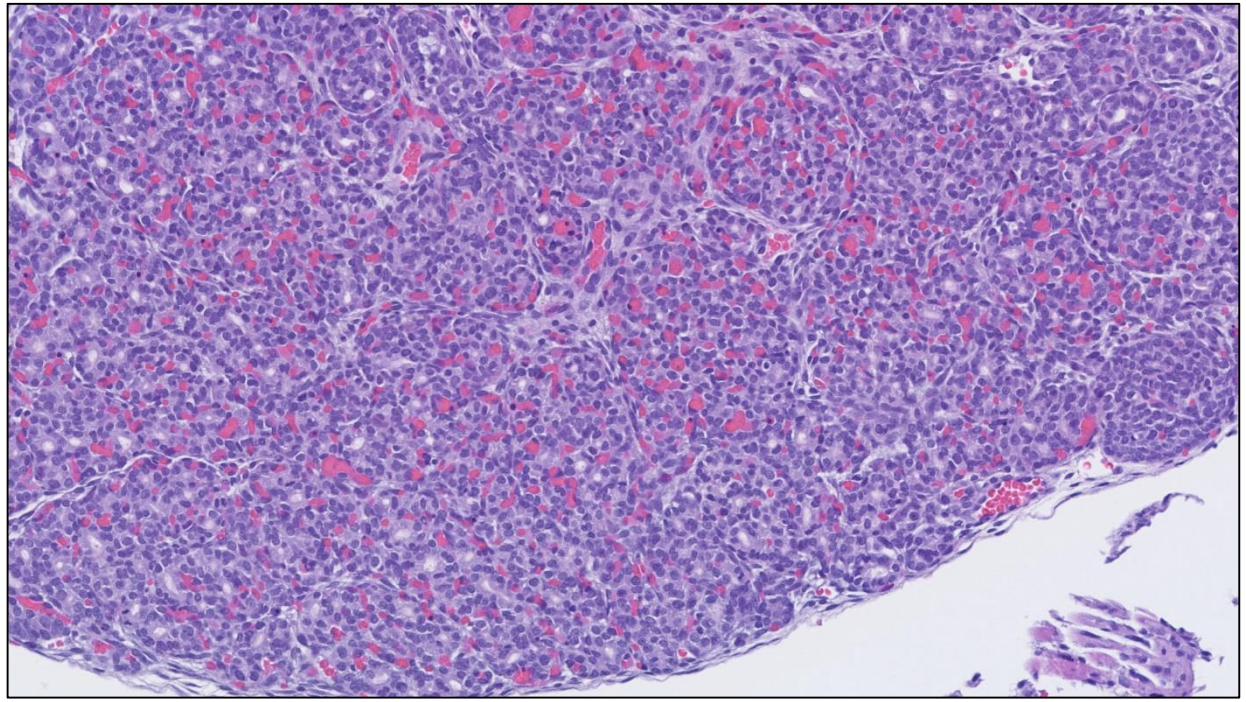

PTU

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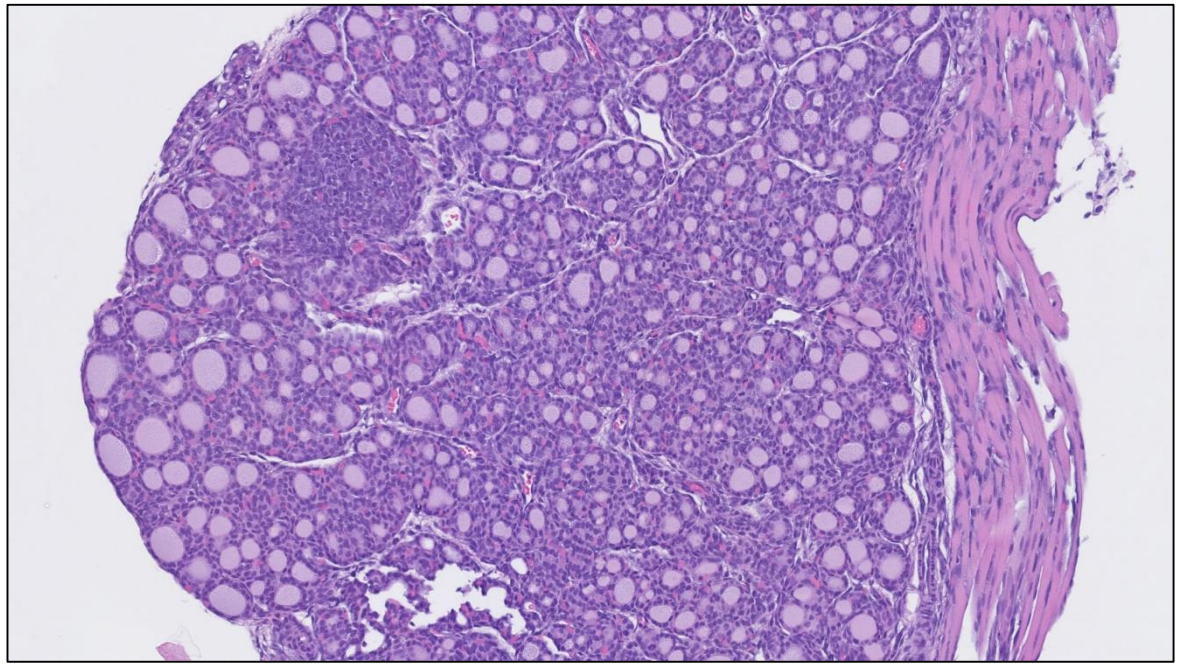
PND 4 MALE



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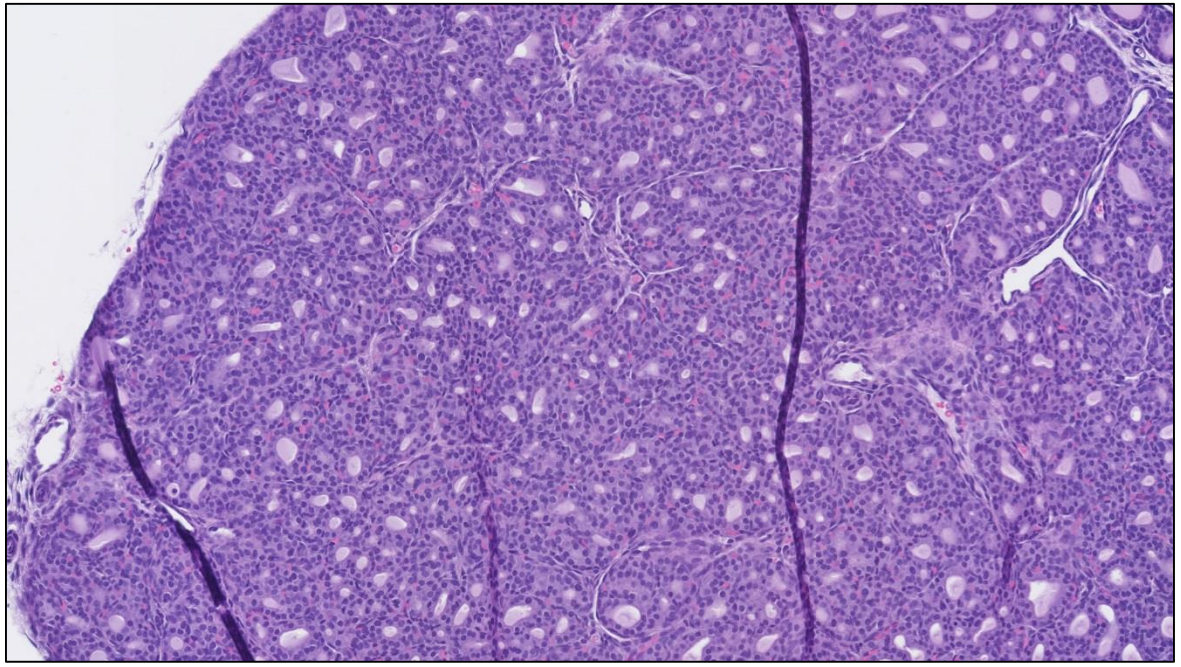
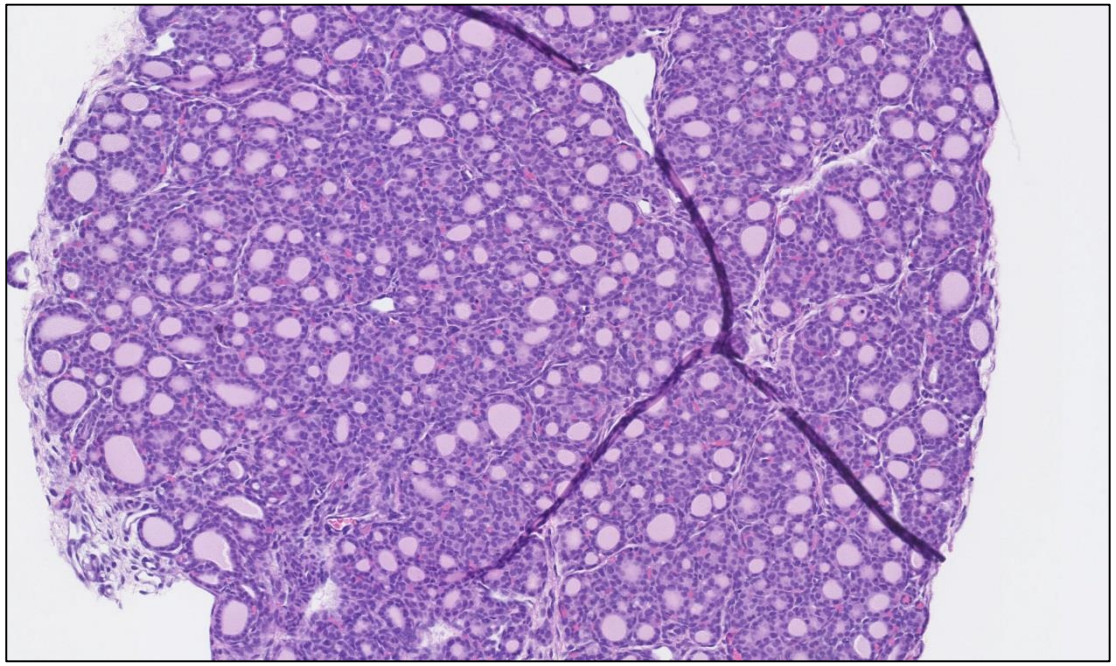
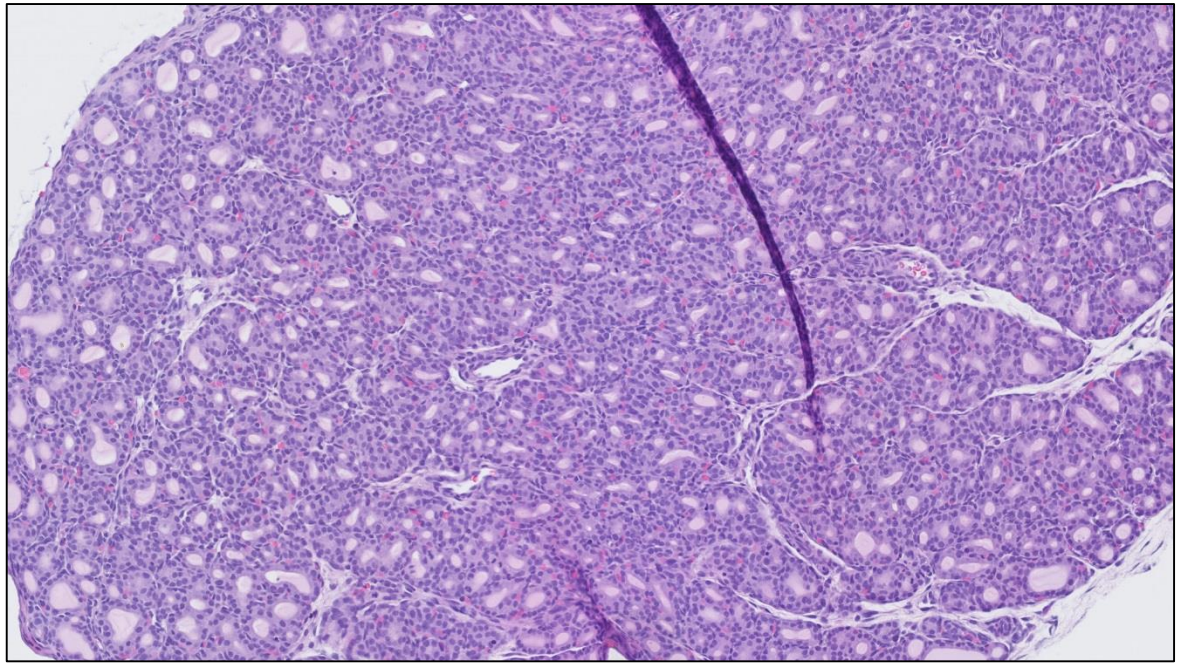


PTU

PND 4 FEMALE



Control



PTU

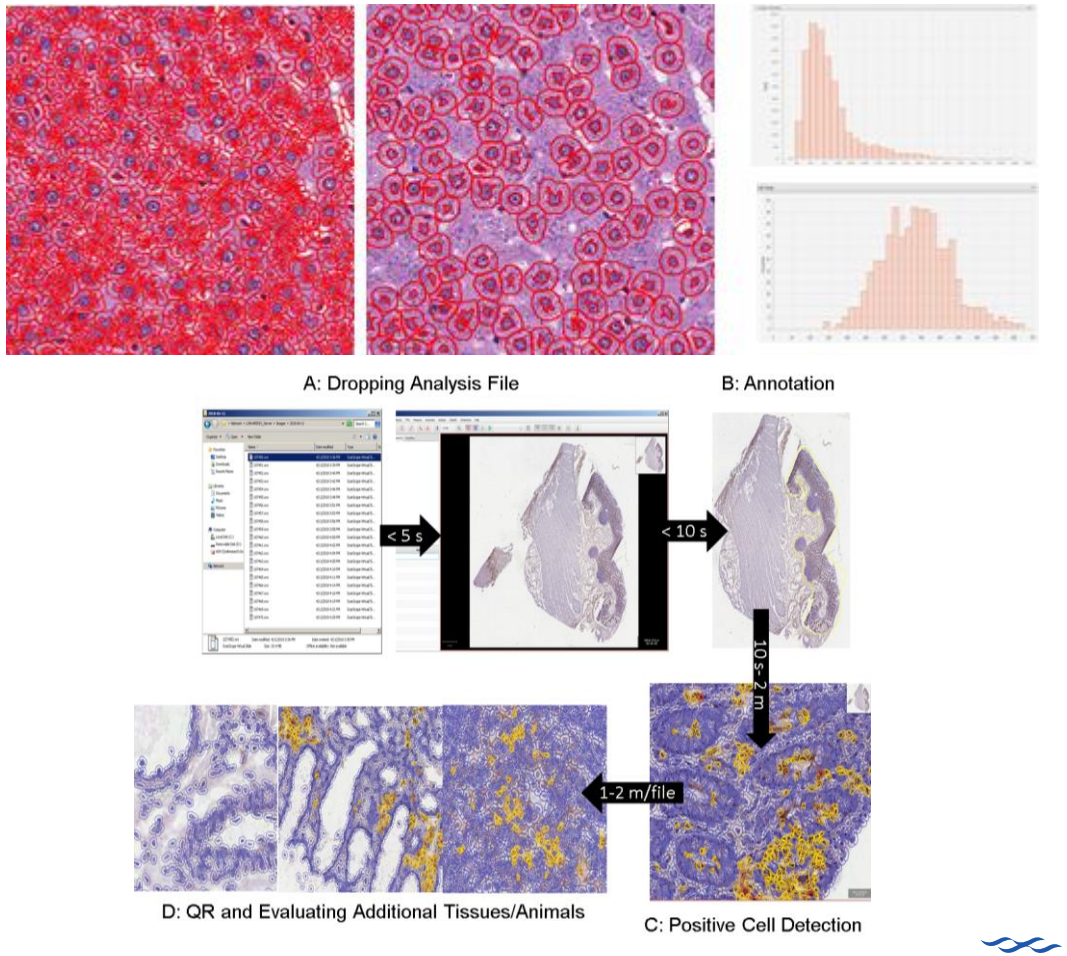
RESULTS

Cohort	Histopathology	Organ weight	Hormone levels
GD 20 Fetuses	No follicular lumen or No colloid formation Follicular cell hypertrophy	↑ thyroid weight	↓ total T_4 ↓ total T_3 ↑ TSH
PND 4 Pups	↑ follicular height ↓ colloid area	↑ thyroid weight	↓ total T_4 ↑ TSH
PND 21 Pups	↑ follicular height ↓ colloid area	↑ thyroid weight	↓ total T_4 ↑ TSH
GD 20 Dams	↑ follicular height ↓ colloid area	↑ thyroid weight	↓ total T_4 ↑ TSH
LD 21 Dams	↑ follicular height ↓ colloid area	↑ thyroid weight	No change

- EPA guidelines applied to PND 4 & 21 pups and to Dams
- Establish the criteria for each grading scheme in each cohort
- For GD 20 fetuses, there were limitations on applying EPA guidelines for histopathology as written
- Modified grading scheme accurately identified PTU-related histopathologic lesions

DIGITAL SLIDE SCANNING AND ANALYSIS

Leicabiosystems.com



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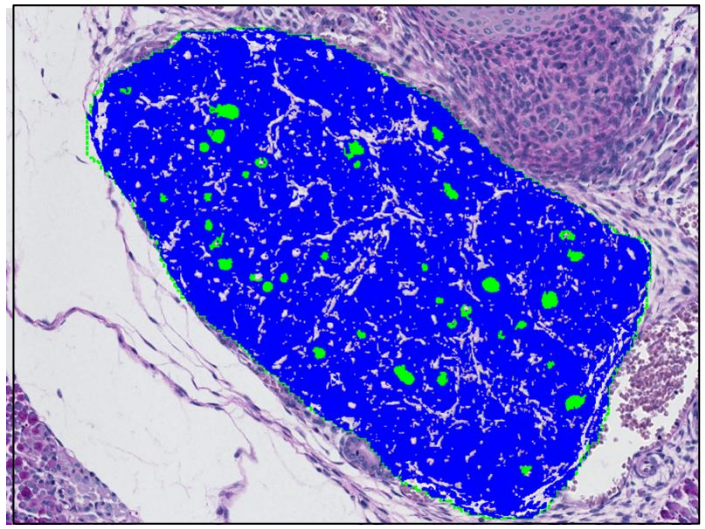
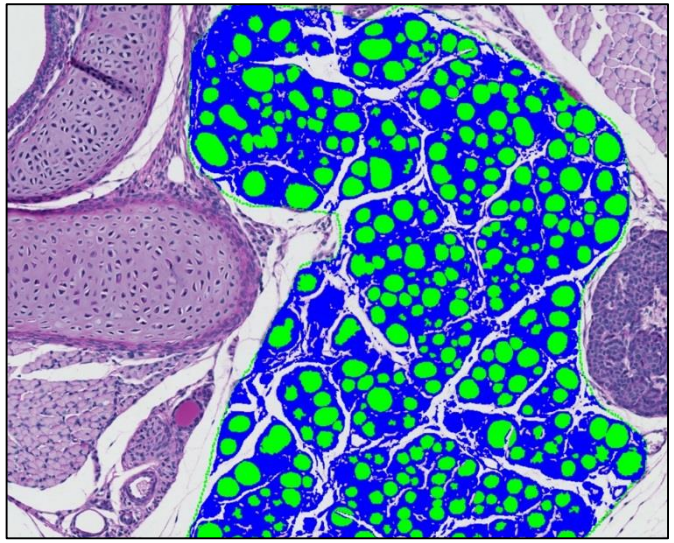
WHOLE TISSUE ANALYSIS

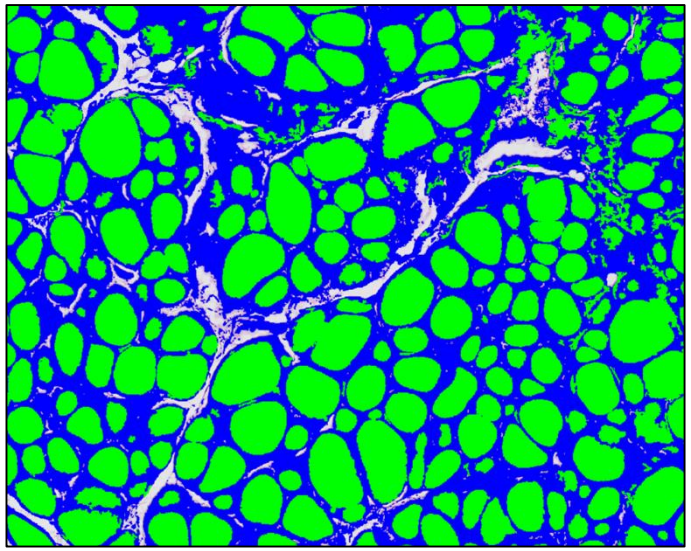
GD 20

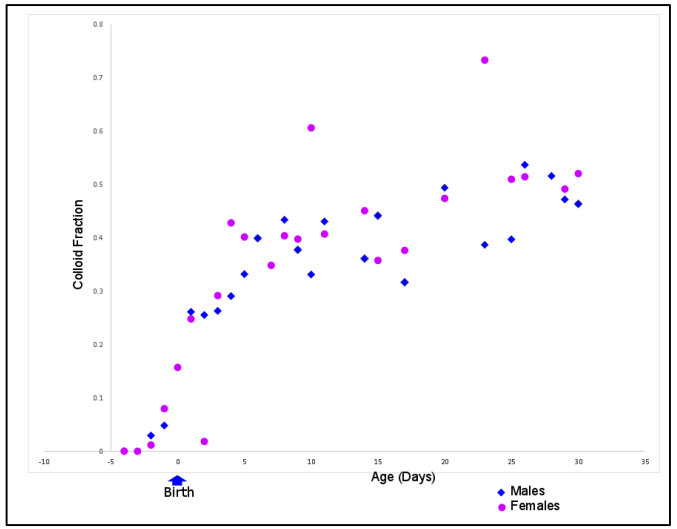


PND 3-4

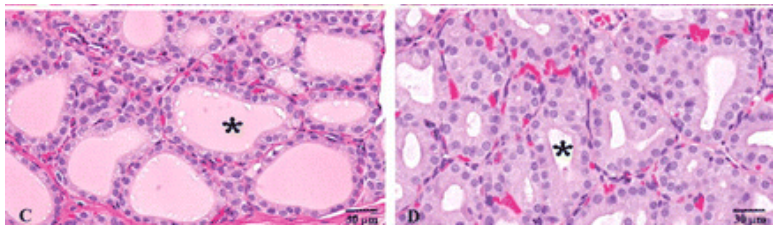


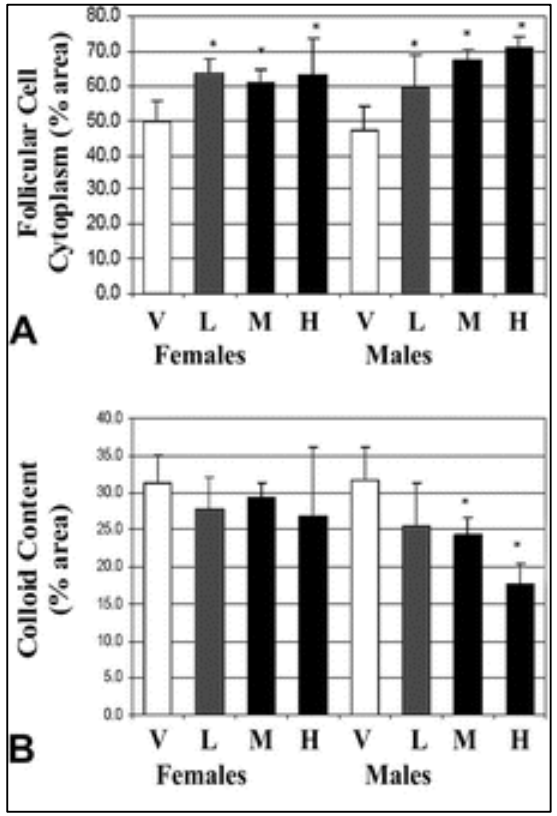
PND 30





Picut et al., 2017





Zabka et al., 2011

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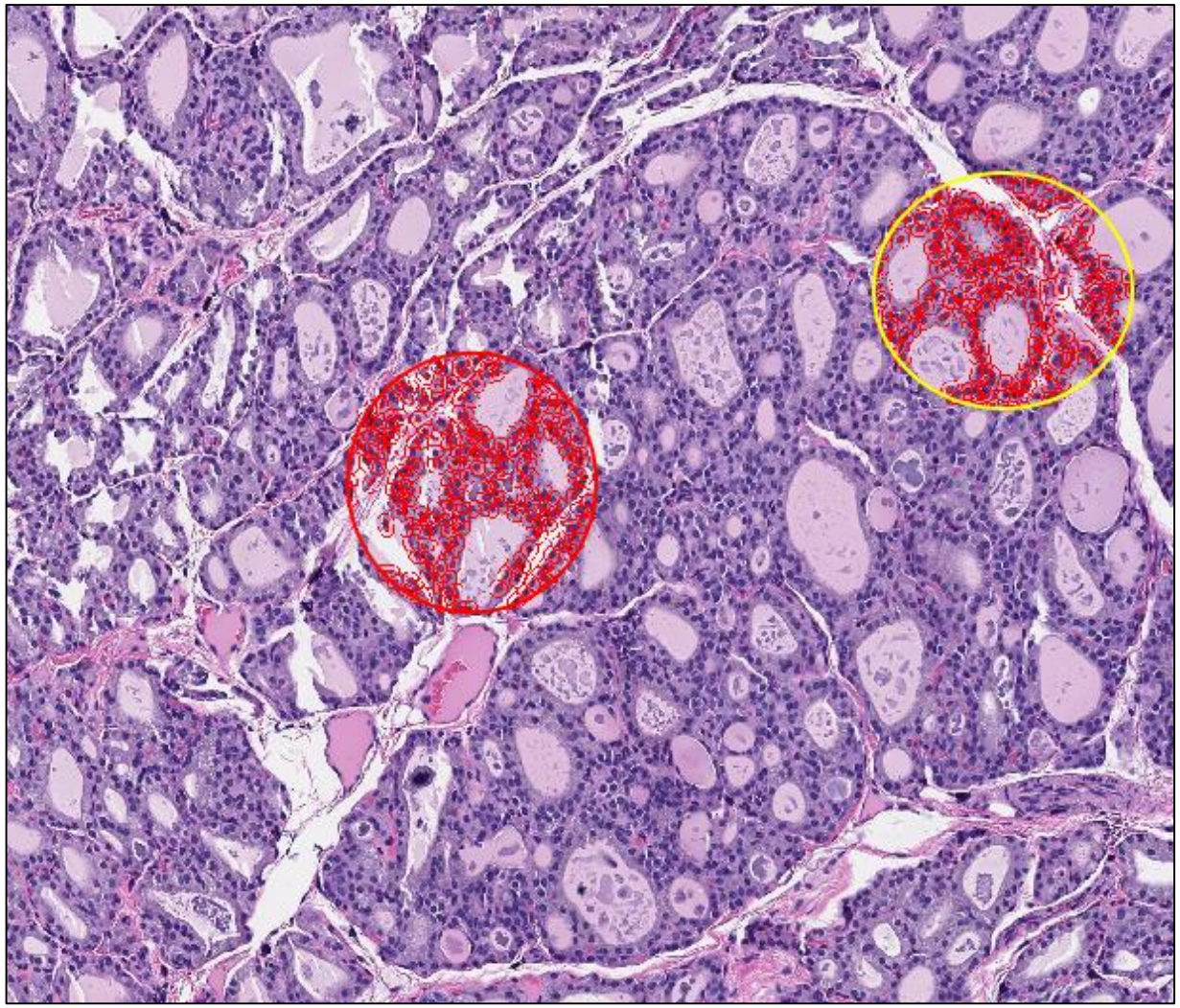
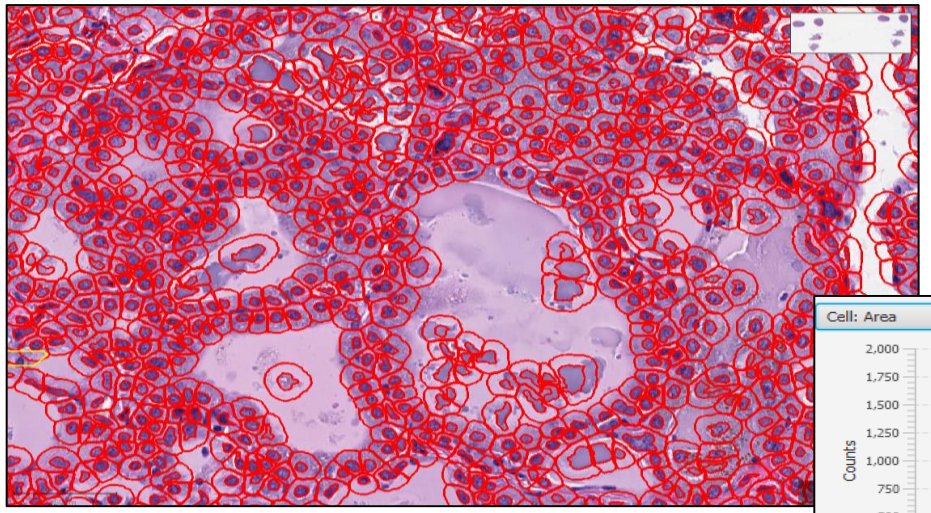
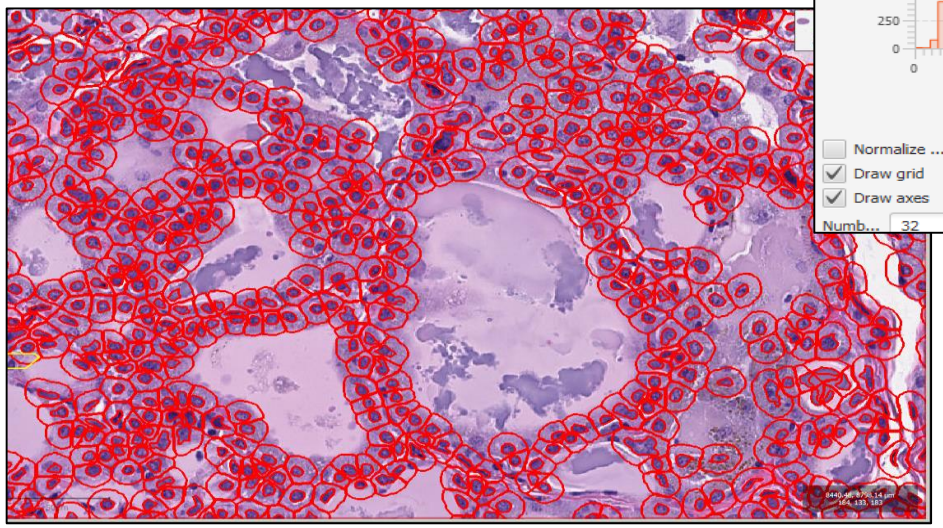
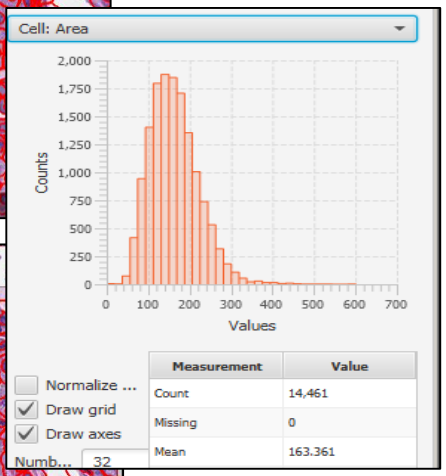
INDIVIDUAL CELL ANALYSIS









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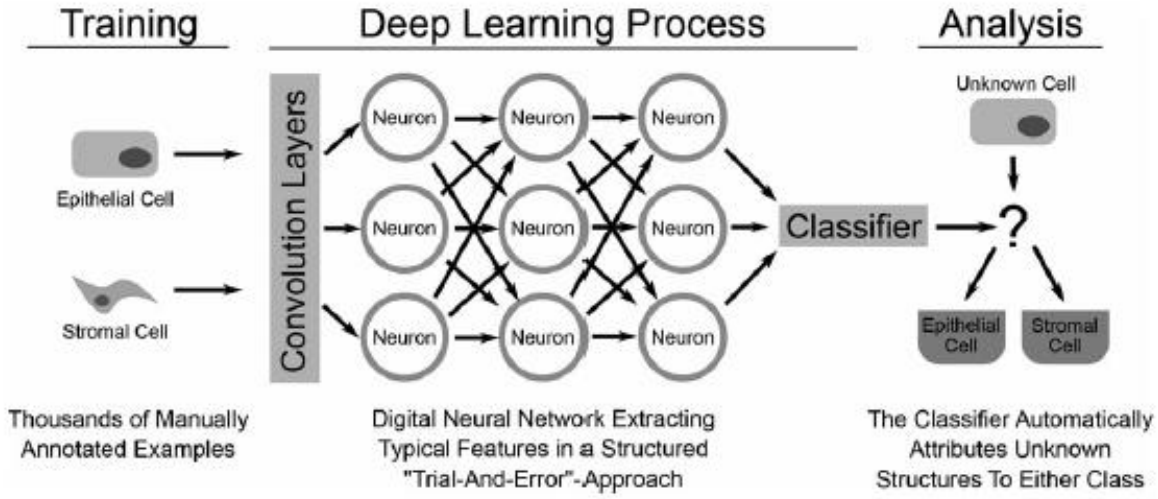
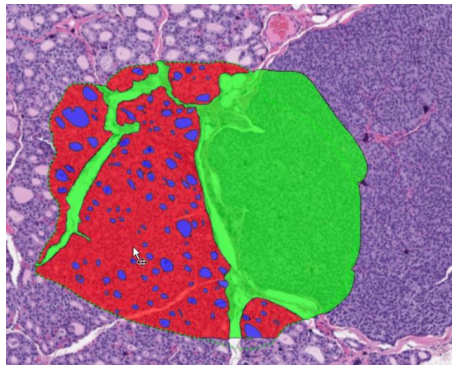
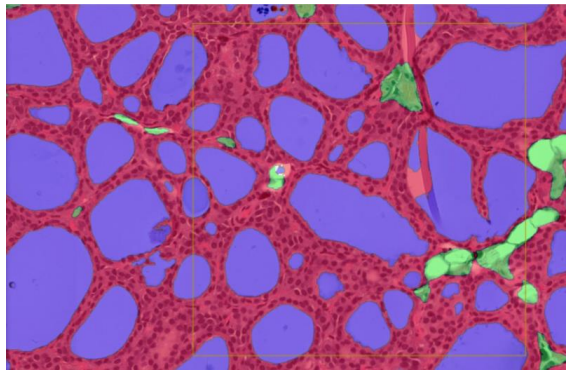
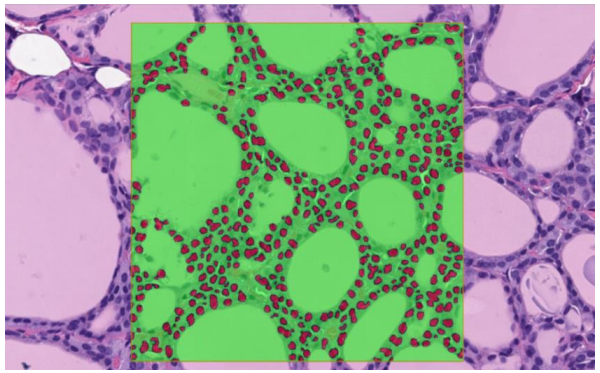
Deep Learning Workflow Example

Visiopharm Artificial Intelligence









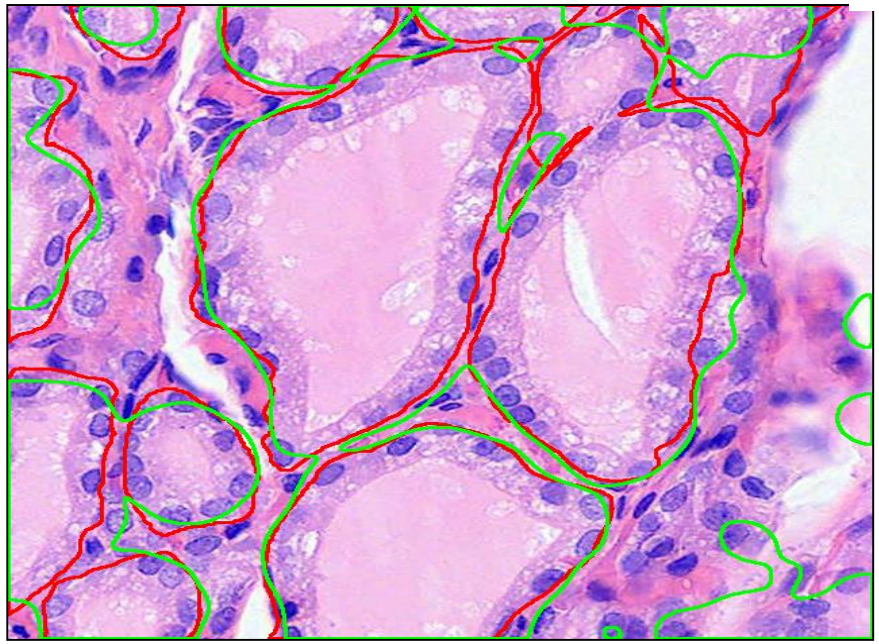
Slide courtesy of Dan Rudmann

OHIO UNIVERSITY COLLABORATION

Thyroid gland Collaboration- Ohio University (Lui and Rosol Laboratories)

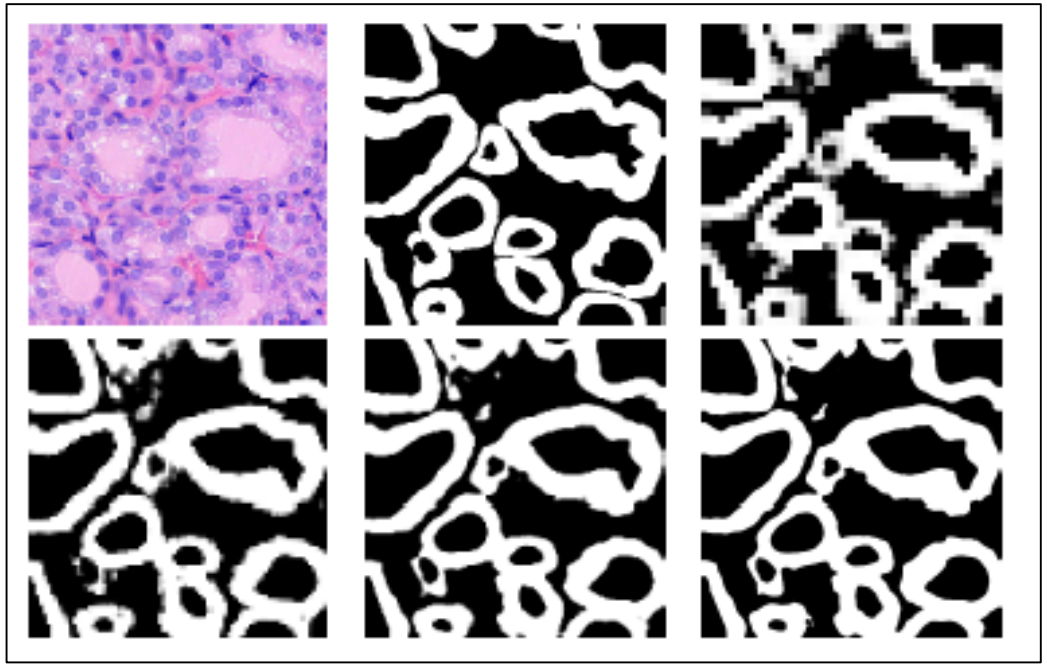
Objective of Collaboration:

1. Develop code that advances QuPath capability to measure colloid area and follicular cell size in the thyroid
2. Build partnership for long term machine learning approach for specific diagnostic endpoints common for toxicologic pathologists



RESIDUAL PYRAMID FCN FOR ROBUST FOLLICLE SEGMENTATION

Zhewei Wang[1] *Weizhen Cai*[1] *Charles D. Smith*[2] *Noriko Kantake*[3] *Thomas J. Rosol*[3] *Jundong Liu*[1]



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CONCLUSIONS

- Disruption of the HPT axis can affect numerous physiologic systems in the fetus, adolescent, and adult animals which can result in adverse and often permanent outcomes and which produce corresponding histopathologic lesions
- With the exception of the brain and, to a lesser extent, the reproductive system, very little published research has included histopathologic endpoints in the results
- DNT studies with earlier time points including histopathology would help characterize early hypothyroid-related lesions
- …not to mention all of the other organ systems…

- Histopathologic guidelines established by the EPA for the evaluation of thyroid function in rats to determine if a pesticide, chemical, or other substance may pose a risk to humans due to disruption of the endocrine system are sensitive and repeatable. Additionally, they can be applied to immature rat pups and rat fetuses as early as gestational day 20.
- Digital imaging and image analysis technology has the potential to enhance histopathologic evaluation of the thyroid gland

THANK YOU

Charles River Laboratories

- Cathy Picut
- Prägati Coder
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Ohio University

- Zhewei Wang
- Weizhen Cai
- Noriko Kantake
- Thomas J. Rosol
- Jundong Liu

University of Kentucky

- Charles D. Smith