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Rat Hippocampal and Dentate Gyrus Prolactin Expression in the Different Stages of Life

International Doctoral Thesis

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Abbreviations

Akt	Protein kinase C
APS	Ammonium peroxydisulfate
Box1	Phosphorylation site 1 in the cytoplasmic prolactin receptor monomer
Box2	Phosphorylation site2 in the cytoplasmic prolactin receptor monomer
BSA	Bovine Albumin Serum
CA	Cornu Ammonis (Ammon's horn) or Hippocampus
CA1	Region 1 of Ammon's horn
CA2	Region 2 of Ammon's horn
CA3	Region 3 of Ammon's horn
CA4	Region 4 of Ammon's horn
$CaCl_2$	Calcium Chloride
cDNA	Complementary deoxyribonucleic acid
Chr	Chromosome
CSF	Cerebrospinal fluid
DG	Dentate Gyrus
DKF	Deutsches Krebsforschungszentrum
ELISA	Enzyme immunosorbent assay
ERK1/2	Protein-serine/threonine kinases that participate in the Ras-Raf-MEK-ERK signal transduction cascade
F	Females
Foxo-1	Forkhead box protein O1
Fyn	SRC-related kinase
Gr	Granular Layer
GRB2	Growth factor receptor bound protein 2
GSK3	Glycogen Synthase Kinase 3
H_2O	Water
HRO	Horseradish peroxidase
IBSAL	Instituto Biosanitario de Salamanca
IL2	Interleukin 2
IL10	Interleukin 10
IFN-γ	Interferon gamma
INCYL	Instituto de Neurociencias de Castilla y León
JAK2	Jabus kinase 2
KCl	Potassium Chloride
KO	Knock-out
LEC	Lateral entorhinal cortex
LH	Luteinizing hormone

M	Males
MAPK	Mitogen-activated protein kinase
MEC	Medial entorhinal cortex
MEK	Methyl ethyl cetone
Mol	Molecular Layer
mRNA	Messenger ribonucleic acid
mTor	Mammalian target of rapamycin
NaCl	Sodium Chloride
$NaHCO_3$	Sodium bicarbonate
NaH_2PO_4	Monosodium phosphate
NIH	National Institute of Health in USA
NS	Statistically no significant
Or	Oriens Layer
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDK-1	Phosphoinositide-dependent protein kinase 1
PI3K	Phosphatidyl inositol 3 kinase
Pol	Polymorphic Layer
Prl or PRL	Prolactin
PRLR	Prolactin receptor
PRLR L1	Large isoform of prolactin receptor
PRLR S1	Short isoform of prolactin receptor
Pyr	Pyramidal Layer
qPCR	Quantitative Polymerase Chain Reaction
Rad	Radiate Layer
RNA	Ribonucleic acid
RNase	Ribonuclease
SDS	Sodium dodecyl sulphate
SOCS	Suppressor of cytokine signaling
SOS	Global response to DNA damage that activates bypass polymerases
Src	Stored response chain protein
SSC	Sodium citrate solution
STAT	Signal transducer and transcriptional trigger
STAT3	Signal transducer and transcriptional trigger 3
STAT5	Signal transducer and transcriptional trigger 5
TB	Trizma buffer
TBS	Trizma buffer saline
TBST	Tris-buffered saline with 1· Tween 20
TEMED	Tetramethylehyneldiamine
VGLUT1	Vesicular glutamate transporter 1

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Funding and Facility Resources

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Introduction

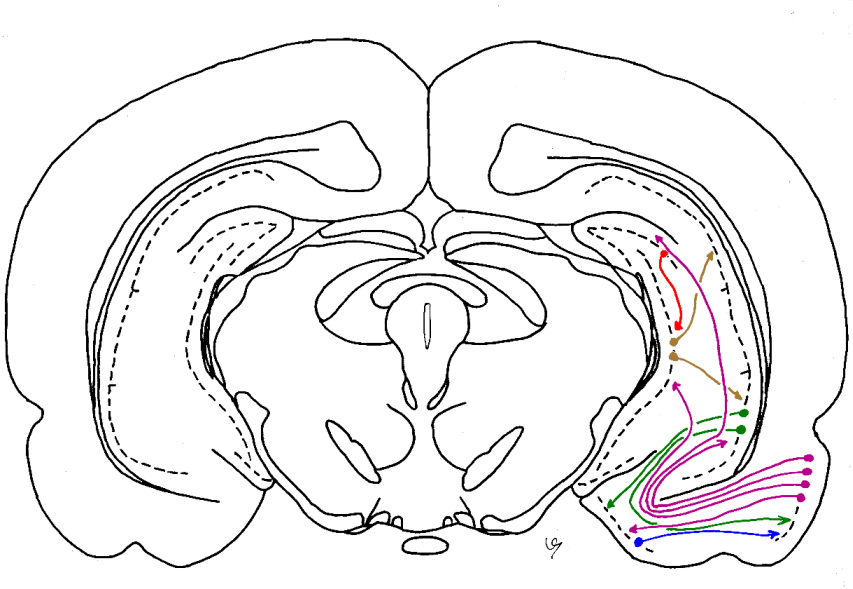
The Hippocampal Formation

Some authors would refer to the hippocampus as the dentate gyrus and Ammon's horn together, for simplicity, as these two regions are studied together, given their connections and functions.

In this thesis, despite the lack of consensus between neuroanatomists, the following brain regions will be referred to as the hippocampal formation: the dentate gyrus, hippocampus (the Ammon's horn subregions), prosubiculum, subiculum, presubiculum, parasubiculum, and entorhinal cortex.

The hippocampus has been used as a neurobiological model for decades because of its anatomical and neurobiological features; all the somas of the neurons are concentrated in a single layer, its connections between different subregions are generally unidirectional, the synapses are very plastic, and hippocampal cells have the capacity to survive for prolonged periods of time in vitro and in cultures.

Regarding the unidirectionality of the information and excitatory pathways in the hippocampal formation, it is widely known that the dentate gyrus connects to the CA3 (Ammon's horn 3), this connects to CA1, which connects directly or indirectly through the subiculum to the entorhinal cortex. The entorhinal cortex connects back to the subiculum, CA1, and dentate gyrus through the perforant pathway, thereby closing the communication loop.

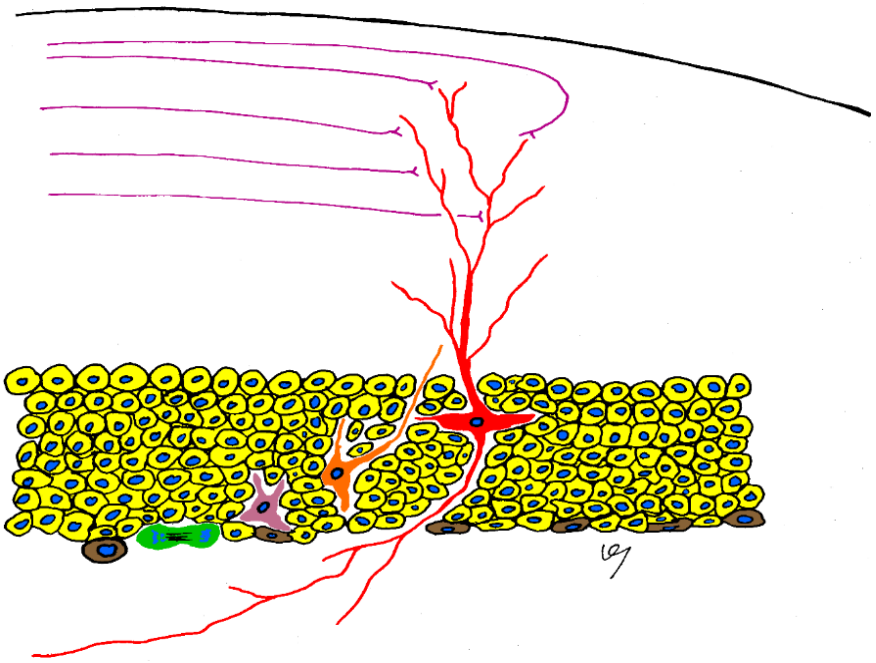


Schema connections inside the hippocampal formation. Purple: entorhinal projections to the subiculum, the horn of Amon and the dentate gyrus -perforant pathway-, Red: projections of the dentate gyrus to the horn of Amon, brown: projections from CA3 to CA1, green: projections from the horn of Amun to the subiculum and the entorhinal cortex, blue: projections from the subiculum to the entorhinal cortex.

The architecture of the hippocampal formation is very similar between mammals, and it is easy to identify different regions in one species using the other as a model, although there are still some significant differences. Comparing the rodent and human hippocampus, the most important difference is the existence of a well-developed dorsal hippocampus arranged below the corpus callosum in rodents while in human is underdeveloped and arranged above the corpus callosum. Moreover, the pyramidal layer of cells in CA1 is thicker in humans, and the entorhinal cortex is divided into two subregions in the rat, while the human has 27 subdivisions, and the commissural connections in the dentate gyrus in rats are massive, while in humans they are nearly non-existent, for example.

The Dentate Gyrus and the Ammon's Horn

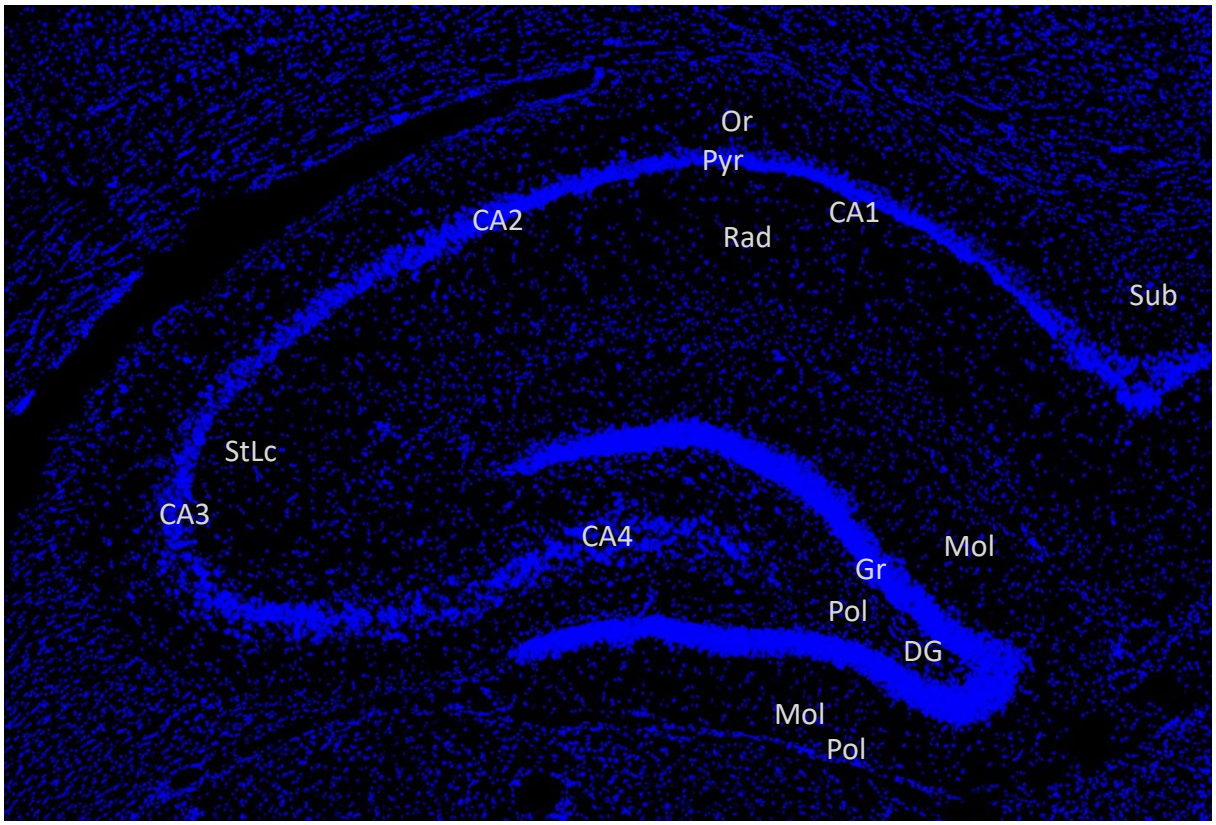
The dentate gyrus has a characteristic V-shape and is composed of four laminate layers of the cortex. The polymorphic layer contains axons from the granular layer that communicate with the CA3. The subgranular layer is composed of stem cells that are responsible for neuronal proliferation, and their somas are slightly more elliptical in shape than granular somas. The granular layer is composed of well-defined neuronal somas that migrate away from the subgranular layer according to their maturity, and more cell generations are formed. The last layer is the molecular layer, which contains dendrites that connect axons of neurons arranged in the subiculum and entorhinal cortex. In this study, we divided the dentate gyrus into ventral, dorsal, and peak regions.



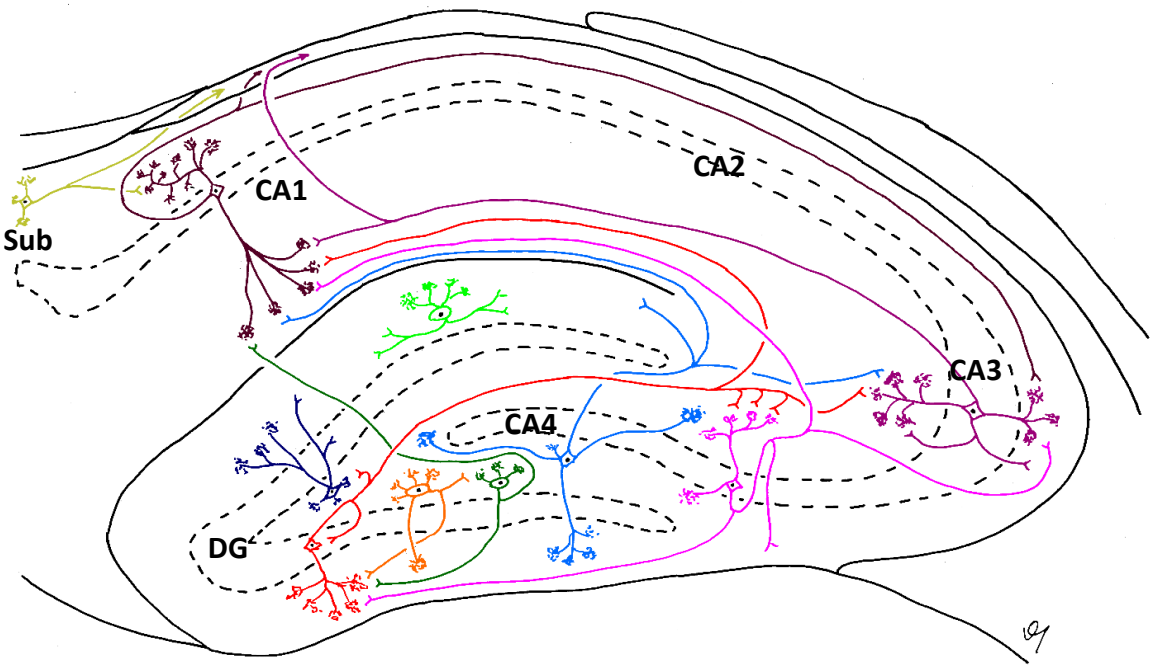
Schema of DG structure. Proliferation and differentiation of granular neurons (purple, orange, red and yellow) from subgranular cells (brown and green) is showed.

All the subregions of the Ammon's Horn have the same architecture, with the layer with the neuronal somas called the pyramidal layer (Stratum Pyramidale), since all the neurons in the hippocampus are pyramidal neurons. The axons from the pyramidal layer are in the Stratum Oriens and the dendrites are in the Stratum Radiatum. Below CA1's dendrite later is the subiculum, another brain region part of the hippocampal formation that is very important for the communication between the hippocampus and the entorhinal cortex. Next to the pyramidal layer of CA3 there is the Stratum Lacunosum, which contains dendrites from CA3, but also axons that come from the entorhinal cortex towards the dentate gyrus (perforant pathway) and axons from dentate gyrus to CA3 region. Moreover, there are secondary neurons (no granular or pyramidal neurons) arranged mainly in polymorphic layer of dentate gyrus.

This study focuses its attention mainly on the dorsal hippocampal formation of the rat brain.



Hoescht staining microphotography showing the different parts of the dorsal hippocampal formation. Mole: molecular layer, Gr: Granular layer, Pol: polymorphic layer, Or: Ories layer, Pyr: Pyramidal layer, Rad: radiate layer, StLc: Stratum Lacunosum layer, Sub: Subiculum, DG: dentate gyrus.



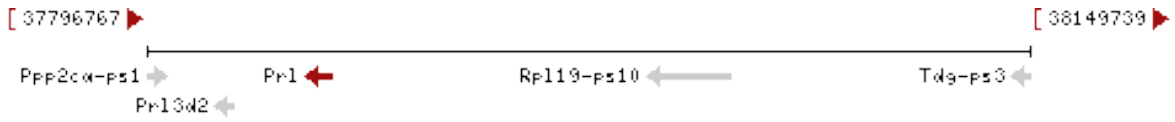
Schema of hippocampus layer structure and their connections. The granular neurons of the dentate gyrus (red) receive projections in the molecular layer from neurons of CA3 (pink) and project to granular cells of the dentate gyrus, to pyramidal neurons of CA3 (purple) which in turn project to the radiated layer of CA1 and out of the hippocampal formation, through the alveus, and to the radiated layer of CA1 on pyramidal neurons (brown). Pyramidal CA1 neurons project to the CA3 pyramidal neurons through the oriens layer and send fibers to the alveus. In the polymorphous layer of the dentate gyrus, the secondary neurons that also exist in CA1 and CA3 are represented (dark blue, light blue, orange, light green and dark green), although to a lesser extent, these secondary neurons are of various types and interconnect with each other and between the granular or pyramidal neurons of the dentate gyrus and the Amon's horn. Some of them receive projections from granular neurons in the dentate gyrus. Although they have not been represented for the sake of simplifying the scheme, CA4 pyramidal neurons have the same afferents and efferents as CA3 neurons.

Prolactin in the Hippocampus

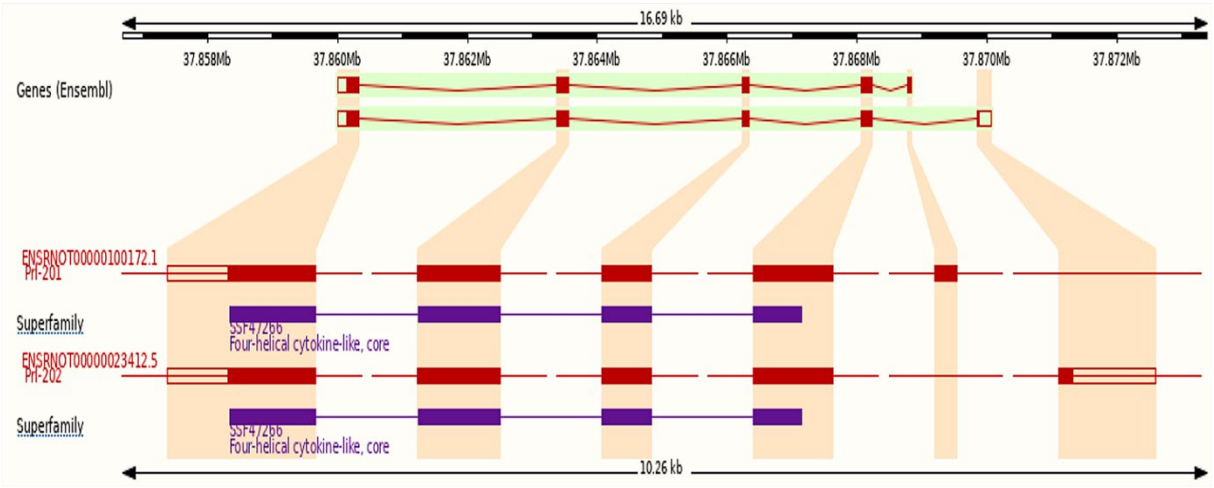
Prolactin is a protein hormone that is common to all the vertebrae species. More than 300 biological functions or roles have been described where prolactin had a role or has been involved. These include neurogenesis stimulation, stress and anxiety modulation, calcium transport, and immune system regulation (Ferraris et al., 2013; Larsen & Grattan, 2012; Patil et al., 2014).

The Gene: expression and synthesis

In the rat (Rattus norvegicus), the gen for prolactin is located in the chromosome 17 (Chr17p12) with the coordinates NC_051352.1 (37859999.37870062) according to the 108th annotation (NCBI ID) published in January 2021 (mRatBN7.2).



According to the Ensembl database, this gene has two transcripts, 315 orthologs, and 24 paralogs, and is associated with 47 phenotypes. Of these two different transcripts (Prl-201 ENSRNOT00000100172.1), the one that is the most complete, conserved, and expressed is considered to be responsible for most of the roles of this hormone.



The gene has five exons, four introns, and 834 pairs of bases (Prl-201 Ensembl). It is mainly expressed in the hypophysis, with more than 90% of the total prolactin originating from the pituitary gland. However, it is worth mentioning other tissues where prolactin has been expressed apart from the pituitary gland, from most expressed to least expressed (Genevisible): the femoral growth plate, seminiferous tubule, tibialis anterior muscle, extraocular muscles, striatum, epididymal adipose tissue, sacral region of the spinal cord, vermis, and other regions of the spinal cord. Other databases, such as those from the NCBI, Ensembl, or Expression Atlas, have registered data for prolactin expression in the adrenal gland and brain.

Prolactin is, then, mostly synthetized in the pituitary gland, specifically in the anterior lobe of adenohypophysis, where it is secreted into the portal-system blood in a pulsating manner.

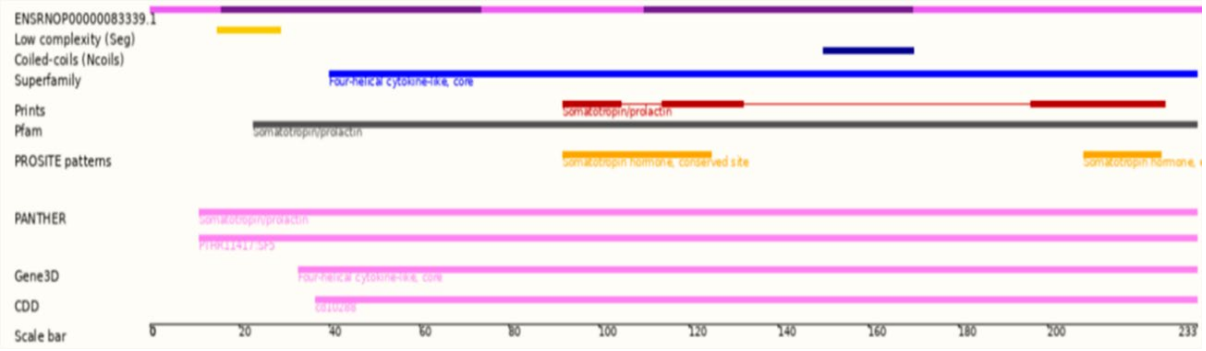
The origin of prolactin in the brain has been controversial over the past decades because it should not be able to pass through the blood-brain barrier because of the hormone size and protein nature. During this time, experiments were conducted to test the following hypotheses:

Some studies suggest that prolactin in the brain comes from the blood, as serum levels are correlated to brain levels (Grattan, 2015; Tejadilla et al., 2010), but experiments with hypophysectomized animals showed that there was still brain prolactin, despite being in much lower levels than in control animals.

Other studies suggested receptor-controlled transport from the blood into the cerebrospinal fluid (CSF), given the high amount of prolactin receptors in the choroid plexus (Clemens & Sawyer, 1974; Ferraris et al., 2013; Hirai et al., 2013; Larsen & Grattan, 2012; Patil et al., 2014; Tejadilla et al., 2010). This was debunked since prolactin receptor knockout animals presented unaltered levels of prolactin in the brain compared to controls (Brown et al., 2016). Recently, it has been suggested that the brain synthesizes its own prolactin. More specifically, the choroid plexus synthetizes brain prolactin, which would be different from the prolactin originated in the pituitary gland (Costa-Brito et al., 2021).

The Protein: sequence and nature

According to the protein sequence published in UniProt (P01237) in October 2022 (version 157), the prolactin protein from the rat consists of 226 amino acids; however, Ensembl Prl-201 consists of 233 amino acids. UniProt information has been revised by Swiss-Prot, and its evidence has been proven at the protein level.



Length 226
Mass (Da) 25,753
Last updated 1986-07-21 v1
Checksum[i] 755E2874A26862C7

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        10         20         30         40         50         60         70         80         90        100
MNSQVSARKA GTLLLLMMSN LLFCQNVQTL PVCSGGDCQT PLPELFDRVV MLSHYIHTLY TDMFIEFDKQ YVQDREFIAK AINDCPTSSL ATPEDKEQAQ

       110        120        130        140        150        160        170        180        190        200
KVPPEVLLNL ILSLVHSWND PLFQLITGLG GIHEAPDAII SRAKEIEEQN KRLLEGIEKI ISQAYPEAKG NEIYLVWSQL PSLQGVDEES KDLAFYNNIR

       210        220
CLRRDSHKVD NYLKFLRCQI VHKNNC
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The Prolactin Receptor

Structure and isoforms.

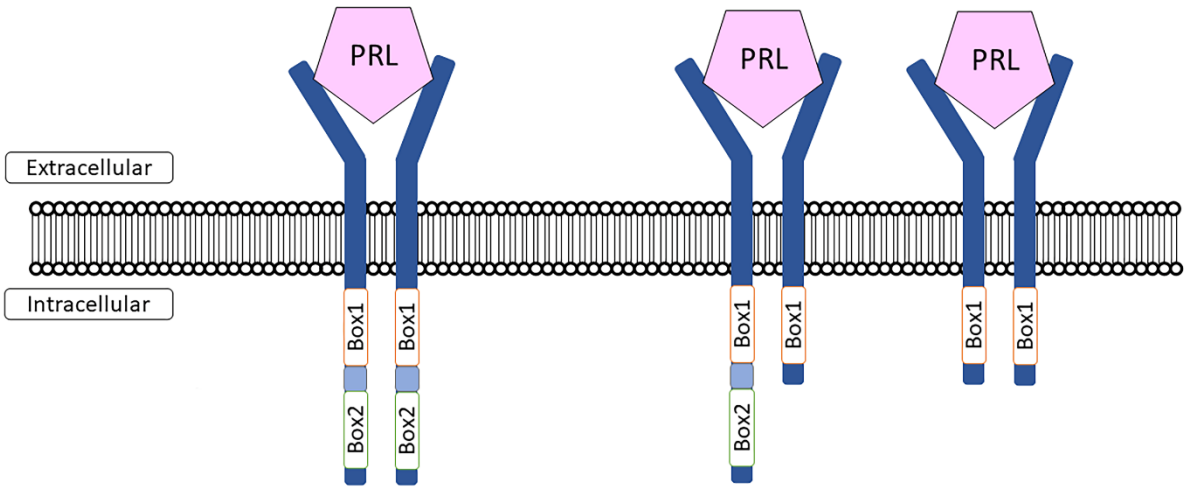
The prolactin receptor is divided into three parts: the extracellular, transmembrane, and intracellular domains. All receptor isoforms have the same structure in the extracellular and transmembrane domains. Therefore, the differences between isoforms depend on the structure of the intracellular domain. This domain is larger than the other two domains and interacts with other cytoplasmic molecules to initiate signaling pathways (Bernard et al., 2015).

Prolactin ligands with two different extracellular interactive sites, called binding domains, inducing conformational changes in the receptor dimer.

The intracellular domain structure can be divided into hydrophobic parts and interactive sites. Some hydrophobic parts can interact with lipids and change interacting with the inner side of the cell membrane after prolactin ligands. On the other hand, interactive sites are called Box1 and Box2(Goffin et al., 1995).

The interactive sites are the responsible of the existence of three isoforms: long, intermediate, and short (Bole-Feysot et al., 1998).

The long isoform of the receptor is a dimerization of two molecules of receptor that have both Box1 and Box2. The intermediate isoform would be the result of dimerization of one molecule with Box1 and Box2 but the other has only Box1. The short isoform would be the dimerization of two molecules that only have box1 (Bugge et al., 2016).



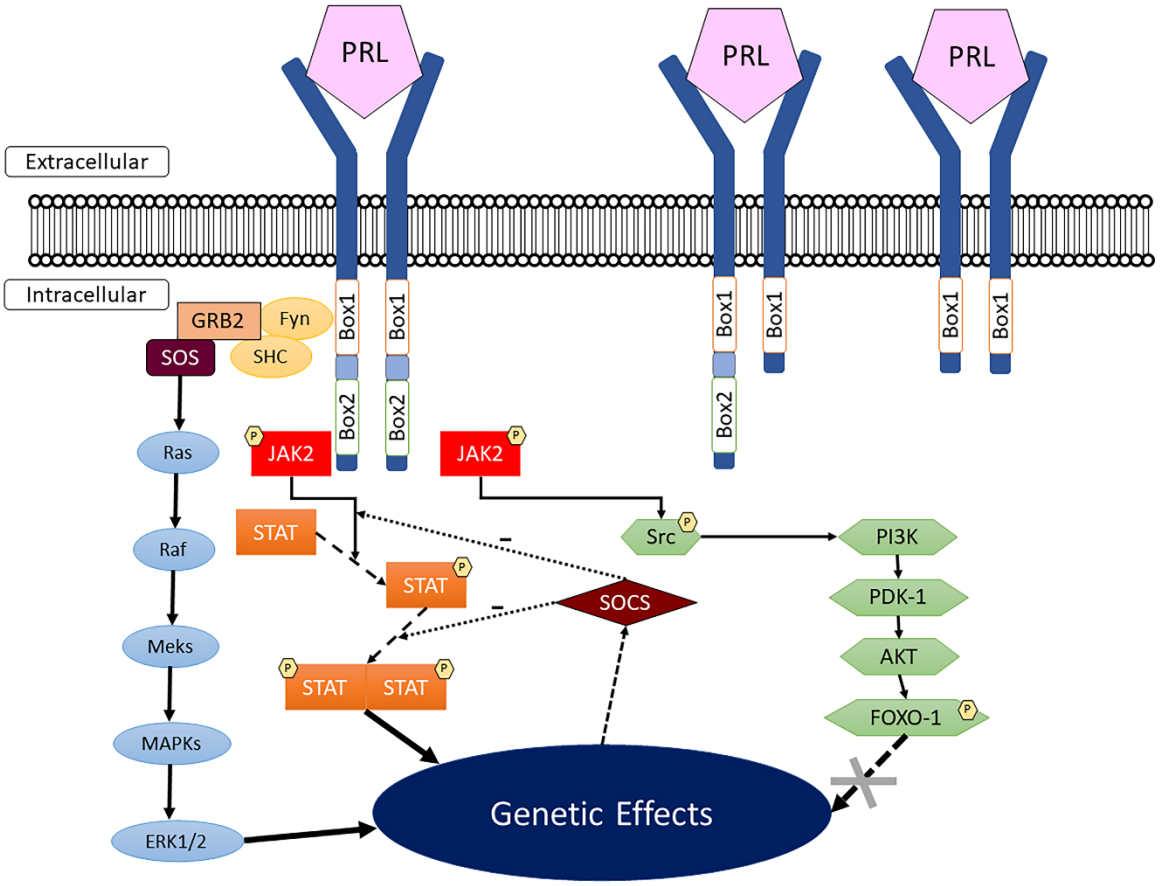
Schematic simplified representation of the prolactin receptor isoforms.

Prolactin ligand with the receptor dimer can be transported and internalized to the nucleus of the cell.

Intracellular Signaling Pathways

Box1 activates JAK2 located nearby in the cytoplasm via phosphorylation. This is the main difference between prolactin activity and other cytokines, because JAK2 is not part of the receptor molecule. JAK2 activation initiates a signaling pathway that can be divided into three pathways: MAPK, STAT5, and Akt (Bugge et al., 2016).

The activation of JAK2 provokes a series of interactions and cascade reactions that would be, in a very simplified way, the phosphorylation of Src, which leads to the activation of Ras, Raf, MEK, and MAPKs, ultimately stimulating cell proliferation. Another consequence of Src activation is the reaction cascade of PI3K, Akt phosphorylation, and mTor, which leads to antiapoptotic effects. The last pathway mentioned is also initiated by the phosphorylation of JAK2, which facilitates the phosphorylation of the STAT family. The most relevant ones are STAT5 and STAT3, which are internalized into the nucleus and activate their transcriptomic functions, typical of cytokines and growth factors.



Summarized representation of prolactin internal signaling pathways.

Roles associated to prolactin in the hippocampus.

Since the identification of the prolactin receptor in the hippocampus, its direct effect on this structure has been analyzed. Whether considering the arrival of prolactin from the pituitary gland through the choroid plexuses, as well as the local synthesis, prolactin triggers different effects on hippocampal cells by activating its own receptor.

Neuroprotection

There is evidence of the neuroprotective effect of prolactin in female rodents during pregnancy and lactation against neuronal damaged associated with excitotoxicity due to the direct action of kainic acid or glutamate (Cabrera et al., 2009; Vanoye-Carlo et al., 2008), while these effects are directly mediated by the presence of estrogens too (Tejadilla et al., 2010). The Ammon's horn pyramidal neurons are one of those that most benefit from the protective role of prolactin against neuronal damage (Cabrera et al., 2009; Vanoye-Carlo et al., 2008). The physiological increase in prolactin causes an overexpression of β-cell lymphoma 2 (Bcl-2) accompanied by a decrease in intracellular calcium concentration, reinforcing the idea of the neuroprotective role of prolactin in the hippocampus (Rivero-Segura et al., 2017).

On the other hand, in the final stages of pregnancy, as well during lactation, an attenuation of the stress response is observed with the consequent modulation of Tau protein phosphorylation in the hippocampus in rodents, mainly associated with the reduction of the expression of Glycogen Synthase Kinase 3 (GSK3) thought Akt signaling, possibly by prolactin receptor activation (Steinmetz et al., 2015). The use of prolactin analogs in transgenic animal models of amyloidosis caused a decrease in the aggregation of β-amyloid as well as the phosphorylation of Tau in the hippocampus (Holubová et al., 2019), a fact that supports the neuroprotective role of prolactin.

The increase in serum prolactin concentration associated with patho-physiological factors, such as the presence of prolactinomas, can be associated not only with impaired cognitive function but also with structural alterations of the hippocampus in adult women. The main effect observed was a decrease in the volume of grey matter evidence by magnetic resonance imaging (Yao et al., 2018). However, pathologies such as schizophrenia are associated with increases in the serum concentration of prolactin, mainly related to the antipsychotic drugs used for its treatment, whose main target is dopaminergic inhibition. In these patients, a decrease in the overall volume of the hippocampus can also be observed (Rametti et al., 2007).

Similar effects are observed when the increase in prolactin is caused by exogenous administration. Cell death due to excitotoxicity is reversed by prolactin, both in female (Morales et al., 2014; Ortiz-Pérez et al., 2019; Reyes-Mendoza & Morales, 2016) and male rodents (Anagnostou et al., 2021). It is not clear which region of the hippocampus benefits the most from the neuroprotective effect of prolactin. Some studies observed that only CA1 (Reyes-Mendoza & Morales, 2016) or CA3 (Anagnostou et al., 2021) benefit from the neuroprotective role of prolactin while others determined that the effect occurs throughout the Ammon's horn, without mentioning the effect produced on the dentate gyrus (Morales et al., 2014; Ortiz-Pérez et al.,

2019). However, this effect may be linked to a decrease in glial activity (Cabrera-Reyes et al., 2019; Reyes-Mendoza & Morales, 2016) and/or an overexpression of VGLUT1, a modulator of the glutamatergic response, in the hippocampus of these rodents (Ortiz-Pérez et al., 2019). These effects are likely to be triggered by an increase in Akt activity, followed by a decrease in MAPKs activity after prolactin receptor activation (Anagnostou et al., 2021; Morales et al., 2014).

Some studies focused on the use of cell lines derived from hippocampal neurons to analyze the neuroprotective role of prolactin against excitotoxicity (Rivero-Segura et al., 2017; Vergara-Castañeda et al., 2016). As observed in in vivo experiments, prolactin can reverse neuronal death produced by the direct action of glutamate (Rivero-Segura et al., 2019; Vergara-Castañeda et al., 2016) through the action of its own receptors. One of the effects could be via the preservation of altered mitochondrial activity because of calcium influx induced by glutamate action and/or by the increase in the expression of one of the neuronal antioxidant defense components, the enzyme superoxide dismutase (Rivero-Segura et al., 2017, 2019).

Neurogenesis

Neurogenesis is a complex process that includes the activation, proliferation, differentiation, and migration of stem and glial cells and the synaptic integration of new neurons into the already formed neural network (Walker et al., 2012). One of the areas in the brain that has been described to have neurogenesis in the adult age is the subgranular layer in the dentate gyrus of the hippocampus (Torner et al., 2009; Walker et al., 2012). Newly formed neurons in the dentate gyrus migrate a short distance into the granular layer and become dentate neurons that are directly related to learning, memory formation, object recognition, and spatial orientation processes. The administration of prolactin in the hippocampus triggers a response of processes associated not only with neuroprotection and neurogenesis but also with glial differentiation, and neuronal and glial migration (Cabrera-Reyes et al., 2019), presumably through the activation of MAPKs, as previously described.

Even though exogenous prolactin does not generate an increase in the number of proliferating cells in some studies (Shingo, 2003; Smeeth et al., 2021; Wagner et al., 2009), it has been associated with the reversion of the neurogenic alterations caused by chronic stress, like the decreasing of the number of proliferating astrocytes in the dentate gyrus (Torner et al., 2009). The reduction in astrogliosis by prolactin was associated with a decrease in neuronal loss of CA1 caused by excitotoxicity, without altering neurogenic processes, according to other studies (Cabrera-Reyes et al., 2019). The effects observed in the initial stages of neuronal differentiation show that prolactin can increase the proportion of immature hippocampal stem cells (Pathipati et al., 2011; Shingo, 2003), without this effect being intravenous dose dependent (Smeeth et al., 2021), possibly indicating that changes in serum prolactin concentration would not greatly affect hippocampal neurogenic control. Exogenous prolactin causes a decrease in the survival of granular cells of the dentate gyrus without increasing the rate of neuronal death. Everything seems to indicate that the effect of increased serum prolactin concentration in neonatal periods is associated with a decrease in neurogenesis in the subgranular zone of the dentate gyrus. These differences between young and adult animals could be associated

with neonatal susceptibility to sudden changes in prolactin concentration (Lajud et al., 2013; Larsen & Grattan, 2010; Shingo, 2003).

Spatial orientation, learning and memory formation.

The hippocampus has been described to be involved with episodic memory in humans (O'Keefe & Nadel, 1978; Scoville & Milner, 1957; Squire et al., 2004) and similarly in other animals, during the memory formation process of the "what", "where" and "when" components (Clayton & Dickinson, 1998; Eichenbaum & Fortin, 2005). The hippocampus receives inputs from other parts of the brain as we have mentioned previously, like the medial entorhinal cortex (MEC) and the lateral entorhinal cortex (LEC), that have been related to specific spatial information and item recognition, respectively (Burwell, 2000; Deshmukh & Knierim, 2011; Knierim, 2006; Manns & Eichenbaum, 2009; W. A. Suzuki et al., 1997; Witter & Amaral, 2004). This way, the hippocampus has been mainly linked to memory formation and consolidation, along with memory retrieval and storage (Barker & Warburton, 2011).

Prolactin role in the hippocampus regarding memory formation and consolidation has been supported by studies where hippocampal prolactin receptor-deleted and prolactin knock-out (PRL KO) mice showed a deficit on learning and memory processes; conditions reversed on PRL KO mice after administration of prolactin directly into the hippocampus (Walker et al., 2012).

However, the recent studies trying to investigate the specific role prolactin may play in the memory formation process have encountered contradictory results. While some authors have indicated that reproductive experience (repetitive pregnancy and post-partum) improves spatial memory in animals (Kinsley et al., 1999; Love et al., 2005; Pawluski et al., 2006) others have described that such physiological stages of hyperprolactinemia in women were related to cognitive impairment of executive actions (Henry & Sherwin, 2012). Human patients with prolactinomas also showed cognitive deficits on verbal memory and executive function tasks (Yao et al., 2018). Furthermore, hyperprolactinemia in male rats showed object recognition impairment, although it did not affect spatial learning (Torner et al., 2013) and elderly men's higher levels of serum prolactin were linked to cognitive and executive performance deficits (Castanho et al., 2014). Prolactin patterns may vary between species (Ben-Jonathan et al., 2008; Freeman et al., 2000; Phillipps et al., 2020) and sex and its changes during physiological or pathological stages might be the reason for these contradictions.

Additionally, other studies supported the role of prolactin having an important role in memory formation and long-term consolidation in the CA1 region of hippocampus during chronic stress conditions (Leem et al., 2019) and by increasing serum levels with exogen prolactin in mice (Moreno-Ruiz et al., 2021). These studies do not work with the hypothesis of the hormone being directly synthesized in the brain and assume that the serum prolactin is capable of somehow passing the blood-brain-barrier.

Anti-inflammatory effects

It is known that females are more resistant to brain injuries than males, especially if it is delayed secondary brain injury. The reason behind this is not very clear but it could be because

of sex steroids since they have effects on vascular stability, as some trials have proven for estrogen and progesterone (Khaksari et al., 2011, 2015; Späni et al., 2018). The level of production and secretion of pituitary hormones is also affected during brain damage, like it is the case for triiodothyronine, cortisol, growth hormone and prolactin (Benvenga, 2005; Benvenga et al., 2000). In the case of prolactin, the levels are increased by three in both serum and cerebrospinal fluid (Dalwadi et al., 2017; Kaulfers et al., 2010; Scranton & Baskin, 2015; Skalny et al., 2017). Prolactin has also been described to increase the proliferation of immune system cells in the brain (Anagnostou et al., 2018) but there are other contradictory studies (Aimaretti et al., 2004; Baxter et al., 2013; Giordano et al., 2005). As an anti-stress hormone, prolactin helps sustain homeostasis by suppressing glucocorticoids and inflammatory mediators, enhancing the immune and inflammatory response regulating the mitogenic signaling in T cells (Yu-Lee, 2002). Inflammation is an important part of neurodegeneration and appears in most of degenerative disorders and brain strokes (Choopankareh et al., 2015). A study in 2018 confirmed that paracrine prolactin helped modulating the neuroinflammatory response (Anagnostou et al., 2018), and it was already known that prolactin could modulate the cytokine reaction during antigenic response increasing IFN-γ, IL-12 and IL-10 while inducing T cell differentiation (Matalka, 2003).

Prolactin during different stages of life

It is known that newborns have elevated levels of serum prolactin and that it decreases over time to reach normal levels when they are 3-4 months old. (Walker et al., 2012) It has been speculated that this happens because of the stimulation of the high estrogen levels from the mother. The level of serum prolactin during childhood are the lowest in the whole life circle and start increasing during puberty. Some studies show that basal serum levels of prolactin keep decreasing over time with age but there are other studies that found no such changes. (Howie et al., 1980; Lévy et al., 2011) It should be noted that these studies only describe serum prolactin and brain prolactin has not been described from this point of view.

Estrous Cycle and prolactin

Some women have been described to have higher levels of serum prolactin in the middle of their menstrual cycle and lower levels during follicular phase compared to the luteal phase. However, there had no correlation with estrogen, progesterone, luteinizing hormone (LH) or follicle stimulating hormone levels (FSH) (Cole et al., 1977; Djursing et al., 1981; Franchimont et al., 1976; Fujimoto et al., 1990; Gordon et al., 1979; McNeilly & Chard, 1974; Tyson & Friesen, 1973). Other authors have made a link between prolactin and LH where both have a simultaneous secretion in the luteal phase (Braund et al., 1984).

Pregnancy and Lactation

The serum levels of prolactin increase over time during pregnancy, correlated with an increase in number of lactotrophs in the pituitary gland, and can reach quantities 10 times higher than non-pregnant women (Ben-David et al., 1973; Poindexter et al., 1977; Rigg et al., 1977; Tyson & Friesen, 1973) This happens in preparation for lactation after birth, where serum levels stay

elevated for the first 4-5 weeks postpartum. Suckling by the newborn while breastfeeding triggers a rapid release of pituitary prolactin that elevates prolactin quantities three to fivefold (Noel et al., 1974; Tyson & Friesen, 1973). After breastfeeding, prolactin levels decrease gradually to pre-nursing levels. The effectiveness of suckling-triggering and basal levels of prolactin decreases gradually during the next 4 to 12 weeks (Johnston & Amico, 1986). Eventually, the amount of milk produced is no correlated with the prolactin serum levels (Howie et al., 1980).

Prolactin and stress

Prolactin secreted during stress usually doubles or triples serum levels and this lasts around 1 hour. It seems that the cortisol that is secreted also during stress suppresses further prolactin secretion, although the prolactin levels will keep stable as long as the stress stimuli is applied (HARMS et al., 1975; Subramanian & Gala, 1978). However, not all forms of stress provoke hyperprolactinemia and how this response is regulated is still to be fully explained.

Motivation, Hypothesis and Objectives

No studies in the scientific literature have analyzed the possible variations in prolactin and its receptor in the hippocampus during aging. It is unknown whether neurodegeneration and memory loss that occur during late age are accompanied by significant variations in prolactin hippocampal levels.

Therefore, we analyzed whether prolactin changes during different stages of life. To determine this, we designed an experimental study in which we analyzed the expression and location of prolactin and its receptor in the rat hippocampus: Ammon's horn and Dentate Gyrus.

Our hypothesis is that there is sexual dimorphism, and that this expression could be related to puberty and the fertile ages of the animals.

The main objective of this thesis was to establish the ground knowledge of rat hippocampal prolactin expression during aging.

For specific objectives, we want to

- Determine the location, quantity, and molecular weight of prolactin in the hippocampus.

  To this end, ELISA, Western Blotting, and Immunohistochemistry techniques have been used.

- Determine whether the hippocampus is able to synthesize prolactin and the location of this synthesis.

  To this end, qPCR and in situ hybridization techniques have been used.

- Determine if there are changes between the expression of the prolactin receptor isoforms.

  To this end, the in situ hybridization technique has been used, differentiating the mRNA from the Box1 or Box2 fragments of the receptor.

- Establish key points in life in which prolactin levels in the hippocampus change significantly.

  To this end, morphometric and densitometric analyses have been developed of the results obtained with the aforementioned techniques.

- Determine where all these variations are sex related.

  For this objective and the previous one, a statistical analysis of the results obtained has been carried out in the previous objectives.

Materials and Methods

Animals and Groups to Study

A total of 100 rats (*Rattus norvergicus*) were used for this study. 50 of them were used for histological analysis and the other 50 were used for molecular analysis. Rats that developed pathologies due to aging like diabetes or tumors, specially prolactinomas, were discarded at the time of sacrifice. The study groups were divided in the following manner:

- Prepubertal group: 4 weeks old, they were already separated from the mother and were not lactating rats, however they did not reach puberty yet.
- Postpubertal group: 6 weeks old, where they reached puberty within 5 days from the day of sacrifice.
- Young Adult: 4-5 months old, virgin and not exposed to mating.
- Adult: 9-10 months old, virgin and not exposed to mating.
- Old: 15+ months, virgin, not exposed to mating and could not reproduce anymore.

Each of these groups had 10 animals of each sex, 5 would be for histological study and the other 5 for molecular studies. Every female rat was sacrificed during their estrus stage of the estrous cycle. All animals were kept in standard conditions for experimental rats, with a light/dark cycle of 12/12h and free access to food and water resources *ad libitum*.

As a control, 2 male and 2 female animals were used that were sacrificed 14 days after transsphenoidal hypophysectomy, served by Charles-River®.

Biological Ethics Committee Approval

All methods and procedures involved in this experiment were approved by the Ethics Committee of the University of Salamanca (Registration No. 1146) and will be conducted in accordance with the animal care guidelines of the European Common Council (86/609/EEC) and Spanish regulations (R. D. 1201/2005), making significant efforts to minimize both suffering and the number of animals used.

Sacrifice of the animals

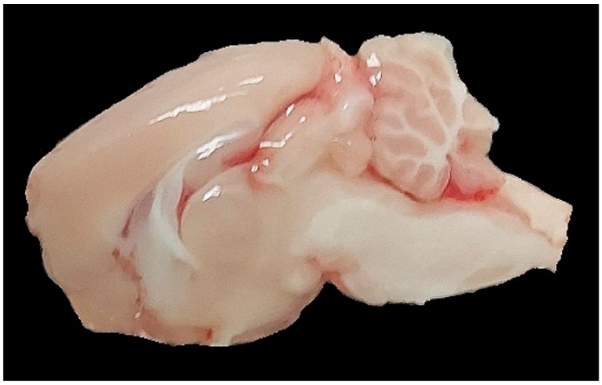
For histological studies, animals were sedated with equitesin. After opening their thorax, they were killed via trans-cardiac perfusion by substitution of the blood by heparinized saline serum (0.9% NaCl) and then perfused with paraformaldehyde (4% in phosphate buffer 0.1M, pH:7.4).

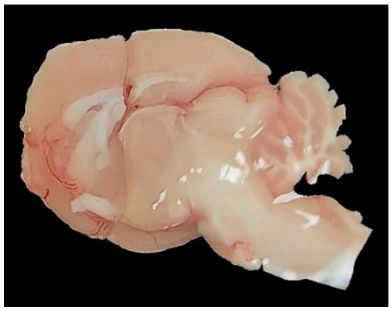
By careful dissection, brains were taken out of their skull and kept in paraformaldehyde (4%) for the next 24h.

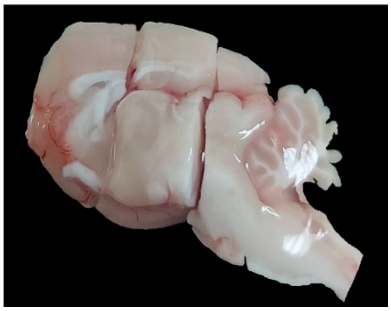
For analytical and molecular studies, animals were sedated with equitesin.

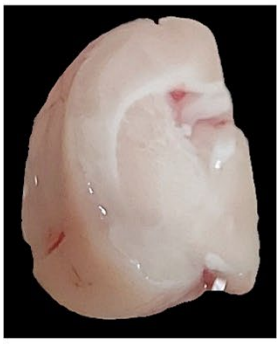
By careful dissection, brains were taken out of their skull and kept in synthetic cerebrospinal fluid for dissection of hippocampus using a protocol designed by Dr. José Carretero specifically for this study.

To obtain the cerebral block containing the hippocampus, according to Patxino's Rat Brain stereotaxic atlas, 2 frontal cuts were made: one cephalic cut in the intersection of fornix column with corpus callosum and one caudal cut in the cephalic part of superior colliculus. In a third step, dissecting below the corpus callosum, towards the lateral ventricle, the striatum and thalamus were moved medially.

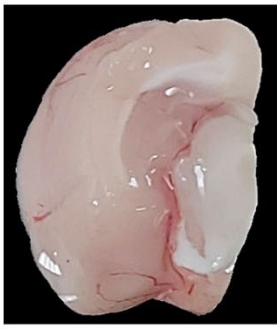


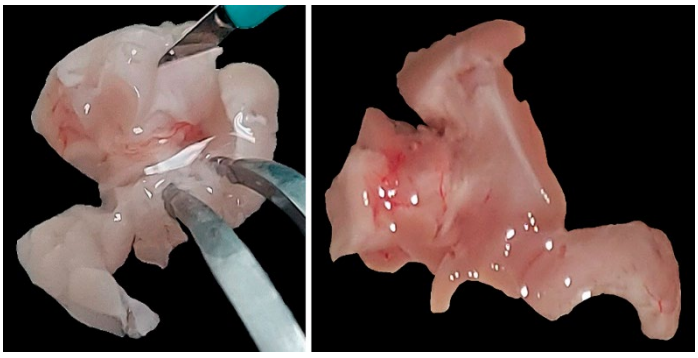






Finally, through careful dissection, the hippocampus was separated from the rest of the cortex following its dorsolateral limit, which is given by the corpus callosum, until it was completely separated. Once isolated, the hippocampus was frozen by immersion in liquid nitrogen and stored in a freezer at -80°C.





Equitesin Solution

For 250mL:

1.	Pentobarbital sodium	2.425 g
2.	Ethanol Absolute	24.125 ML
3.	Chloral Hydrate	10.625 g
4.	Propylene glycol	103.25 mL
5.	Bidistilled H2O	87.5 mL
6.	Mg Sulfate	5.25 g

Mix and shake by heating slightly and add 28.2 mL of bidistilled water.

Dose used: 1 mL / 250 g body weight (intraperitoneal administration).

Synthetic cerebrospinal fluid for brain dissection

Dilution buffer: Aqueous solution of KCl (3 mM), NaH_2PO_4 (1.25 mM), $NaHCO_3$ (28mM).

Immediately before use, to 200 mL of the dilution buffer, gasified with air for 1 minute, the following are added: 14.56 g of Sucrose, 0.3 6 g of Dextrose, 2 mL of $MgCl_2$ (1M), 100 mL of $CaCl_2$ (1M) and 1 mL of sodium pyruvate (600mM).

Immunohistochemical Study

Tissue Fixations and Slicing

To preserve the tissues for freezing, brains were submerged in increasing solutions of sucrose in Phosphate saline buffer (PBS 0.1M, 0.9% NaCl, pH 7.4) and then frozen and serial sections of 20 µm thickness were obtained with a Microm® cryostat. They were introduced in wells with PBS and then fished to be placed in microscope slides.

Immunostaining for Prolactin

After drying the frozen slides, they were washed in TBS (0.05M Trizma base Sigma®, 0.8% NaCl, pH 7.5). Blocking was made with Bovine Albumin Serum (BSA Sigma® Diluted at 1/30 in TBS). The samples were incubated over night at 4°C in humidity chamber with Rabbit primary antibody for human-prolactin (DAKO. A0269/lot. 076, reactive for human, rat, and mouse) diluted at 1/250 in TBS. After 2 washes in TBS for 5 minutes at room temperature, the samples were incubated for 3 hours at room temperature in dark chamber with Goat Anti-Rabbit conjugated with AlexaFluor 488 (Abcam ® ab150077) diluted at 1/800 in TBS. After washing the slides on TBS three times for 5 minutes each, the samples were incubated with Hoechst 33342 (Cayman®. 15547) diluted at 1/1500 for 15 minutes at room temperature. After 2 washes in TBS and 1 wash in distilled water, the slides were covered with Fluoromont (Sigma-Aldrich) medium and sealed.

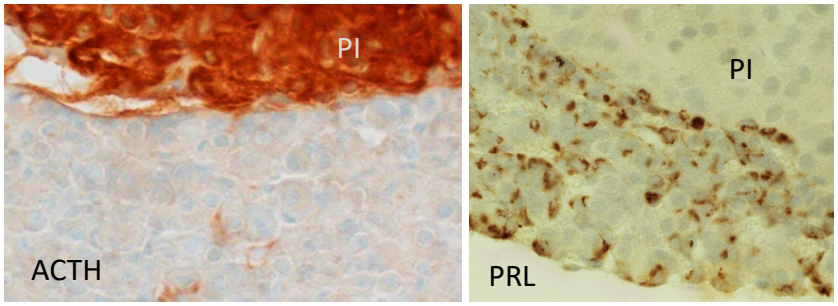
Immunostaining for Pituitary Prolactin and ACTH (Opiomelanocortin)

Positive control for prolactin and comparison between the reaction pattern of the antibody used for the prolactin study and that of the reaction for opiomelanocortin were carried out on 7mm rat pituitary sections, of untreated adult rats taken from the laboratory's specimen archive. After deparaffinizing and rehydrating the slices, endogenous peroxidase was inhibited in a methanol-3% hydrogen peroxide solution (200 mL/20 mL, vv) for 30 minutes, followed by two 5-minute TBS (0.05M Trizma base Sigma®, 0.8% NaCl, pH 7.5) washes each. Blocking was made with Bovine Albumin Serum (BSA Sigma® Diluted at 1/30 in TBS). The samples were incubated over night at 4°C in humidity chamber with Rabbit primary antibody for prolactin (DAKO. A0269/lot. 076) diluted at 1/250 in TBS or ACTH (Dako, M3501) diluted at 1/500 in TBS. After 2 washes in TBS for 5 minutes at room temperature, the samples were incubated for 45 minutes at room temperature in humidity chamber with biotinylated-Goat Anti-Rabbit (Abcam ® ab150077) diluted at 1/800 in TBS. After 2 washes in TBS for 5 minutes each, the samples were incubated with Streptoavidin complex (Abcam®. 15547) diluted at 1/1500 for 45 minutes at room temperature. After 2 washes in TBS the reaction was developed with 3-3' diaminobenzidine (25mg /100 mL of TB (0.05M pH 7.4) plus 150 mL hydrogen peroxide al 3%, under microscopic control, and then washed in distilled water, the slides were counterstained with Mayer's hematoxylin washed in water and covered with Aquaplast (Sigma-Aldrich).

Controls for immunohistochemistry

For immunohistochemistry, the following were used as negative controls:

- Substitution of the primary antibody with TBS.
- Substitution of secondary antibody with TBS.
- Preabsorption of the primary antibody with human prolactin (Sigma®).
- Positive pituitary control.
- Contrast with immunostaining between prolactin and ACTH (opiomelanocortin) in the pituitary pars intermedia (PI).



In-situ Hybridization

After drying the frozen slides, they were submerged in acetylated buffer for 10 minutes. After that, they were washed with distilled water and dried in a stove at 37°C for 20 minutes. Prehybridization was made with Omnibuffer® in the HybAid Omnislide thermocycler® with

humidity chamber for 1 hour at 37°C. Hybridization was performed in the same chamber at 37ºC overnight with the following 5' biotinylated probes:

Sense Probes	Sequence (5'-3')
Rat PRL	{Btn}CATTACAACTTTCAGCACATGCTTAAGT
Rat PRLR_S1 (short isoform)	{Btn}TCTTGGGTAAAACTCTCCGAGGATAAAC
Rat PRLR_L1 (long isoform)	{Btn}AGAAACCTTACCCTACCGTAATCGGCA
Antisense Probes	Sequence (5'-3')
Rat PRL	{Btn}GTAATGTTGAAAGTCGTGTACGAATTCA
Rat PRLR_S1	{Btn}AGAACCCATTTTGAGAGGCTCCTATTTG
Rat PRLR_L1	{Btn}TCTTTGGAATGGGATGGCATTAGCCGCT

Slides were submerged two times in the HybAid Omnislide washing module® for 5 minutes in astringent washes with 0.1% SSC buffer (sodium citrate solution) at 37°C. Then again washed one last time with the same buffer at room temperature for another 5 minutes. To avoid false positive detection, after hybridization and astringent washes the samples were treated with RNase.

Biotin was detected by immunohistochemistry. For this propose, the samples were washed twice in TBS for 5 minutes at room temperature. Blocking was made with BSA for 30 minutes and then incubated, overnight at 4°C in humidity chamber, with primary mouse antibody Anti-Biotin (DAKO. M0743) at a dilution 1/350 in TBS.

After washing on TBS twice for 5 minutes at room temperature, the samples were incubated, for 3 hours at room temperature, with Goat Anti-Mouse antibody conjugated with AlexaFluor 488 (Abcam. ab150113) diluted at 1/800 in TBS. Then washed twice with TBS and Hoechst 33342 (Cayman. 15547) at a dilution of 1/1500 was incubated for 15 minutes. Finally, after washing with TBS twice and leaving the slides for another 5 minutes on distilled water, Fluoromont (Sigma-Aldrich) was applied, and cover was placed and sealed.

Summary table of Antibodies

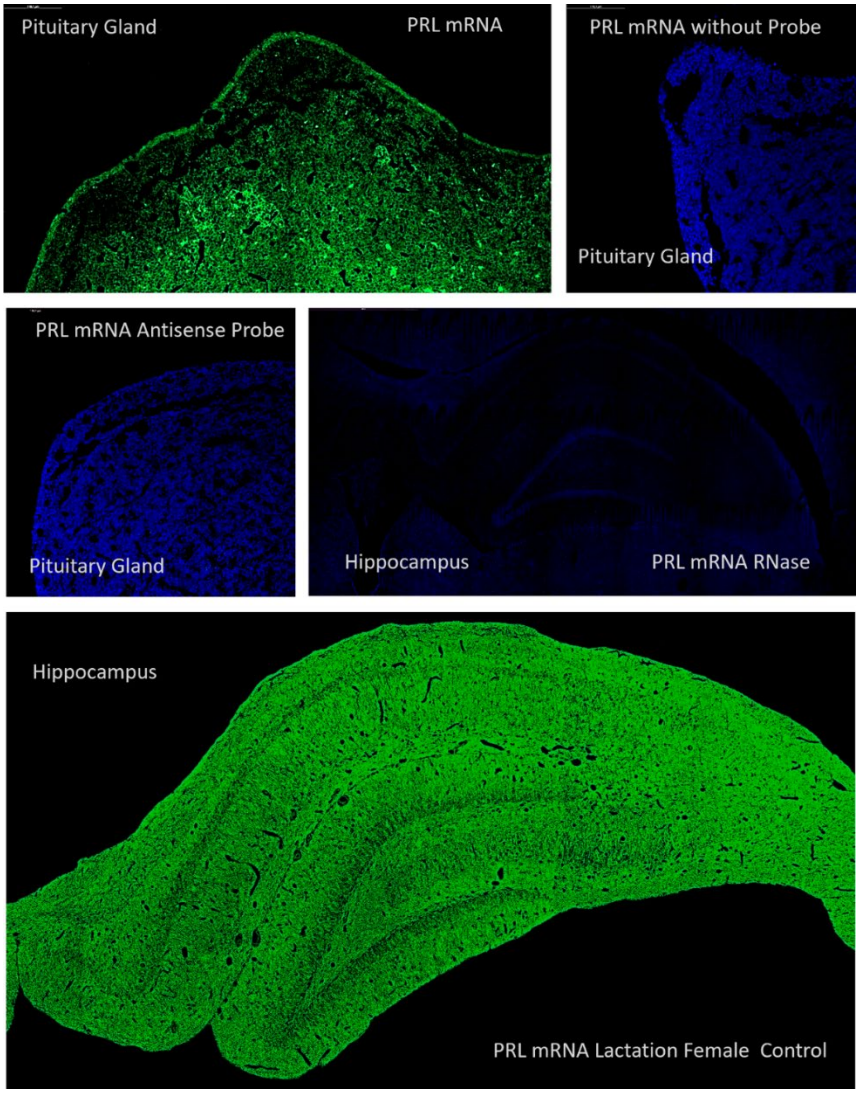
Antibody	Manufacturer	Code ID	Dilution
Anti-prolactin	DAKO	A0269	1/250
Anti-biotin	DAKO	M0743	1/350
Anti-doublecortin	Abcam	ab18723	1/200
Anti-NeuN	Abcam	ab177487	1/200
Anti-ACTH	DAKO	M3501	1/500
Goat Anti-Rabbit Ig G + Alexa488	Abcam	ab150077	1/800
Goat Anti-Mouse Ig G + Alexa488	Abcam	ab150113	1/800
Biotinylated Goat Anti-Rabbit IgG	Abcam	ab150077	1/800
Streptavidin-Peroxidase complex	Abcam	ab15547	1/500

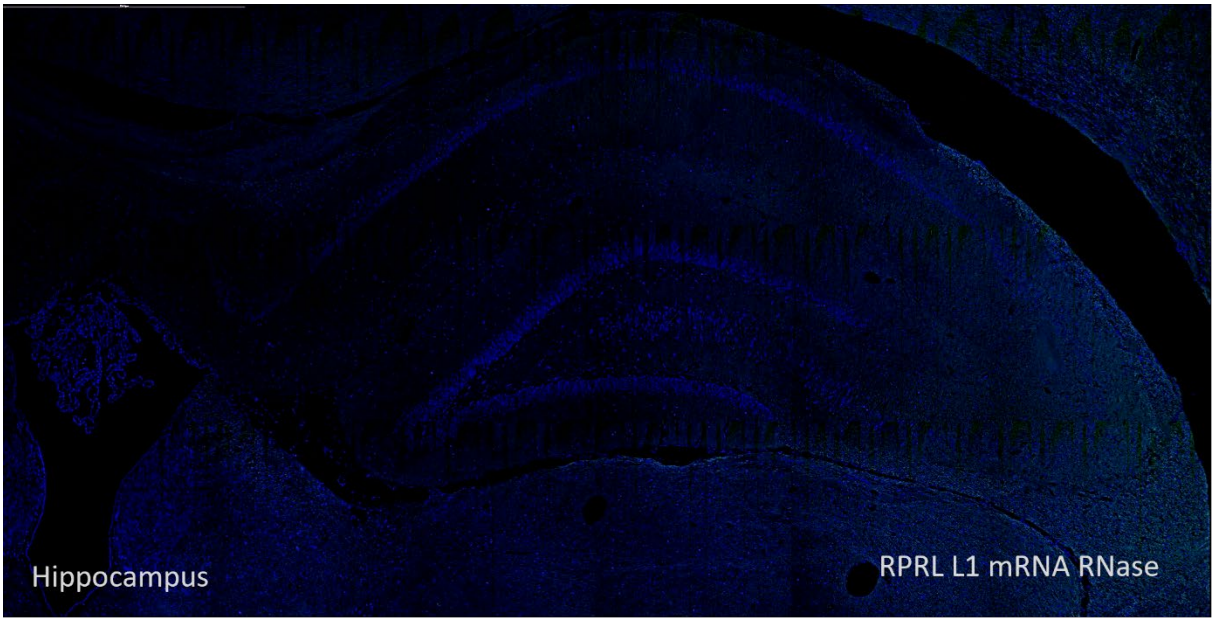
Controls for in situ Hybridization

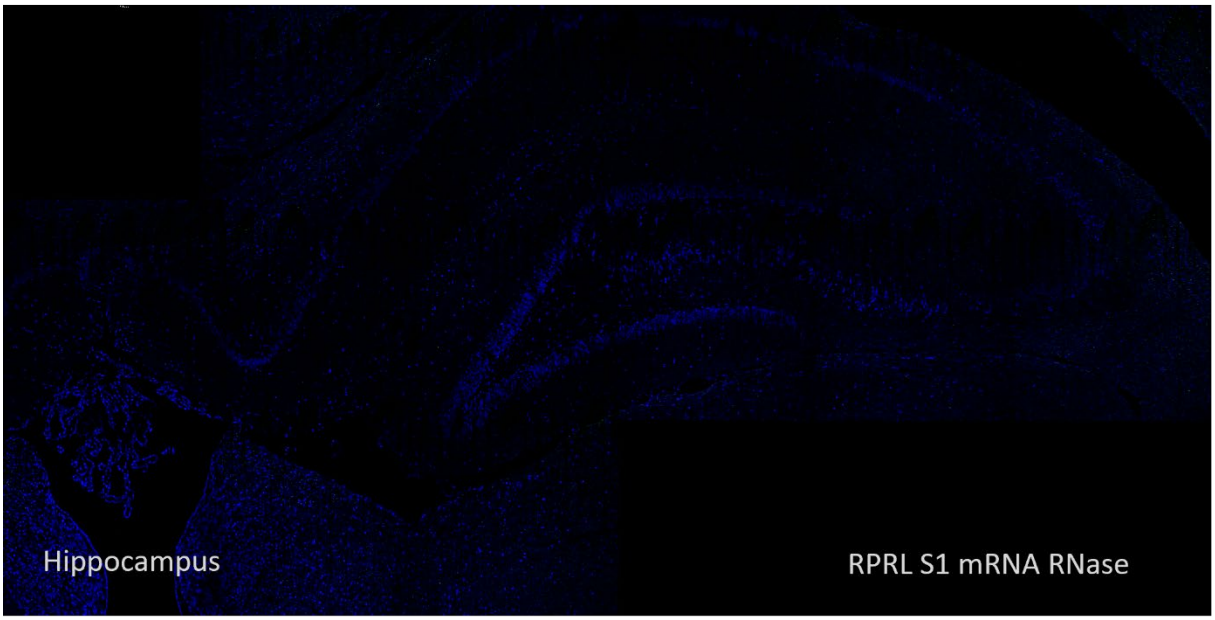
Hybridization controls were performed under the following conditions:

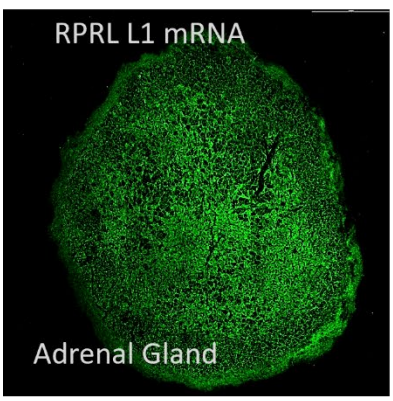
- Positive control for rat PRL mRNA: pituitary gland and hippocampus, obtained from female (48 hours of lactation) rat.
- Positive control for rat PRLR-L1 and RPRL-S1: adrenal gland, obtained from untreated female rat in estrous cycle.
- Negative control: pituitary gland, adrenal gland, and hippocampus.
- Negative control: same quantity of sense and antisense probes.
- Negative control: pre-treatment of tissue with RNase enzyme.

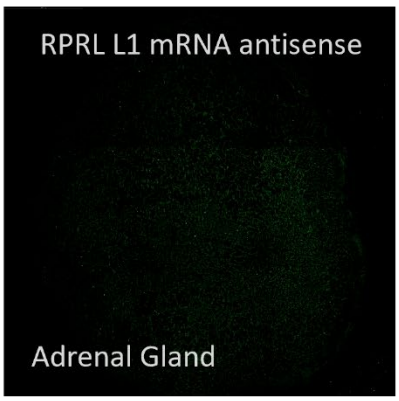
The following images show some of the results obtained for positive and negative controls for in situ hybridization in the pituitary gland, hippocampus, and adrenal gland.




Hippocampus
RPRL L1 mRNA RNase


Hippocampus
RPRL S1 mRNA RNase


RPRL L1 mRNA
Adrenal Gland


RPRL L1 mRNA antisense
Adrenal Gland

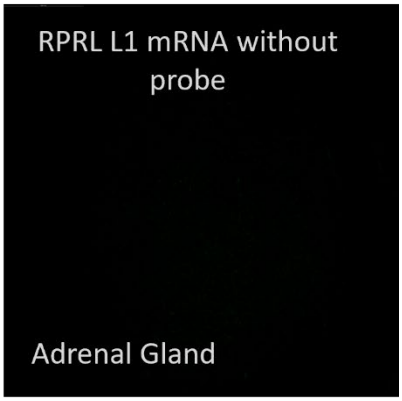

RPRL L1 mRNA without probe
Adrenal Gland

Image Analysis

Images were obtained with a confocal microscope Leica Stellaris located in the Castille and Leon Institute of Neurosciences. Magnification was x20 and resolution 2048x2048. The following hippocampal regions were photographed: CA1, CA3, and DG. A Z-Stack was performed and exported images were max exposure composition of all the photos made of the Z axis in the same X and Y position. Same pinhole, gain, brightness, and contrast were used to obtain the digital microphotographs.

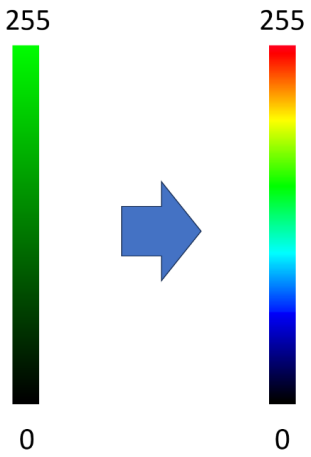
Densitometric and morphoplanimetric analysis

Using ImageJ-Fiji® (free software from NIH, USA. See features in the western blotting section) a densitometric and morphoplanimetric analysis of the digital images was carried out, determining:

- From 10 microphotographs per animal (to analyze fluorescence intensity for prolactin in situ hybridization, prolactin immunohistochemistry, prolactin receptor long isoform L1 in situ hybridization, and prolactin receptor short isoform S1 in situ hybridization):
  - The grey mean values for the oriens, pyramidal and radiate layers in CA1 and CA3 regions of Cornu Ammonis.
  - The grey mean values for the molecular, granulate and polymorphic layers in DG.
- From 400 positive neurons per animal:
  - The grey mean values for the pyramidal or granular mRNA-PRL-positive neurons in CA1 and CA3 regions or DG.
  - The cellular area of the pyramidal or granular mRNA-PRL-positive neurons in CA1 and CA3 regions or DG.
- From 4000 neurons (positive or negative) per animal:
  - The percentage of positive neurons of the pyramidal or granular mRNA-PRL-positive neurons in CA1 and CA3 regions or DG.

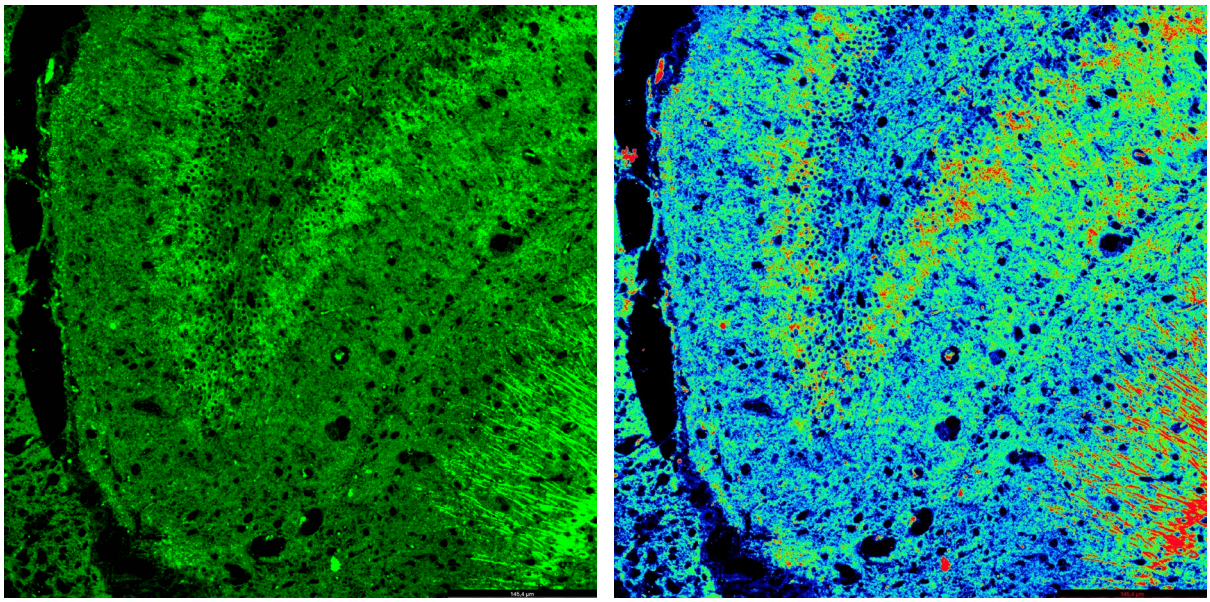
Immunofluorescence Gradient Python Script

Immunofluorescence images were processed using a custom Python script to generate the intensity gradient images. The script assigns an intensity value that ranges from 0 to 255 in the original photo and replaces that pixel on a scale where “cold” colors represent low intensity values and “warm” colors are higher values, resulting in an image where the most intense pixels can be easily detected. The main difference between this gradient and others used in the industry is that the purple color has been removed from the scale, as it usually appears between blue and teal colors and we consider purple a “warm” color, confusing the interpretation of the images. The script was programmed in Python 3.8.6 by Julian Happel (Happel, J. (2022). IF-Grad: Fluorescent image



gradient converter (Version 0.1.2) [Computer software]. https://github.com/chaotix1992/IF-Grad).

The following images show the conversion from a microscopic fluorescent picture into a gradient image.



Molecular Study

Western Blotting Technique

Protein Extraction and BCA Essay

After tissue collection, fresh samples were immediately frozen in liquid nitrogen and stored at -80 °C until protein extraction. At the time of the assay, the samples were unfrozen, and they were then placed in 1,5 ml tubes with 100 µL of RIPA buffer (Cell Signaling Technology®). #9806S), diluted with protease and phosphatase inhibitors (Life Technologies®. #A32957 and #A32955). The tubes were shock-frozen at least three times by placing them in dry ice to freeze and scratch on a grid. Once the tissue was completely dissolved, the samples were centrifuged for 15 minutes at 12.000 rpm at 4 °C.

The protein concentration was calculated using a bovine serum albumin (BSA) kit (Thermo Fisher Scientific Pierce™ BCA Protein Assay Kit. #23227) following the manufacturer's instructions.

Electrophoresis Gel and Blotting

To visualize all possible prolactin bands, the concentration of acrylamide in the separating gel was 8%, while the concentration of the stacking gel was 5%.

	Separating Gel	Stacking Gel
Acrylamide Concentration	8%	5%
Water	4,62 ml	3,09 ml
1,5 M Tris pH=8,8	2,5 ml	0,57 ml (pH=6,8)
30% Acrylamide, 29:1	2,67 ml	0,75 ml
10% SDS	100 µl	45 µl
10% APS	100 µl	45 µl
TEMED	10 µl	4,5 µl
Total	10 ml	4,5 ml

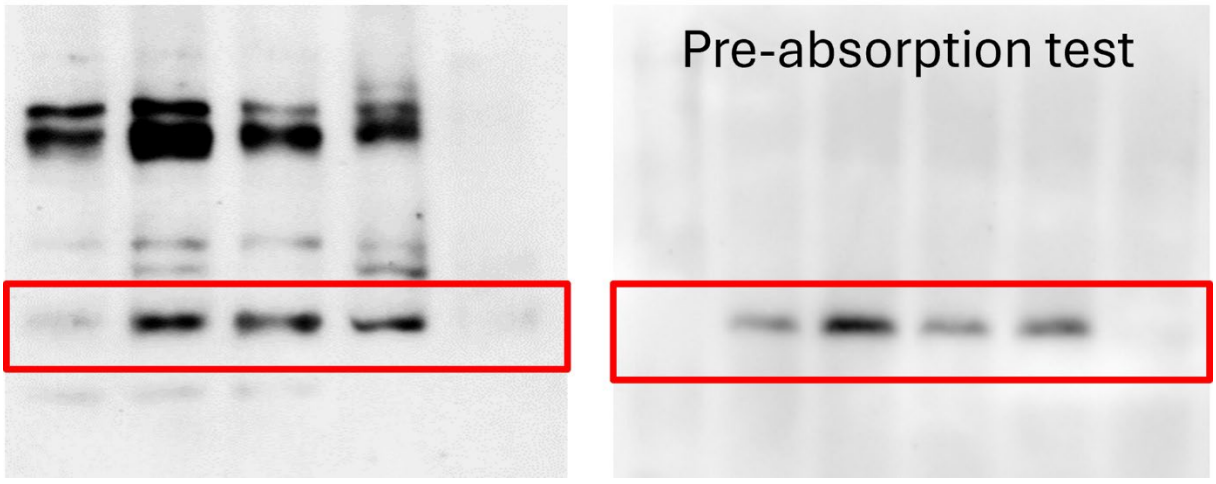
The protein load was 50 µg in 30µl, previously boiled in Laemmli buffer (BioRad®. #1610747) for 5 minutes at 95°C. Each gel had 15 wells, of which 10 were used for samples and the other 2 were used as label markers (load of 8 µl of BioRad Precision Plus Protein Dual Color Standards®. #1610374). SDS-PAGE was performed at a constant 100V for 1.5 hours. The separated proteins were blotted onto nitrocellulose membranes (Bio-Rad® #1620115) at constant 0,37 A for 1 h and 45 min.

Membrane Incubation

The membrane was blocked with TBST 5% skim milk solution (GERBU®) #1.602.500) for 1 hour. Primary antibody Anti-Prolactin (abcam®. ab183967) was diluted in TBST + 5% milk at a concentration of 1/1000 and incubated with the membrane overnight at 4 °C in a mixer. After three washes, the cells were incubated with the secondary antibody goat anti-rabbit + HRP (…®A16110) at a 1/5000 concentration for at least 2 h. Band detection was achieved using Super ECL solution (Thermo Fisher Scientific SuperSignal™ West Pico PLUS Chemiluminescent Substrate. #34580) using ChemiDoc (Bio-Rad®).

Control of the technique. Pre-absorption

Because six different bands were detected in repeated experiments, an additional pre-absorption test was performed: prior to incubation with the primary antibody, 10 ml of antibody were incubated with 3 µg rat prolactin protein (Abcam® ab54140). All bands, except for one around 60-65 kDa disappeared (see the following image).



Therefore, this band will not be considered prolactin, and thus will not be mentioned in the results.

Reagents

Reagent	Manufacturer	ID
TBST	Carl Roth GmbH	1061.1
Tris Buffer	Carl Roth GmbH	5429.36
APS	VWR International	486-25G
Glycine	Sigma-Aldrich	33226-1kg
Methanol	ThermoFisher Scientific	11976961

Running Buffer (x10 Stock)

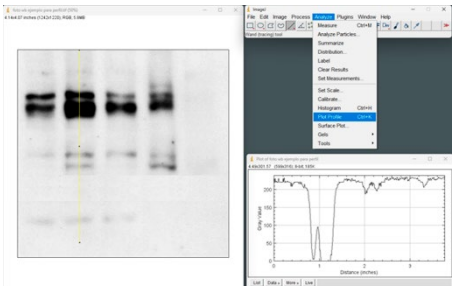
Component	Concentration	Weight or Volume
Tris base	26 mM	30,3 g
Glycine	192 mM	144,4 g
20% SDS	0,1 %	50 ml
Water		To 1 litter

Transfer Buffer

10x Concentration Stock:	When diluting to 1x Concentration:
30,3 g Tris Base	100 ml of 10xStock
144 g Glycine	200 ml Methanol
Water up to 1 litter	700 ml Water

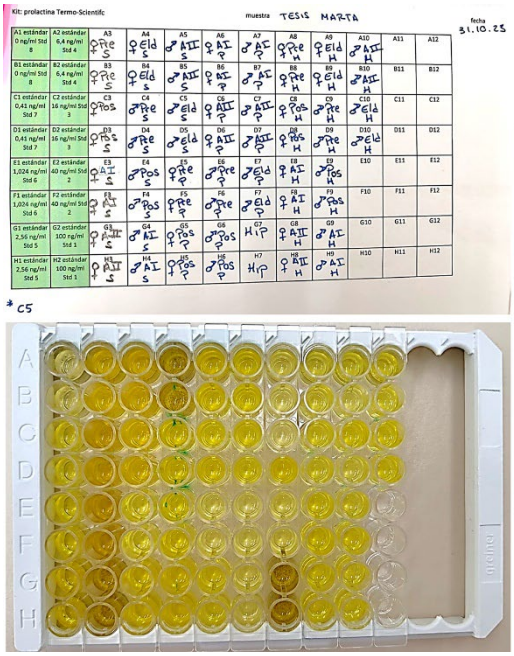
Densitometric analysis of western blotting

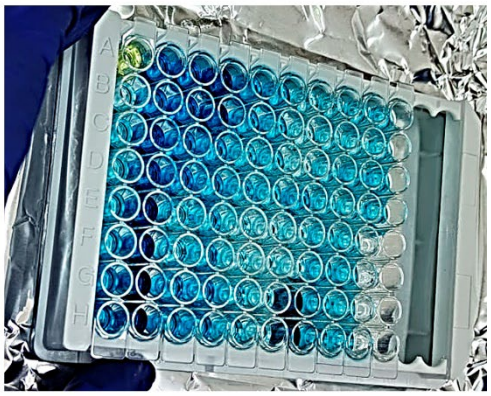
Because the determination of prolactin levels was performed by ELISA, no quantification of the bands was made, and instead, a qualitative analysis of the profiles of each sample group was performed. To do this, ImageJ-Fiji® application has been used. ImageJ-Fiji® is a public domain Java image processing program inspired by NIH Image. It can display, edit, analyze, process, save and print 8-bit, 16-bit and 32-bit images. It can calculate area and pixel value statistics of user-defined selections. It can measure distances and angles. It can create density histograms and line profile plots. It supports standard image processing functions such as contrast manipulation, sharpening, smoothing, edge detection and median filtering. Spatial calibration is available to provide real world dimensional measurements in units such as millimeters. Density or grey scale calibration is also available. The adjacent image shows an example of the process to obtaining a densitometric profile with ImageJ-Fiji®.



ELISA Analysis Technique

To determine serum and hippocampal prolactin levels, a prolactin ELISA rat kit (Invitrogen® ERA50EB provided by Thermo Fisher Scientific®) was used, according to the manufacturer's instructions.





q-PCR Technique

Messenger RNA Extraction and Complementary DNA Synthesis

After brain dissection, the tissue was immediately frozen in liquid nitrogen and stored at -80°C till RNA isolation.

Isolation was performed using RNeasy Mini and QIAshredder Kits (Qiagen®. #74104 and #79654) according to the manufacturer's instructions. RNA was stored at -80°C, and cDNA was obtained using an RNA-to-cDNA kit (Thermo Fisher Scientific. High-capacity RNA-to-cDNA™ kit #4387406) in a thermal cycler with the following settings:

Mix per Sample		Program settings	
Reagent	Quantity	Time	Temperature
2xBuffer Mix	10 µl	60 minutes	37°C
20x Enzyme	1 µl	5 minutes	95°C
RNA sample	Up to 2 µg or Up to 9 µl	For ever	4°C
Nuclease-free water	To a total of 20 µl		

The cDNA obtained was stored at -20°C till the qPCR experiment.

Cycling Chain Reaction

Primer concentration was verified after reconstitution by calculating the real concentration according to its absorbance at 260 nm using a NanoDrop spectrophotometer. Given every base has a molar extinction coefficient, and knowing the sequence of every primer, the concentration was calculated in the following manner: an example.

Prolactin Primer FORWARD sequence 5'-3'		CCACCTAGTCCAGTTATTAGTTGA	
Molar Extinction coefficient per base		Number bases	Total coefficient
A	15200	6	91200
C	7050	6	42300
G	12010	3	48040
T	8400	4	67200
Total		24	257700
Primer Absorbance at 250 nm (A)	7,925	Concentration (µM)	30,75
Concentration = (absorbance/total coefficient)*1000000			

Experiment settings

Mix per well: total volume 20 µl

- cDNA previously diluted to 20ng/µl: 2 µl
- SYBR™ Select Master Mix (Applied Biosystems™4472908) 10 µl
- Forward primer previously diluted at 20 µM: 0,1 µl
- Reverse primer previously diluted at 20 µM: 0,1 µl
- Double-distilled water: 7,8 µl

Standard cycling mode

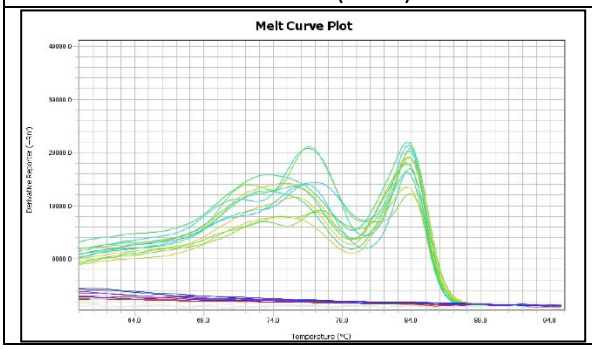
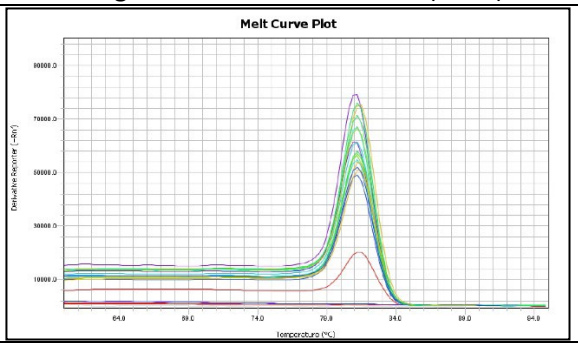
Step	Temperature	Duration	Stage
1	50 °C	2 minutes	Hold
2	95 °C	10 minutes	
1	95 °C	15 seconds	PCR x40 cycles
2	60 °C	1 minute	

Dissociation curve (Melting curve)

Step	Ramp rate	Temperature	Time
1	1,6 °C/sec	95 °C	15 seconds
2	1,6 °C/sec	60 °C	1 minute
3 (dissociation)	0,15°C/sec	95°C	5 seconds

Primer Design and BLAST tool

The primers were first tested to determine whether they were optimal for our experiments. In this test experiment, primers for prolactin were tested in pituitary gland and 48h post-birth lactation female hippocampus. The primers for prolactin receptor were tested in the adrenal gland and the same hippocampus. All primers were optimal, except for one of the selected primers for prolactin, which was previously used by a research group in Portugal (Costa-Brito et al., 2021), as the melting curve showed several peaks, and its product was over 800. To detect prolactin mRNA in our samples, we decided to use another designed by Vergara-Castañeda and Grattan in 2016 (Vergara-Castañeda et al., 2016).

Costa-Brito (2021)	Vergara-Castañeda & Grattan (2016)
	

Data Analysis

The ratio was calculated using a housekeeping gene (HKG) as the calibrator, Hprt1, which was stable in all samples, using the following formula:

$$\text{Ratio (\%)} = 2^{(-\Delta Ct)} \times 100, \text{ where } \Delta Ct = \text{Average target Ct} - \text{Average HKG Ct}$$

Primer sequences 5'-3'

Prolactin forward	CCACCTAGTCCAGTTATTAGTTGA
Prolactin reverse	CCCTAGCTACTCCTGAAGACA
Prolactin receptor Box 1 forward	AAAGTATCTTGTCCAGACTCGCTG
Prolactin receptor Box 1 reverse	AGCAGTTCTTCAGACTTGCCCTT
Prolactin receptor Box 2 forward	GCAGGTGAATGTTTCCTTGTC
Prolactin receptor Box 2 reverse	CTTGCTTTCGTCCTACTTGTTC
Housekeeping gene Hprt1 forward	CCCAGCGTCGTGATTAGCGAT
Housekeeping gene Hprt1 reverse	CGAGCAAGTCTTTCAGTCCTGTCCATA

Statistic Treatment of Data

The values obtained were statistically analyzed using the GraphPad Prims (version 8.4.3).

Descriptive statistics (minimum, maximum, range, arithmetical mean, standard deviation, and standard error of the mean were calculated), and one-way ANOVA analysis was carried out.

Significance of multiple comparisons was checked by a Tukey test. The confidence interval was 95%, where p value was significant if $p<0.05$ and very significant if $p<0.01$ or more.

Prolactin levels obtained by ELISA in serum and hippocampus were compared by correlation analysis using Pearson r with a 95% confidence interval.

Results

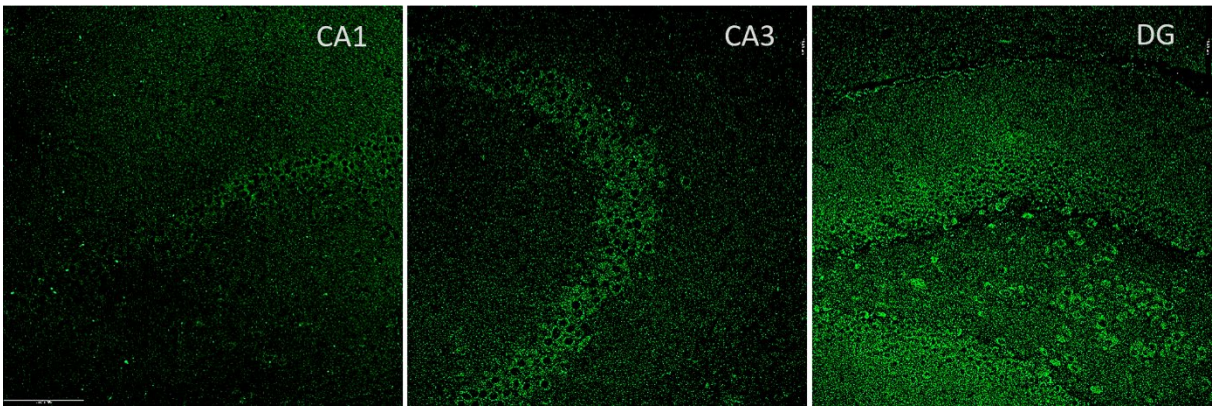
Prolactin-positive Cells in Hypophysectomized Rats

Immunohistochemical Study

The hypophysectomized rat is an excellent experimental model to rule out that prolactin positivity in the hippocampus is due to the possibility that blood prolactin secreted by the pituitary gland reaches the hippocampus via some route.

Taking advantage of samples from previous studies conducted in our laboratory, immunohistochemistry for prolactin was performed in the brain region of postpubertal male animals.

As shown in the following images, the neurons of CA1, CA3, and DG appeared reactive after hypophysectomy, which allowed us to ensure that the origin of the hormone was not the pituitary gland.



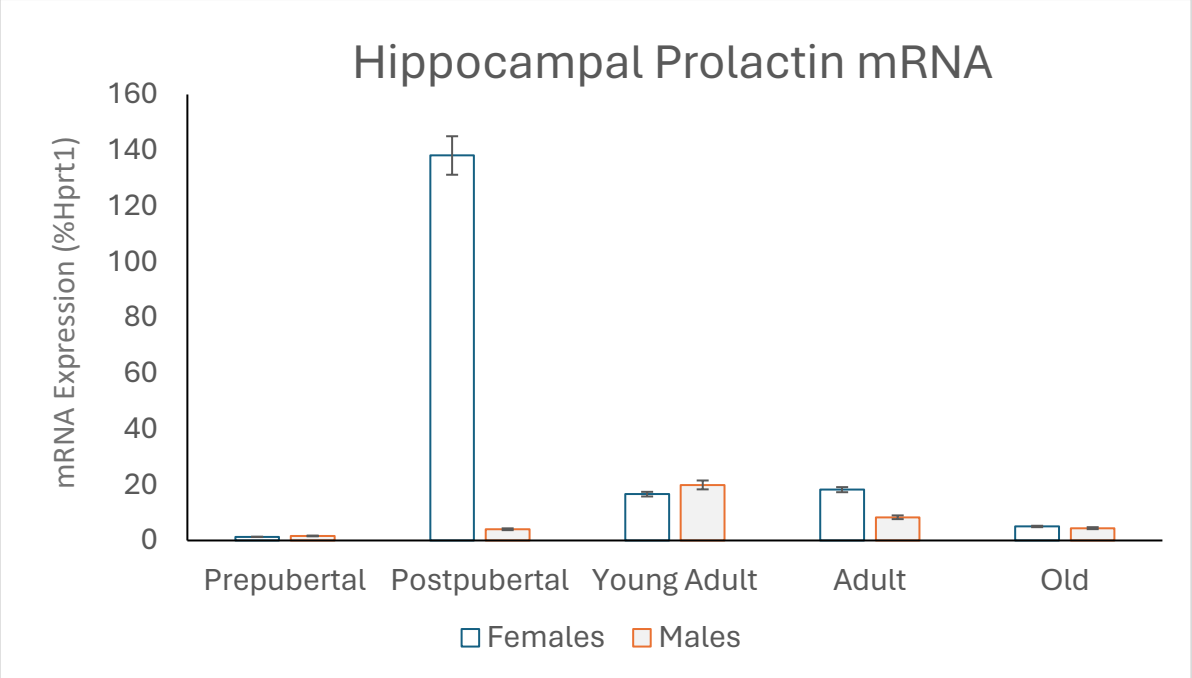
Prolactin-Synthesizing Cells. qPCR study

Hippocampal Prolactin mRNA

Messenger RNA was detected in both sexes and in all age groups. However, the results observed in both, males and females, were different. In general, the most striking value was observed in female postpubertal rats, which showed a peak in prolactin mRNA levels that was seven times greater than that observed in the other groups. In the female groups, pre-puberty and old age had very low mRNA levels, whereas in adulthood, it became more stable. In the male groups, there was a peak in the young adult group, reaching similar levels as the adult females, and it decreased to half of that in the adult group. In both males and females, the differences observed were always significant (p<0.01), except for the differences observed in Prolactin-mRNA levels among postpubertal and old-age males (no significant difference).

When sex differences were analyzed, no significant differences were found between prepubertal males and females. The other age groups showed significant differences (p<0.01); however, the statistical significance between the old female and male groups was much lower than that observed between males and females at the postpubertal, young adult, and adult stages.

Interestingly, the hippocampus that was used to adjust the protocol that belonged to a young female rat that was breastfeeding her pups, the quantities for mRNA prolactin were 77 times greater than the quantity for the Hprt1 housekeeping gene mRNA. Taking the same age group, this rat had from our samples as a reference, and the breast-feeding rat had more than 464 times more prolactin mRNA.



The following tables show the results of the One-way ANOVA test and Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**<0.0001**	Postpubertal F		
Young Adult F	**<0.0001**	**<0.0001**	Young Adult F	
Adult F	**<0.0001**	**<0.0001**	**<0.0001**	Adult F
Old F	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	**<0.0001**	**<0.0001**	Young Adult M	
Adult M	**<0.0001**	**<0.0001**	**<0.0001**	Adult M
Old M	**<0.0001**	**0.1681, NS**	**<0.0001**	**<0.0001**

	P value	
Prepubertal F	**0.3331, NS**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**<0.0001**	Adult M
Old F	**0.0063**	Old M

Prolactin-Synthesizing Cells. In situ Hybridization Study

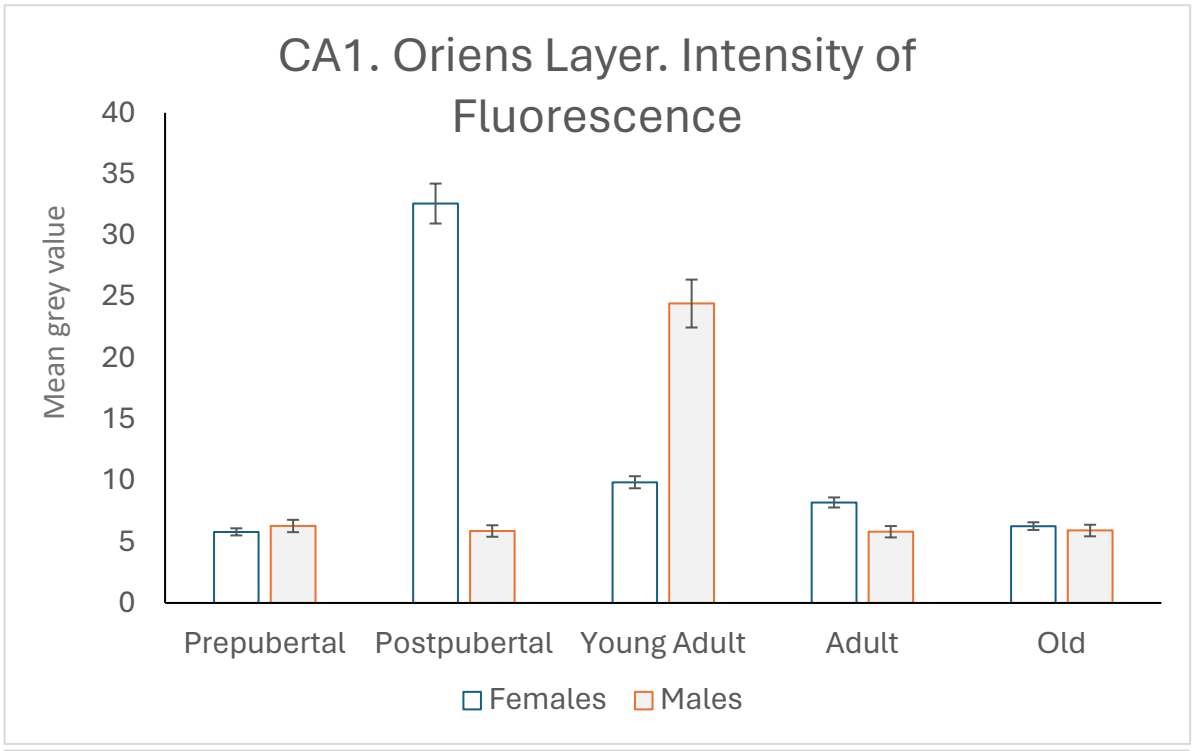
Hippocampus (Cornu Ammonis). CA1 Region

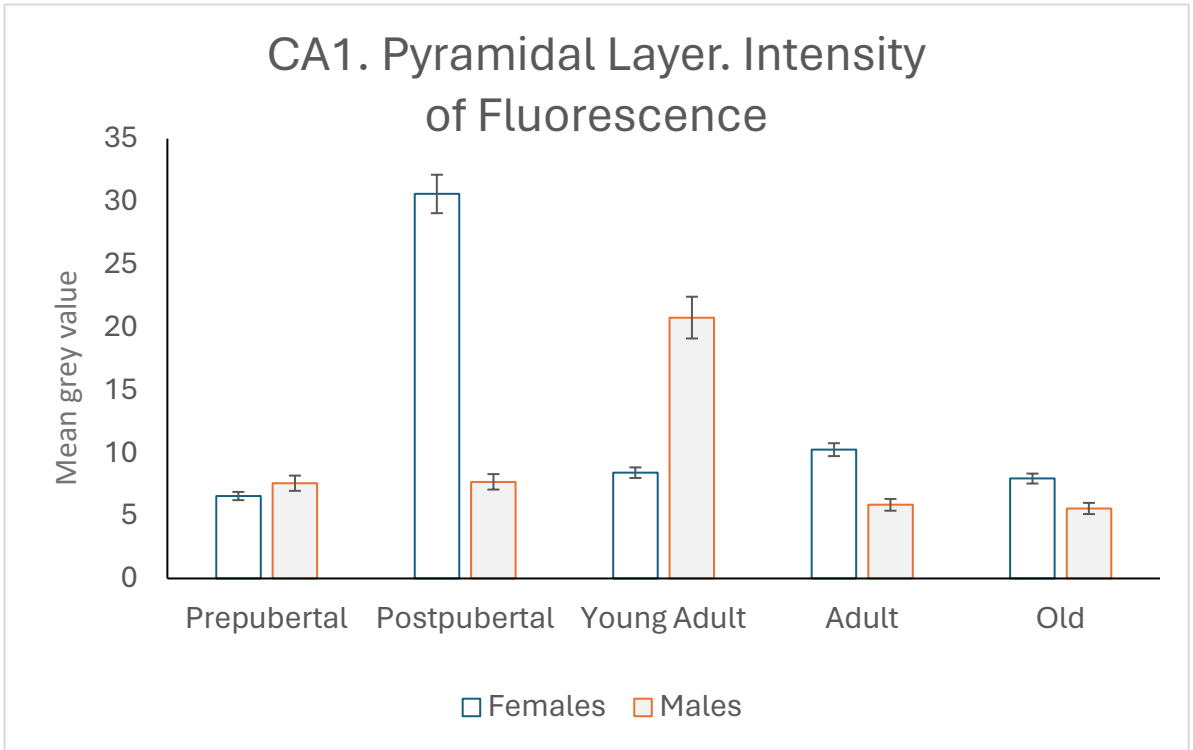
Topographic Distribution of Prolactin-mRNA

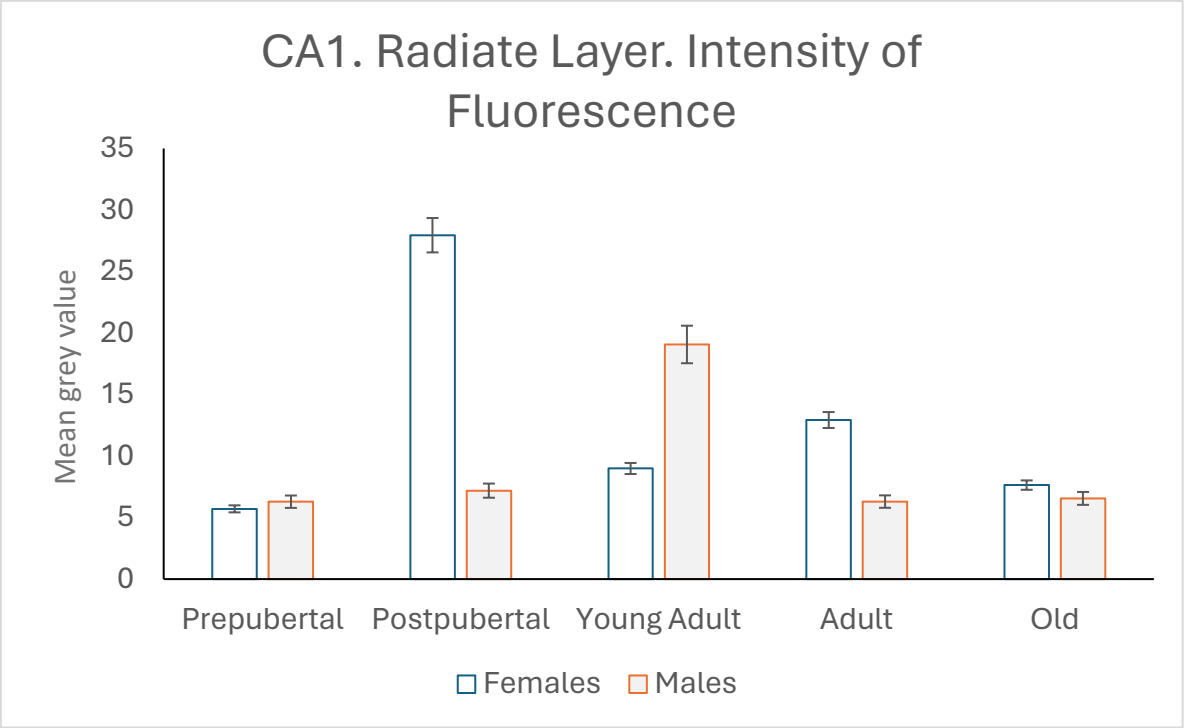
The presence of prolactin mRNA in the hippocampal CA1 region was evident in the oriens, pyramidal, and radiate layers studied. Densitometric analysis of the fluorescence intensity in the three layers revealed the existence of sexually dysmorphic results with variations related to the age of the animals. As shown in the attached graphs, these differences followed a similar pattern in the three layers.

The intensity was low in the prepubertal animals but increased significantly in the postpubertal females ($p<0.001$), with respect to the other groups of females and the prepubertal males, but not in the postpubertal males that showed similar values to the prepubertal males. In young adult animals, the fluorescence intensity decreased significantly in females, whereas in males, there was a significant increase ($p<0.001$, with respect to the other groups of males and young adult females). In the pyramidal layer, the fluorescence intensity was higher in females than in males ($p<0.05$), The intensity was low in the prepubertal animals but increased significantly in the postpubertal females ($p<0.001$), with respect to the other groups of females and the prepubertal males, but not in the postpubertal males that showed similar values to the

prepubertal males. In young adult animals, the fluorescence intensity decreased significantly in females, whereas in males, there was a significant increase ($p<0.001$, with respect to the other groups of males and young adult females). In the pyramidal layer, the fluorescence intensity was higher in females than in males ($p<0.05$), except in the young adult animals.







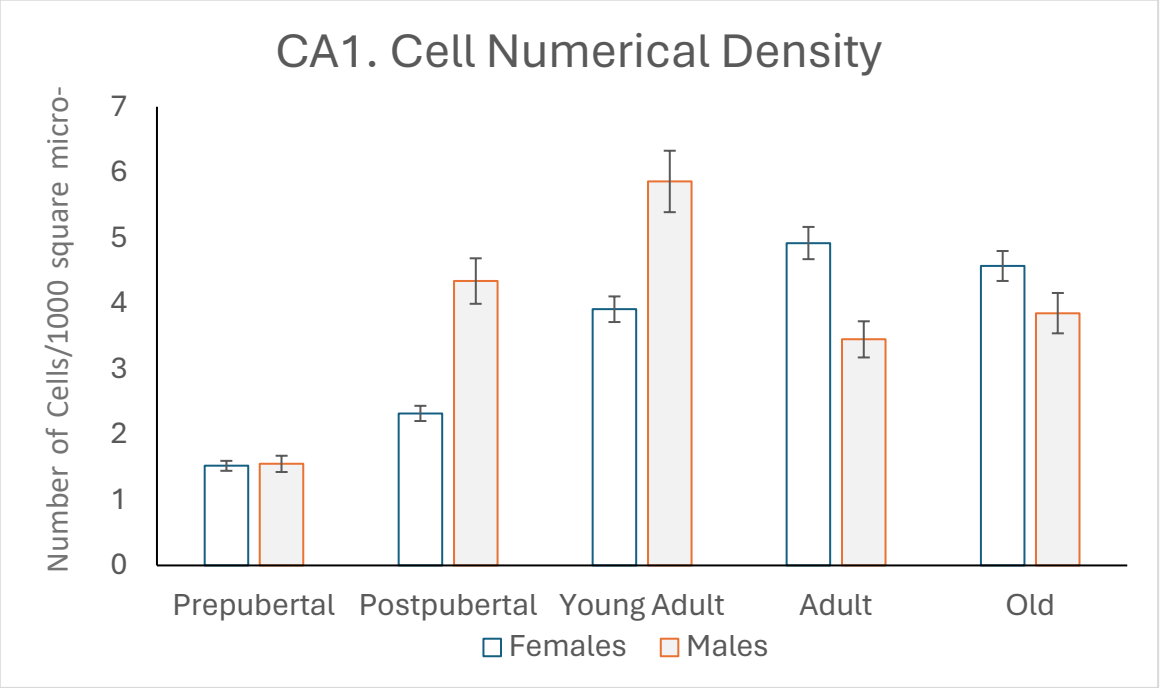
Cell Numerical Density of Pyramidal Layer

As the graph and accompanying tables show, the number of cells per thousand square micrometers of the surface area in the pyramidal layer of the CA1 region varied greatly in relation to age and sex.

The lowest numerical density was observed in prepubertal animals, with no significant differences between males and females. From the postpubertal stage, numerical density began to increase in both sexes, but in a different manner. The differences observed between males and females, except for the prepubertal stage, were always statistically significant. Numerical density was higher in postpubertal males and young adults than in females. In contrast, it was higher in adult and older females than in males.

In females, in a statistically significant manner, the numerical density increased from the postpubertal stage to the adult stage, in which the increase was slowed and decreased slightly, but not significantly, in old female rats.

In males, in a statistically significant way, the numerical density increased from the postpubertal stage to the young adult stage, in which the increase slowed and decreased significantly from the adult stage onwards and remained in the old male rats.



The following tables show the results of the One-way ANOVA test and Tukey test (F: Female, M: Male, NS: No significant).

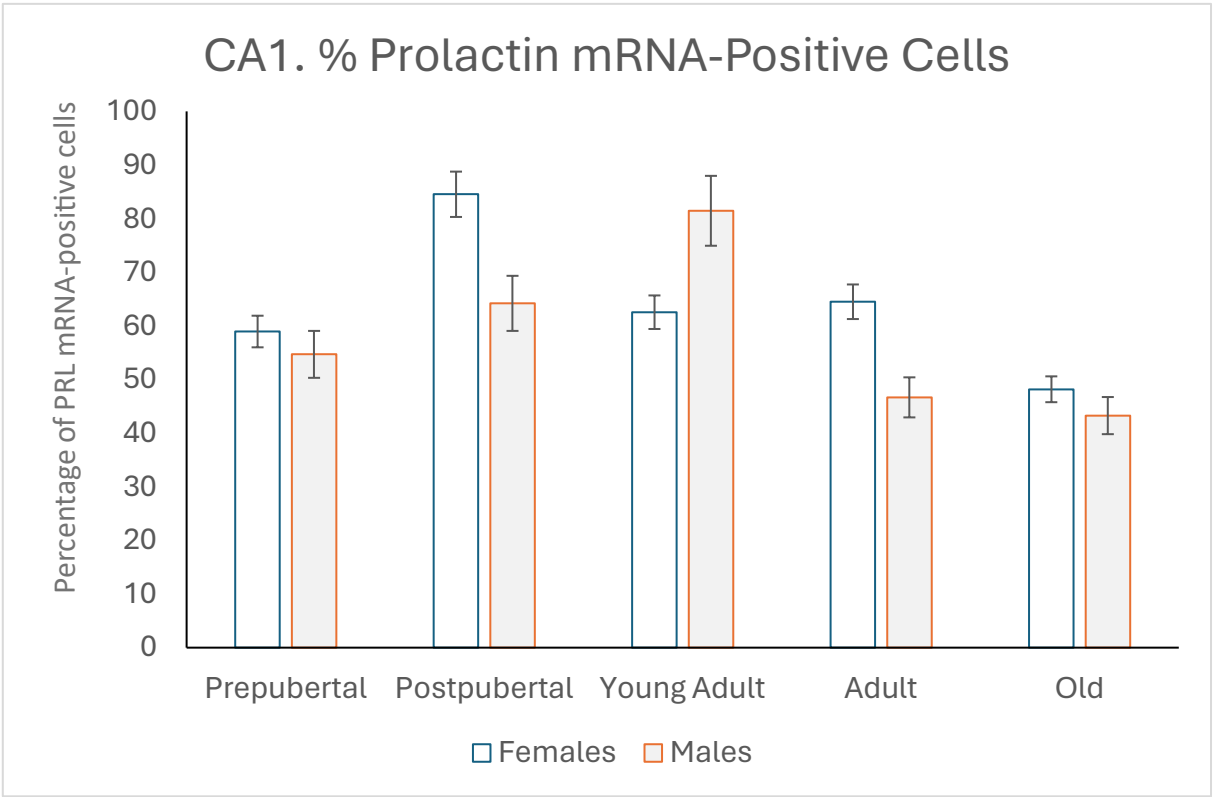
P value	Prepubertal F			
Postpubertal F	**<0.0004**	Postpubertal F		
Young Adult F	**<0.0001**	**<0.0001**	Young Adult F	
Adult F	**<0.0001**	**<0.0001**	**<0.0001**	Adult F
Old F	**<0.0001**	**<0.0001**	**0.0048**	**0.4587, NS**
P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	**<0.0001**	**<0.0001**	Young Adult M	
Adult M	**<0.0001**	**<0.0001**	**<0.0001**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**0.2790, NS**

	P value	
Prepubertal F	**>0.9999, NS**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**<0.0001**	Adult M
Old F	**0.0017**	Old M

Percentage of Prolactin mRNA-Positive-Cells

When analyzing the percentage of neurons in the pyramidal layer of the CA1 region that express prolactin mRNA (see graph and attached tables), a high percentage of prolactin mRNA-positive neurons was observed in prepubertal animals, with no significant differences between males and females. In postpubertal female rats, the highest percentage of neurons expressing the messenger was observed, which was statistically significant compared to the rest of the studied female groups. The percentage decreased significantly in young adult females, remained the same in adult females, and decreased in old females below prepubertal values.

In male rats, from the postpubertal stage onwards, a statistically significant increase in the percentage of positive neurons was observed, reaching its maximum value in young adult males. From the adult stage, the percentage decreased significantly, even below the prepubertal values, and remained the same in old males. When comparing the sexes, it was observed that there were no differences between males and females in the prepubertal and old stages, while the other differences were significant. The values observed in adult males were similar to those observed in older females.



The following tables show the results of the One-way ANOVA test and Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**<0.0001**	Postpubertal F		
Young Adult F	**0.3525, NS**	**<0.0001**	Young Adult F	
Adult F	**<0.0202**	**<0.0001**	**0.9478, NS**	Adult F
Old F	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	**<0.0001**	**<0.0001**	Young Adult M	
Adult M	**<0.0001**	**<0.0001**	**<0.0001**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**>0.9999, NS**

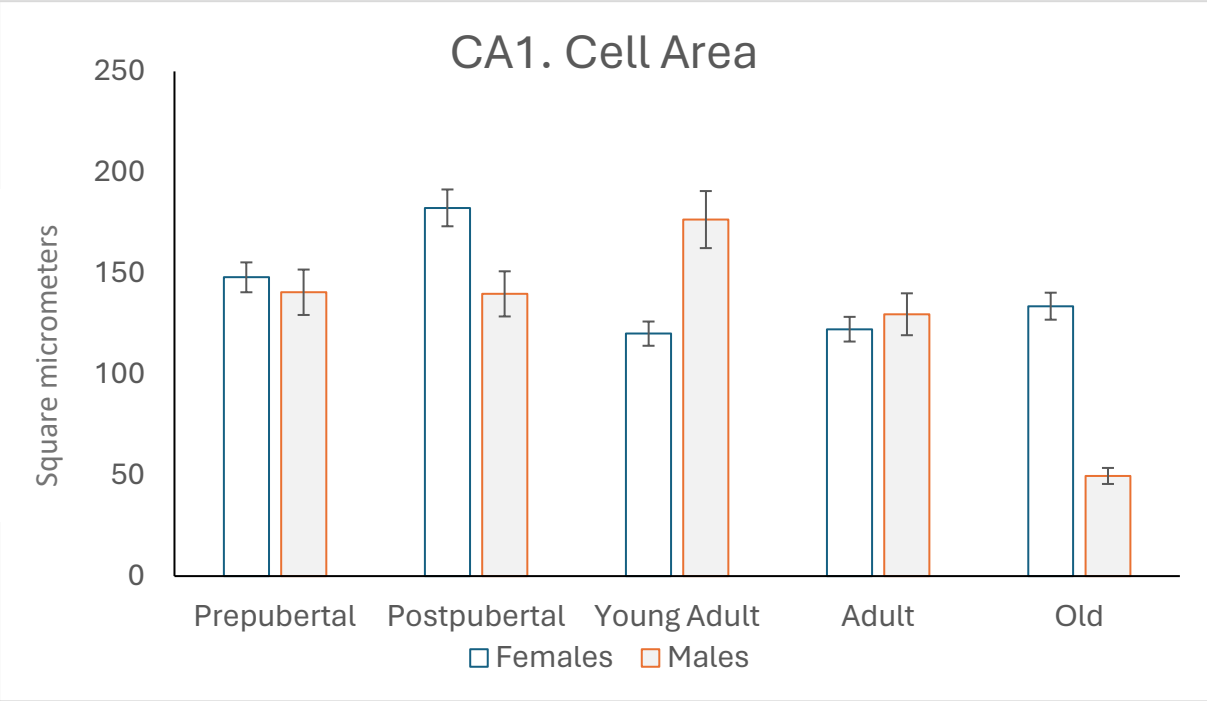
	P value	
Prepubertal F	**0.1576, NS**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**<0.0001**	Adult M
Old F	**0.0598, NS**	Old M

Cell Size of Prolactin mRNA-Positive-Neurons

In female rats, the cell area of prolactin mRNA-positive pyramidal neurons was very similar at all ages studied, with no statistically significant differences, except for the group of postpubertal females in which prolactin mRNA-positive pyramidal neurons were significantly larger than those observed in the rest of the groups studied (see graph and attached tables).

In male rats, prolactin mRNA-positive pyramidal neurons exhibit two important age-related variations. First, young adult animals had significantly larger prolactin mRNA-positive pyramidal neurons than those in the other age groups. The second variation consisted of a significant decrease in the cellular area of the prolactin mRNA-positive pyramidal neurons of old male rats (see graphs and attached tables).

The prolactin mRNA-positive pyramidal neurons of prepubertal and adult rats showed no difference between males and females. In the case of postpubertal and old rats, prolactin mRNA-positive neurons were significantly larger in females than in males, whereas in young adult rats, prolactin mRNA-positive neurons were significantly larger in males than in females, presenting a size very similar to that observed in postpubertal female rats.



The following tables show the results of the One-way ANOVA test and Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**<0.01**	Postpubertal F		
Young Adult F	**0.10, NS**	**<0.0001**	Young Adult F	
Adult F	**0.18, NS**	**<0.0001**	**>0.99, NS**	Adult F
Old F	**0.89, NS**	**<0.0001**	**0.92, NS**	**<0.97, NS**
P value	Prepubertal M			
Postpubertal M	**>0.99, NS**	Postpubertal M		
Young Adult M	**<0.007**	**<0.005**	Young Adult M	
Adult M	**0.98, NS**	**0.99, NS**	**<0.0001**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

	P value	
Prepubertal F	**0.99, NS**	Prepubertal M
Postpubertal F	**<0.0004**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**>0.99, NS**	Adult M
Old F	**<0.0001**	Old M

Morphological Features of Prolactin mRNA-Positive-Cells

Female Rats

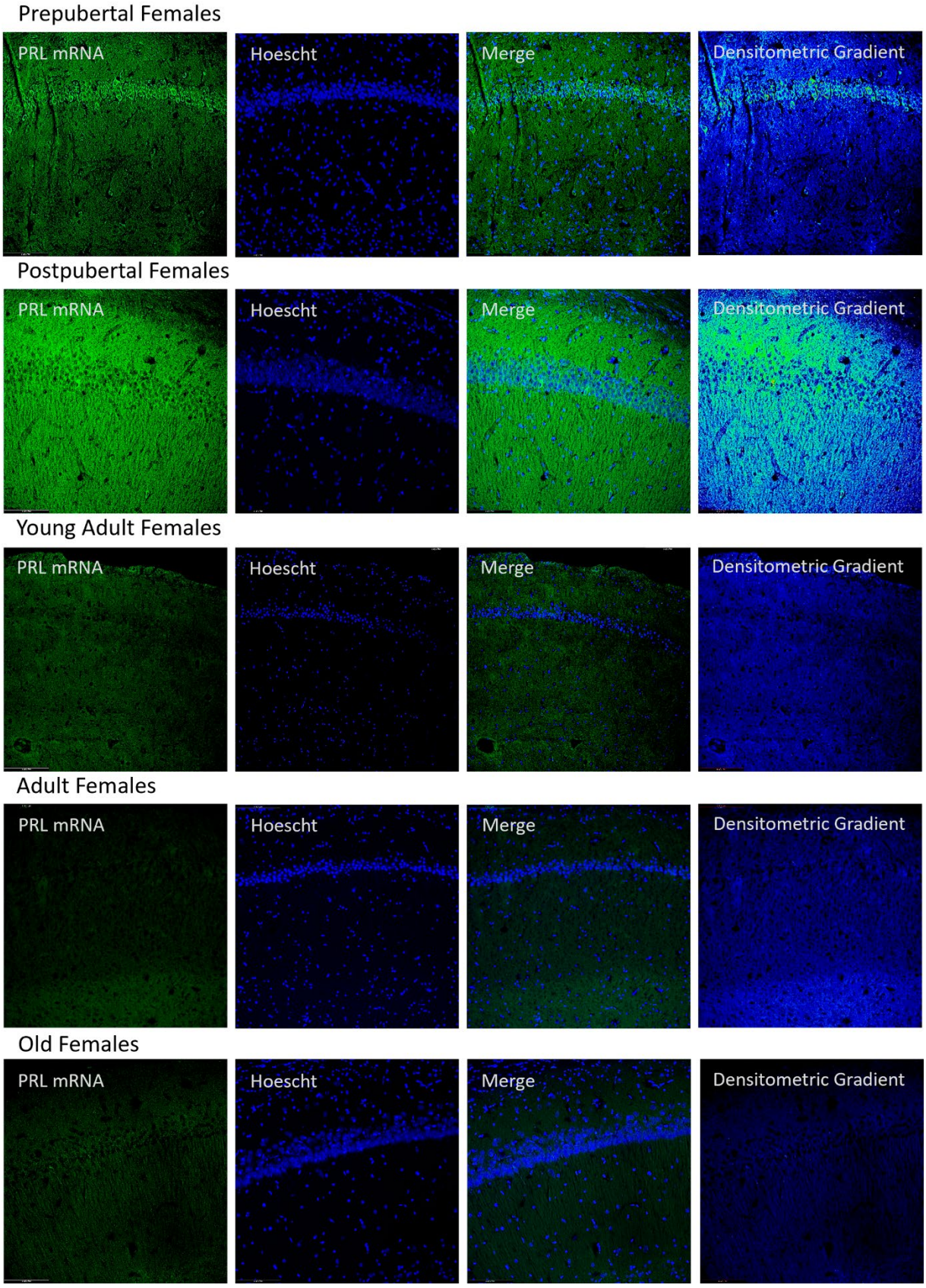
In prepubertal females, most prolactin mRNA-positive neurons were in the pyramidal layer of this region and were characterized by a punctiform or granular reaction in their cytoplasm. It was striking that among homogeneous and weakly pyramidal reactive neurons, more reactive and isolated cells appeared indistinctly close to the radiated or molecular layer. In the CA1 region of these animals, secondary neurons were observed, isolated, and scattered in the molecular layer and were smaller in size in the radiated layer. The dendrites and axons of these neurons hardly reacted, although the densitometric gradient showed that the areas closest to the pyramidal layer of the oriens and radiated layers had a greater reaction intensity.

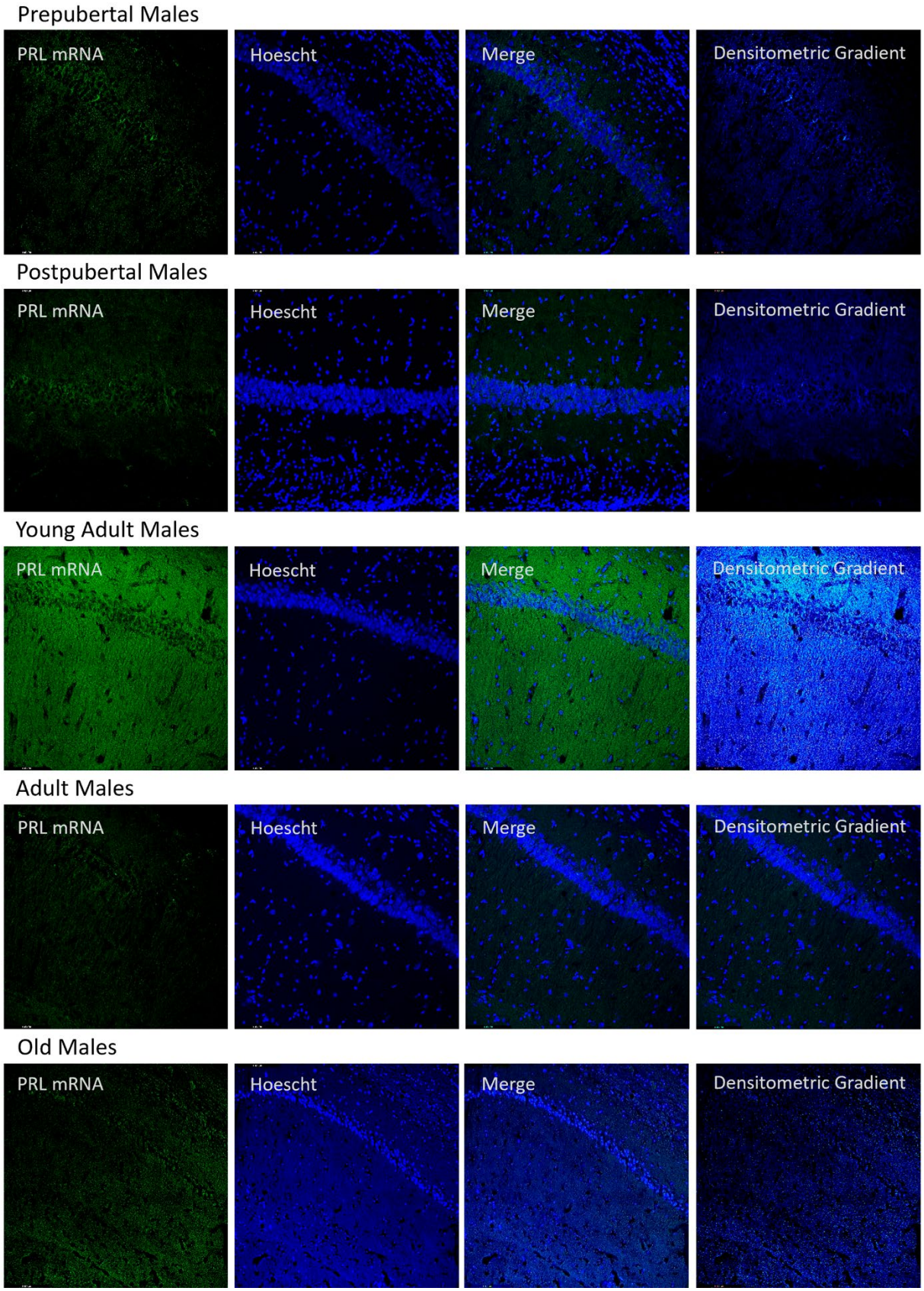
Very different results were observed in post-pubertal females. Practically all CA1 pyramidal neurons were positive for prolactin mRNA, and the positivity in dendrites and axons was very striking, affecting the orientation and radiated layers. As shown by the densitometric gradient, the fluorescence intensity was considerably higher than that observed in prepubertal females. In these animals, some secondary neurons of the oriens and radiate layers and even the lacunous layer were strongly positive.

The hyperexpression of prolactin mRNA observed in postpubertal females decreased in young adult females, and even more so in adult and old females. In all cases, a punctiform reaction appeared, which made it difficult to identify and delimit the neurons. These animals had hardly any positive secondary neurons and only pyramidal neurons were positive. Densitometric gradients showed a decrease in prolactin mRNA expression in young to old adulthood.

Male Rats

The appearance and distribution pattern of prolactin mRNA in prepubertal males was like that observed in female rats.
The reaction was almost exclusively limited to the pyramidal layer, where some highly reactive neurons appeared, with positive extensions to the radiated and oriens layers. However, the reaction was not homogeneous, with areas that were more reactive than others.
The morphological pattern in postpubertal males, contrary to what happened in females, was like that of prepubertal males, with a predominance of reaction in the pyramidal layer in which highly positive neurons appeared. However, postpubertal males showed fewer reactive prolongations and some positive secondary neurons, mainly in the radiated layer.
Also, unlike what happened in females, young adult males were the ones who showed the highest positivity for prolactin mRNA. The increased reaction affected all CA1 layers equally, with many strongly positive neurons in the pyramidal layer and positivity in almost all neuronal prolongations in the radiated and oriens layers. Positive secondary neurons were not observed in these animals.


Prepubertal Females
PRL mRNA
Hoescht
Merge
Densitometric Gradient
Postpubertal Females
PRL mRNA
Hoescht
Merge
Densitometric Gradient
Young Adult Females
PRL mRNA
Hoescht
Merge
Densitometric Gradient
Adult Females
PRL mRNA
Hoescht
Merge
Densitometric Gradient
Old Females
PRL mRNA
Hoescht
Merge
Densitometric Gradient


Prepubertal Males
PRL mRNA
Hoescht
Merge
Densitometric Gradient
Postpubertal Males
PRL mRNA
Hoescht
Merge
Densitometric Gradient
Young Adult Males
PRL mRNA
Hoescht
Merge
Densitometric Gradient
Adult Males
PRL mRNA
Hoescht
Merge
Densitometric Gradient
Old Males
PRL mRNA
Hoescht
Merge
Densitometric Gradient

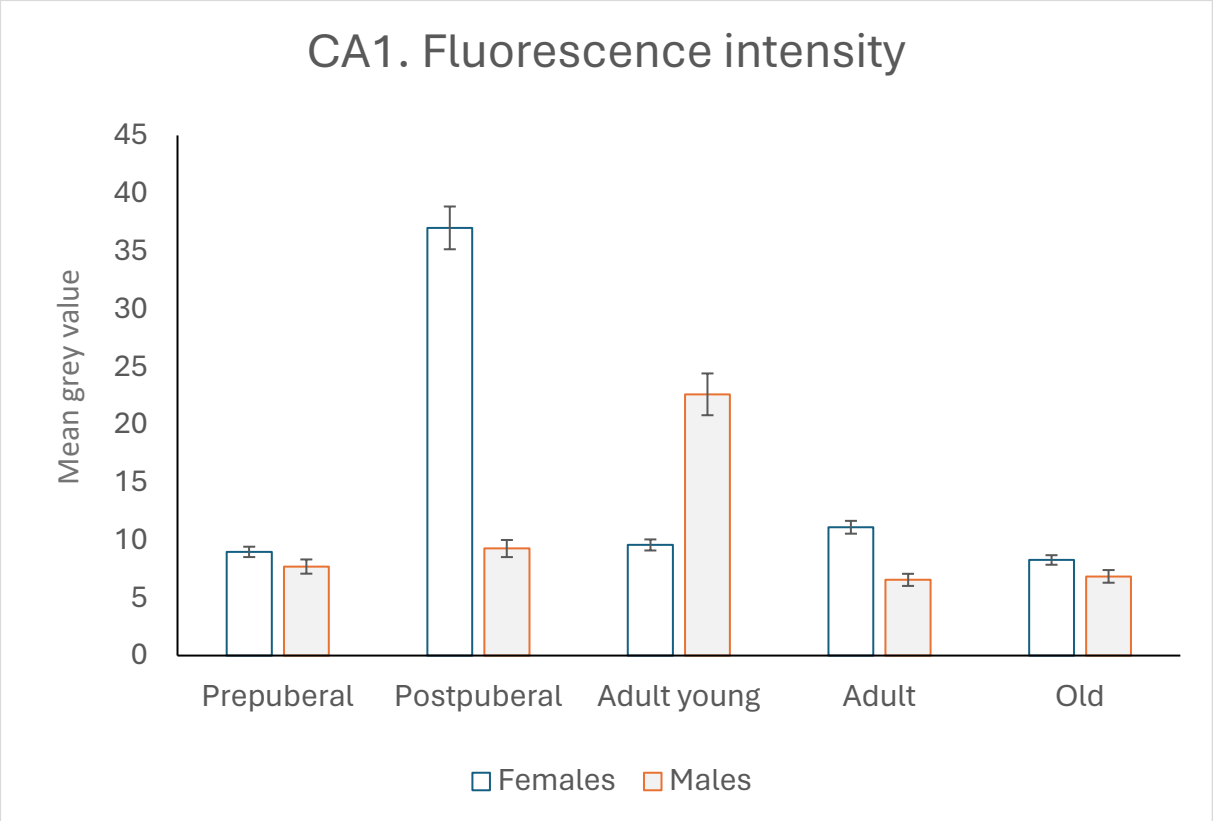
Densitometric Analysis

Densitometric analysis of prolactin mRNA positivity in CA1 pyramidal neurons revealed important differences between age and sex (see the graph and attached tables).

The densitometric values observed in the prepubertal, young adult, and adult females were not significantly different. However, post-pubertal females showed significantly higher intensities than those found in the other groups of female rats. The decrease in intensity observed between adult and old females was also statistically significant.

In male rats, an increase in intensity was observed in young adults, with statistically significant differences in relation to the other age groups. In addition, the decrease observed in adult and old male rats was statistically significant with respect to that in postpubertal males. There were no significant differences between prepubertal, adult, and old males.

The prepubertal and old differences observed between female and male animals were not significant.



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	*Prepubertal F*			
Postpubertal F	**<0.0001**	*Postpubertal F*		
Young Adult F	**>0.9999, NS**	**<0.0001**	*Young Adult F*	
Adult F	**0.06, NS**	**<0.0001**	**0.38, NS**	*Adult F*
Old F	**0.98, NS**	**<0.0001**	**0.49, NS**	**<0.002**
P value	*Prepubertal M*			
Postpubertal M	**0.35, NS**	*Postpubertal M*		
Young Adult M	**<0.0001**	**<0.0001**	*Young Adult M*	
Adult M	**0.74, NS**	**0.002**	**<0.0001**	*Adult M*
Old M	**0.96, NS**	**0.0134**	**<0.0001**	**>0.9999, NS**

	P value	
Prepubertal F	**0.62, NS**	*Prepubertal M*
Postpubertal F	**<0.0001**	*Postpubertal M*
Young Adult F	**<0.0001**	*Young Adult M*
Adult F	**<0.0001**	*Adult M*
Old F	**0.52, NS**	*Old M*

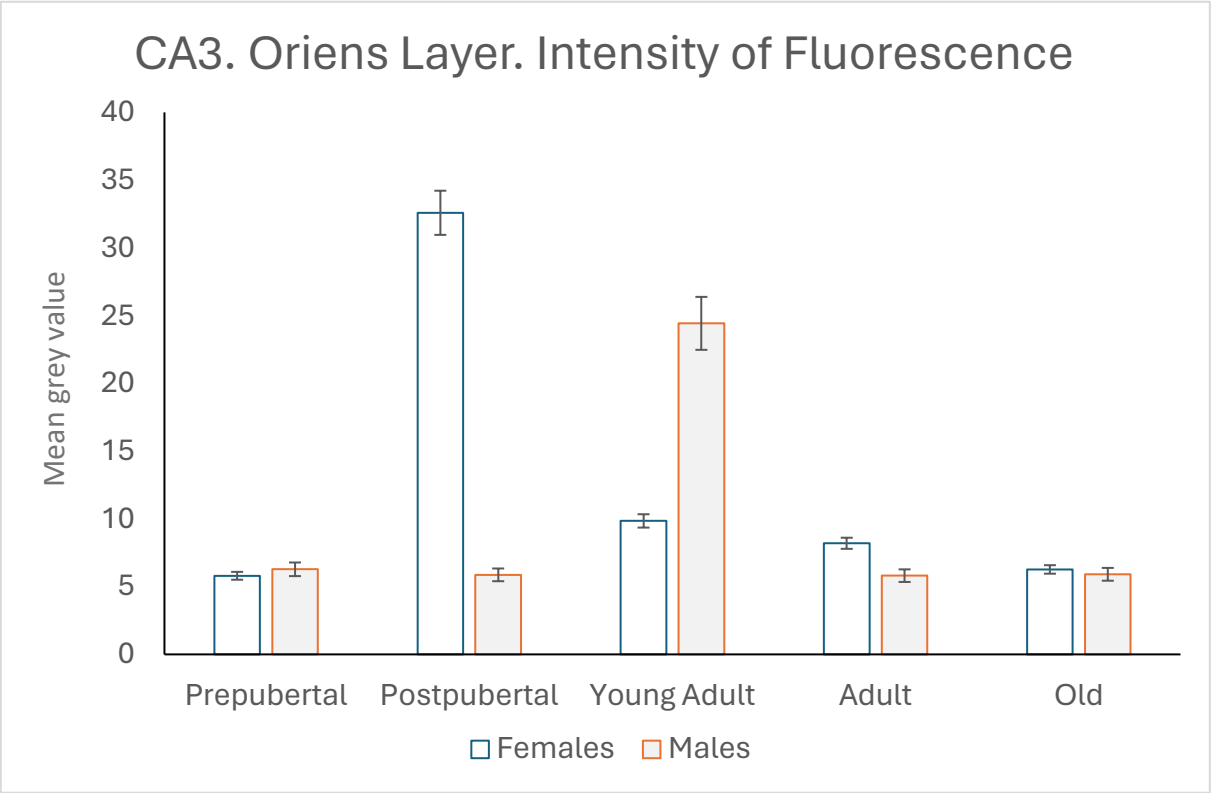
Hippocampus (Cornu Ammonis). CA3 Region

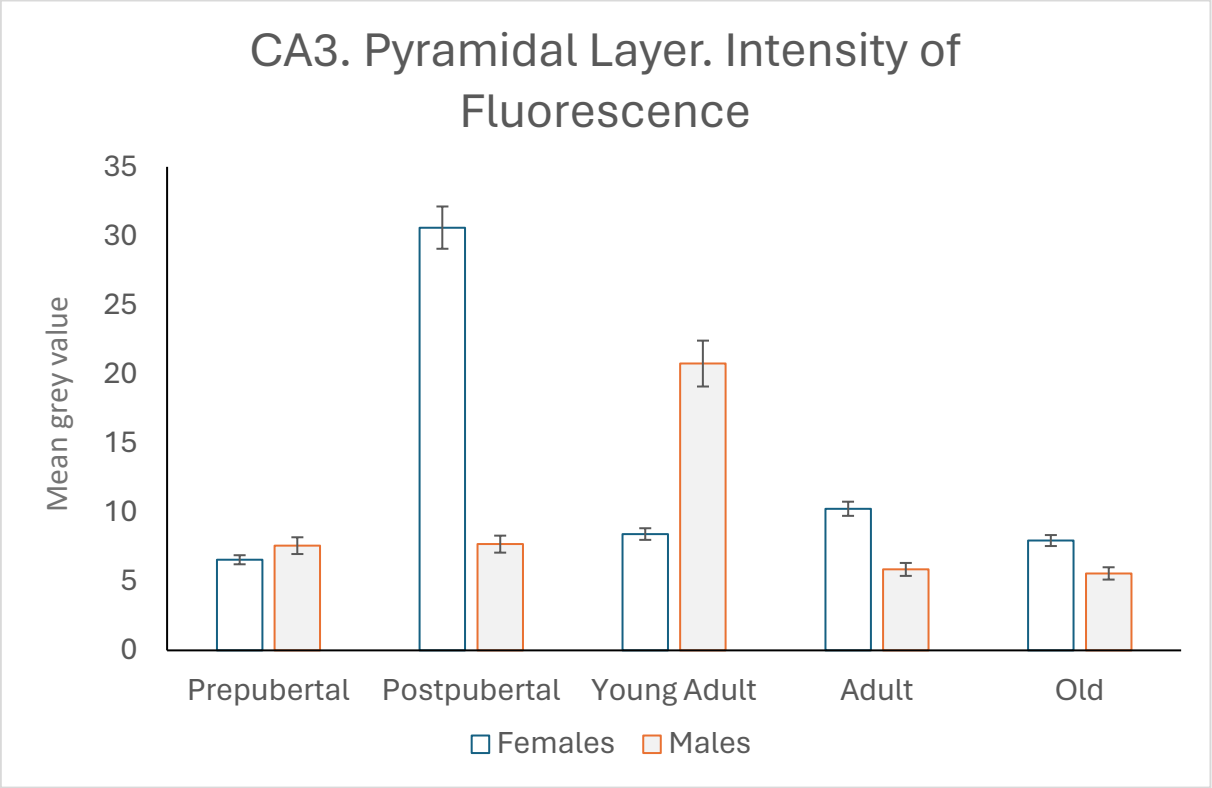
Topographic Distribution of Prolactin-mRNA

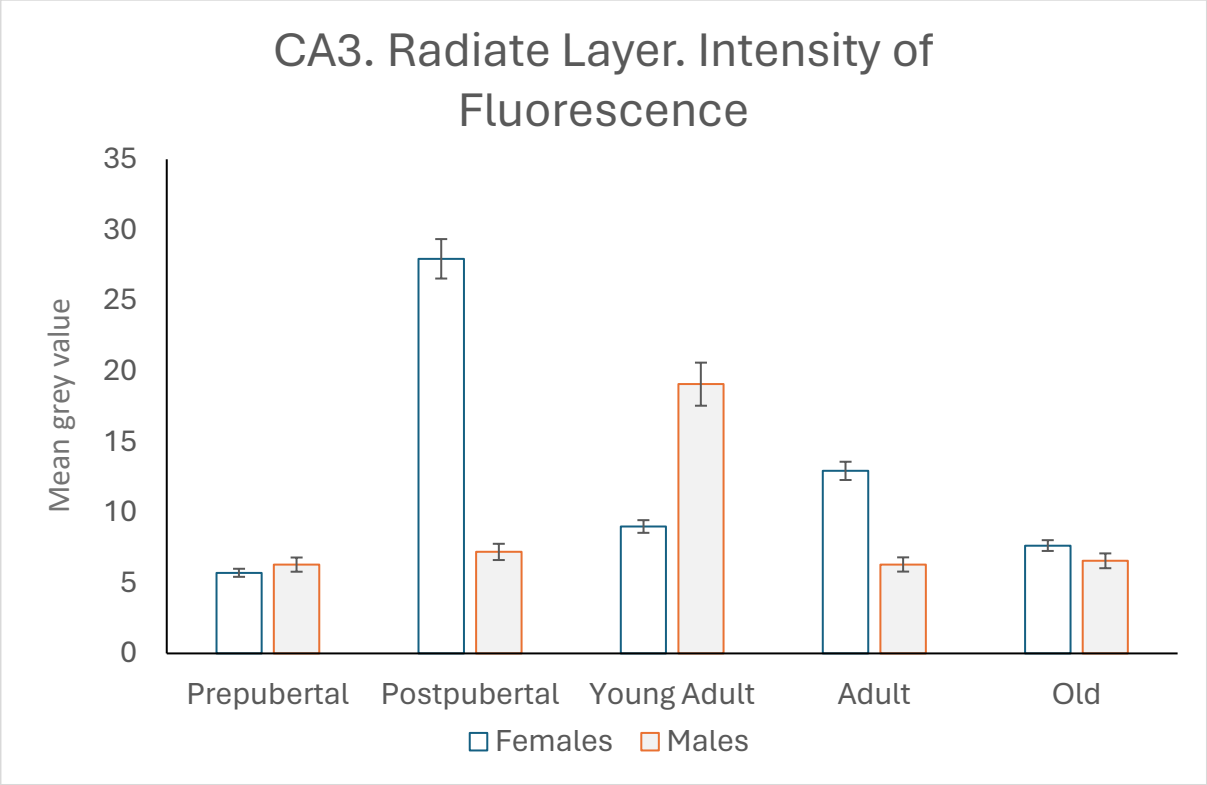
The presence of prolactin mRNA in the hippocampal CA3 region was evident in three layers studied: oriens, pyramidal, and radiate. Densitometric analysis of fluorescence intensity in the three layers revealed sexually dysmorphic results with variations related to the age of the animals.

As shown in the attached graphs, these differences followed a similar pattern in the three layers. The intensity was low in the prepubertal animals but increased significantly in the post-pubertal females ($p<0.001$, with respect to the other groups of females and the prepubertal males), but not in the post-pubertal males that showed similar values to the prepubertal ones. In young adult animals, the fluorescence intensity decreased significantly in females, whereas in males, there was a significant increase ($p<0.001$ compared to the other groups of males and young adult females).

In the pyramidal layer, the fluorescence intensity was higher in females than in males ($p<0.05$), except in young adult animals.


CA3. Oriens Layer. Intensity of Fluorescence
Mean grey value
40
35
30
25
20
15
10
5
0
Prepubertal
Postpubertal
Young Adult
Adult
Old
Females
Males


CA3. Pyramidal Layer. Intensity of Fluorescence
Mean grey value
35
30
25
20
15
10
5
0
Prepubertal
Postpubertal
Young Adult
Adult
Old
Females
Males



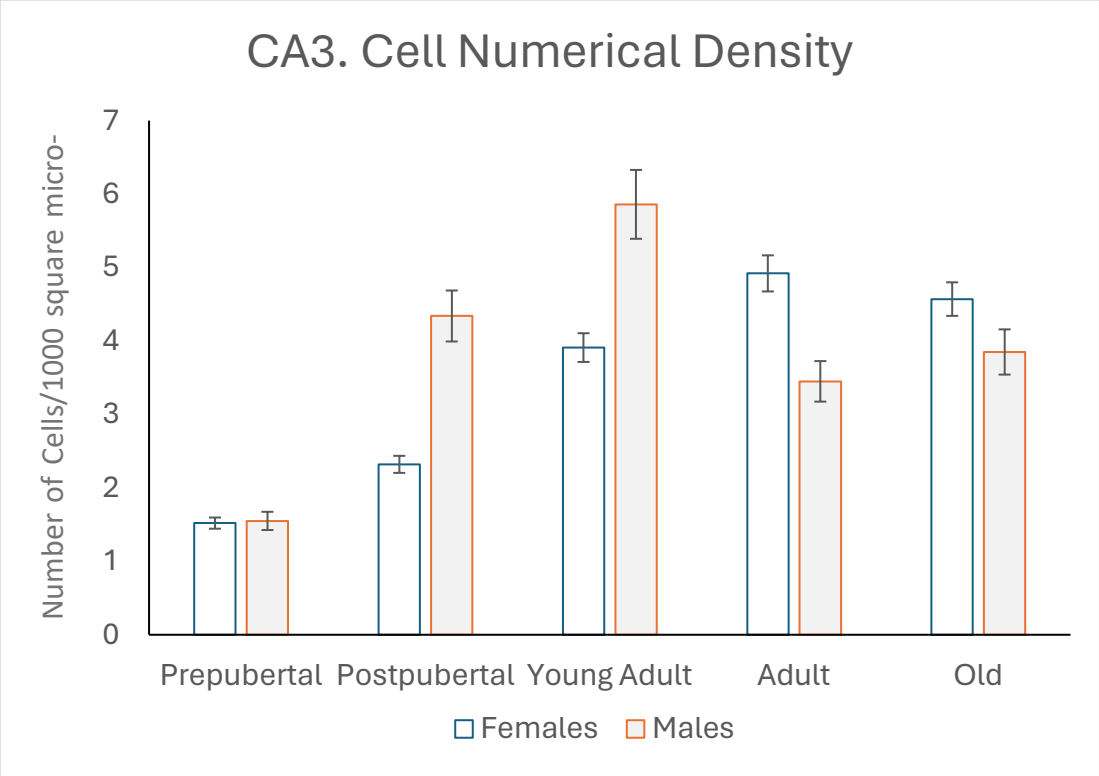
Cell Numerical Density of Pyramidal Layer

As the graph and accompanying tables show, the number of cells per thousand square micrometers of surface area in the pyramidal layer of the CA3 region varied significantly in relation to age and sex.

The lowest numerical density was observed in prepubertal animals, with no significant differences between males and females. From the postpubertal stage, numerical density began to increase in both sexes, but in a different manner. The differences observed between males and females, except for the prepubertal stage, were always statistically significant. Numerical density was higher in postpubertal males and young adults than in females. In contrast, it was higher in adult and older females than in males.

In females, in a statistically significant manner, the numerical density increased from the postpubertal stage to the adult stage, in which the increase was slowed and decreased slightly, but not significantly, in old female rats.

In males, in a statistically significant way, the numerical density increased from the postpubertal stage to the young adult stage, in which the increase slowed and decreased significantly from the adult stage onwards and remained in the old male rats.



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**<0.0004**	Postpubertal F		
Young Adult F	**<0.0001**	**<0.0001**	Young Adult F	
Adult F	**<0.0001**	**<0.0001**	**<0.0001**	Adult F
Old F	**<0.0001**	**<0.0001**	**<0.0048**	**0.4587, NS**
P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	**<0.0001**	**<0.0001**	Young Adult M	
Adult M	**<0.0001**	**<0.0001**	**<0.0001**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**0.2790, NS**

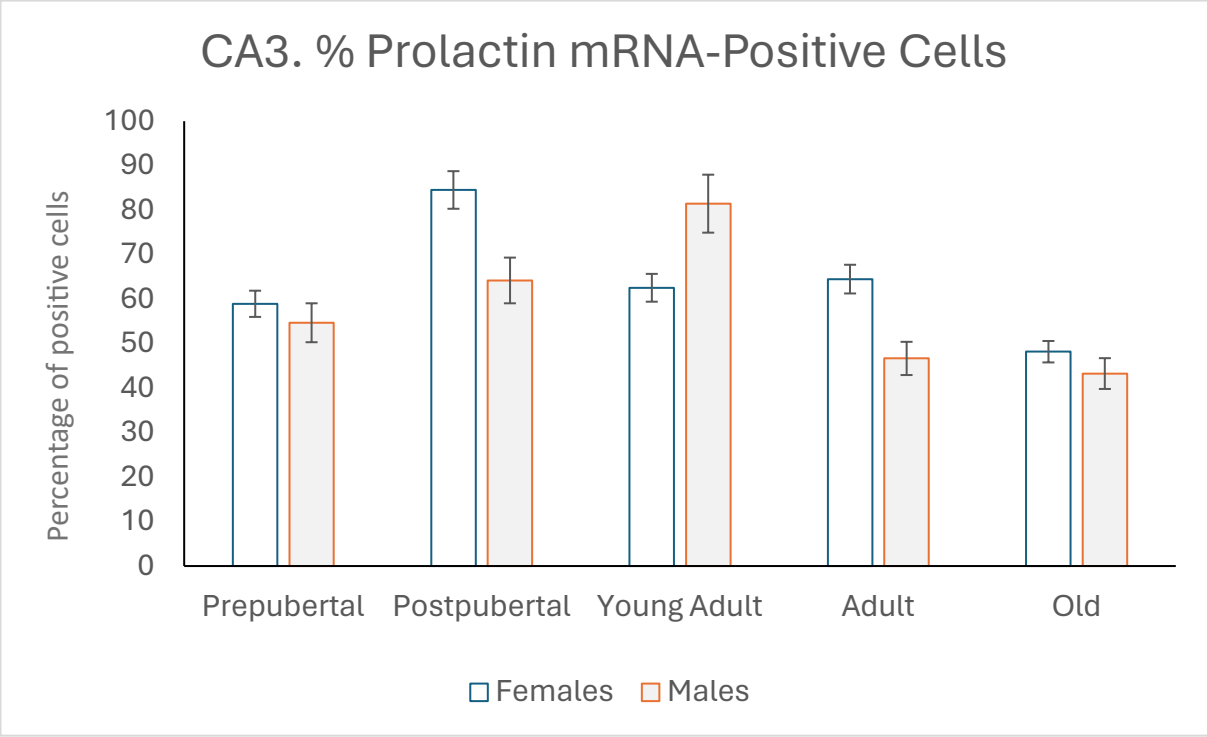
	P value	
Prepubertal F	**>0.9999, NS**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**<0.0001**	Adult M
Old F	**<0.0017**	Old M

Percentage of Prolactin mRNA-Positive-Cells

When analyzing the percentage of neurons in the pyramidal layer in the CA3 region that express prolactin mRNA (see graph and attached tables), a high percentage of prolactin mRNA-positive neurons was observed in prepubertal animals, with no significant differences between males and females. In postpubertal female rats, the highest percentage of neurons expressing the messenger was observed, which was statistically significant compared to the rest of the studied female groups. The percentage decreased significantly in young adult females, remained the same in adult females, and decreased in old females below prepubertal values.

In male rats, from the postpubertal stage onwards, a statistically significant increase in the percentage of positive neurons was observed, reaching its maximum value in young adult males. From the adult stage, the percentage decreased significantly, even below the prepubertal values, and remained the same in old males.

When comparing the sexes, it was observed that there were no differences between males and females in the prepubertal and old stages, while the other differences were significant. The values observed in adult males were similar to those observed in older females.



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**<0.0001**	Postpubertal F		
Young Adult F	**0.3525, NS**	**<0.0001**	Young Adult F	
Adult F	**<0.0202**	**<0.0001**	**0.9478, NS**	Adult F
Old F	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	**<0.0001**	**<0.0001**	Young Adult M	
Adult M	**<0.0001**	**<0.0001**	**<0.0001**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**>0.9999, NS**

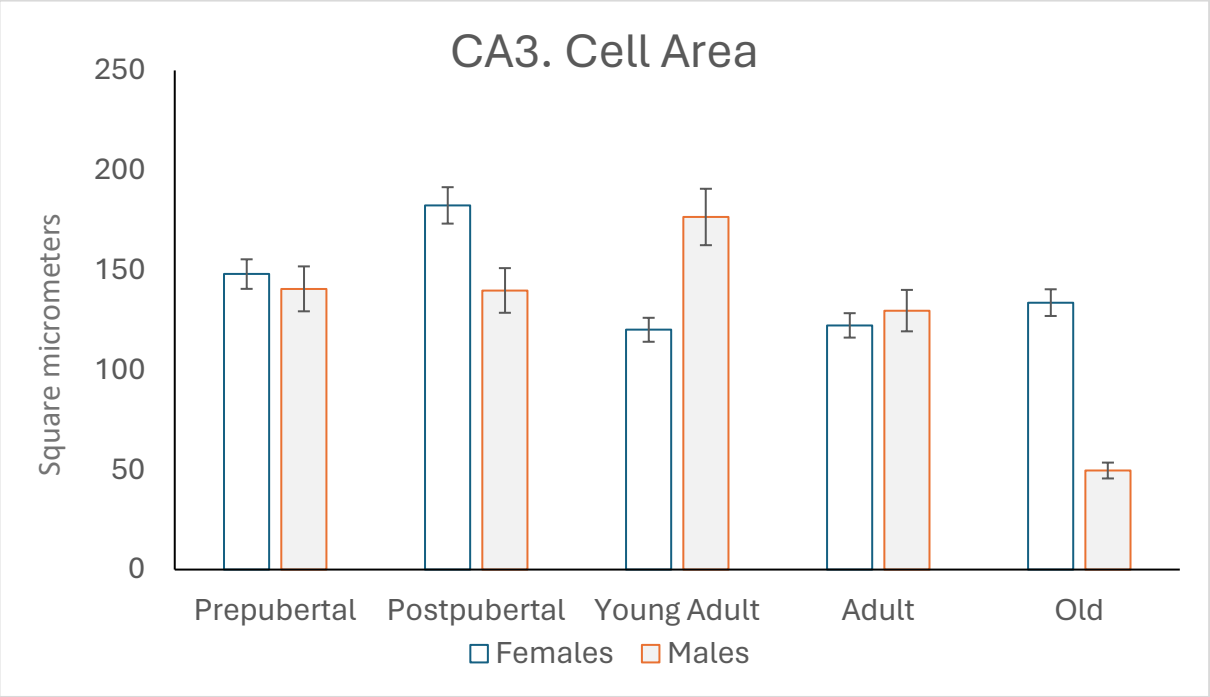
	P value	
Prepubertal F	**0.1576, NS**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**<0.0001**	Adult M
Old F	**0.0598, NS**	Old M

Cell Size of Prolactin mRNA-Positive-Neurons

In female rats, the cell area of prolactin mRNA-positive pyramidal neurons was very similar at all ages studied, with no statistically significant differences, except for the group of postpubertal females in which prolactin mRNA-positive pyramidal neurons were significantly larger than those observed in the rest of the groups studied (see graph and attached tables).

In male rats, prolactin mRNA-positive pyramidal neurons exhibit two important age-related variations. First, young adult animals had significantly larger prolactin mRNA-positive pyramidal neurons than those in the other age groups. The second variation consisted of a significant decrease in the cellular area of the prolactin mRNA-positive pyramidal neurons of old male rats (see graphs and attached tables).

The prolactin mRNA-positive pyramidal neurons of prepubertal and adult rats showed no difference between males and females. In the case of postpubertal rats and old rats, prolactin mRNA-positive neurons were significantly larger in females than in males, while in young adult rats, prolactin mRNA-positive neurons were significantly larger in males than in females, presenting a size very similar to that observed in postpubertal female rats.



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**<0.01**	Postpubertal F		
Young Adult F	**0.10, NS**	**<0.0001**	Young Adult F	
Adult F	**0.18, NS**	**<0.0001**	**>0.99, NS**	Adult F
Old F	**0.89, NS**	**<0.0001**	**0.92, NS**	**<0.97, NS**
P value	Prepubertal M			
Postpubertal M	**>0.99, NS**	Postpubertal M		
Young Adult M	**<0.007**	**<0.005**	Young Adult M	
Adult M	**0.98, NS**	**0.99, NS**	**<0.0001**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

	P value	
Prepubertal F	**0.99, NS**	Prepubertal M
Postpubertal F	**<0.0004**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**>0.99, NS**	Adult M
Old F	**<0.0001**	Old M

Morphological Features of Prolactin mRNA-Positive-Cells

Females

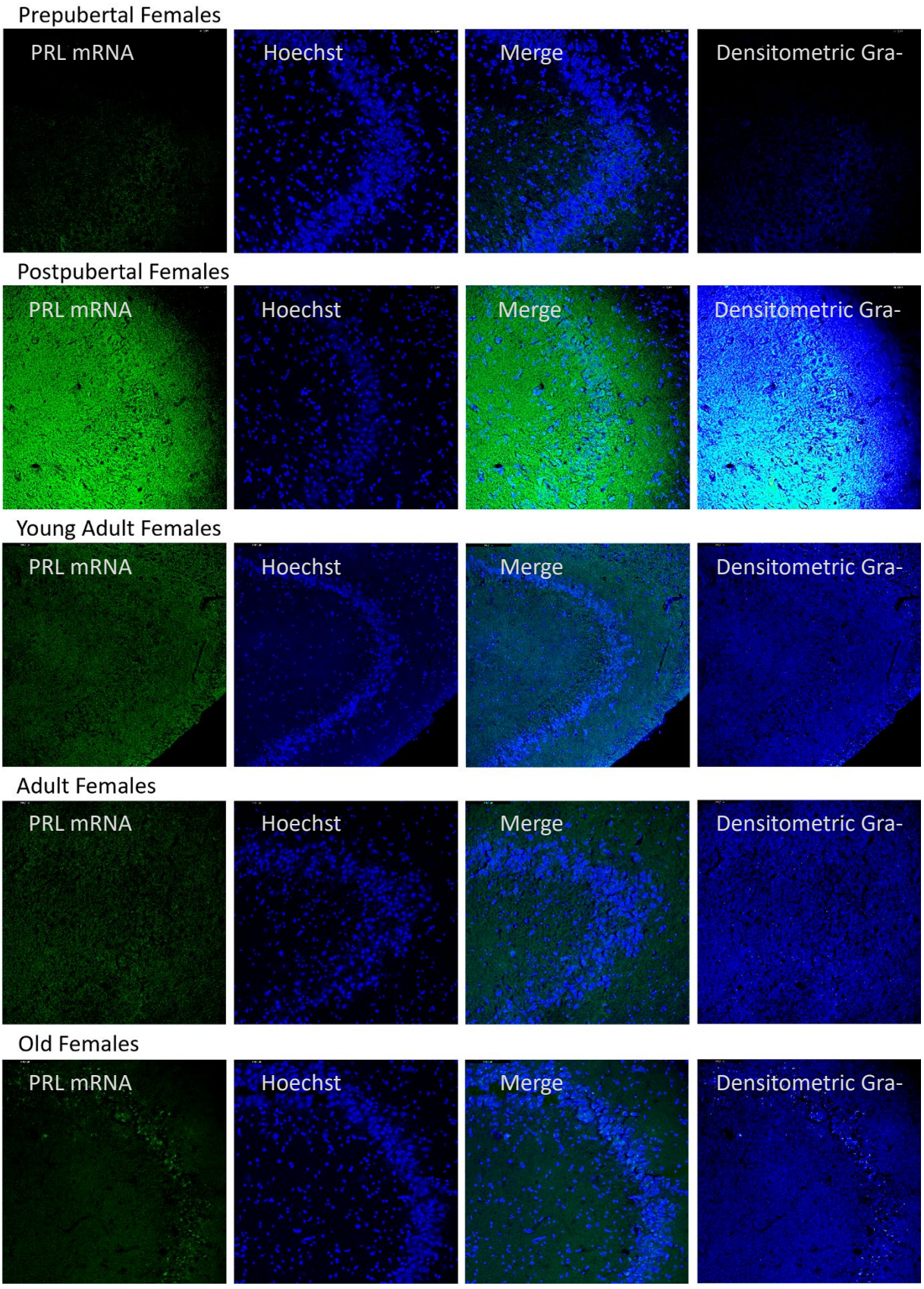
All pyramidal neurons in the CA3 region of postpubertal female rats were prolactin mRNA-positive neurons, with a highly reactive cytoplasm, a reaction that was also observed in some neurons located in the oriens and radiate layers. The expression of the mRNA extended to the dendrites and axons of the oriens and radiated layers, which gave a homogeneous appearance of great intensity when analyzing the densitometric gradient.

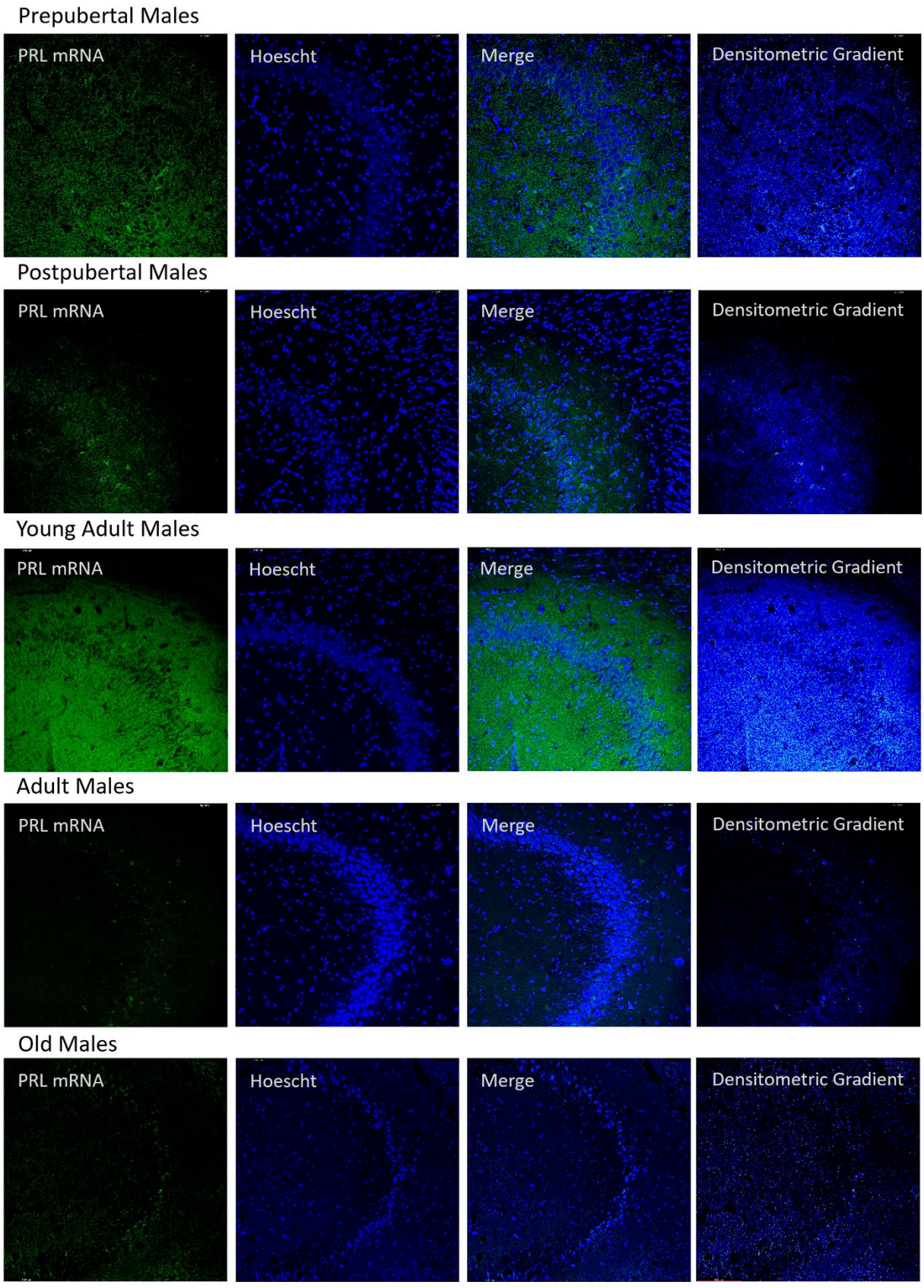
The densitometric gradient showed that the prepubertal animals were the least reactive. Although some neurons isolated from the pyramidal layer of these animals appeared reactive, in general, the reaction was limited to a punctiform reaction of the pyramidal layer and some areas of the radiated layer. Young adult and adult rats had punctiform reaction in the three layers studied, although they were more concentrated in the neuronal somas of the pyramidal layer. Old females presented with reactive neuronal somas in the pyramidal layer, and the reaction in the dendrites or axons of the oriens and radiated layers was almost negligible.

Males

In all age groups studied, most prolactin mRNA-positive neurons were in the pyramidal layer of this region and were characterized by a punctiform or granular reaction in the cytoplasm. In the CA3 region of prepubertal, postpubertal, and young adult animals, secondary neurons were observed, isolated, and scattered in the molecular layer and were smaller in size in the radiated layer. The CA3 region in old males had hardly any positive neurons, although a punctiform reaction appeared in the pyramidal layer.

In general, the dendrites and axons of these neurons hardly reacted, and the densitometric gradient showed that the areas closest to the pyramidal layer of the oriens and radiated layers had a greater reaction intensity. The highest presence of reactive axons and dendrites in the CA3 occurred in young adult males, followed by prepubertal males, and disappeared almost entirely in adult and old males.


Prepubertal Females
PRL mRNA
Hoechst
Merge
Densitometric Gra-
Postpubertal Females
PRL mRNA
Hoechst
Merge
Densitometric Gra-
Young Adult Females
PRL mRNA
Hoechst
Merge
Densitometric Gra-
Adult Females
PRL mRNA
Hoechst
Merge
Densitometric Gra-
Old Females
PRL mRNA
Hoechst
Merge
Densitometric Gra-

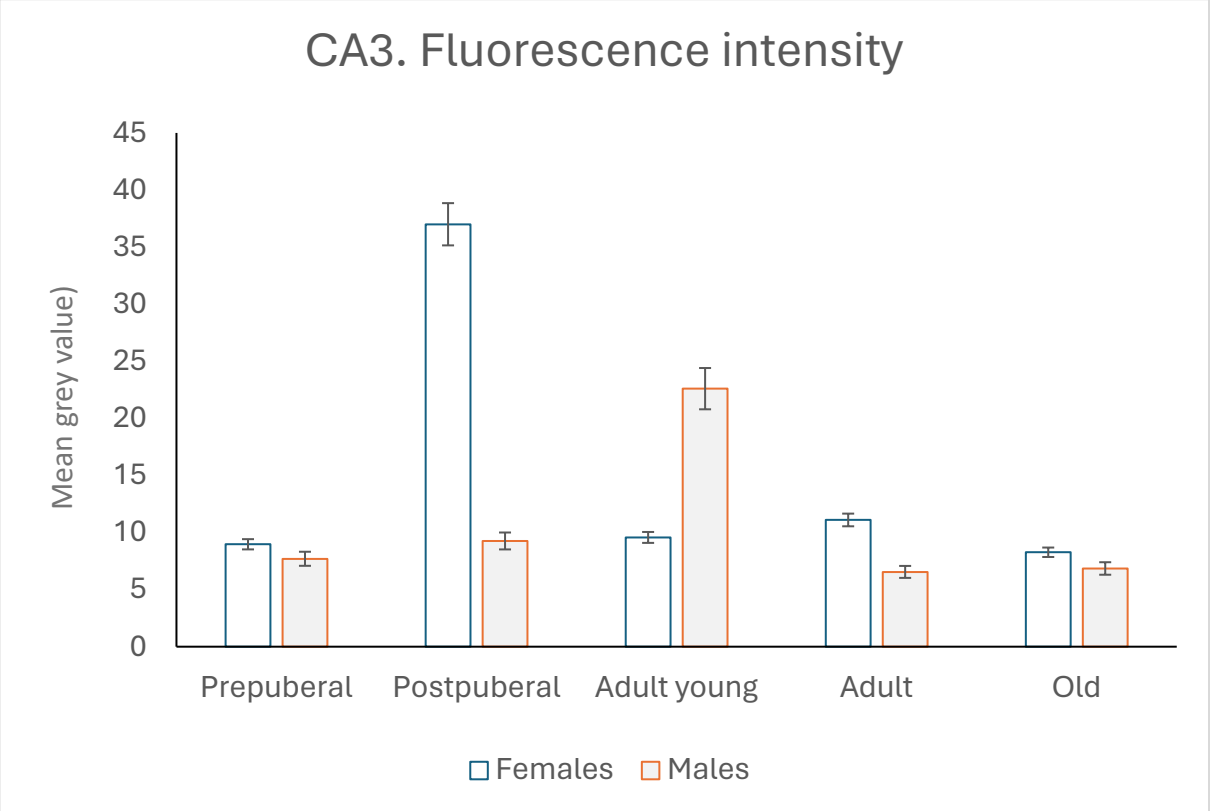

Prepubertal Males
PRL mRNA
Hoescht
Merge
Densitometric Gradient
Postpubertal Males
PRL mRNA
Hoescht
Merge
Densitometric Gradient
Young Adult Males
PRL mRNA
Hoescht
Merge
Densitometric Gradient
Adult Males
PRL mRNA
Hoescht
Merge
Densitometric Gradient
Old Males
PRL mRNA
Hoescht
Merge
Densitometric Gradient

Densitometric Analysis

Densitometric analysis of prolactin mRNA positivity in CA3 pyramidal neurons revealed significant differences between age and sex (see the graph and attached tables).

The densitometric values observed in the prepubertal, young adult, and adult females were not significantly different. However, post-pubertal females showed significantly higher intensities than those found in the other groups of female rats. The decrease in intensity observed between adult and old females was also statistically significant.

In male rats, an increase in intensity was observed in young adults, with statistically significant differences in relation to the other age groups. In addition, the decrease observed in adult and old male rats was statistically significant with respect to that in postpubertal males. There were no significant differences between prepubertal, adult, and old males.



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	*Prepubertal F*			
Postpubertal F	**<0.0001**	*Postpubertal F*		
Young Adult F	**0.99, NS**	**<0.0001**	*Young Adult F*	
Adult F	**0.06, NS**	**<0.0001**	**0.38, NS**	*Adult F*
Old F	**0.98, NS**	**<0.0001**	**0.49, NS**	**<0.002**
P value	*Prepubertal M*			
Postpubertal M	**0.35, NS**	*Postpubertal M*		
Young Adult M	**<0.0001**	**<0.0001**	*Young Adult M*	
Adult M	**0.74, NS**	**0.002**	**<0.0001**	*Adult M*
Old M	**0.96, NS**	**0.0134**	**<0.0001**	**>0.99**

	P value	
Prepubertal F	**0.62, NS**	*Prepubertal M*
Postpubertal F	**<0.0001**	*Postpubertal M*
Young Adult F	**<0.0001**	*Young Adult M*
Adult F	**<0.0001**	*Adult M*
Old F	**0.52, NS**	*Old M*

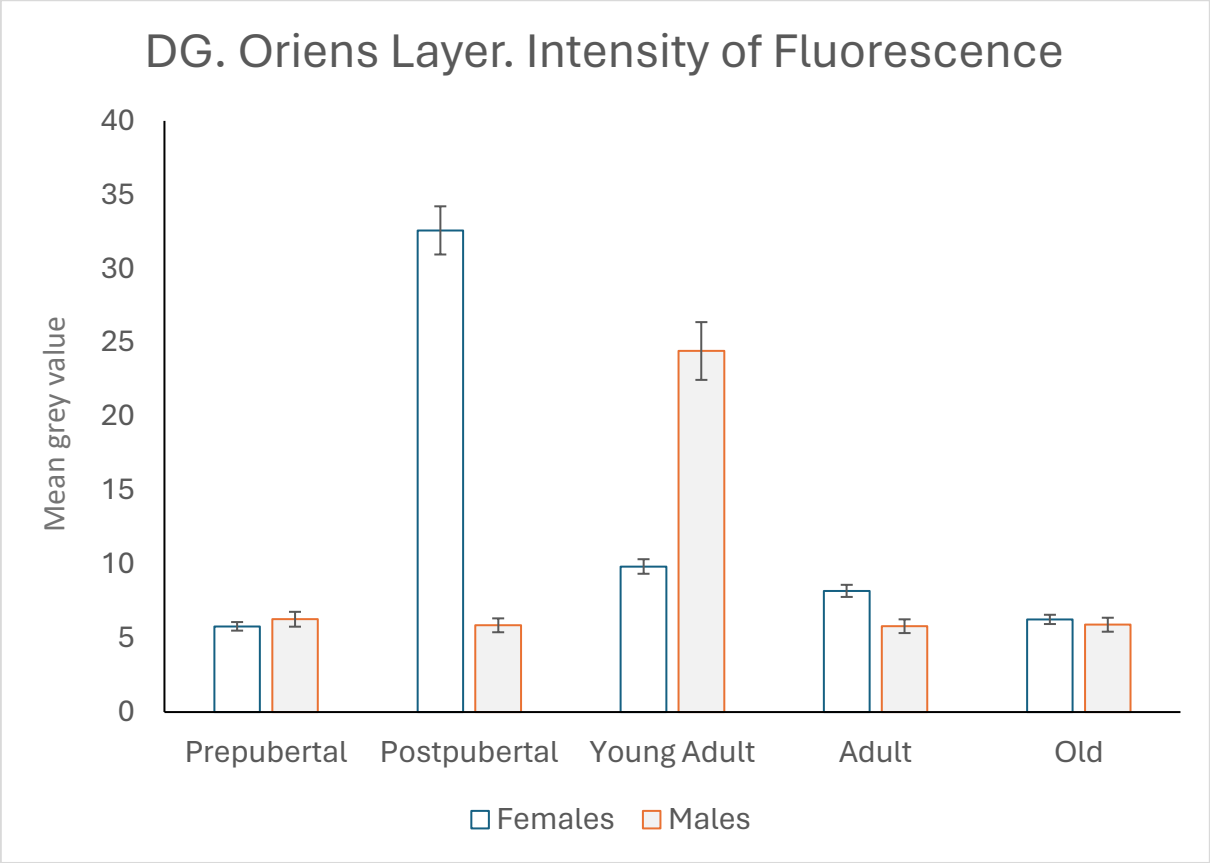
Dentate Gyrus

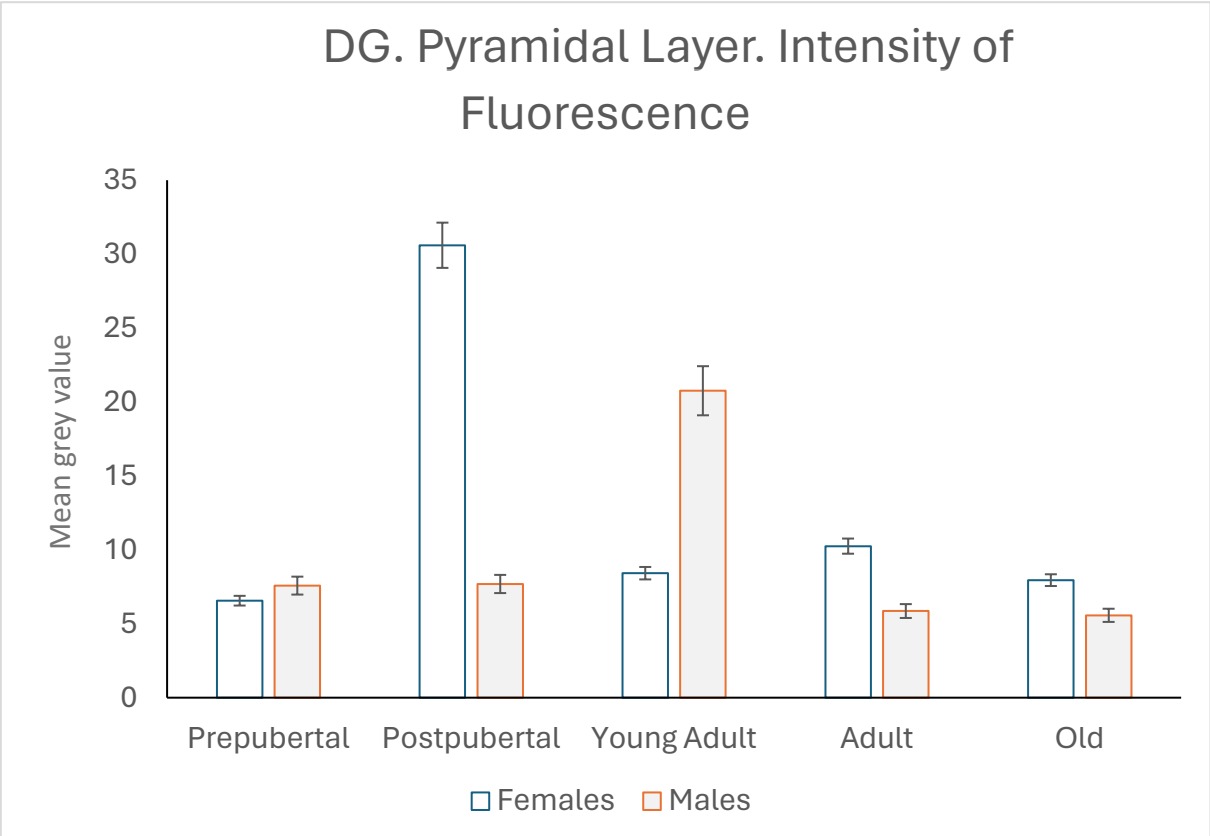
Topographic Distribution of Prolactin-mRNA

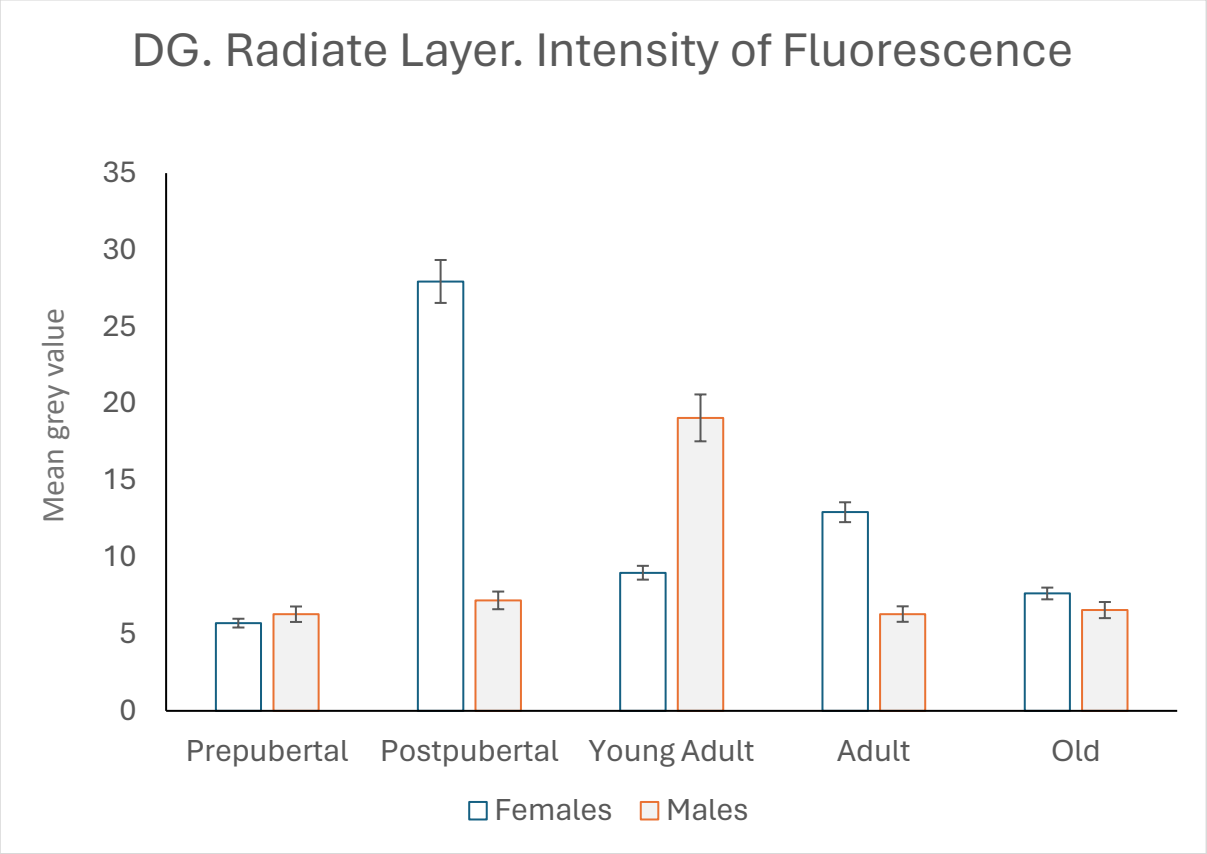
The presence of prolactin mRNA in the hippocampal CA3 region was evident in three layers studied: oriens, pyramidal, and radiate. Densitometric analysis of the fluorescence intensity in the three layers revealed the existence of sexually dysmorphic results with variations related to the age of the animals.

As shown in the attached graphs, these differences followed a similar pattern in the three layers. The intensity was low in the prepubertal animals, it increased significantly in the post-pubertal females ($p<0.001$, with respect to the other groups of females and the prepubertal males), but not in the post-pubertal males that showed similar values to the prepubertal ones.

In young adult animals, the fluorescence intensity decreased significantly in females, whereas in males, there was a significant increase ($p<0.001$, with respect to the other groups of males and young adult females). In the pyramidal layer, the fluorescence intensity was higher in females than in males ($p<0.05$), except in young adult animals.


DG. Oriens Layer. Intensity of Fluorescence
Mean grey value
40
35
30
25
20
15
10
5
0
Prepubertal
Postpubertal
Young Adult
Adult
Old
Females
Males


DG. Pyramidal Layer. Intensity of Fluorescence
Mean grey value
35
30
25
20
15
10
5
0
Prepubertal
Postpubertal
Young Adult
Adult
Old
Females
Males

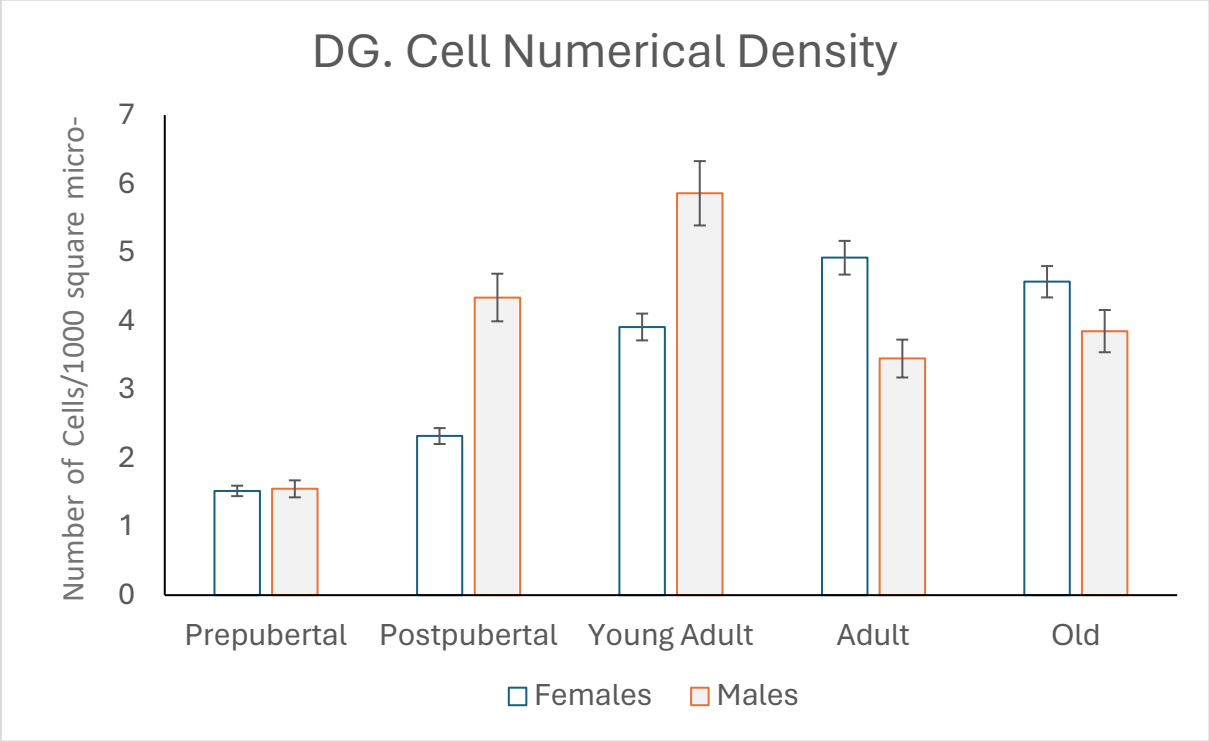


Cell Numerical Density of Granular Layer

As the graph and accompanying tables show, the number of cells per thousand square micrometers of the surface area in the pyramidal layer of the CA3 region varied greatly in relation to age and sex. The lowest numerical density was observed in prepubertal animals, with no significant differences between males and females. From the postpubertal stage, numerical density began to increase in both sexes, but in a different manner. The differences observed between males and females, except for the prepubertal stage, were always statistically significant. Numerical density was higher in postpubertal males and young adults than in females. In contrast, it was higher in adult and older females than in males.

In females, in a statistically significant manner, the numerical density increased from the postpubertal stage to the adult stage, in which the increase was slowed and decreased slightly, but not significantly, in old female rats.

In males, in a statistically significant way, the numerical density increased from the postpubertal stage to the young adult stage, in which the increase slowed and decreased significantly from the adult stage onwards and remained in the old male rats.



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**<0.0004**	Postpubertal F		
Young Adult F	**<0.0001**	**<0.0001**	Young Adult F	
Adult F	**<0.0001**	**<0.0001**	**<0.0001**	Adult F
Old F	**<0.0001**	**<0.0001**	**<0.0048**	**0.4587, NS**
P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	**<0.0001**	**<0.0001**	Young Adult M	
Adult M	**<0.0001**	**<0.0001**	**<0.0001**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**0.2790, NS**

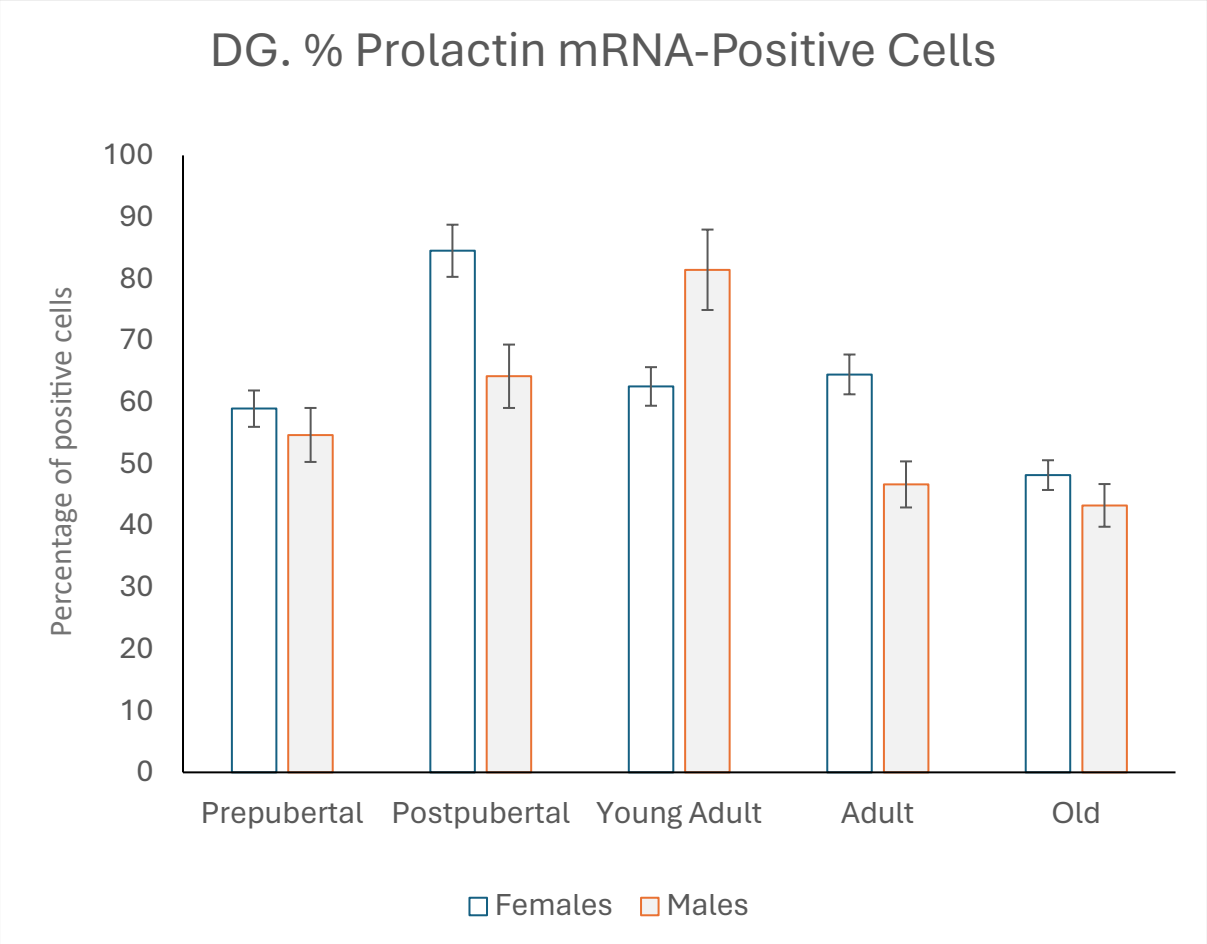
	P value	
Prepubertal F	**>0.9999, NS**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**<0.0001**	Adult M
Old F	**<0.0017**	Old M

Percentage of Prolactin mRNA-Positive-Cells

When analyzing the percentage of neurons of the pyramidal layer in the CA3 region that express prolactin mRNA (see graph and attached tables), a high percentage of prolactin mRNA-positive neurons was observed in prepubertal animals, with no significant differences between males and females. In postpubertal female rats, the highest percentage of neurons expressing the messenger was observed, which was statistically significant compared to the rest of the studied female groups. The percentage decreased significantly in young adult females, remained the same in adult females, and decreased in old females below prepubertal values.

In male rats, from the postpubertal stage onwards, a statistically significant increase in the percentage of positive neurons was observed, reaching its maximum value in young adult males. From the adult stage, the percentage decreased significantly, even below the prepubertal values, and remained the same in old males.

When comparing the sexes, it was observed that there were no differences between males and females in the prepubertal and old stages, while the other differences were significant. The values observed in adult males were similar to those observed in older females.



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**<0.0001**	Postpubertal F		
Young Adult F	**0.3525, NS**	**<0.0001**	Young Adult F	
Adult F	**<0.0202**	**<0.0001**	**0.9478, NS**	Adult F
Old F	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	**<0.0001**	**<0.0001**	Young Adult M	
Adult M	**<0.0001**	**<0.0001**	**<0.0001**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**>0.9999, NS**

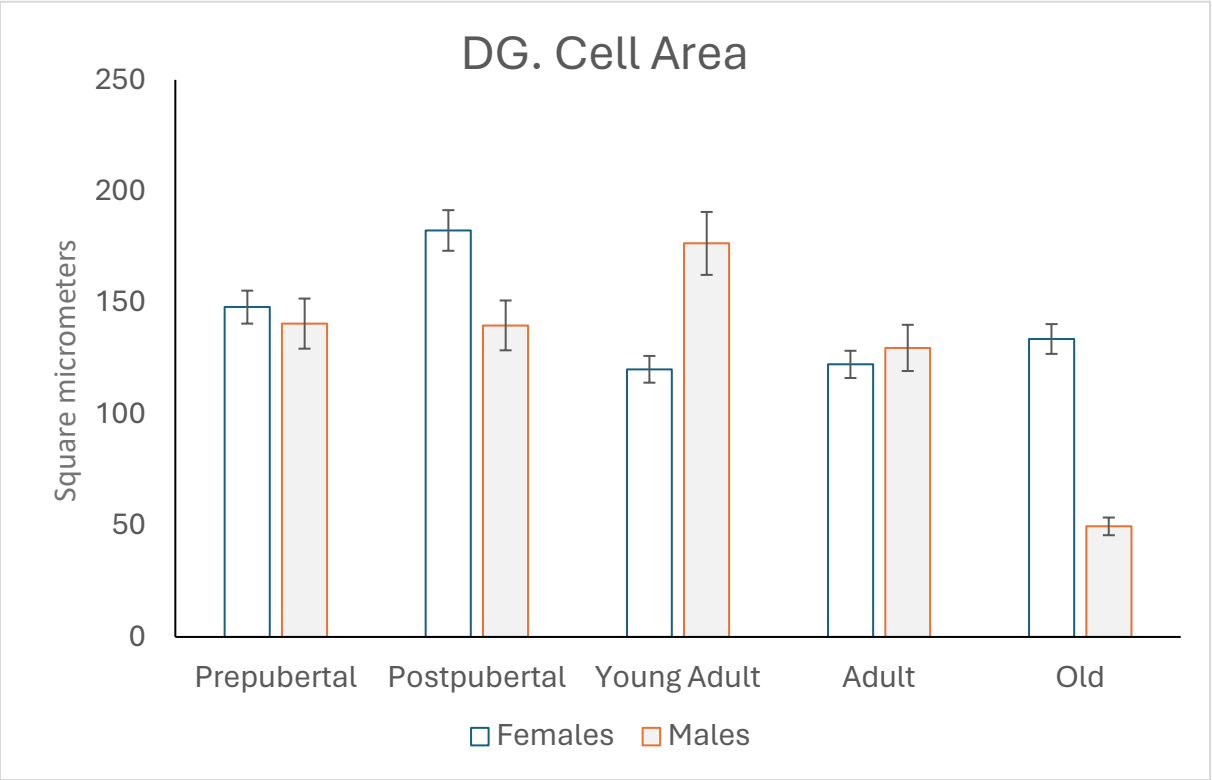
	P value	
Prepubertal F	**0.1576, NS**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**<0.0001**	Adult M
Old F	**0.0598, NS**	Old M

Cell Size of Prolactin mRNA-Positive-Neurons

In female rats, the cell area of prolactin mRNA-positive pyramidal neurons was very similar at all ages studied, with no statistically significant differences, except for the group of postpubertal females in which prolactin mRNA-positive pyramidal neurons were significantly larger than those observed in the rest of the groups studied (see graph and attached tables).

In male rats, prolactin mRNA-positive pyramidal neurons exhibit two important age-related variations. First, young adult animals had significantly larger prolactin mRNA-positive pyramidal neurons than those in the other age groups. The second variation consisted of a significant decrease in the cellular area of the prolactin mRNA-positive pyramidal neurons of old male rats (see graphs and attached tables).

The prolactin mRNA-positive pyramidal neurons of prepubertal and adult rats showed no difference between males and females. In the case of postpubertal rats and old rats, prolactin mRNA-positive neurons were significantly larger in females than in males, while in young adult rats, prolactin mRNA-positive neurons were significantly larger in males than in females, presenting a size very similar to that observed in postpubertal female rats.



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**<0.01**	Postpubertal F		
Young Adult F	**0.10, NS**	**<0.0001**	Young Adult F	
Adult F	**0.18, NS**	**<0.0001**	**>0.9999, NS**	Adult F
Old F	**0.89, NS**	**<0.0001**	**0.92, NS**	**0.97, NS**
P value	Prepubertal M			
Postpubertal M	**>0.9999, NS**	Postpubertal M		
Young Adult M	**<0.007**	**<0.005**	Young Adult M	
Adult M	**0.98, NS**	**>0.9999, NS**	**<0.0001**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

	P value	
Prepubertal F	**>0.9999, NS**	Prepubertal M
Postpubertal F	**<0.0004**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**>0.9999, NS**	Adult M
Old F	**<0.0001**	Old M

Morphological Features of Prolactin mRNA-Positive-Cells

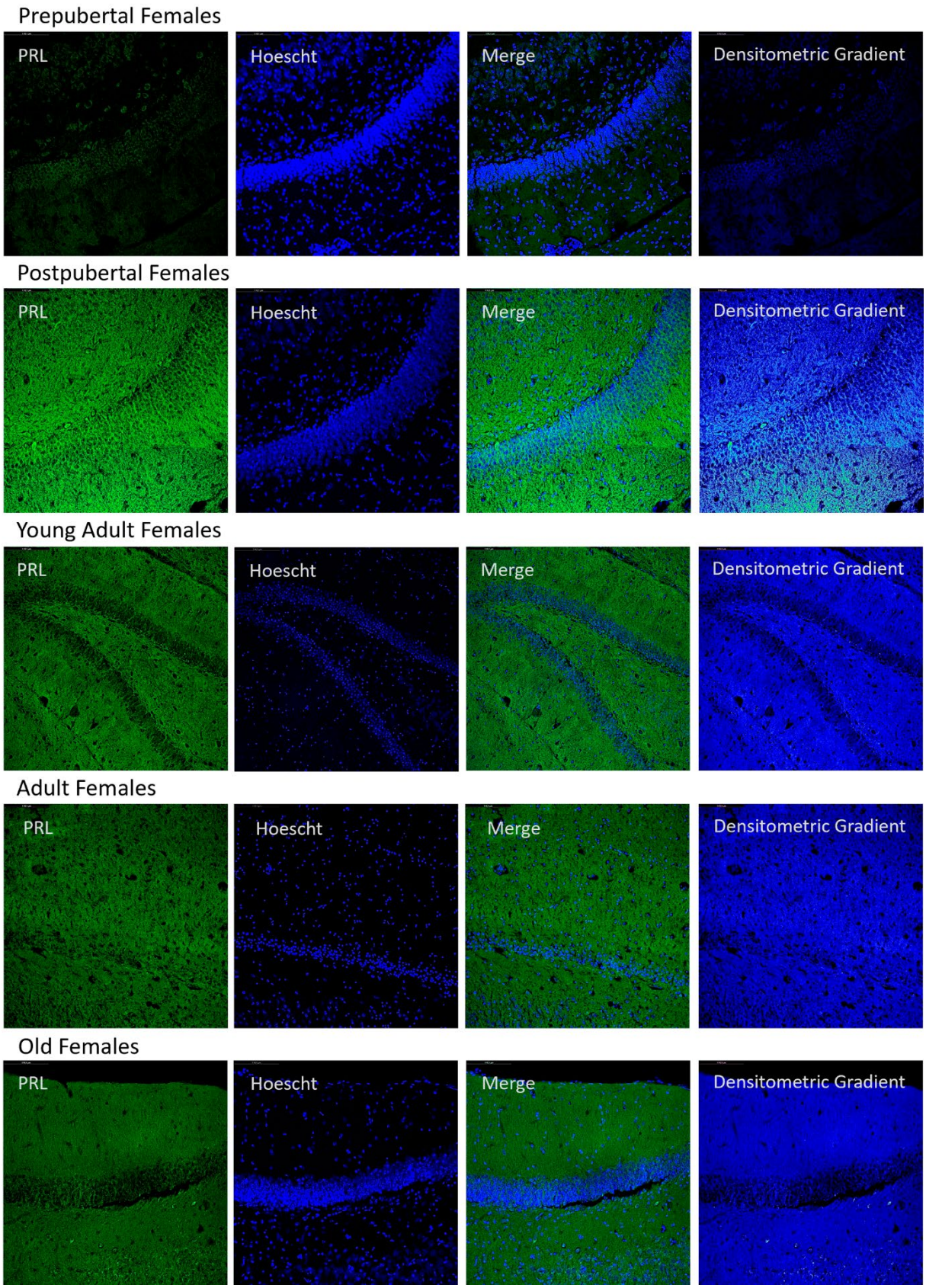
In the dentate gyrus, three different areas were analyzed: the dorsal, the ventral and the beak. However, apart from the differences in their shapes, there were no differences in the positive signals after in situ hybridization between the groups studied.

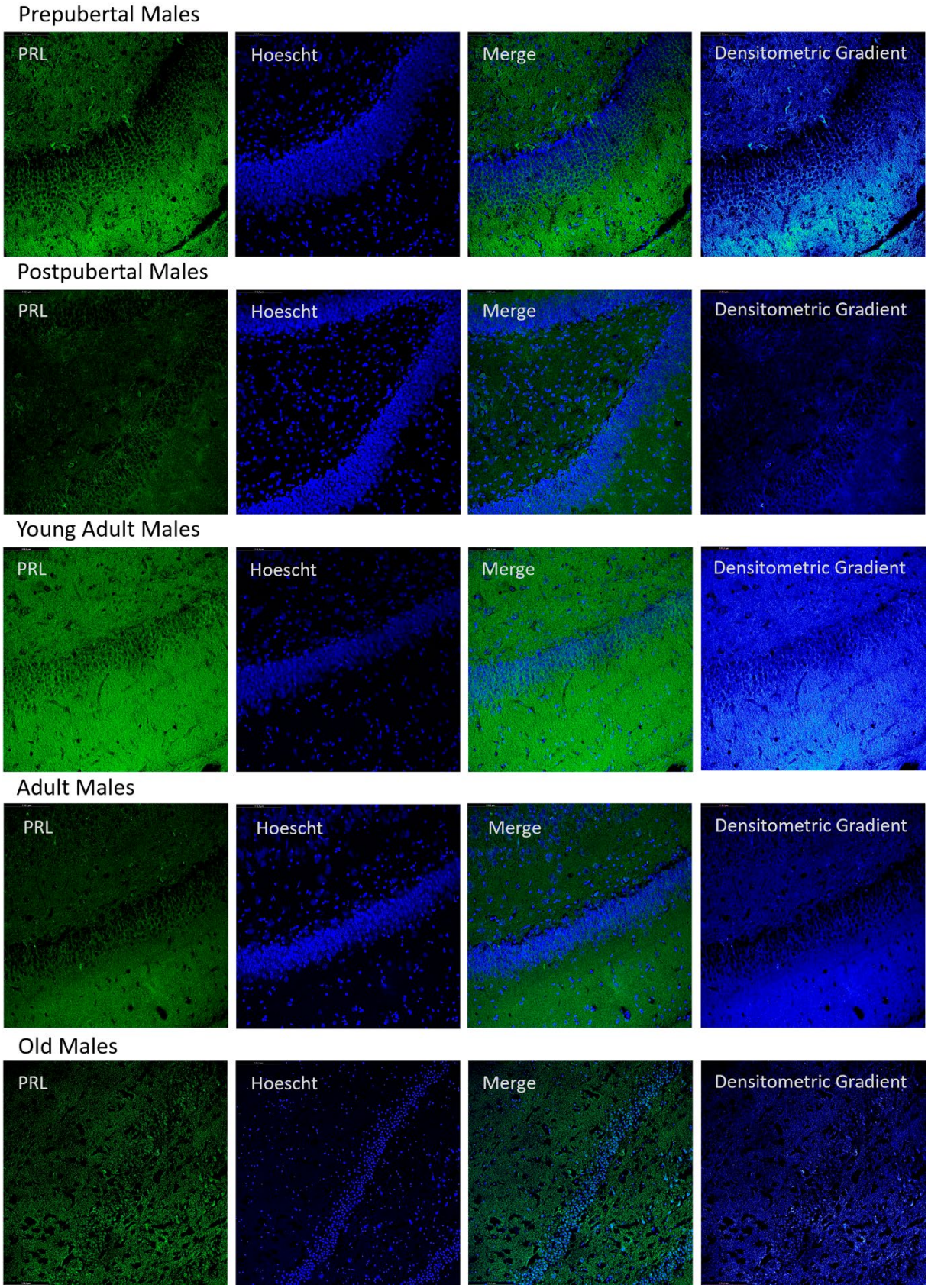
Females

In females, the greatest reactivity affecting the neuronal somas of the granular layer and the fibers of the molecular and polymorphic layers was observed in postpubertal females. In these animals, subgranular cells arranged between the granular and polymorphic layers were observed with a greater reaction intensity than the rest of the cells in the dentate gyrus. Prepubertal females had a reaction in the granular layer and in secondary neurons of the polymorph, with some positive subgranular cells, but their reaction intensity was lower than in postpubertal females; The oriens and polymorphic layers had hardly any reactive fibers. Young adults had a reaction that affected all three layers, although the granular layer was less reactive than that in the previous age stages, and the number of positive subgranular cells was lower. In the molecular layer, two zones with different reactions were differentiated, with the marginal zone being more reactive than that adjacent to the granular layer. Positivity in the fibers of the molecular and polymorphic layers progressively decreased with age in adult and old females, as did the presence of subgranular cells. Old females had positive zones next to negative zones in the granular layer, and some secondary neurons in the polymorphic layer were strongly positive. All of these differences became evident when analyzing the densitometric gradients.

Males

In male rats, the layered reaction patterns between pre- and post-pubertal ages appear inverted when compared to females. In males, the prepubertal and young adult stages were the two age stages with the greatest sign of hybridization. In prepubertal males, almost all granular cells were positive, and the fibers of the molecular layer were very positive. Very positive secondary neurons also appeared in the polymorphic layer, and very positive subgranular cells stood out, some penetrating the granular layer. Postpubertal males lose positivity in the fibers of the molecular and polymorphic layers. There were fewer reactive granular cells and no positive subgranular cells, although isolated positive secondary neurons were observed in the polymorphic layer. In young adults, the hybridization signal was recovered, with strong positivity in all three layers, even higher than that in prepubertal animals, but without subgranular cells or positive secondary neurons. As in females, the intensity of the hybridization signal in the three layers of the dentate gyrus decreased with age. The pattern of reaction in adult males is reminiscent of that in prepubertal males, but with a lower reaction intensity and no subgranular cells. In the old males, the signal appeared intense in some isolated granular cells, along with others that were not reactive (see merge and densitometric gradient), there were no positive subgranular cells, and there were secondary neurons in the polymorphic layer.


Prepubertal Females
PRL
Hoescht
Merge
Densitometric Gradient
Postpubertal Females
PRL
Hoescht
Merge
Densitometric Gradient
Young Adult Females
PRL
Hoescht
Merge
Densitometric Gradient
Adult Females
PRL
Hoescht
Merge
Densitometric Gradient
Old Females
PRL
Hoescht
Merge
Densitometric Gradient

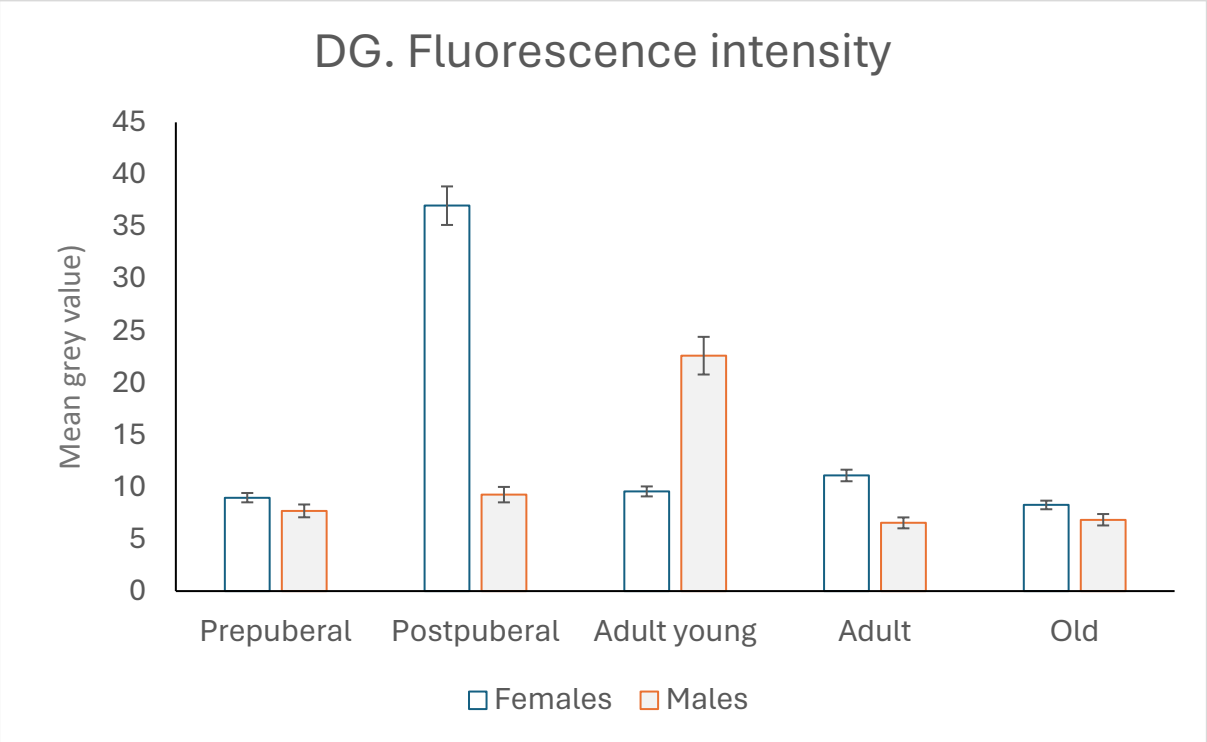

Prepubertal Males
PRL
Hoescht
Merge
Densitometric Gradient
Postpubertal Males
PRL
Hoescht
Merge
Densitometric Gradient
Young Adult Males
PRL
Hoescht
Merge
Densitometric Gradient
Adult Males
PRL
Hoescht
Merge
Densitometric Gradient
Old Males
PRL
Hoescht
Merge
Densitometric Gradient

Densitometric Analysis

Densitometric analysis of prolactin mRNA positivity in CA3 pyramidal neurons revealed significant differences between age and sex (see the graph and attached tables).

The densitometric values observed in the prepubertal, young adult, and adult females were not significantly different. However, post-pubertal females showed significantly higher intensities than those found in the other groups of female rats. The decrease in intensity observed between adult and old females was also statistically significant.

In male rats, an increase in intensity was observed in young adults, with statistically significant differences in relation to the other age groups. In addition, the decrease observed in adult and old male rats was statistically significant with respect to that in postpubertal males. There were no significant differences between prepubertal, adult, and old males.



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	*Prepubertal F*			
Postpubertal F	**<0.0001**	*Postpubertal F*		
Young Adult F	**0.99, NS**	**<0.0001**	*Young Adult F*	
Adult F	**0.06, NS**	**<0.0001**	**0.38, NS**	*Adult F*
Old F	**0.98, NS**	**<0.0001**	**0.49, NS**	**<0.002**
P value	*Prepubertal M*			
Postpubertal M	**0.35, NS**	*Postpubertal M*		
Young Adult M	**<0.0001**	**<0.0001**	*Young Adult M*	
Adult M	**0.74, NS**	**0.002**	**<0.0001**	*Adult M*
Old M	**0.96, NS**	**0.0134**	**<0.0001**	**>0.99, NS**

	P value	
Prepubertal F	**0.62, NS**	*Prepubertal M*
Postpubertal F	**<0.0001**	*Postpubertal M*
Young Adult F	**<0.0001**	*Young Adult M*
Adult F	**<0.0001**	*Adult M*
Old F	**0.52, NS**	*Old M*

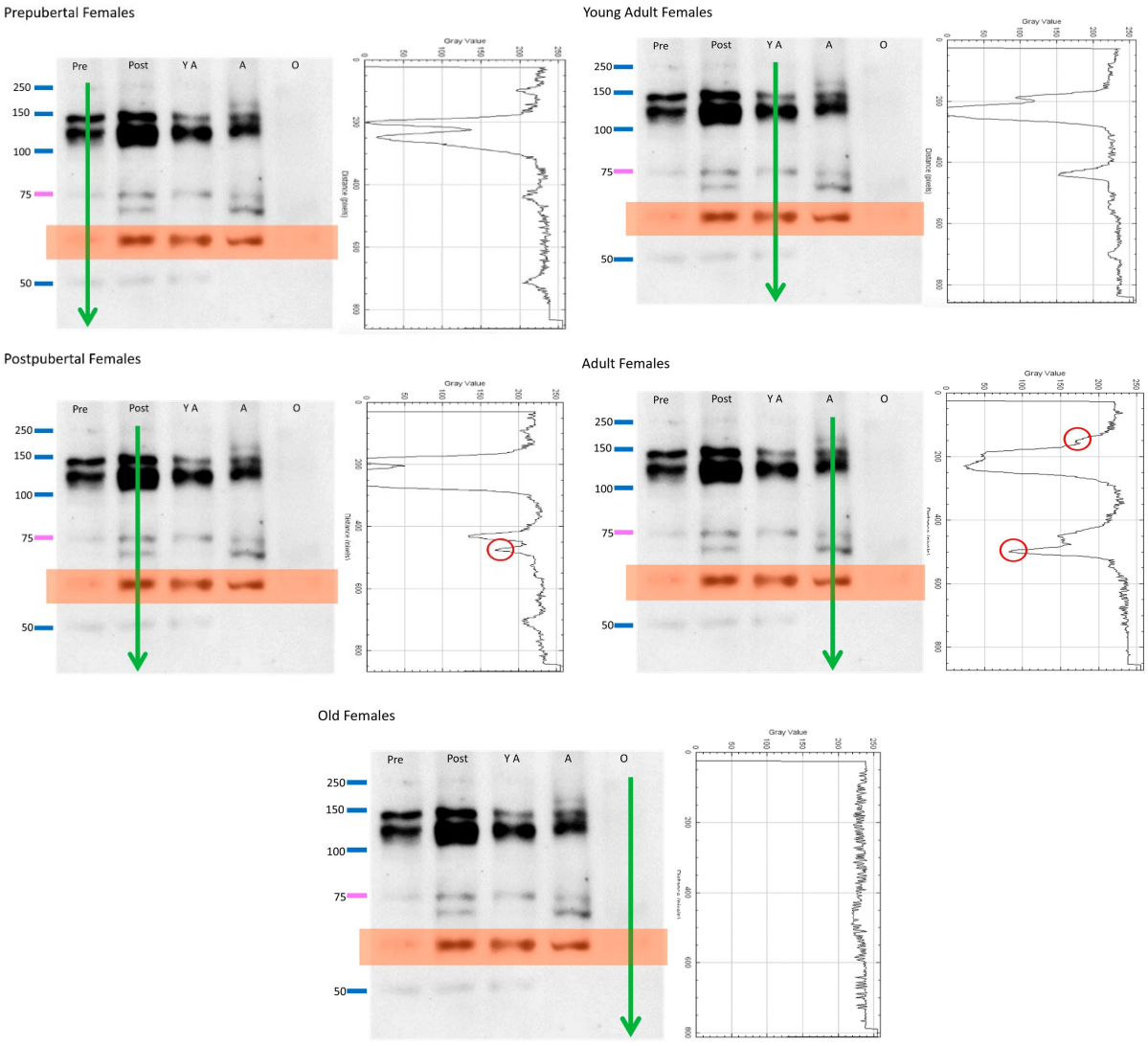
Hippocampal Prolactin-System. Western Blotting

Description and Densitometric Profiles

Females

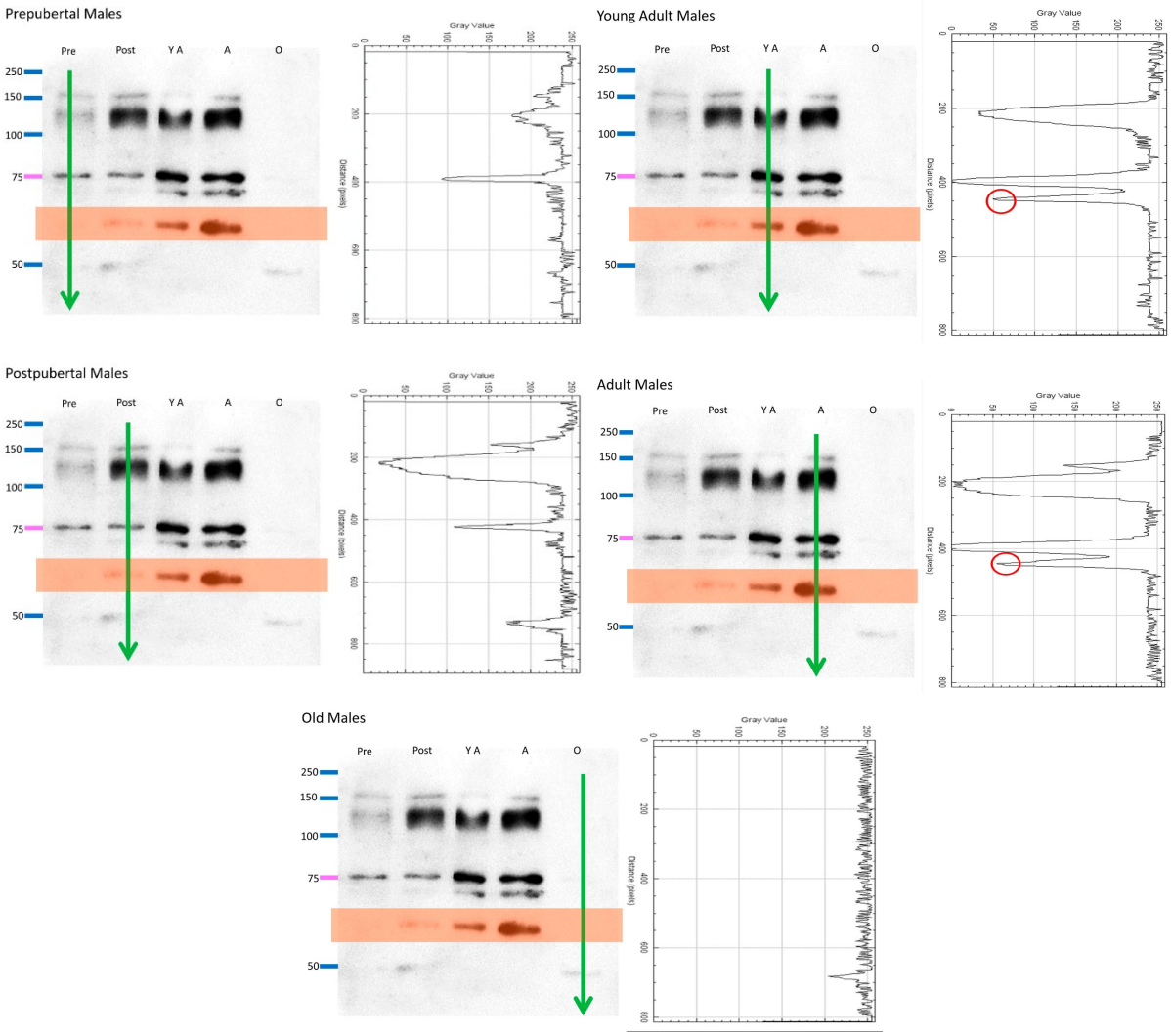
A total number of 5 different bands were detected in all the samples. However, the predominant bands detected in females were 150 kDa and 125 kDa. The band at 75 kDa was present in all groups, but very faintly in prepubertal and old rats. An additional band appeared immediately below approximately 70 kDa in the postpubertal and adult groups. Finally, a band at 50 kDa was detected in the prepubertal, postpubertal, and young adult groups. The strongest band appears to be 125 kDa prolactin. In none of the groups was a monomer band of 25 kDa detected. No bands were detected in the old rats.

The double bands are marked with red circles in the profile graphics.



Males

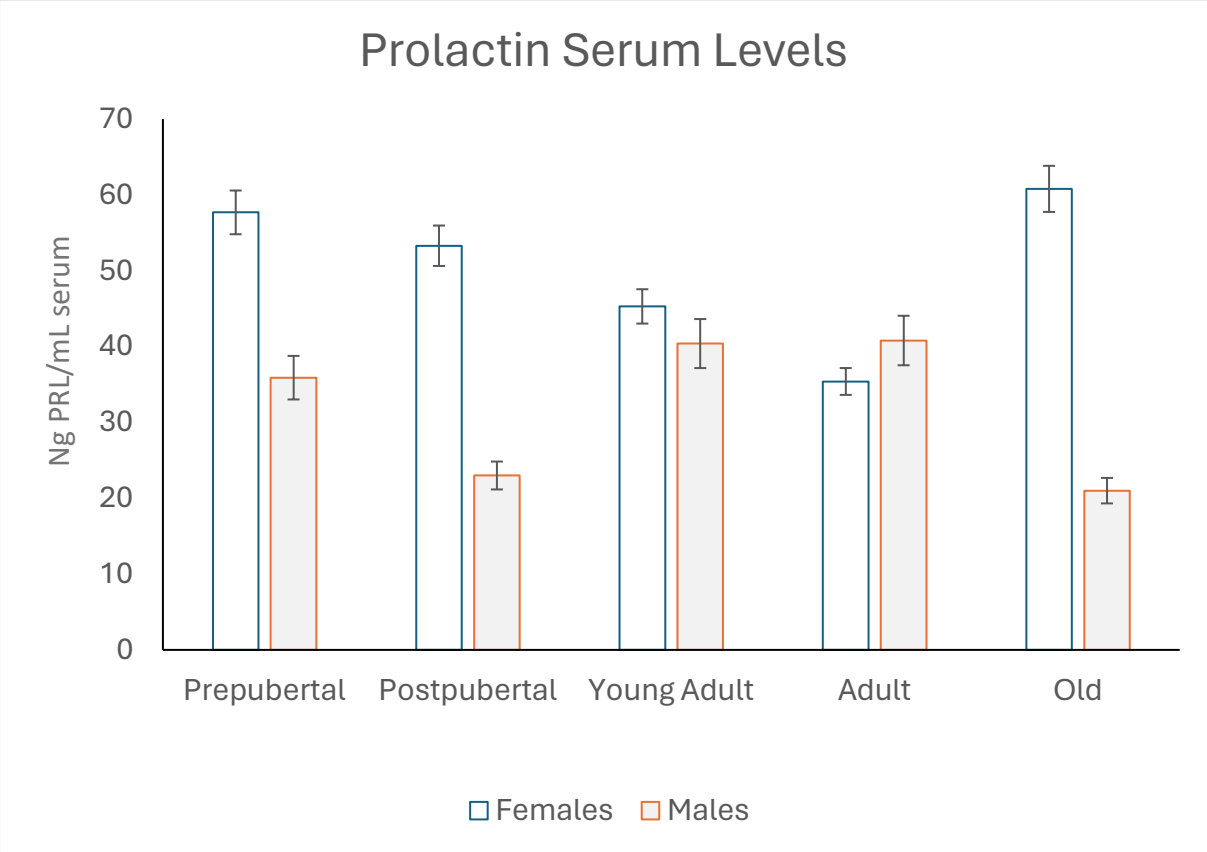
In the case of the male rat groups, the prolactin bands were very different when compared to those of the female rats. There would still be a total of five bands of prolactin detected, but the predominant bands would be at 125 kDa and 75 kDa. Bands at 70 kDa would also appear at specific ages, but this time they are easily detected during adulthood and very faintly during pre- and post-puberty. Similar to the female rats, the old male rats barely showed any prolactin bands, except for males with a faint band at 50 kDa. The strongest and most consistent band was observed at 75 kDa. Again, no band for a prolactin monomer could be detected at 25 kDa.



Hippocampal Prolactin-System. ELISA Study

Prolactin serum levels

The serum prolactin levels were significantly higher in females than in males, except in the adult group. However, the differences between males and females observed in young and older adults were not statistically significant. Within the female groups, the differences between prepubertal and postpubertal, prepubertal and old, postpubertal and young adults, and postpubertal and old adults were not significant. In the male groups, there was a gradual decrease in hormone concentration, reaching its lowest level at young adult age, which increased again with age. Female rats showed more stable serum levels than did male rats. The differences between prepubertal and young, prepubertal and old adults, postpubertal and old, and young adults and adults were not significant. Female animals showed greater uniformity in serum prolactin levels than did male animals at the age stages studied.



The following tables show the results of the One-way ANOVA and Tukey tests (F: Female, M: Male, NS: Not significant).

P value	Prepubertal F			
Postpubertal F	0.6904, NS	Postpubertal F		
Young Adult F	0.0013	0.0676, NS	Young Adult F	
Adult F	<0.0001	<0.0001	0.0126	Adult F
Old F	0.9388, NS	0.1020, NS	<0.0001	<0.0001
P value	Prepubertal M			
Postpubertal M	0.0008	Postpubertal M		
Young Adult M	0.6650, NS	<0.0001	Young Adult M	
Adult M	0.5615, NS	<0.0001	>0.9999, NS	Adult M
Old M	0.0001	0.9965, NS	<0.0001	<0.0001

	P value	
Prepubertal F	<0.0001	Prepubertal M
Postpubertal F	<0.0001	Postpubertal M
Young Adult F	0.5615, NS	Young Adult M
Adult F	0.4359, NS	Adult M
Old F	<0.0001	Old M

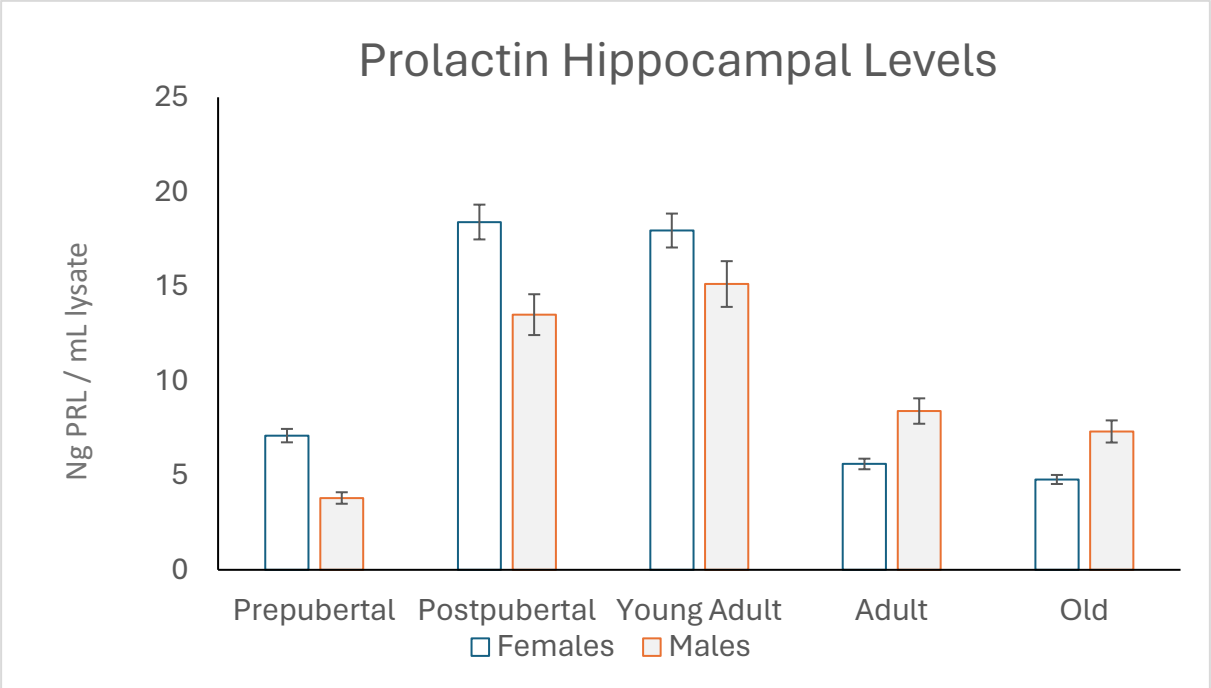
Prolactin levels in the hippocampus

Hippocampal prolactin levels behave similarly between sexes, but there are still some differences. The amount of prolactin also appeared to be higher in females than in males ($p<0.01$), except for adult and old male rats, which had significantly higher levels of prolactin than female animals ($p<0.01$).

When looking at the sexes separately, hormone levels increased gradually in females until they reached their peak in their young adult age, and decreased again with age ($p<0.01$, for all age groups studied).

In male rats, hippocampal prolactin levels increase suddenly when they reach puberty, stabilize during post-puberty and adulthood, and drastically decrease when they reach old age. However, the differences observed between postpubertal and young adult females, prepubertal and old males, and postpubertal and young adult males were not statistically significant.

In both sexes, there was a correlation between serum prolactin levels in the blood and hippocampus, which was more evident in females than in males.



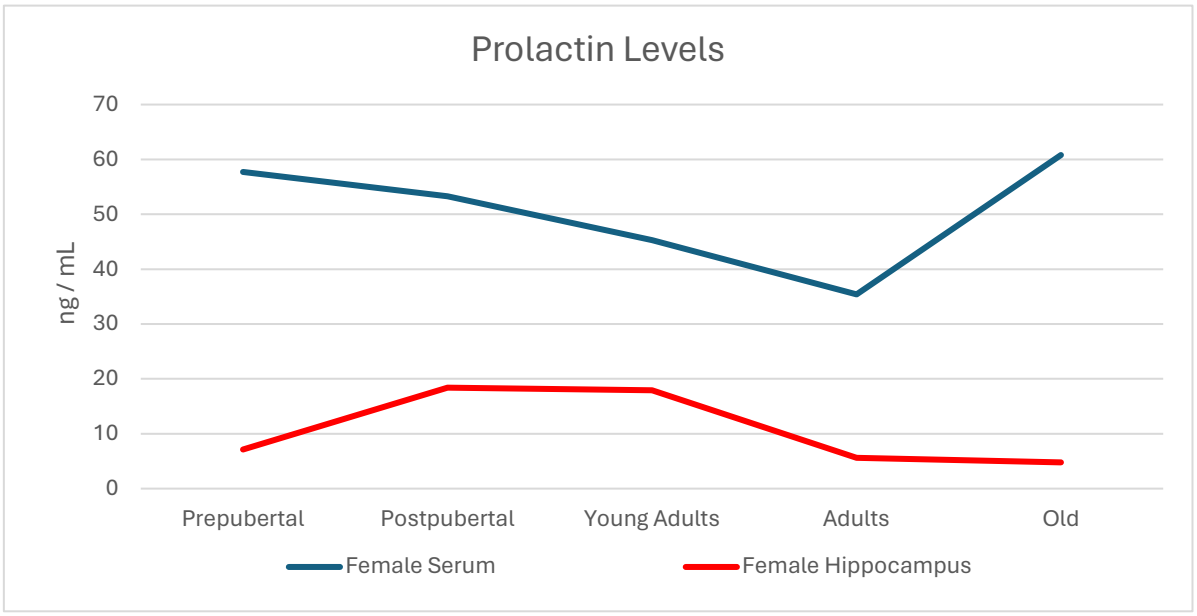
The following tables show the results of the One-way ANOVA and Tukey tests (F: Female, M: Male, NS: Not significant).

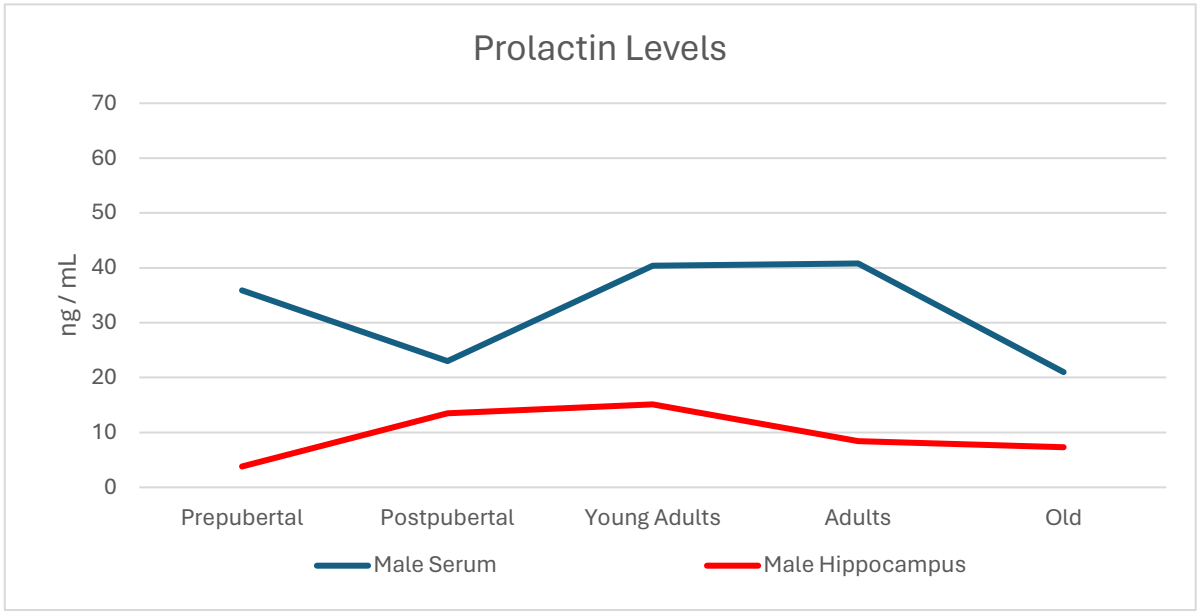
P value	Prepubertal F			
Postpubertal F	**<0.0001**	Postpubertal F		
Young Adult F	**<0.0001**	**0.2738, NS**	Young Adult F	
Adult F	**<0.0001**	**<0.0001**	**<0.0001**	Adult F
Old F	**<0.0001**	**<0.0001**	**<0.0001**	**0.0040**
P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	**<0.0001**	**0.3055, NS**	Young Adult M	
Adult M	**<0.0001**	**<0.0001**	**<0.0001**	Adult M
Old M	**0.3730, NS**	**<0.0001**	**<0.0001**	**<0.0001**

	P value	
Prepubertal F	**<0.0001**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**<0.0001**	Adult M
Old F	**<0.0001**	Old M

There was no correlation between serum and hippocampal levels in either sex or age groups.

Pearson r	Males	Females
r	0.2402	-0.09306
95% confidence interval	-0.8147 to 0.9262	-0.9013 to 0.8598
R squared	0.05769	0.00888661
P value		
P (two-tailed)	**0.6971**	**0.8817**
P value summary	**NS**	**NS**
Significant? (alpha=0.05)	No	No



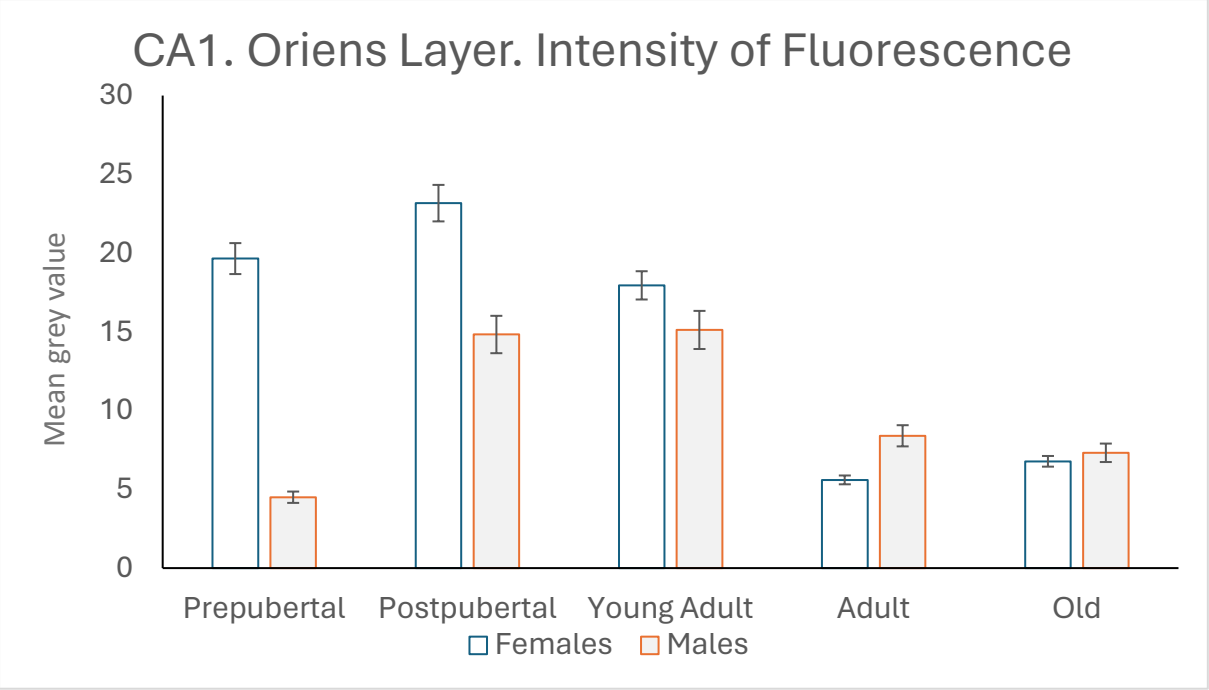


Prolactin-Positive Cells. Immunohistochemical Study

Topographic Distribution of Prolactin. Densitometric Analysis

Prolactin positivity was observed in all groups of animals studied and in all strata of the CA1 region of the Cornu Ammonis analyzed (oriens, pyramidal, and radiated). However, there were statistically significant differences in the densitometric analysis, from age to age, and between females and males. The intensity of the reaction was always higher in prepubertal females than in males ($p<0.01$), whereas in postpubertal animals, the intensity was higher in males than in females ($p<0.01$), especially in the radiated layer. In young adult and adult animals, the intensity was higher in females than in males in all layers ($p<0.01$). However, in old animals, the intensity was higher in males than in females ($p<0.01$).

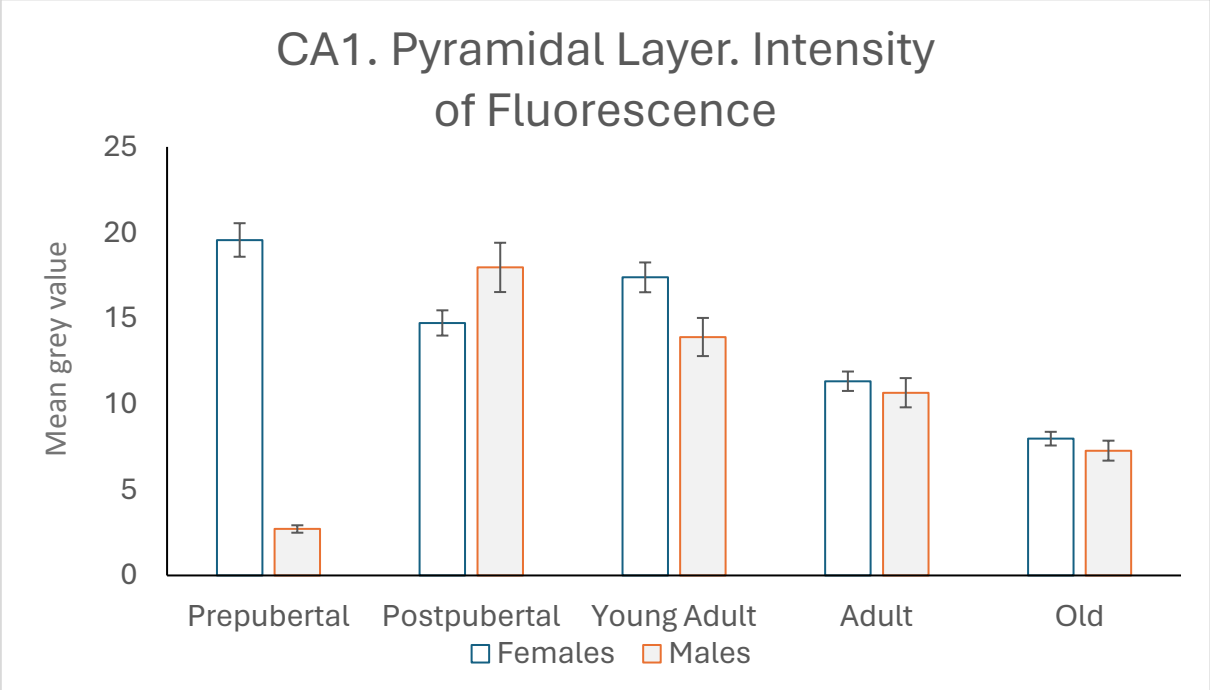
In the oriens layer, the reaction intensity observed in prepubertal and postpubertal females was very similar, and although the ANOVA showed significant differences, the F test to compare variances showed that there were no significant differences (>0.99). The highest reaction intensity was observed in young adult females, which was significantly higher than that in the other female groups ($p<0.01$). Adult females showed more reaction than other groups of females, except for young adults ($p<0.01$). The lowest intensity was observed in older females ($p<0.01$, with respect to all groups of females). In males, the lowest reaction intensity was observed in the prepubertal stage ($p<0.01$); it increased significantly in postpubertal animals ($p < 0.01$), remaining at similar values in young adult males ($p < 0.05$, ANOVA; $p > 0.99$, F test to compare variances), decreased in adult males ($p < 0.01$),) and was maintained in old males ($p<0.01$ in the ANOVA and $p>0.99$ in the F test to compare variances).



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**<0.0001**	Postpubertal F		
Young Adult F	**<0.0001**	**<0.0001**	Young Adult F	
Adult F	**<0.0001**	**<0.0001**	**<0.0001**	Adult F
Old F	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	**<0.0001**	**<0.0489**	Young Adult M	
Adult M	**<0.0001**	**<0.0001**	**<0.0001**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

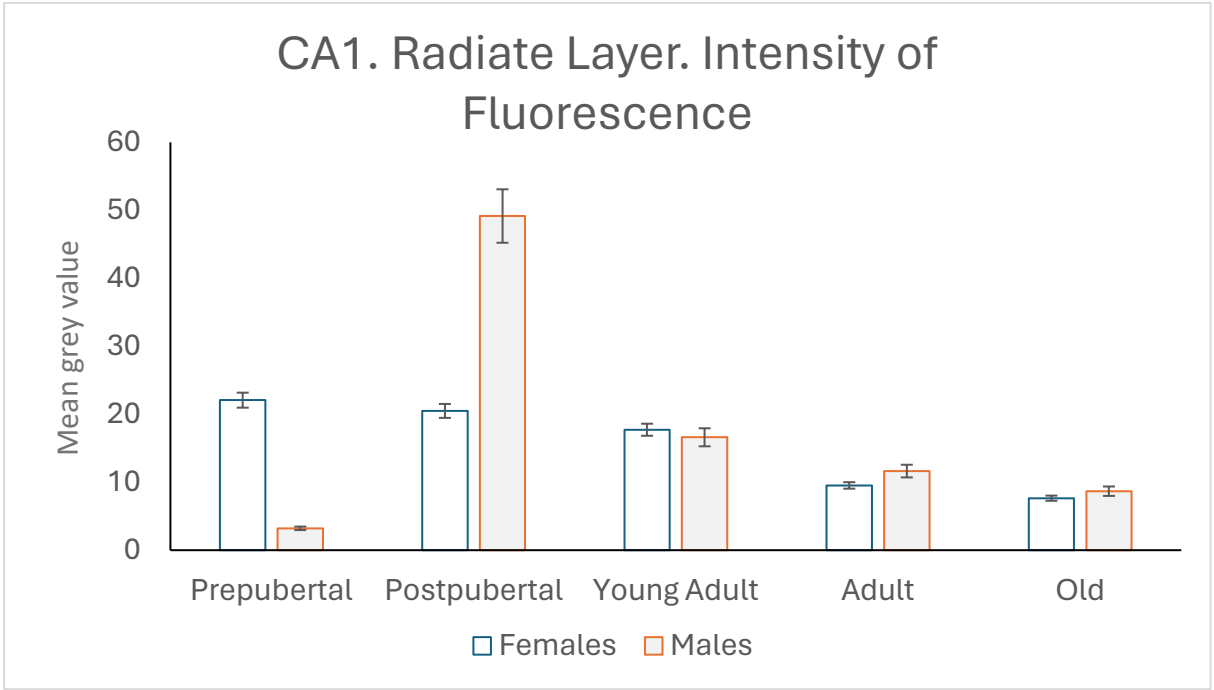
	P value	
Prepubertal F	**<0.0001**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**0.9704, NS**	Adult M
Old F	**0.0130**	Old M



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**<0.0001**	Postpubertal F		
Young Adult F	**<0.0001**	**<0.0001**	Young Adult F	
Adult F	**<0.0001**	**<0.0001**	**<0.0001**	Adult F
Old F	**<0.0001**	**<0.0001**	**0.0181**	**<0.0001**
P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	**<0.0001**	**<0.0001**	Young Adult M	
Adult M	**<0.0001**	**<0.0001**	**<0.0001**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

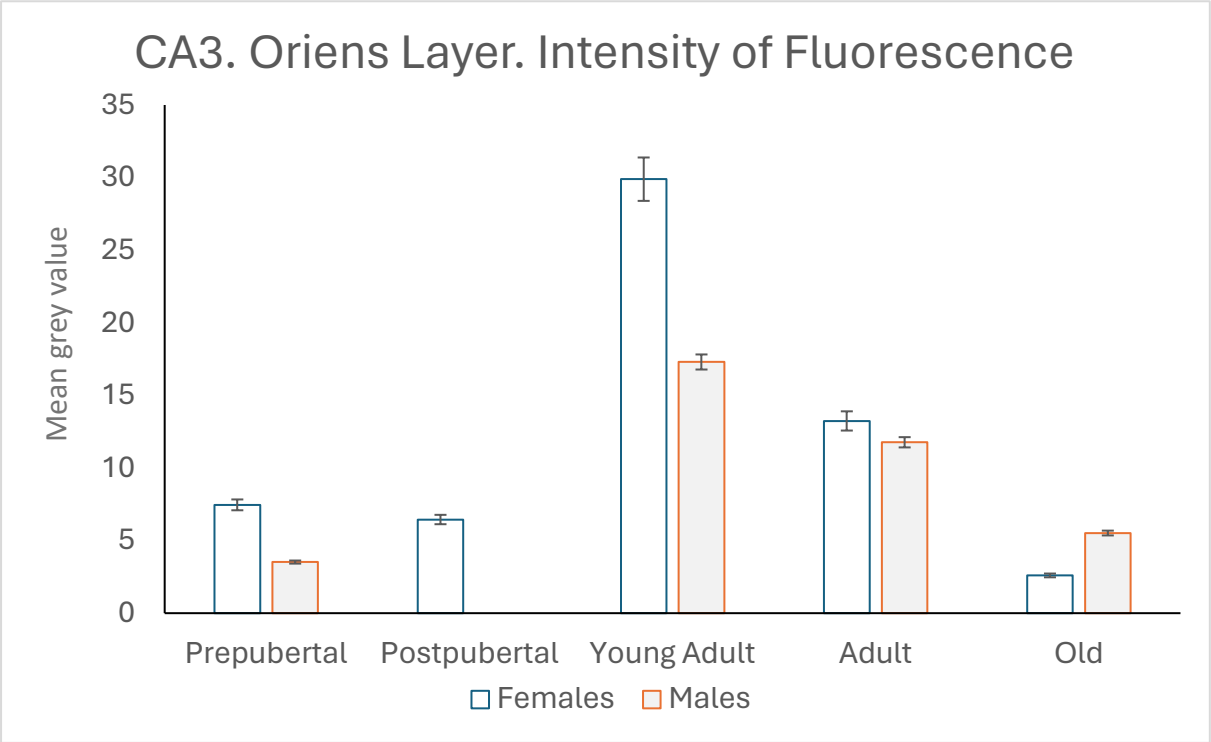
	P value	
Prepubertal F	**<0.0001**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**0.0009**	Adult M
Old F	**0.0009**	Old M



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**<0.0001**	Postpubertal F		
Young Adult F	**<0.0001**	**<0.0001**	Young Adult F	
Adult F	**<0.0001**	**<0.0001**	**<0.0001**	Adult F
Old F	**<0.0001**	**<0.0001**	**0.0004**	**<0.0001**
P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	**<0.0001**	**<0.0001**	Young Adult M	
Adult M	**<0.0001**	**<0.0001**	**<0.0001**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

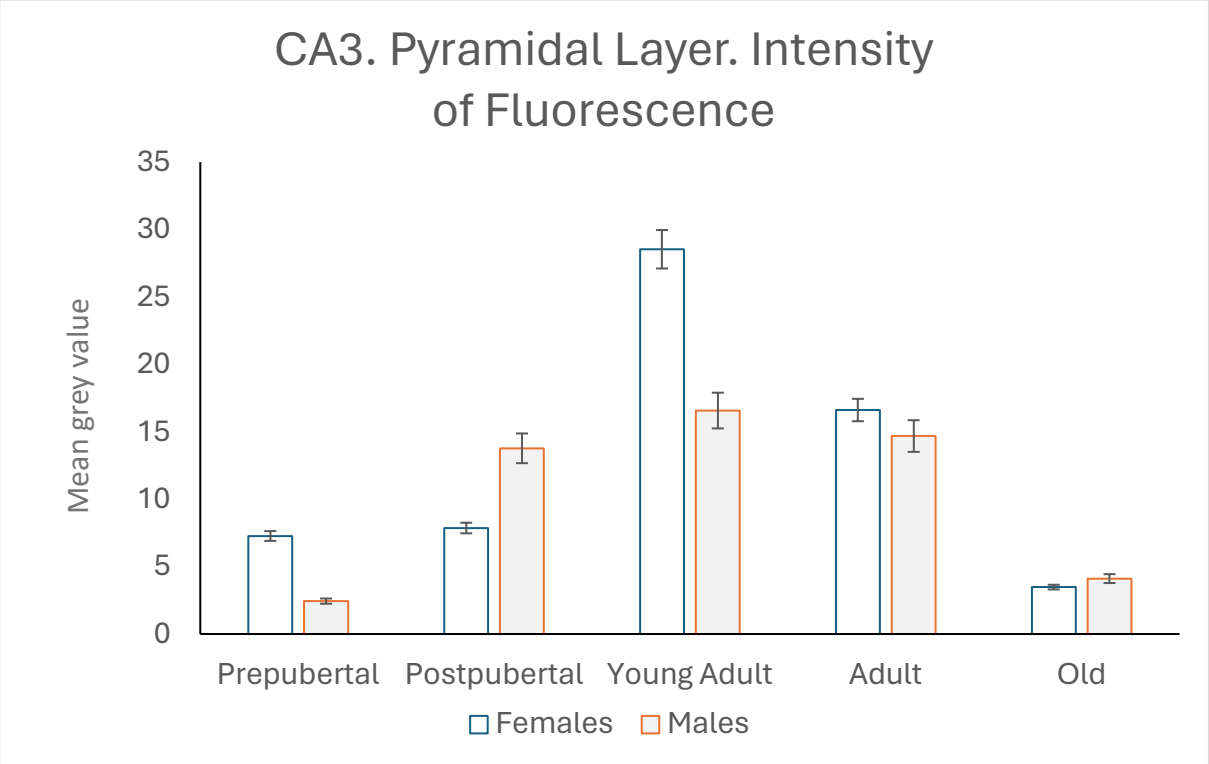
	P value	
Prepubertal F	**<0.0001**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**0.2192, NS**	Adult M
Old F	**0.0016**	Old M



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**0.0002**	Postpubertal F		
Young Adult F	**<0.0001**	**<0.0001**	Young Adult F	
Adult F	**<0.0001**	**<0.0001**	**<0.0001**	Adult F
Old F	**<0.0001**	**0.0005**	**<0.0001**	**<0.0001**
P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	**<0.0001**	**<0.0028**	Young Adult M	
Adult M	**<0.0001**	**<0.0001**	**<0.0001**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

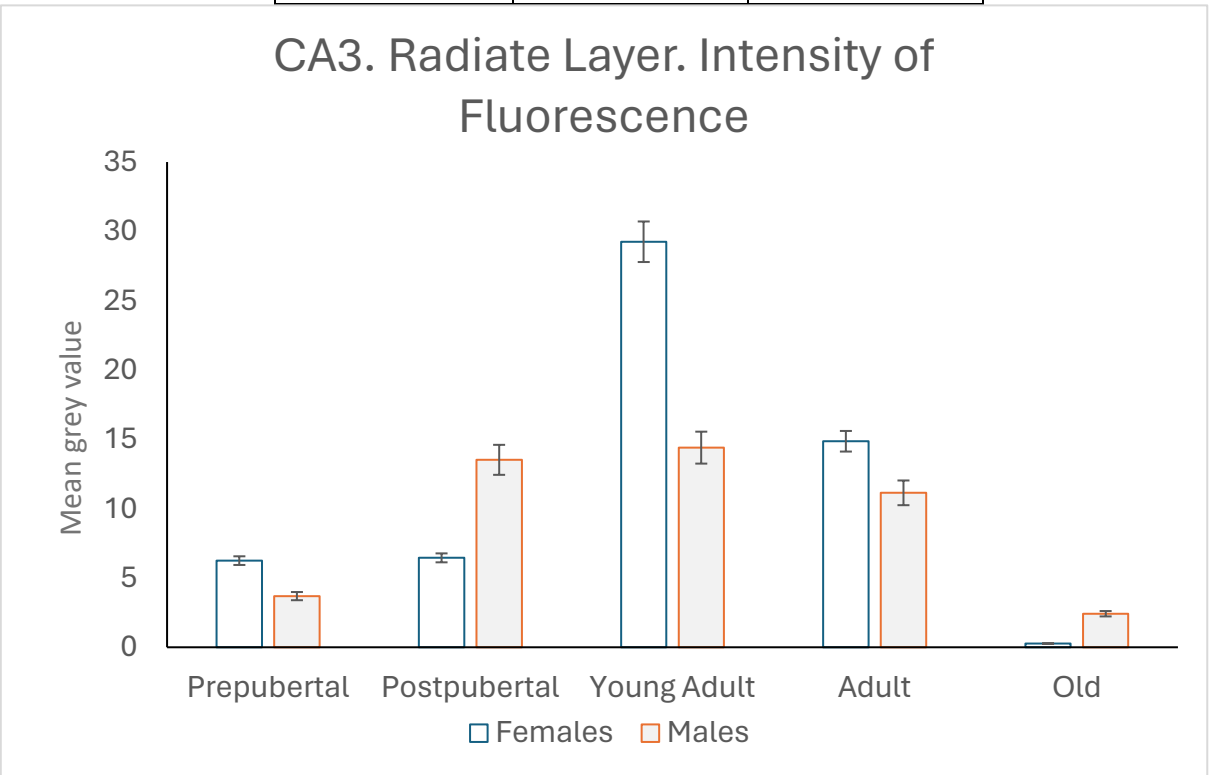
	P value	
Prepubertal F	**<0.0001**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**<0.0001**	Adult M
Old F	**<0.0001**	Old M



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**0.0432**	Postpubertal F		
Young Adult F	**<0.0001**	**<0.0001**	Young Adult F	
Adult F	**<0.0001**	**<0.0001**	**<0.9592, NS**	Adult F
Old F	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	**<0.0001**	**<0.0001**	Young Adult M	
Adult M	**<0.0001**	**0.0006**	**<0.0001**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

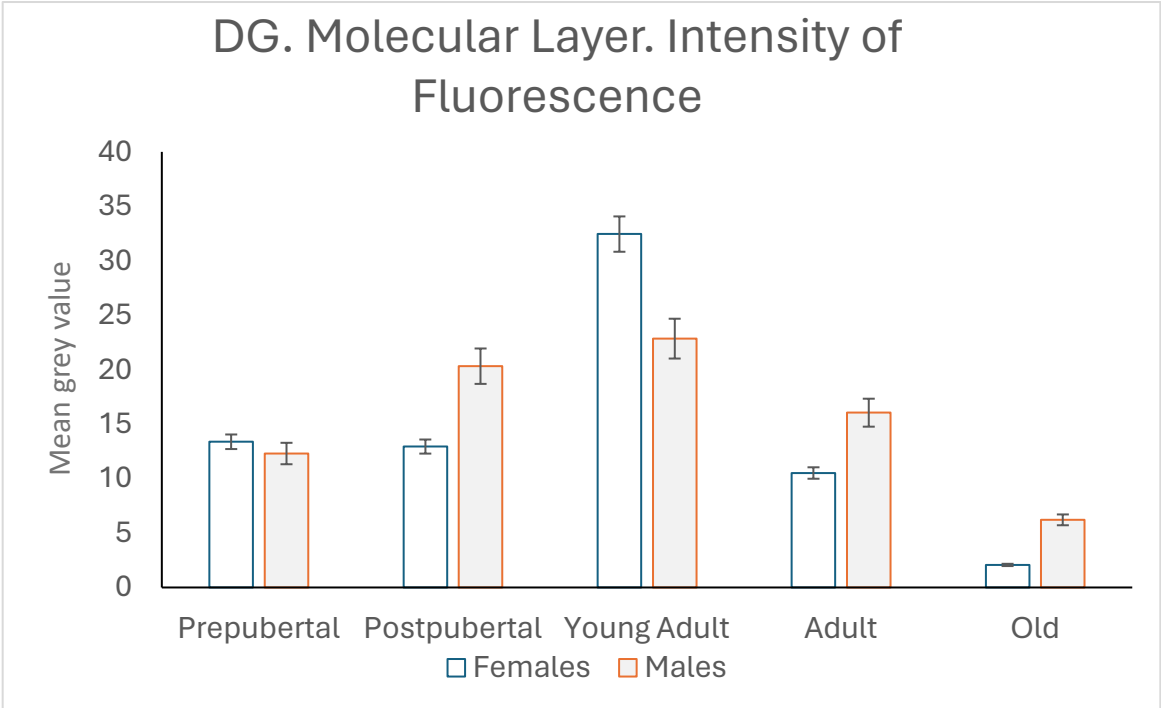
	P value	
Prepubertal F	**<0.0001**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**<0.0001**	Adult M
Old F	**0.0260**	Old M



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	0.9487, NS	Postpubertal F		
Young Adult F	<0.0001	<0.0001	Young Adult F	
Adult F	<0.0001	<0.0001	<0.0001	Adult F
Old F	<0.0001	<0.0001	<0.0001	<0.0001
P value	Prepubertal M			
Postpubertal M	<0.0001	Postpubertal M		
Young Adult M	<0.0001	0.0006	Young Adult M	
Adult M	<0.0001	<0.0001	<0.0001	Adult M
Old M	<0.0001	<0.0001	<0.0001	<0.0001

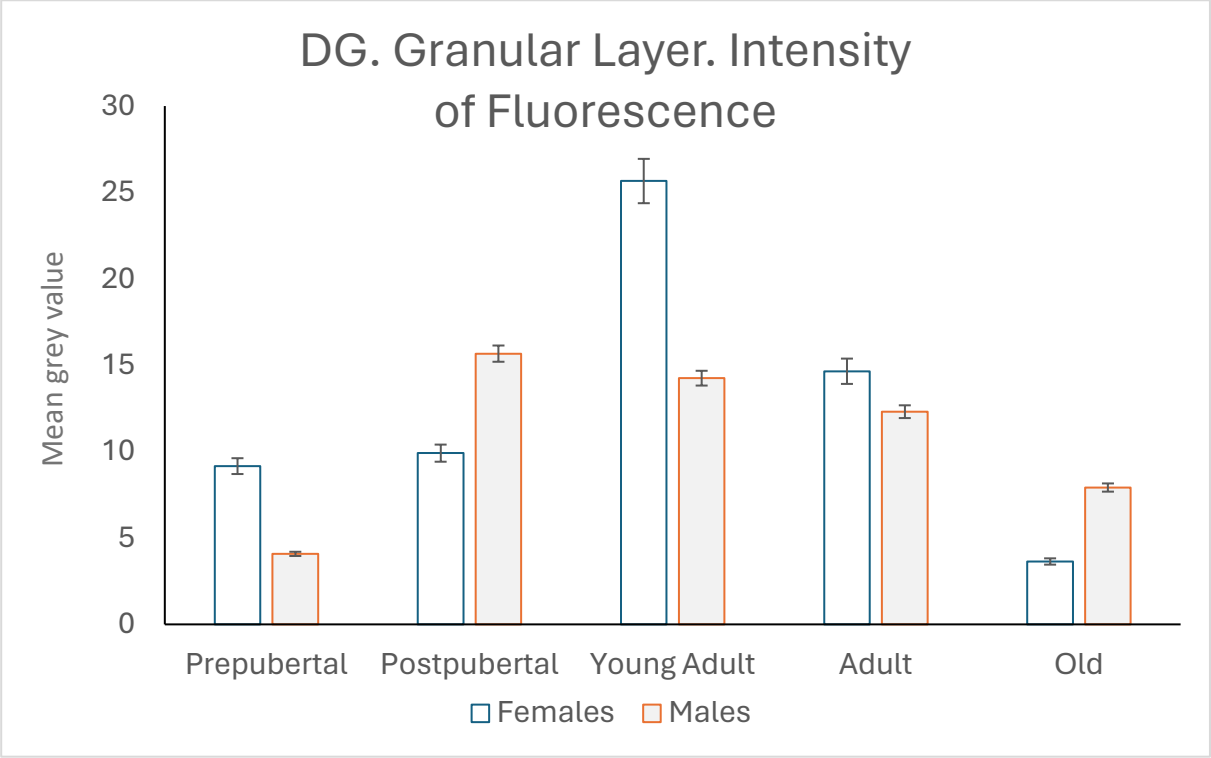
	P value	
Prepubertal F	<0.0001	Prepubertal M
Postpubertal F	<0.0001	Postpubertal M
Young Adult F	<0.0001	Young Adult M
Adult F	<0.0001	Adult M
Old F	<0.0001	Old M



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	0.1630, NS	Postpubertal F		
Young Adult F	<0.0001	<0.0001	Young Adult F	
Adult F	<0.0001	<0.0001	<0.0001	Adult F
Old F	<0.0001	0.0005	<0.0001	<0.0001
P value	Prepubertal M			
Postpubertal M	<0.0001	Postpubertal M		
Young Adult M	<0.0001	<0.0001	Young Adult M	
Adult M	<0.0001	<0.0001	<0.0001	Adult M
Old M	<0.0001	<0.0001	<0.0001	<0.0001

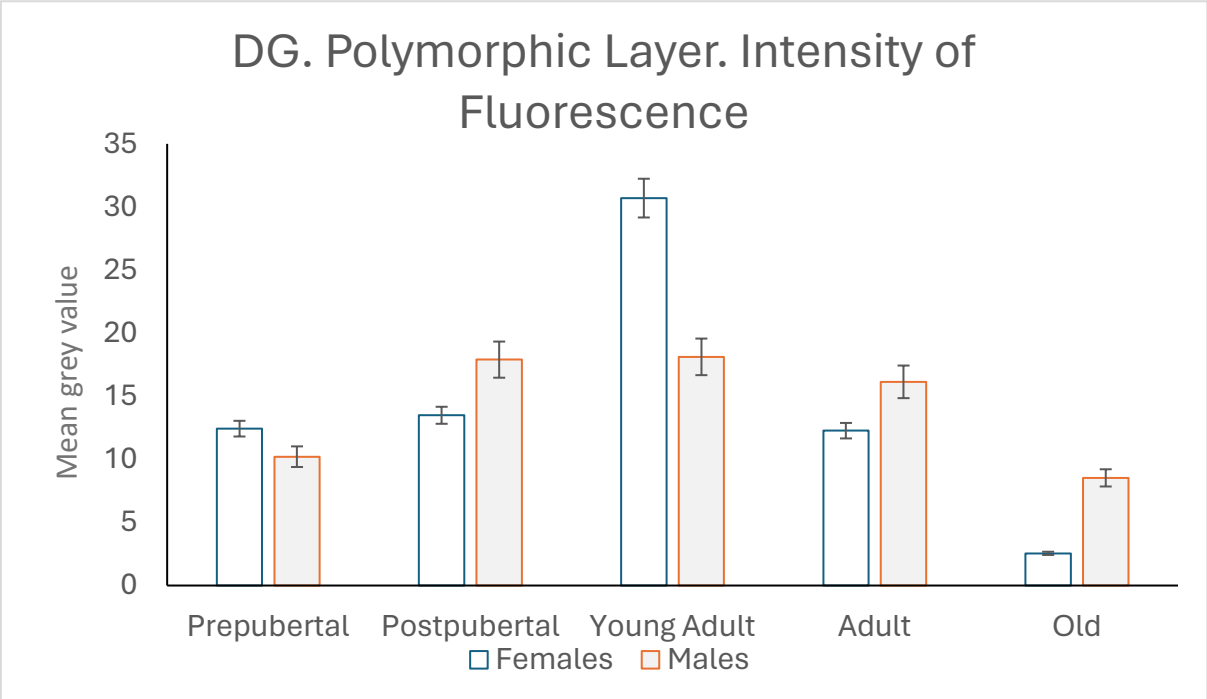
	P value	
Prepubertal F	<0.0001	Prepubertal M
Postpubertal F	<0.0001	Postpubertal M
Young Adult F	<0.0001	Young Adult M
Adult F	<0.0001	Adult M
Old F	<0.0001	Old M



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**0.0066**	Postpubertal F		
Young Adult F	**<0.0001**	**<0.0001**	Young Adult F	
Adult F	**<0.0001**	**<0.0001**	**<0.0001**	Adult F
Old F	**<0.0001**	**0.0005**	**<0.0001**	**<0.0001**
P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	**<0.0001**	**<0.0028**	Young Adult M	
Adult M	**<0.0001**	**<0.0001**	**0.0001**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

	P value	
Prepubertal F	**<0.0001**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**<0.0001**	Adult M
Old F	**<0.0001**	Old M



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**0.0004**	Postpubertal F		
Young Adult F	**<0.0001**	**<0.0001**	Young Adult F	
Adult F	**0.9957, NS**	**<0.0001**	**<0.0001**	Adult F
Old F	**<0.0001**	**0.0005**	**<0.0001**	**<0.0001**
P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	**<0.0001**	**0.9637, NS**	Young Adult M	
Adult M	**<0.0001**	**<0.0001**	**<0.0001**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

	P value	
Prepubertal F	**<0.0001**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**<0.0001**	Adult M
Old F	**<0.0001**	Old M

Morphological Features of Prolactin-Positive-Cells

CA1 Region

The immunohistochemical reaction pattern observed in the CA1 region of female rats was consistent with the hormone levels detected by ELISA and the hybridization signal for prolactin mRNA in this region.

In prepubertal females, the reaction is mainly localized to neurons in the pyramidal layer. In the postpubertal periods, the reaction predominated in the Oriens layer, although there was an increase in all layers, as shown by the densitometric gradient. Young adult females showed less reaction than postpubertal females and were mainly located in the pyramidal layer and basal lacunous stratum to the radiated layer. Immunostaining decreased in adult rats and even more so in older rats. In adults, the oriens layer was the most affected, while in old women, a punctiform reaction was observed in the neurons of the pyramidal layer and in some dendrites of the radiated layer.

The distribution of prolactin in the CA1 region of male rats, unlike in females, presented an unexpected finding when compared with the distribution of the hybridization signal, since the pattern and intensity of the reaction in postpubertal males was very similar to that observed in young adults. In prepubertal males, a punctate reaction appeared distributed through the

three layers of the CA1 region in an isolated and similar manner. Postpubertal males and young adults had a reaction in all three layers. As shown by the densitometric gradient, the reaction was greater in young adults and similarly throughout the CA1 region, whereas in postpubertal adults, the reaction was mainly located in the pyramidal layer and the areas close to it of the oriens and radiated layers. The reaction in adult males was isolated predominantly in pyramidal neurons. In old males, the reaction was limited to the pyramidal layer and isolated areas of the radiated layer, being almost non-existent in the oriens layer.

CA3 Region

In both sexes, the reaction patterns in each layer studied, oriens, pyramidal, and radiated in CA3, presented characteristics similar to those described for the CA1 reaction.

However, immunostaining for prolactin was more intense in young adult females than in postpubertal females, affecting the oriens and radiate layers more than the pyramidal layer. In females, the fibrillar reaction observed in young adults decreased in adult females and disappeared in older females that only had a reaction in the pyramidal layer.

In males, the reaction was preferentially located in the pyramidal layer, although postpubertal males and young adults also had a fibrillar reaction in the oriens and radiated layers. Unlike females, old males reacted in the oriens layer as well as in the pyramidal layer of CA3.

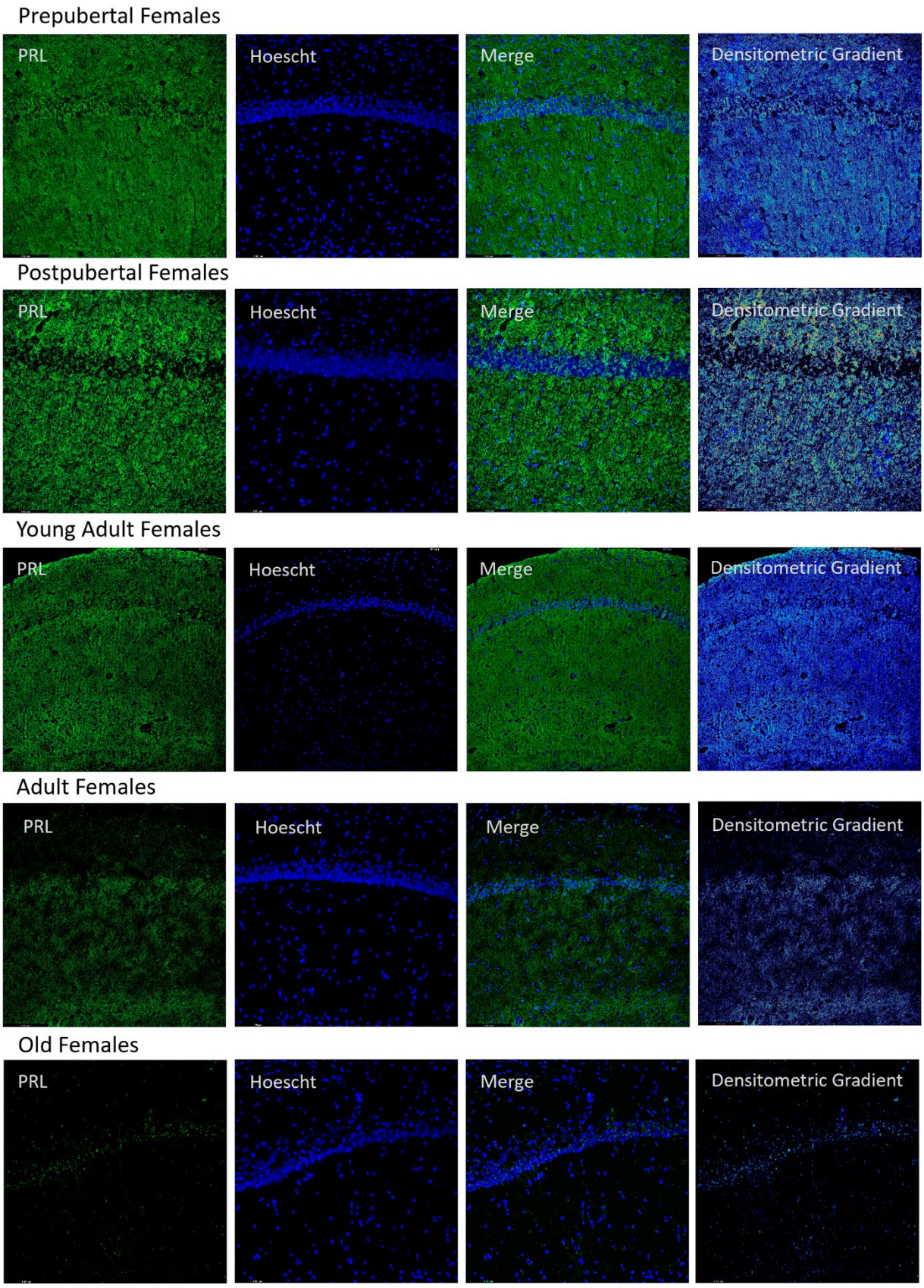
Dentate Gyrus

In the dentate gyrus of prepubertal and old females, immunostaining was very poor. Males, however, showed an evident reaction in the prepubertal stage and, as in postpubertal males, presented positive secondary neurons in the polymorphic layer.

Another difference observed in the dentate gyrus between females and males is that the former did not present positive subgranular cells, while prepubertal and postpubertal males presented strongly positive granular cells, being more abundant in the postpubertal males, in which some of these cells began to invade the granular layer.

Young adult females reacted preferentially in the polymorphic layer close to the granular layer, although no positive subgranular cells were identified, and the granular layer alternated zones of positive cells with negative cells. Adult females showed a greater reaction in the molecular layer adjacent to the granular layer, and in older females, the reaction was limited to a few positive granular cells.

Prepubertal males showed a reaction in the molecular and polymorphic layers, being very scarce in the granular layer, in which scattered reactive neurons appeared. Old males had a sparse reaction in the granular cells and a reactive band in the polymorphic layer that was interposed between a non-reactive zone near the granular layer and another non-reactive zone near the CA4 neurons that invade the hilum of the dentate gyrus.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PRL
Hoescht
Merge
Densitometric Gradient
Postpubertal Females
PRL
Hoescht
Merge
Densitometric Gradient
Young Adult Females
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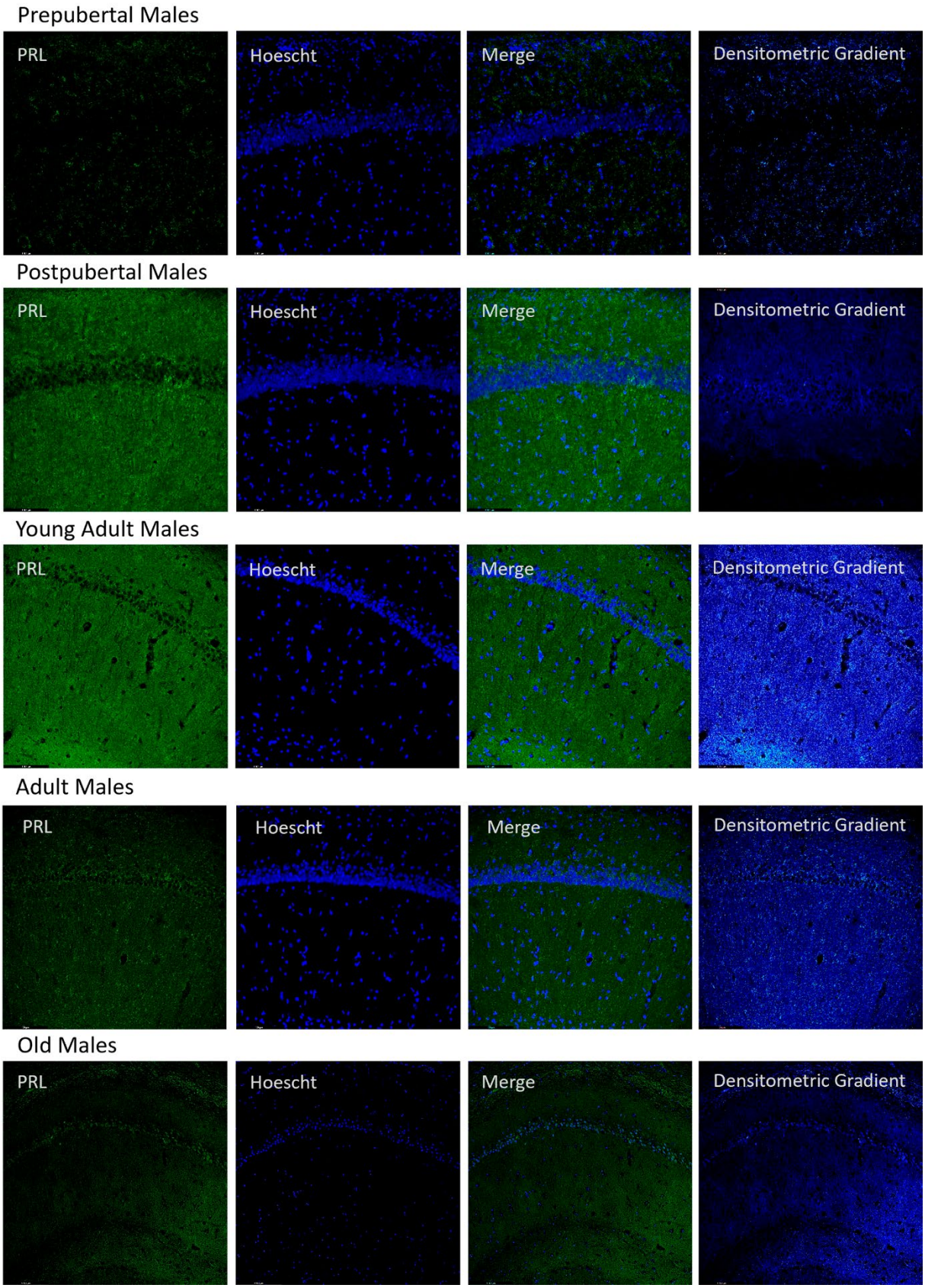
Merge
Densitometric Gradient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Densitometric Gradient
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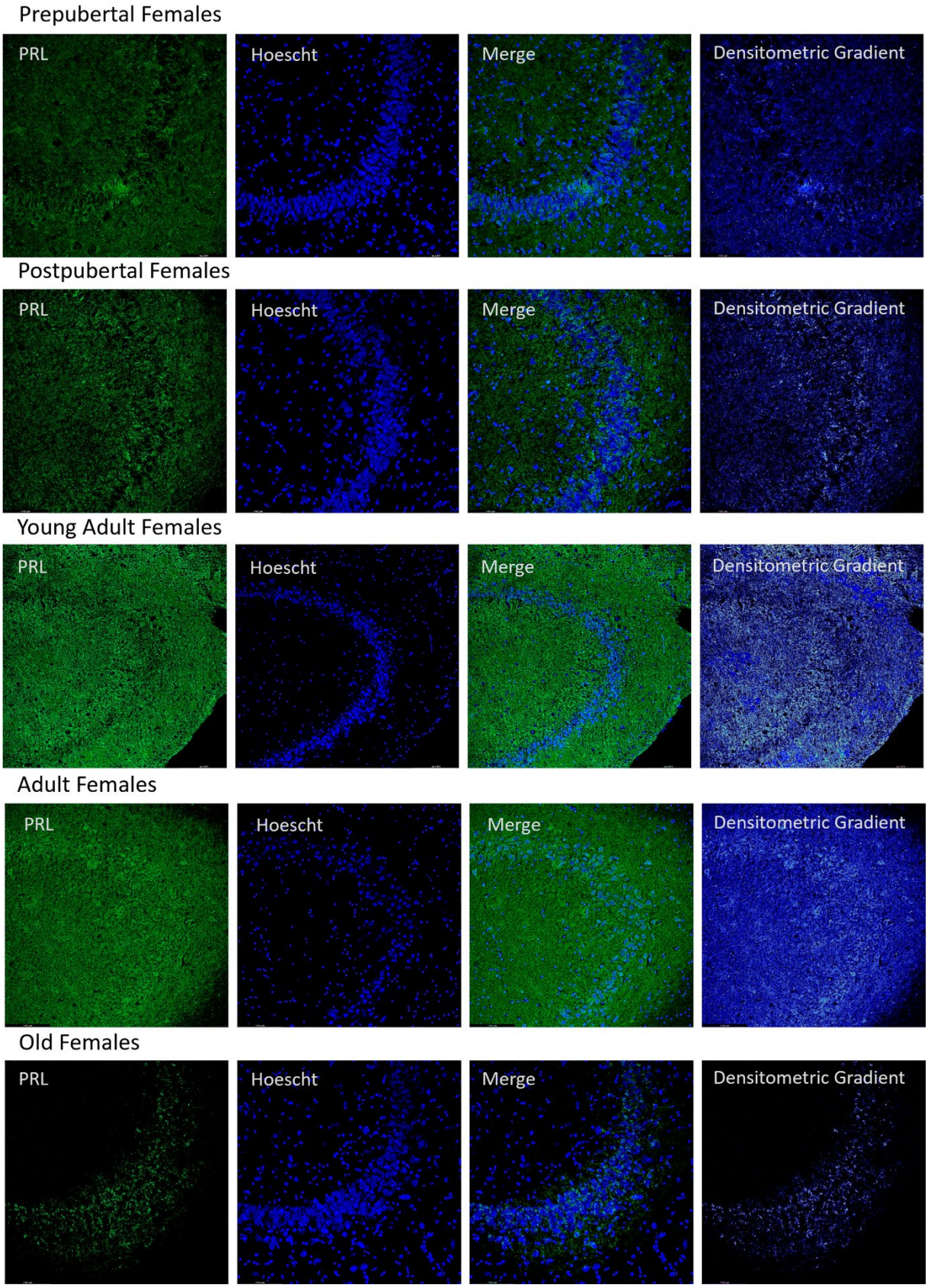
PRL
Hoescht
Merge
Densitometric Gradient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Densitometric Gradient
Postpubertal Females
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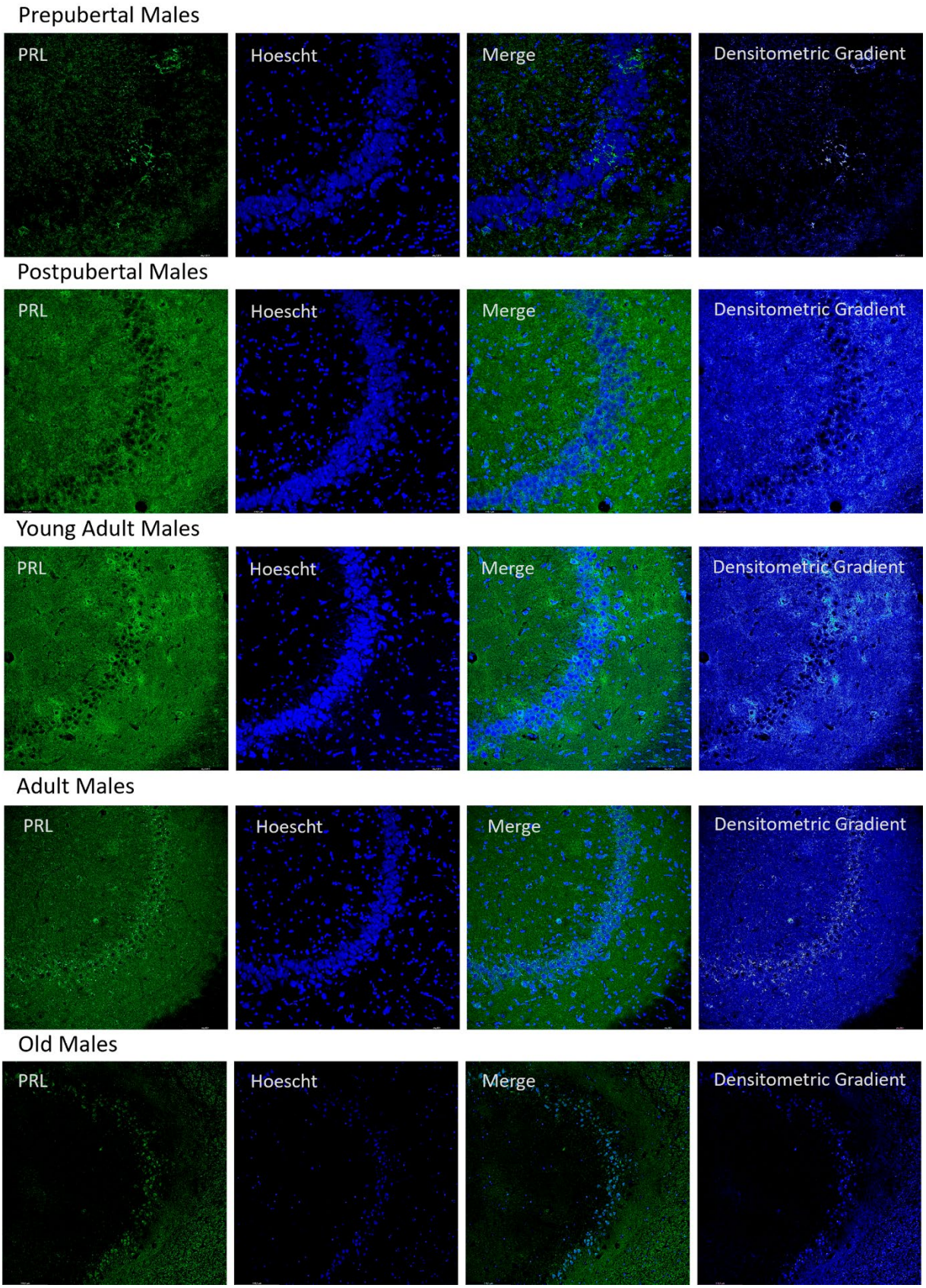
Densitometric Gradient


Prepubertal Males
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Postpubertal Males
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Young Adult Males
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Densitometric Gradient
Adult Males
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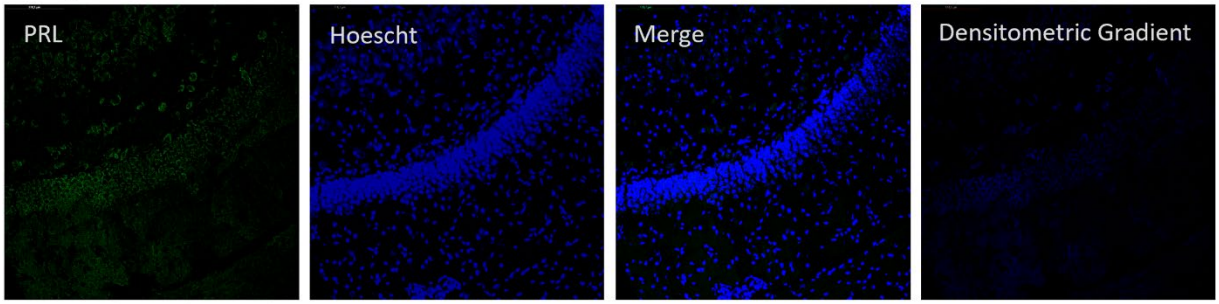
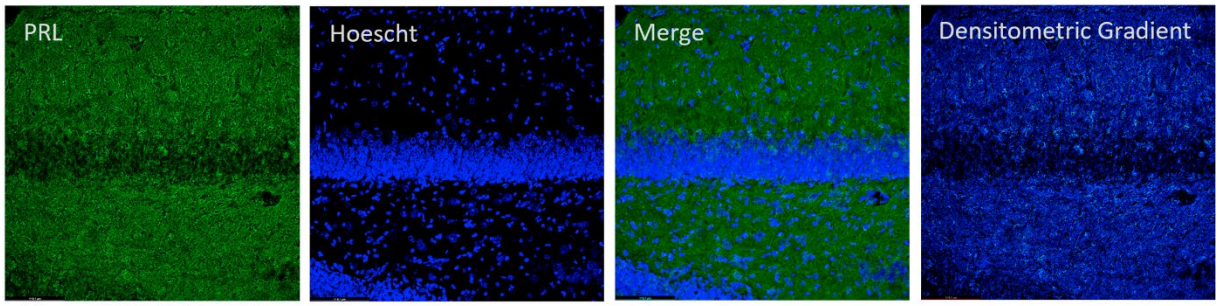
Densitometric Gradient
Old Males
PRL
Hoescht
Merge
Densitometric Gradient

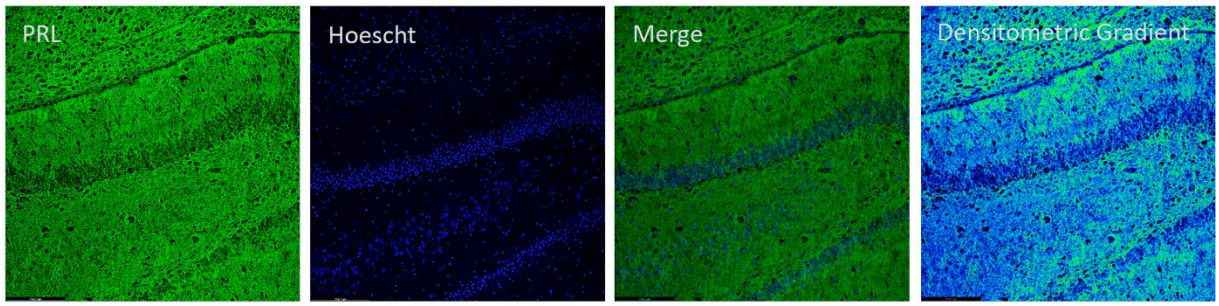
Prepubertal Females



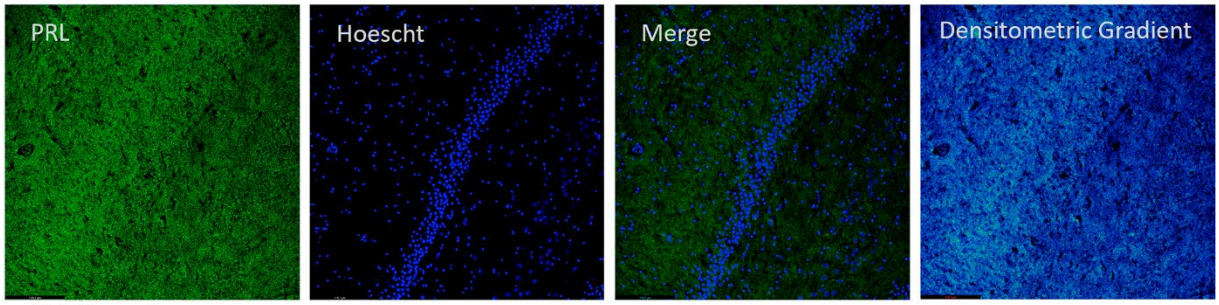
Postpubertal Females



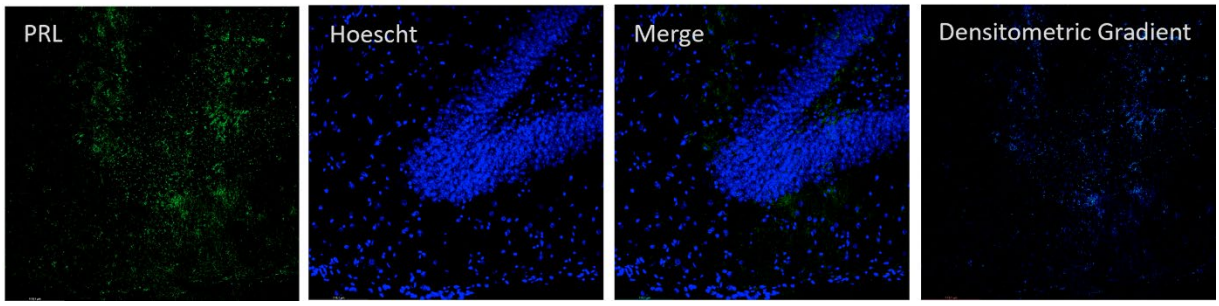
Young Adult Females

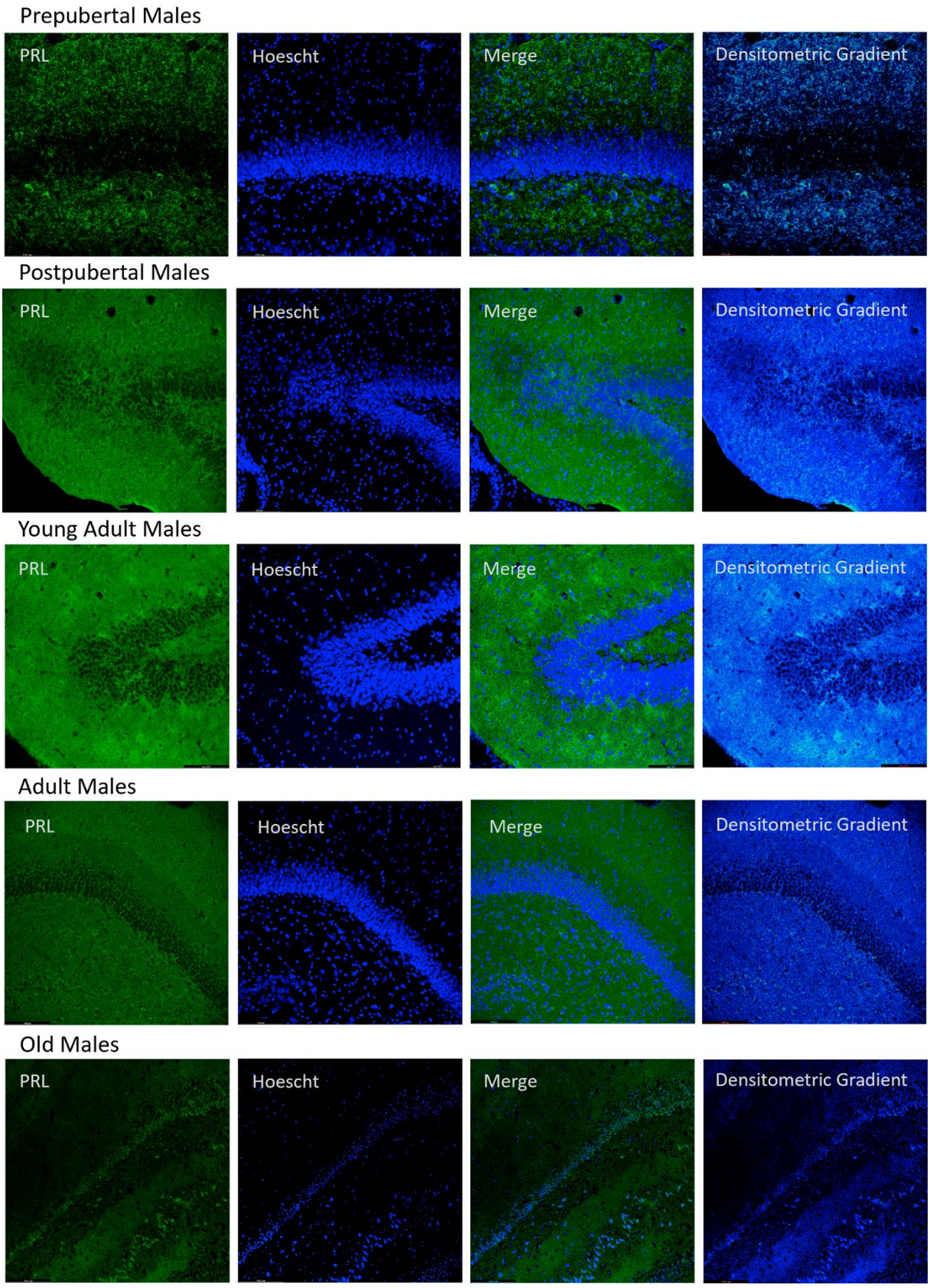


Adult Females



Old Females




Prepubertal Males
PRL
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Postpubertal Males
PRL
Hoescht
Merge
Densitometric Gradient
Young Adult Males
PRL
Hoescht
Merge
Densitometric Gradient
Adult Males
PRL
Hoescht
Merge
Densitometric Gradient
Old Males
PRL
Hoescht
Merge
Densitometric Gradient

Synthesis of the Prolactin-Receptors in the Hippocampus

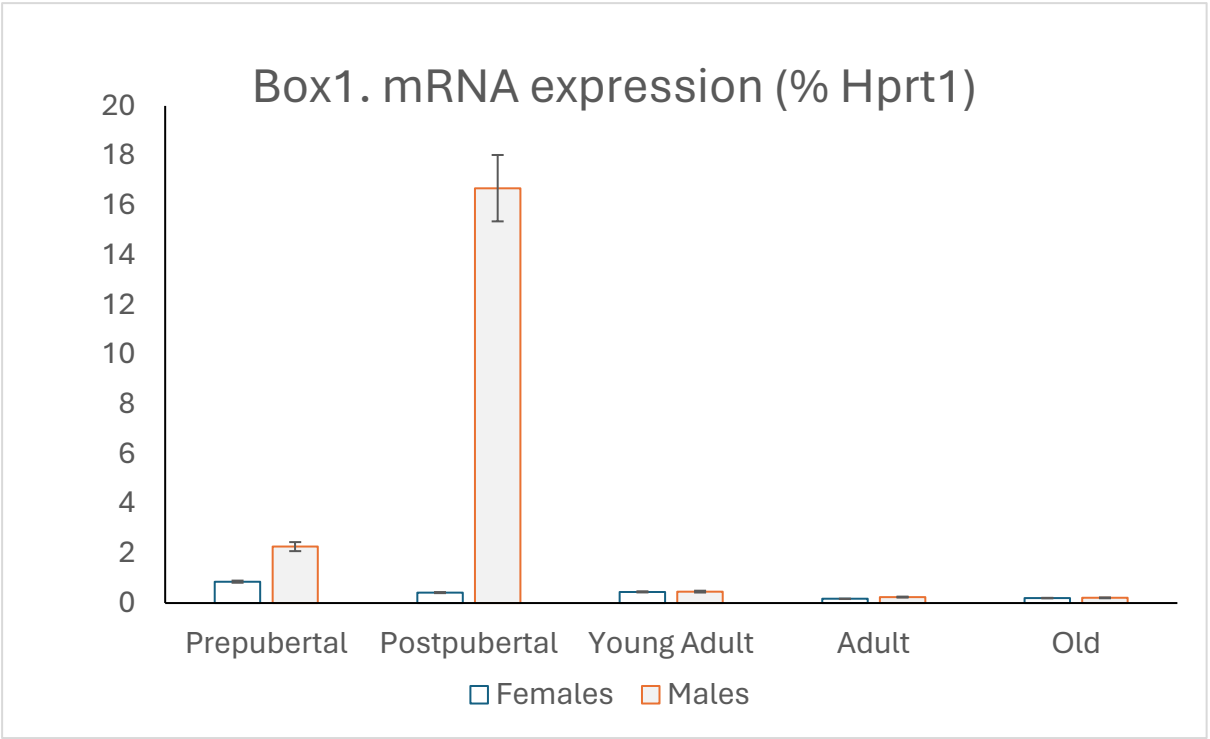
qPCR study of Box1 (PRLR S1) and Box2 (PRLR L1)

Prolactin receptor mRNA was detected in both sexes and age groups. Although the expression was relatively low in most of them, differential expression was observed among males and females. In both Box1 and Box2, female rats showed the highest expression in the prepubertal stage, which gradually decreased with age. There were no significant differences in the expression of Box1 among the groups studied, except when prepubertal females were compared with adult and old females ($p<0.01$).

The expression of Box 2 in prepubertal females showed significant differences ($p<0.01$) compared to the rest of the studied female groups. No significant differences were observed between the other groups. In males, significant differences were observed when comparing postpubertal males with the other study groups ($p<0.01$), and there were no significant differences among the other groups.

When comparing males and females, significant differences in Box2 expression were observed in the pre- and post-pubertal age groups, with no significant differences between young, adult, and old adult males and females. In male rats, the expression of Box1 and Box2 was higher in prepubertal males than in females ($p<0.01$) and had a very significant ($p<0.001$) peak when they reached puberty, being 47 times higher than in their young adult age.

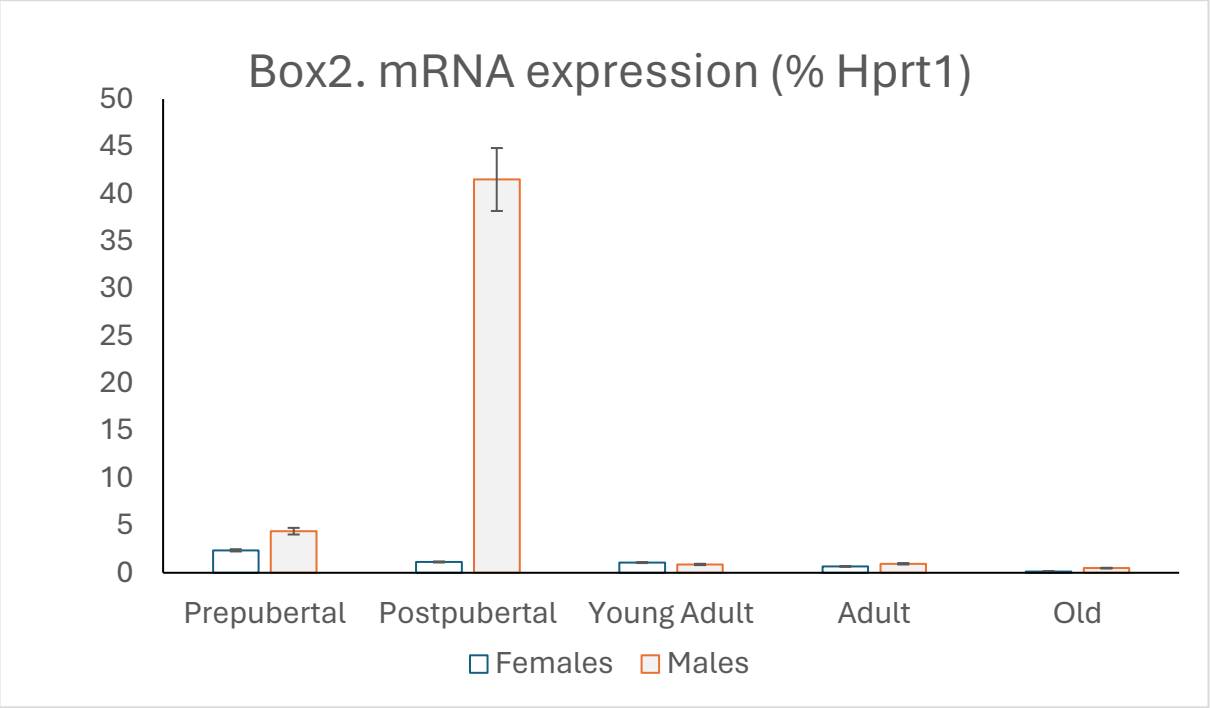
Interestingly, both isoforms of the receptor behave in the same way, but the expression of box2 was at least twice that of box1 for all groups ($p<0.01$) in male rats, while in females, the difference was not significant.



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	0.1211, NS	Postpubertal F		
Young Adult F	0.1758, NS	>0.9999, NS	Young Adult F	
Adult F	0.0031	0.7542, NS	0.6303, NS	Adult F
Old F	0.0049	0.8590, NS	0.7542, NS	>0.9999, NS
P value	Prepubertal M			
Postpubertal M	<0.0001	Postpubertal M		
Young Adult M	<0.0001	<0.0001	Young Adult M	
Adult M	<0.0001	<0.0001	0.8590, NS	Adult M
Old M	<0.0001	<0.0001	0.7542, NS	>0.9999, NS

	P value	
Prepubertal F	<0.0001	Prepubertal M
Postpubertal F	<0.0001	Postpubertal M
Young Adult F	0.9999, NS	Young Adult M
Adult F	>0.9999, NS	Adult M
Old F	>0.9999, NS	Old M



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**0.0044**	Postpubertal F		
Young Adult F	**0.0037**	**>0.9999, NS**	Young Adult F	
Adult F	**0.0030**	**>0.9999, NS**	**>0.9999, NS**	Adult F
Old F	**0.0023**	**>0.9999, NS**	**>0.9999, NS**	**>0.9999, NS**
P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	**0.9968, NS**	**<0.0001**	Young Adult M	
Adult M	**0.9972, NS**	**<0.0001**	**>0.9999, NS**	Adult M
Old M	**0.9931, NS**	**<0.0001**	**>0.9999, NS**	**>0.9999, NS**

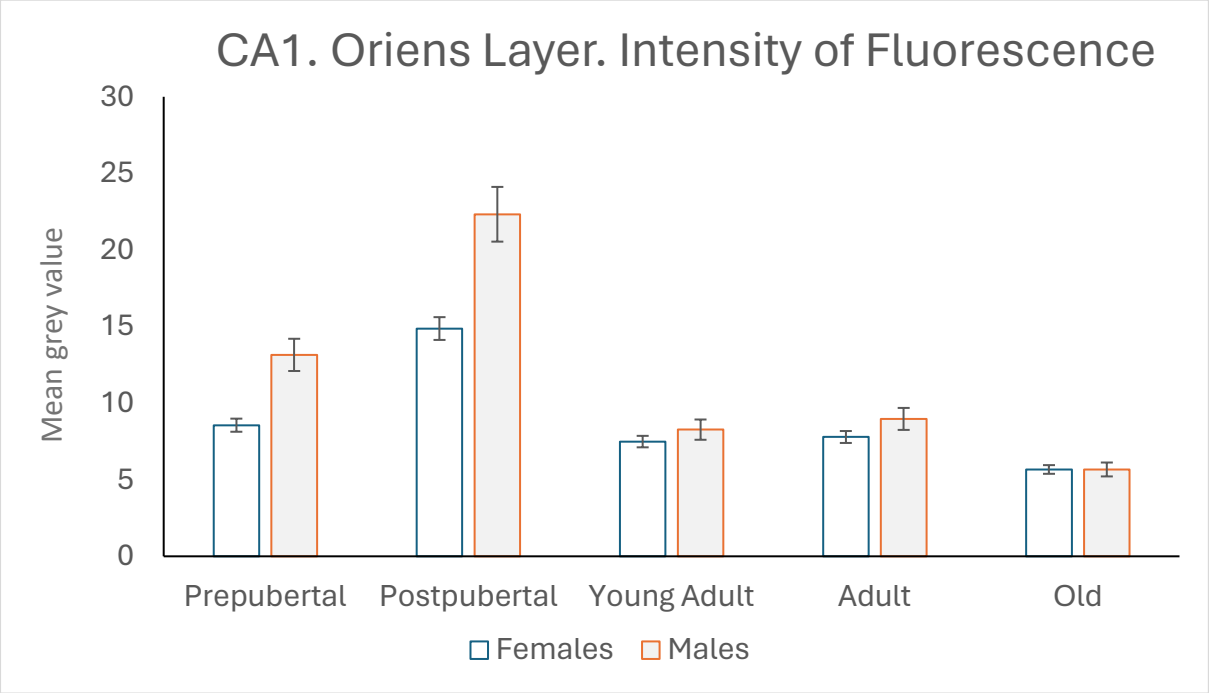
	P value	
Prepubertal F	**0.0206**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**>0.9999, NS**	Young Adult M
Adult F	**>0.9999, NS**	Adult M
Old F	**>0.9999, NS**	Old M

Prolactin Receptor L1-Synthesizing Cells. In situ Hybridization Study

The presence of box2 mRNA was detected by in situ hybridization in all regions and in all groups studied. The oriens layer of the CA1 region showed age-related variations that followed a similar pattern in males and females. However, there were significant differences ($p<0.01$) between males and females in the pre- and post-pubertal stages, and the values observed in males were significantly higher than those in females ($p<0.01$). There were no significant differences between the sexes in the other age groups. In both sexes, the value of the middle grey color increased significantly from the pre- to post-pubertal stages, decreased significantly in young adults, remained in adulthood, and decreased significantly again in older animals. There were no significant differences between the young adults and adults. The densitometric pattern of the pyramidal layer, which was modified with age, was very similar to that of the oriens layer. The greatest differences were observed in the prepubertal stage, as the mean gray value observed in females was significantly higher than that in males ($p<0.01$). The variations observed with age in the radiated layer were similar to those described in the oriens layer, although in the radiated layer, the differences observed between males and females were always significant ($p<0.01$), and the males presented higher mean gray values than the females, except for the old animals.

In the CA3 oriens layer of female rats, the values of medium gray were very similar from the prepubertal stage to the young adults, with no significant differences among them, decreasing in the adult animals ($p<0.01$) and even more in the old animals ($p<0.01$). In males, the values observed for the pre- and post-pubertal stages were similar, decreasing significantly in young adults ($p<0.01$), increasing slightly in adults ($p<0.01$), and decreasing significantly in older adults ($p<0.01$). The densitometric patterns observed in the pyramidal layer of CA3 and the radiate layer of CA3 were similar to those described for the radiated layer of CA1, presenting higher values in males than in females for all age stages studied, except for older animals that were lower ($p<0.01$). The highest values were found in postpubertal males ($p<0.01$ compared with the rest of the groups studied). In females, the values observed in the young adult, adult, and old stages were not significantly different. The radiate layer in females was characterized by the fact that the observed values decreased from prepubertal to old age, striking that postpubertal animals had significantly lower values than prepubertal animals ($p<0.01$). In this layer, the only non-significant difference was observed between young adult and adult females.

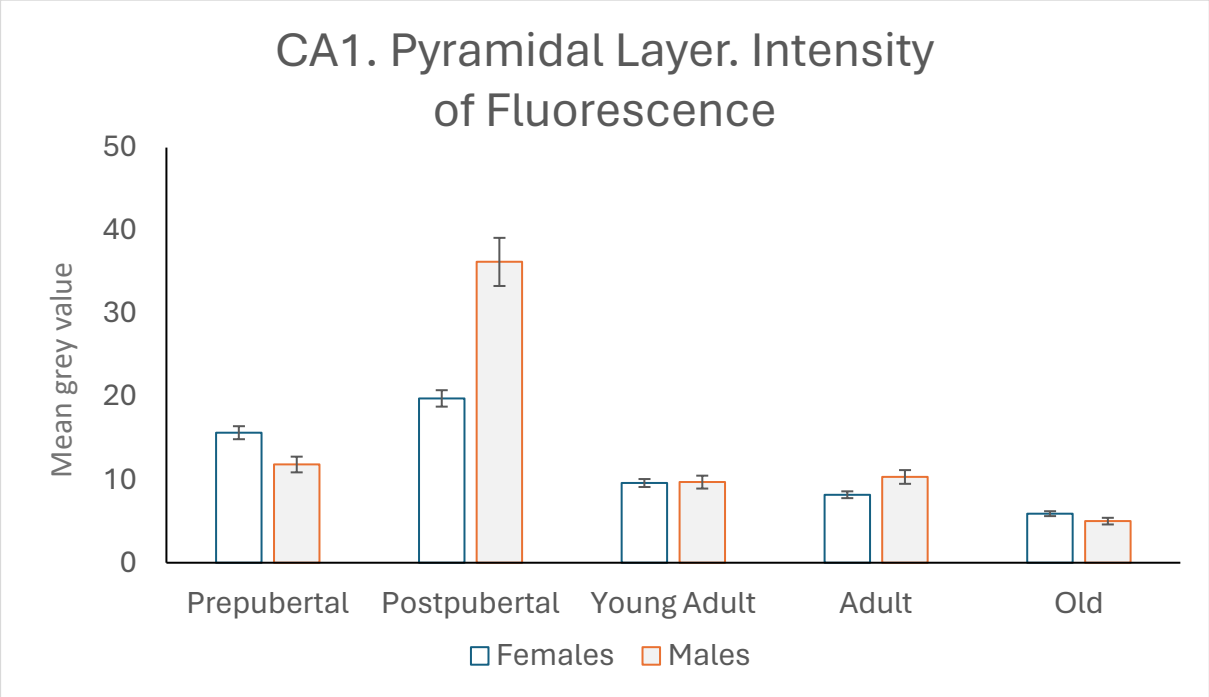
In the three layers of the dentate gyrus of males, it was observed that the mean grey values were modified in sawtooth according to age. Thus, they increased from prepubertal to postpubertal ($p<0.01$), decreased dramatically in young adults ($p<0.01$), increased again in adults ($p<0.01$), and finally decreased in old animals ($p<0.01$). Similar results were observed in females, with values that were almost always lower than those observed in males, although the differences between males and females were not always significant, drawing attention to the fact that the values were higher in females than in males in the granular and polymorphic layers of young adults ($p<0.01$), and in the granular layer of old animals ($p<0.01$).



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**<0.0001**	Postpubertal F		
Young Adult F	**0.4965, NS**	**<0.0001**	Young Adult F	
Adult F	**0.8464, NS**	**<0.0001**	**0.9997, NS**	Adult F
Old F	**0.0003**	**<0.0001**	**<0.0350**	**<0.0095**
P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	**<0.0001**	**<0.0001**	Young Adult M	
Adult M	**<0.0001**	**<0.0001**	**0.9037, NS**	Adult M
Old M	**<0.0001**	**<0.0001**	**0.0011**	**<0.0001**

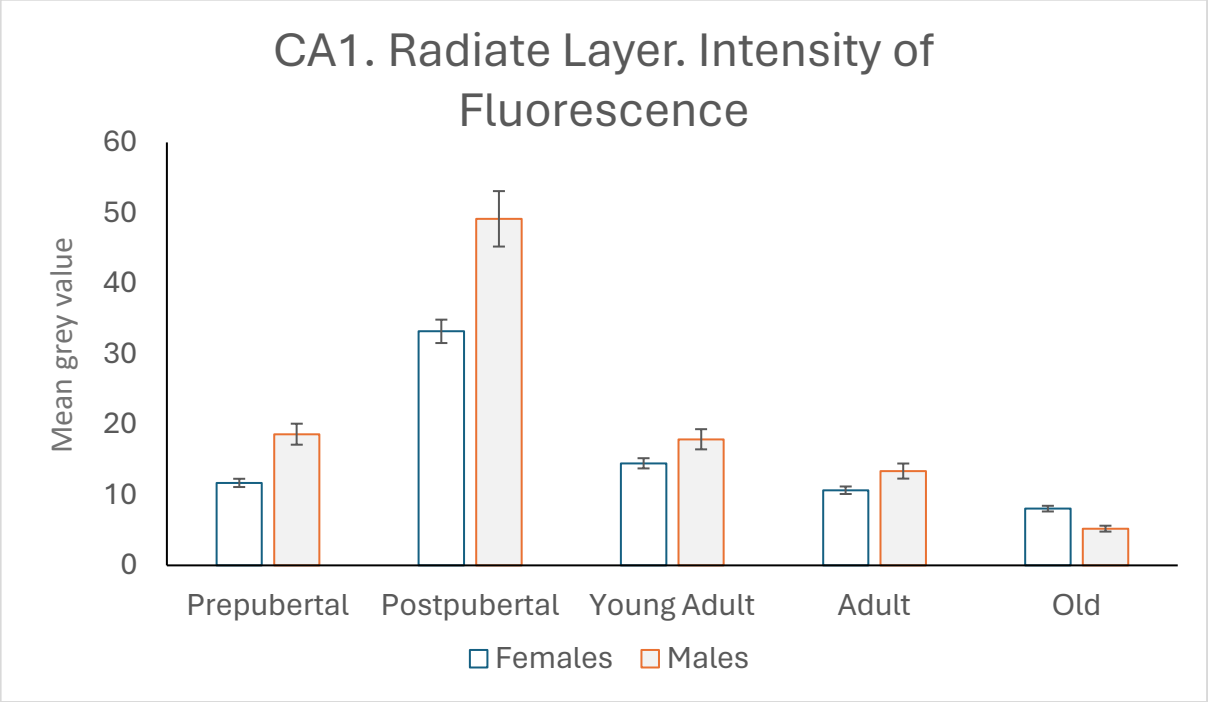
	P value	
Prepubertal F	**<0.0001**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**0.8371, NS**	Young Adult M
Adult F	**0.3706, NS**	Adult M
Old F	**>0.9999, NS**	Old M



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**<0.0001**	Postpubertal F		
Young Adult F	**<0.0001**	**<0.0001**	Young Adult F	
Adult F	**<0.0001**	**<0.0001**	**0.1770, NS**	Adult F
Old F	**<0.0001**	**<0.0001**	**<0.0001**	**0.0047**
P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	0.0096	**<0.0001**	Young Adult M	
Adult M	**0.1274, NS**	**<0.0001**	**0.9508, NS**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

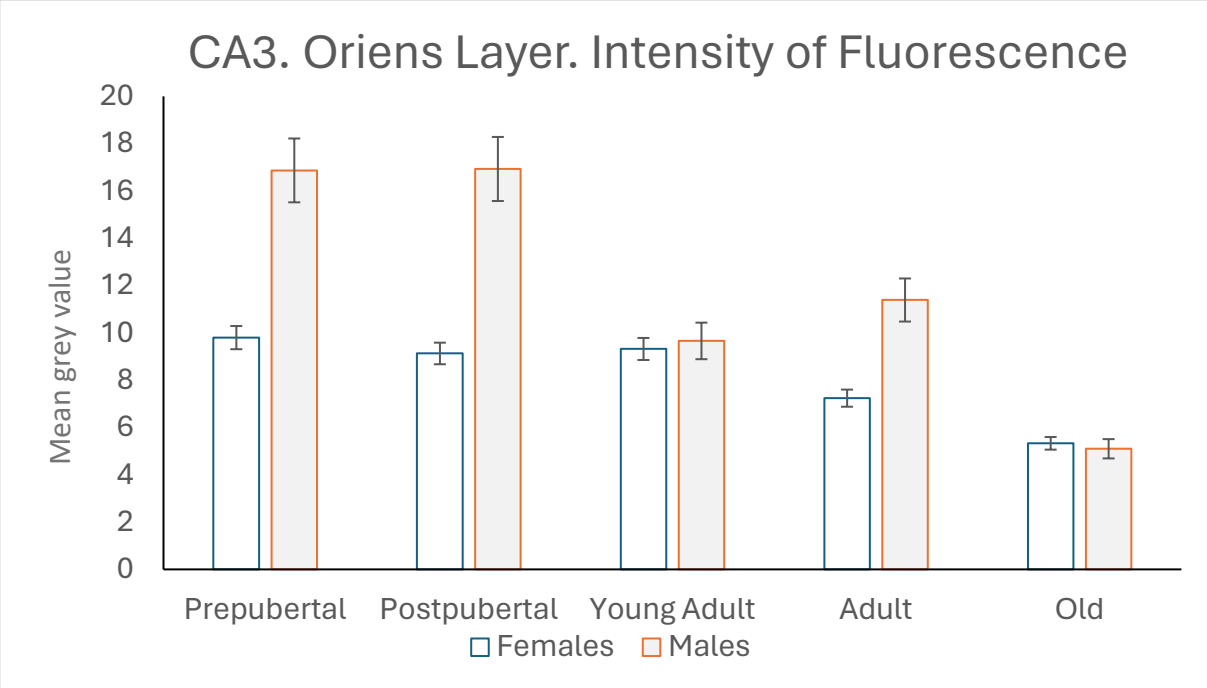
	P value	
Prepubertal F	**<0.0001**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**>0.9999, NS**	Young Adult M
Adult F	**0.0088**	Adult M
Old F	**0.7075, NS**	Old M



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**<0.0001**	Postpubertal F		
Young Adult F	**0.0004**	**<0.0001**	Young Adult F	
Adult F	**0.5133, NS**	**<0.0001**	**<0.0001**	Adult F
Old F	**<0.0001**	**<0.0001**	**<0.0001**	**0.0009**
P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	**0.8800, NS**	**<0.0001**	Young Adult M	
Adult M	**<0.0001**	**<0.0001**	**<0.0001**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

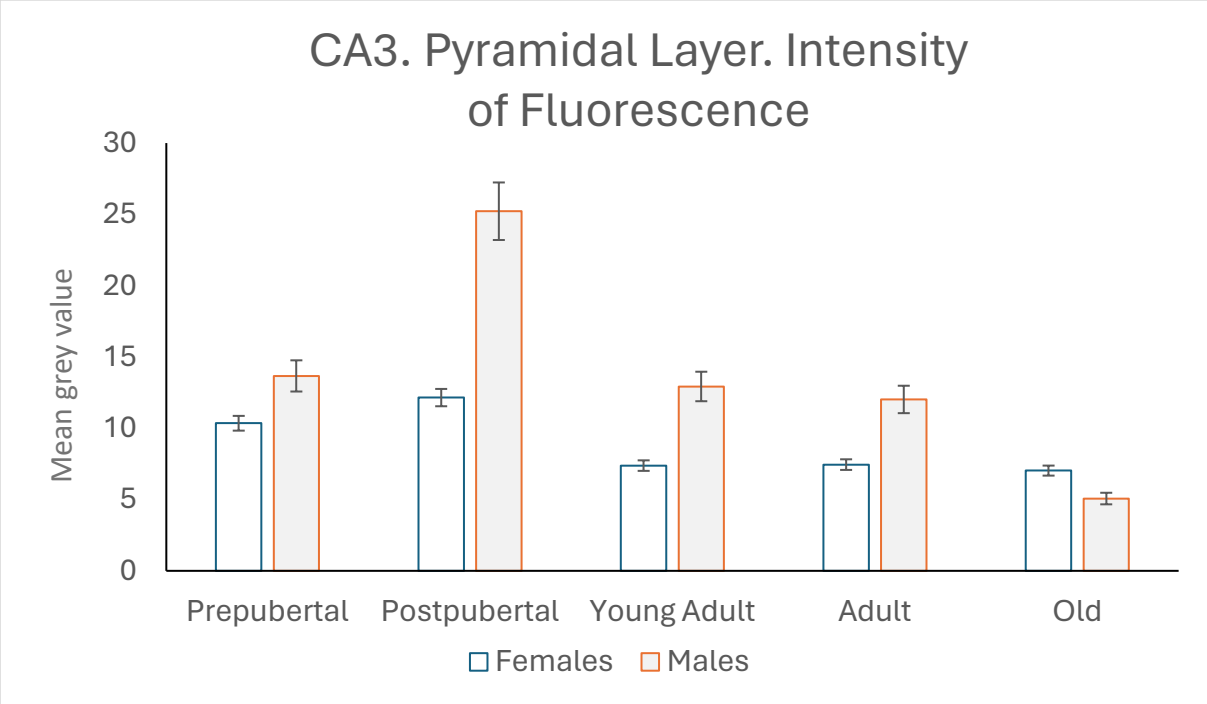
	P value	
Prepubertal F	**<0.0001**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**0.0005**	Adult M
Old F	**0.0003**	Old M



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	0.0620, NS	Postpubertal F		
Young Adult F	0.3528, NS	0.9895, NS	Young Adult F	
Adult F	<0.0001	<0.0001	<0.0001	Adult F
Old F	<0.0001	<0.0001	<0.0048	<0.0001
P value	Prepubertal M			
Postpubertal M	>0.9999, NS	Postpubertal M		
Young Adult M	<0.0001	<0.0001	Young Adult M	
Adult M	<0.0001	<0.0001	<0.0001	Adult M
Old M	<0.0001	<0.0001	<0.0001	<0.0001

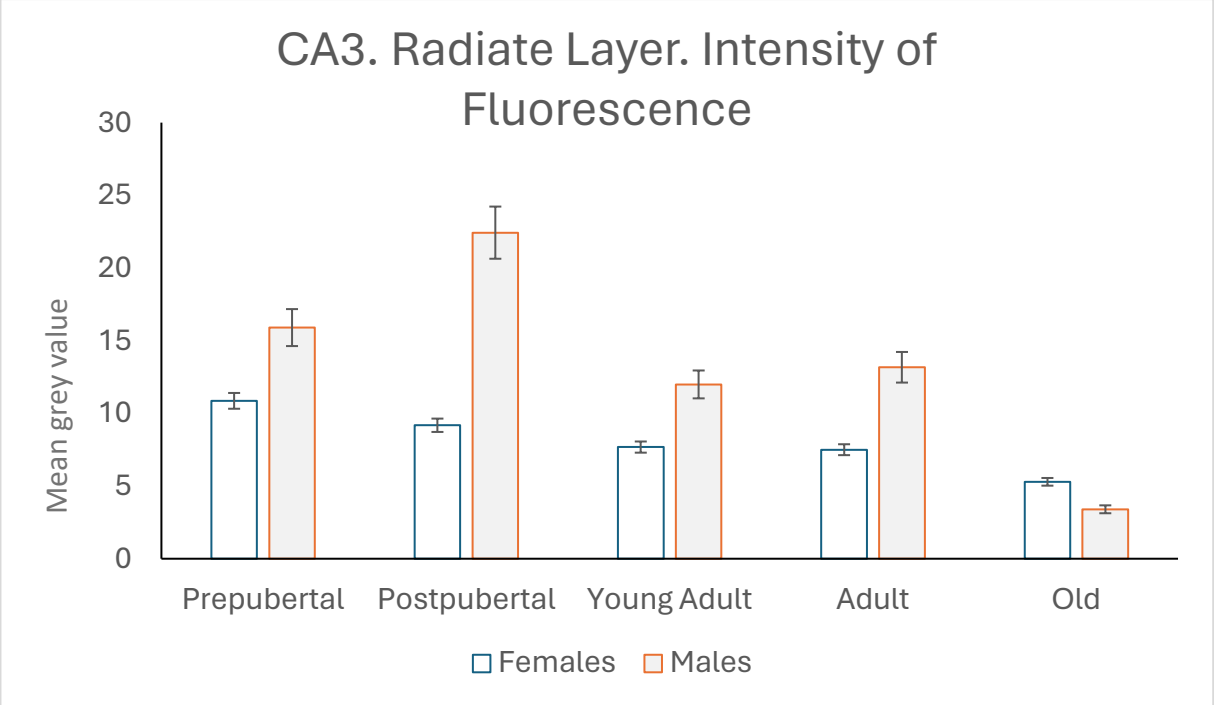
	P value	
Prepubertal F	<0.0001	Prepubertal M
Postpubertal F	<0.0001	Postpubertal M
Young Adult F	0.7696, NS	Young Adult M
Adult F	<0.0001	Adult M
Old F	0.9676, NS	Old M



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**<0.0004**	Postpubertal F		
Young Adult F	**<0.0001**	**<0.0001**	Young Adult F	
Adult F	**<0.0001**	**<0.0001**	**>0.999, NS**	Adult F
Old F	**<0.0001**	**<0.0001**	**0.8668, NS**	**<0.7107, NS**
P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	**0.1195, NS**	**<0.0001**	Young Adult M	
Adult M	**<0.0001**	**<0.0001**	**0.0157**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

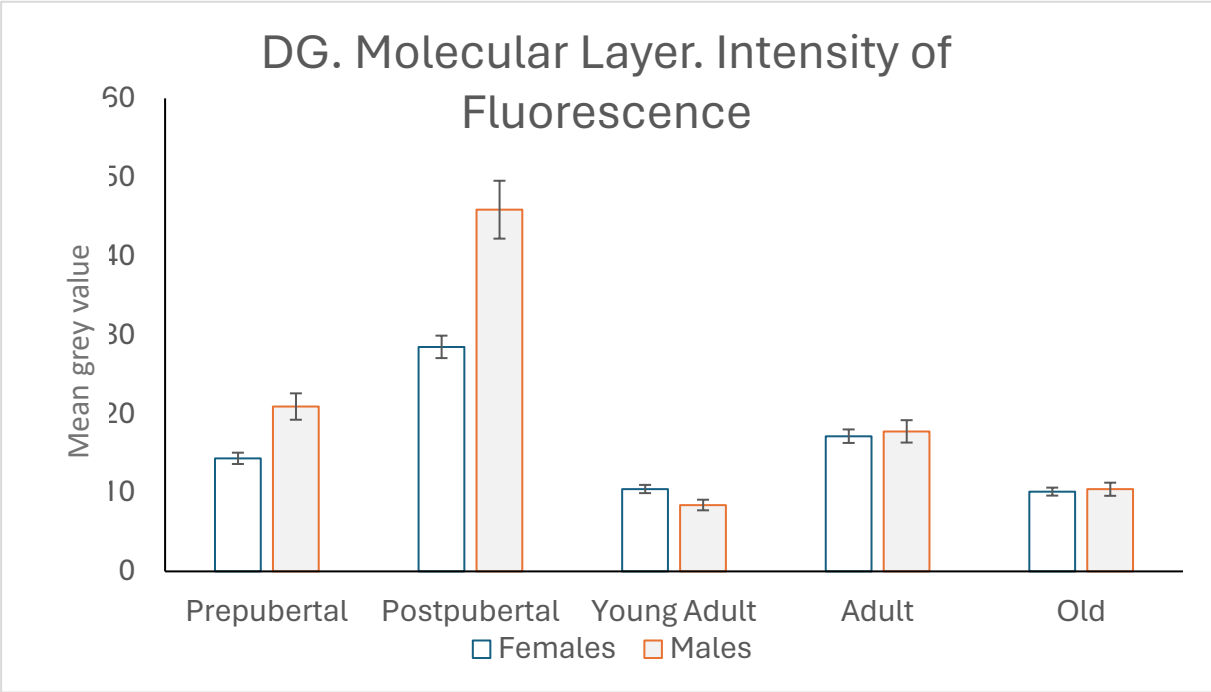
	P value	
Prepubertal F	**<0.0001**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**<0.0001**	Adult M
Old F	**<0.0001**	Old M



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**<0.0001**	Postpubertal F		
Young Adult F	**<0.0001**	**<0.0001**	Young Adult F	
Adult F	**<0.0001**	**<0.0001**	**0.9924, NS**	Adult F
Old F	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	**<0.0001**	**<0.0001**	Young Adult M	
Adult M	**<0.0001**	**<0.0001**	**0.0002**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

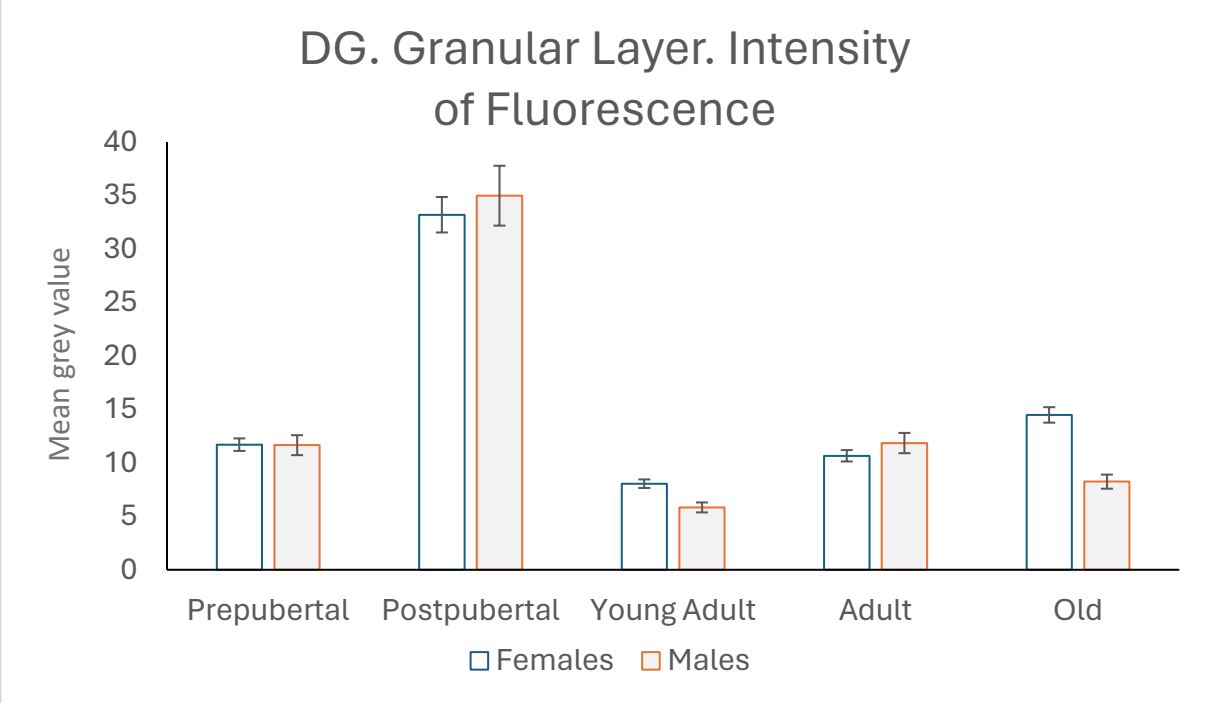
	P value	
Prepubertal F	**<0.0001**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**<0.0001**	Adult M
Old F	**<0.0001**	Old M



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**<0.0004**	Postpubertal F		
Young Adult F	**<0.0001**	**<0.0001**	Young Adult F	
Adult F	**<0.0001**	**<0.0001**	**<0.0001**	Adult F
Old F	**<0.0001**	**<0.0001**	**0.9929, NS**	**<0.0001**
P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	**<0.0001**	**<0.0001**	Young Adult M	
Adult M	**<0.0001**	**<0.0001**	**<0.0001**	Adult M
Old M	**<0.0001**	**<0.0001**	**0.0005**	**<0.0001**

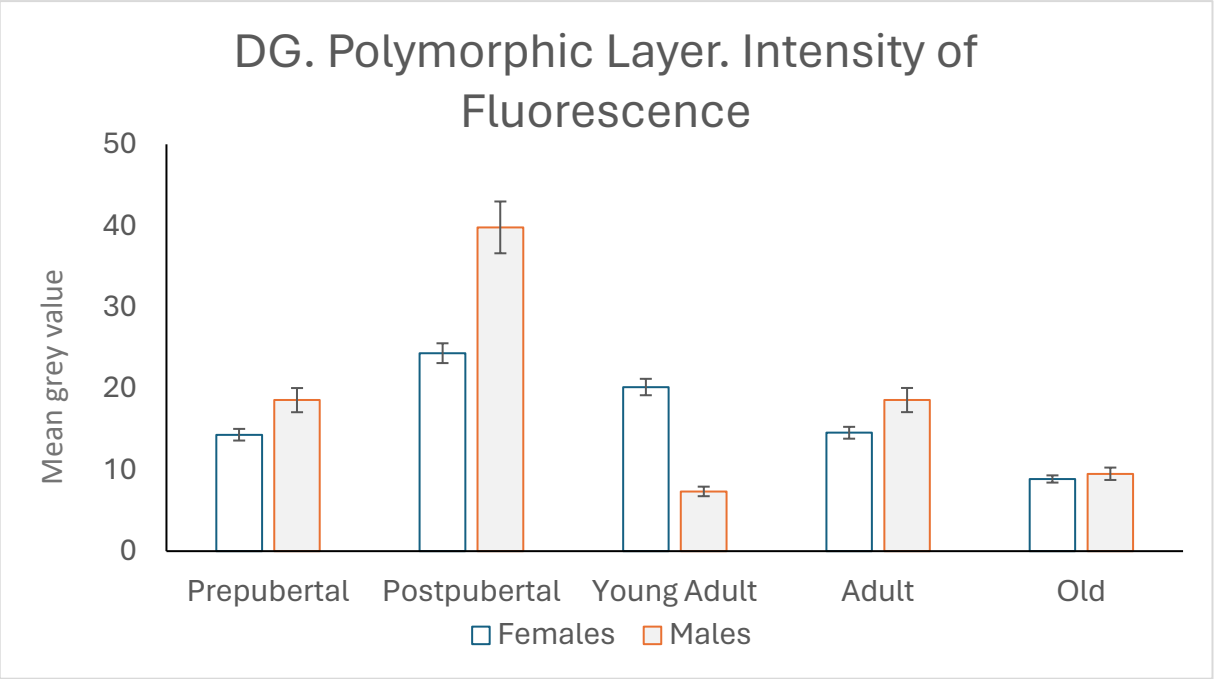
	P value	
Prepubertal F	**<0.0001**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**0.0004**	Young Adult M
Adult F	**0.7664, NS**	Adult M
Old F	**0.9964, NS**	Old M



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**<0.0004**	Postpubertal F		
Young Adult F	**<0.0001**	**<0.0001**	Young Adult F	
Adult F	**<0.0001**	**<0.0001**	**<0.0001**	Adult F
Old F	**<0.0001**	**<0.0001**	**0.0306**	**<0.0001**
P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	**<0.0001**	**<0.0001**	Young Adult M	
Adult M	**0.9993, NS**	**<0.0001**	**<0.0001**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

	P value	
Prepubertal F	**0.7358, NS**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**0.0012**	Adult M
Old F	**0.0010**	Old M



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**<0.0001**	Postpubertal F		
Young Adult F	**<0.0001**	**<0.0001**	Young Adult F	
Adult F	**0.8625, NS**	**<0.0001**	**<0.0001**	Adult F
Old F	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	**<0.0001**	**<0.0001**	Young Adult M	
Adult M	**>0.9999, NS**	**<0.0001**	**<0.0001**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

	P value	
Prepubertal F	**<0.0001**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**<0.0001**	Adult M
Old F	**0.0157**	Old M

Morphological Features of Box2 mRNA-Positive-Cells

CA1 Region

In both sexes and at all age stages analyzed, an in situ hybridization positive signal for box2 mRNA (long isoform of the prolactin receptor) can be found. Prepubertal females showed a positive reaction in the three layers analyzed, but in an irregular shape, with highly reactive neurons and fibers. Next to them, areas in which the reaction disappeared were found. In postpubertal females, the reaction increased and was homogeneously distributed, affecting all pyramidal neurons and fibers of the oriens and radiate layers, the densitometric gradient showed that the area of greatest reaction was the radiate layer, especially the area adjacent to the lacunous stratum. In contrast, the CA1 region of young adult females showed virtually no positive reaction in any of the three layers. The positive signal in adult females was also low, but more evident than in young adult females and less evident than in old females. The reaction in the adult and old females was homogeneous across the three studied layers. Comparing the presence of box2 mRNA between males and females, the reaction in prepubertal males was homogeneous, very similar in postpubertal males, and decreased in young adults, although some pyramidal neurons appeared to be intensely reactive in males. In adult males, the reaction increased, and unlike in females, the presence of positive pyramidal neurons was evident. In addition, unlike what was observed in females, old males showed a decrease in the reaction, even less than that observed in young adult males. Although there were some areas of oriens and radiated positive layers, the reaction was more evident and homogeneous in the pyramidal layer.

CA3 Region

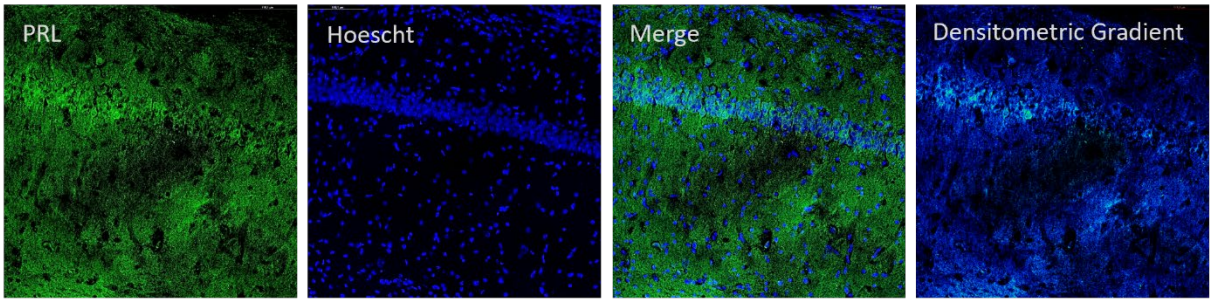
The variations in the results of box2 in situ hybridization in the CA3 region were similar to those described for the CA1 region, except that the intensity of the reaction in postpubertal females was less intense and some areas of the oriens and radiated layers were less positive than the rest. In prepubertal males, higher positivity was observed in CA3 than in CA1 and postpubertal males, and positivity in the pyramidal layer was predominant over the rest of the layers, as shown by the densitometric gradient.

Dentate Gyrus

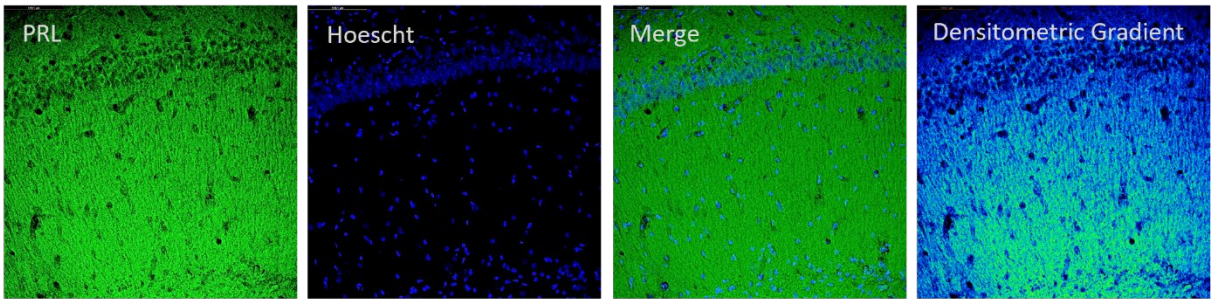
The results found in the dentate gyrus almost overlap with those described for CA3, now in the molecular, granular, and polymorphic layers. In postpubertal males, positivity was higher in the molecular layer.

In pre- and post-pubertal females, box2 mRNA-positive subgranular cells and secondary neurons of the polymorphous layer appeared. In the postpubertal stages, all subgranular cells were positive, and some presented extensions towards the granular layer. In the rest of the age groups, this positivity disappeared, except in some subgranular cells isolated from old females. The findings regarding subgranular cells and secondary neurons in pre- and postpubertal males were the same as those in females. However, they were not observed in old males, and although weakly positive, subgranular cells appeared in young adult and adult males.

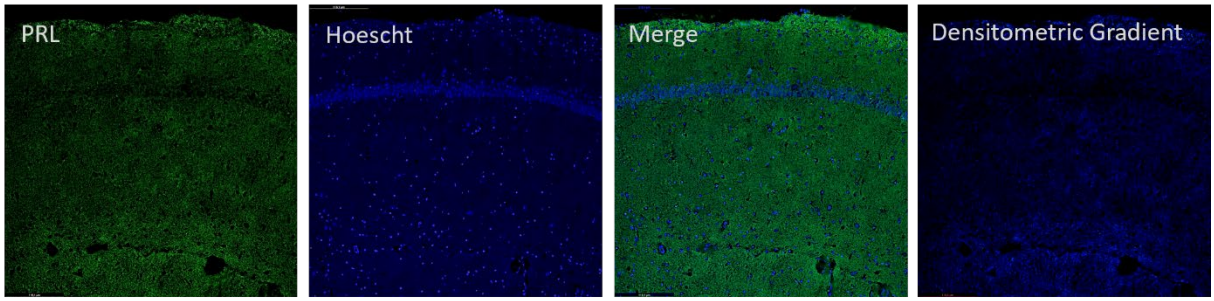
Prepubertal Females



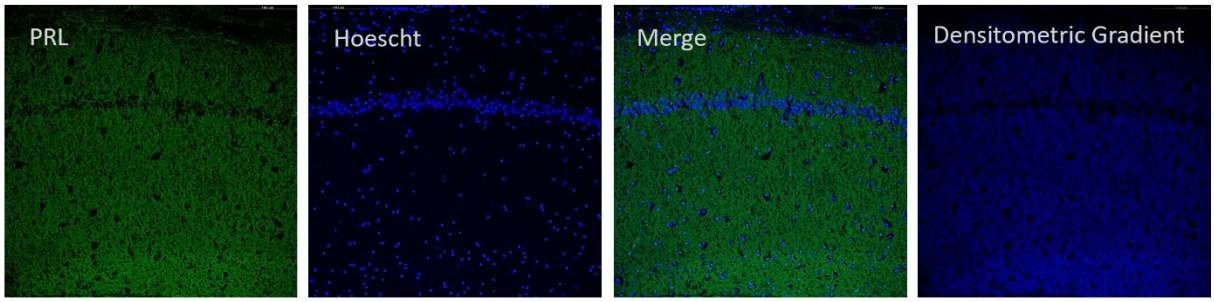
Postpubertal Females



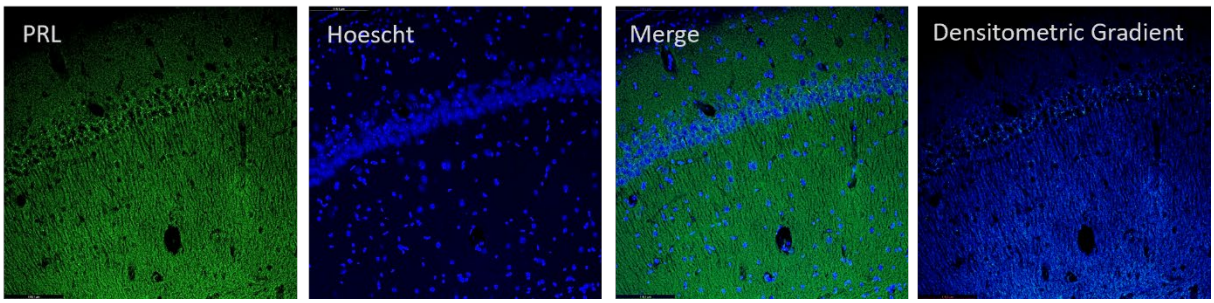
Young Adult Females



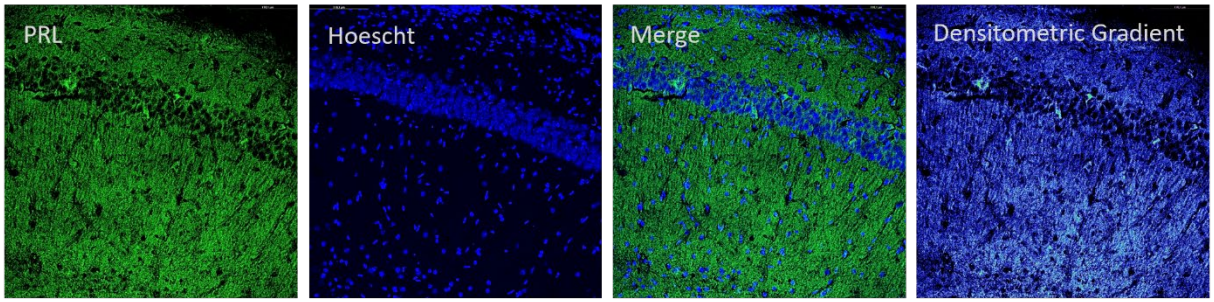
Adult Females



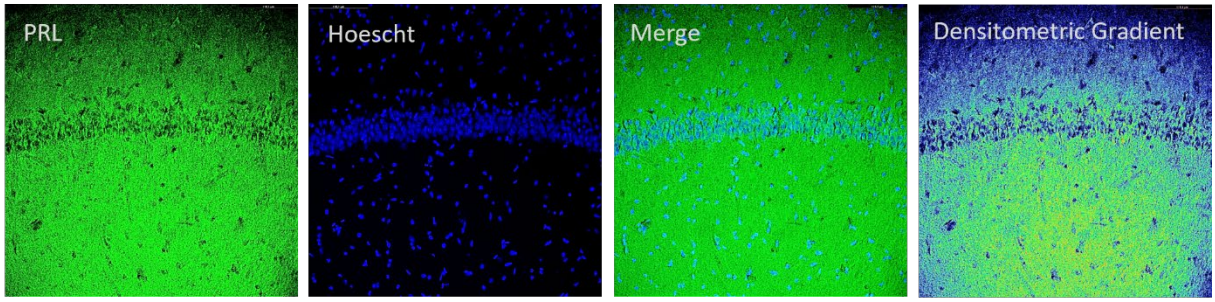
Old Females



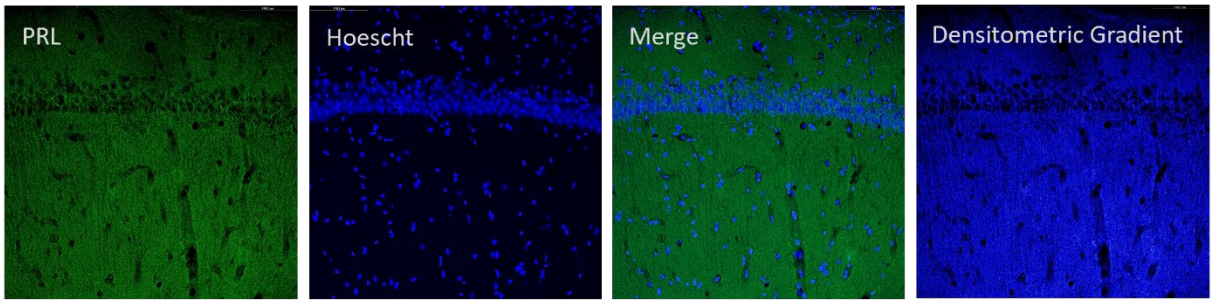
Prepubertal Males



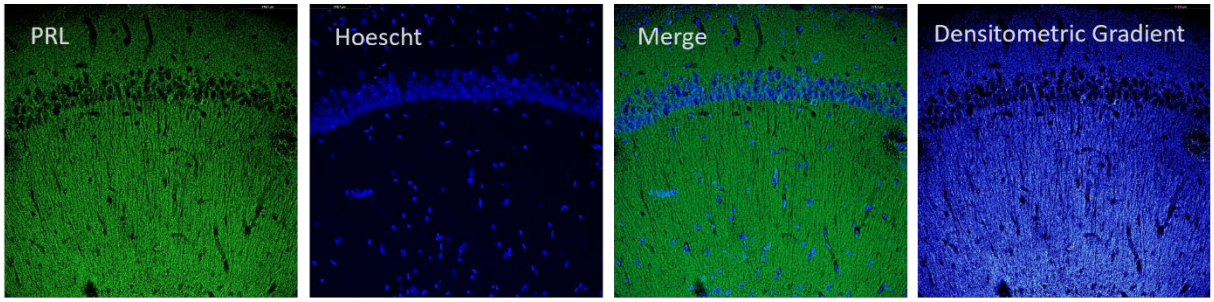
Postpubertal Males



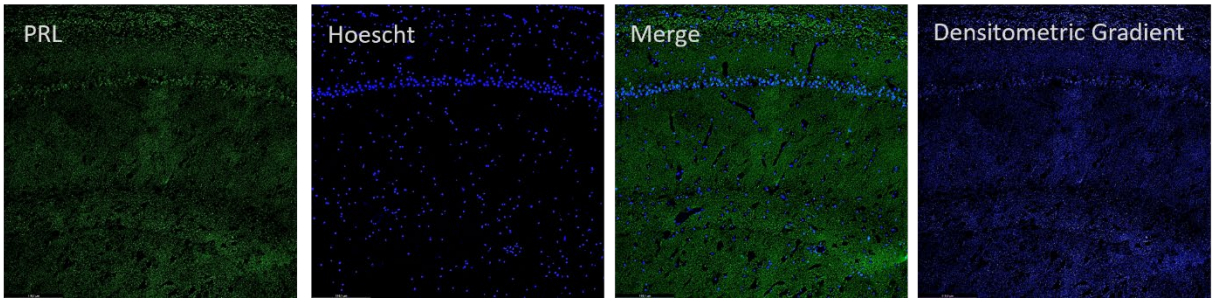
Young Adult Males

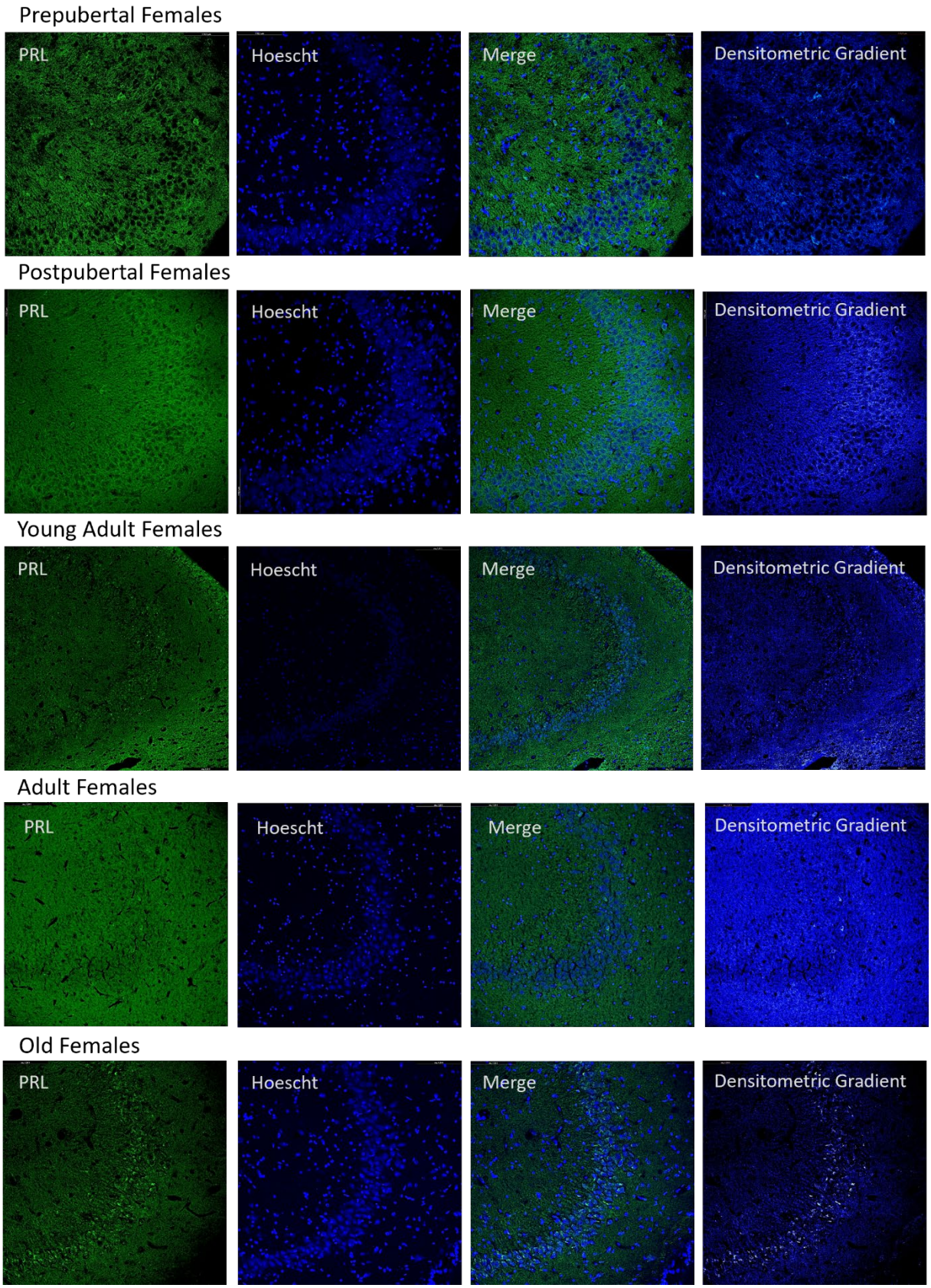


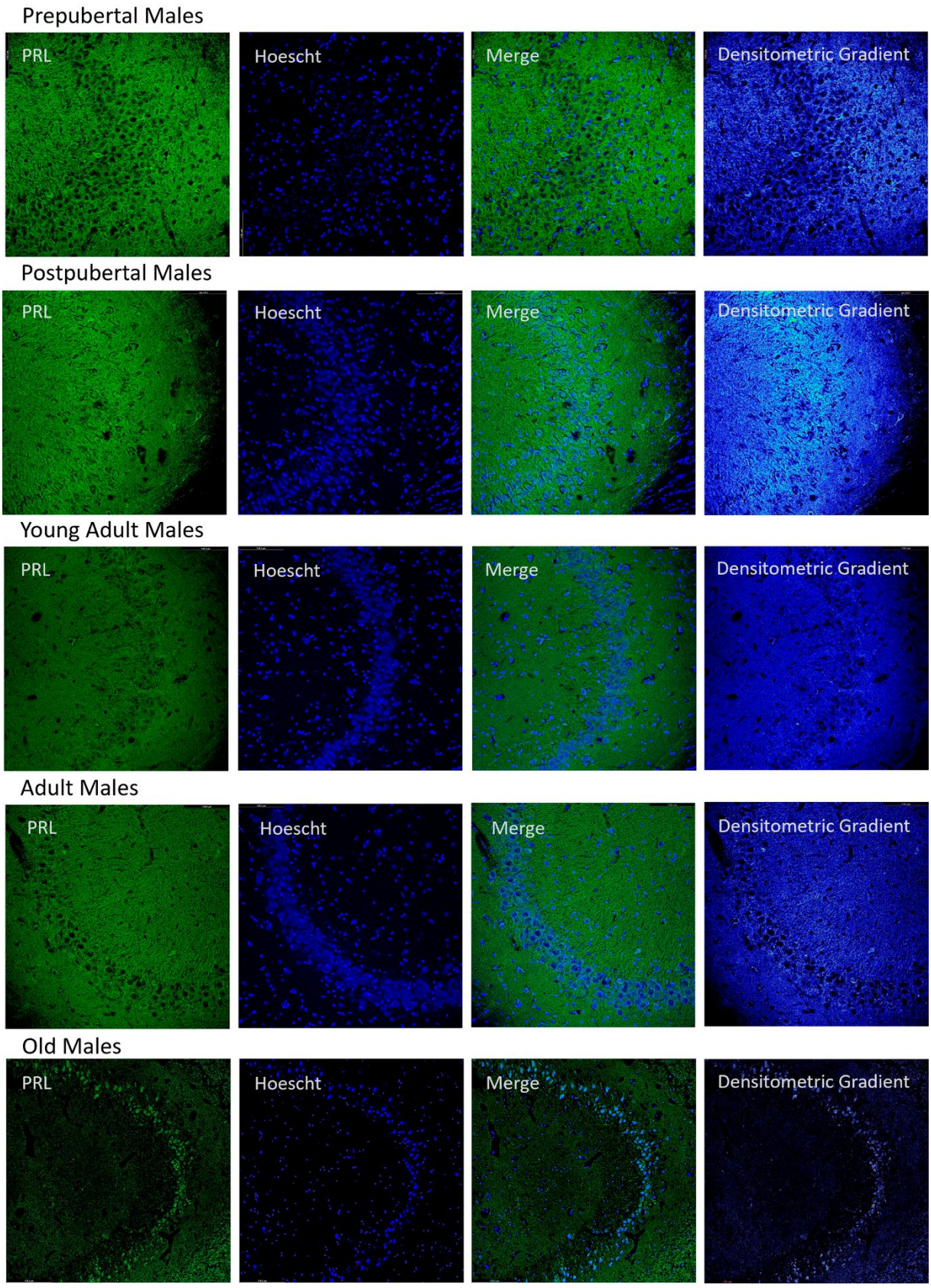
Adult Males



Old Males




Prepubertal Females
PRL
Hoescht
Merge
Densitometric Gradient
Postpubertal Females
PRL
Hoescht
Merge
Densitometric Gradient
Young Adult Females
PRL
Hoescht
Merge
Densitometric Gradient
Adult Females
PRL
Hoescht
Merge
Densitometric Gradient
Old Females
PRL
Hoescht
Merge
Densitometric Gradient


Prepubertal Males
PRL
Hoescht
Merge
Densitometric Gradient
Postpubertal Males
PRL
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Merge
Densitometric Gradient
Young Adult Males
PRL
Hoescht
Merge
Densitometric Gradient
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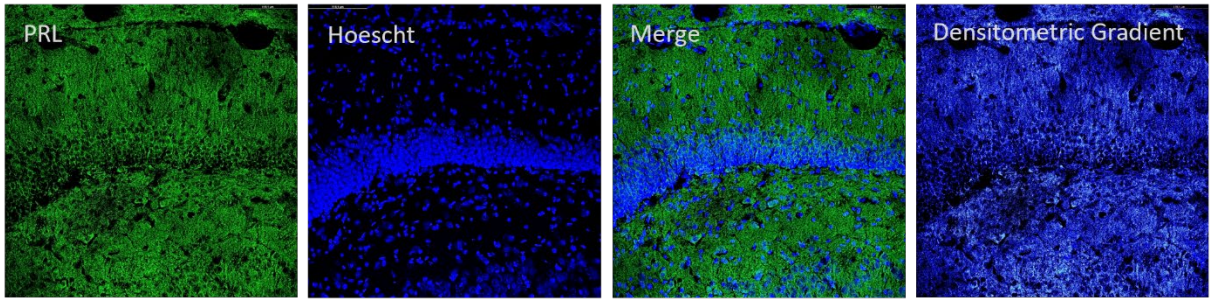
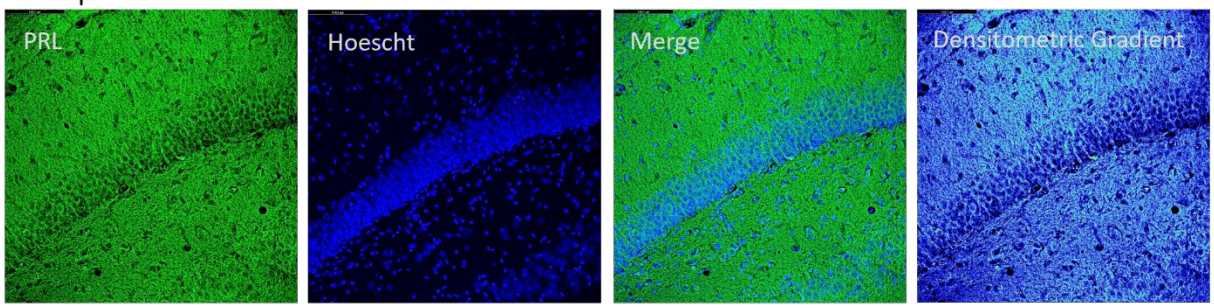
PRL
Hoescht
Merge
Densitometric Gradient
Old Males
PRL
Hoescht
Merge
Densitometric Gradient

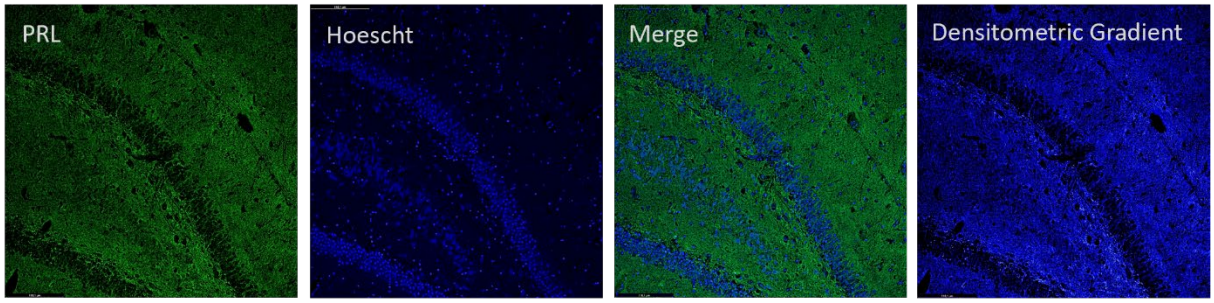
Prepubertal Females



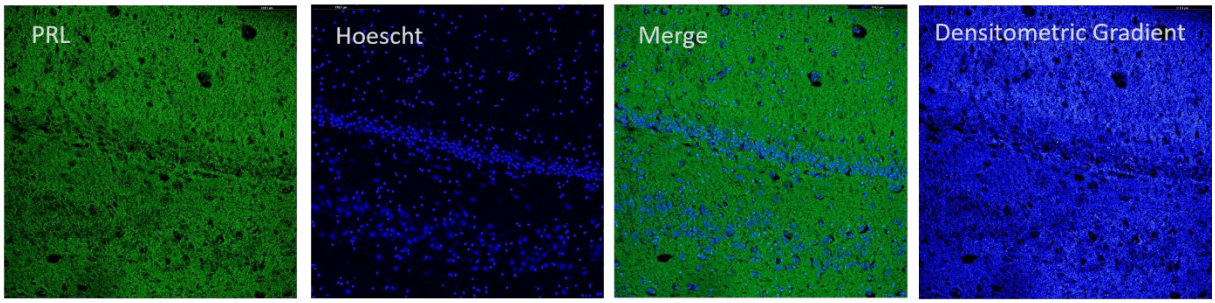
Postpubertal Females



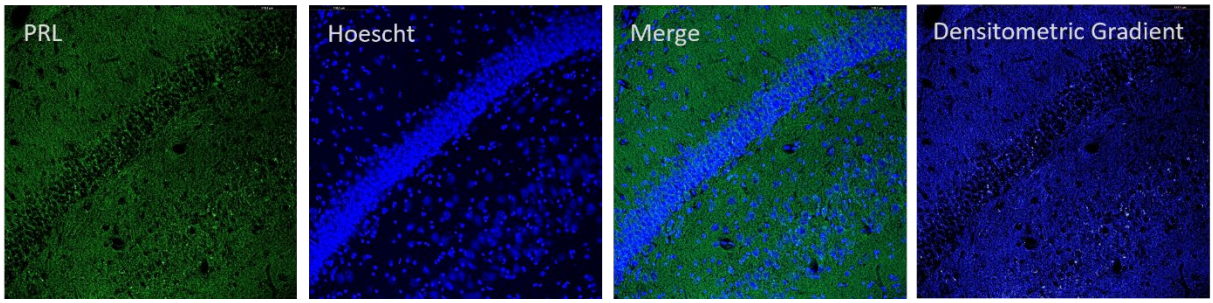
Young Adult Females



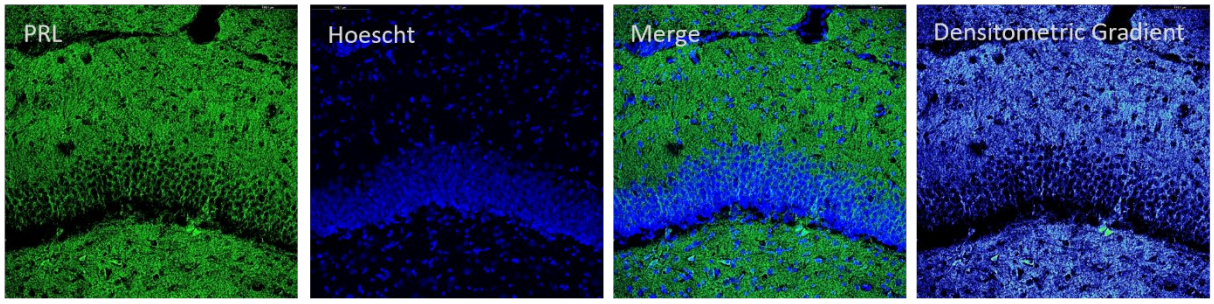
Adult Females



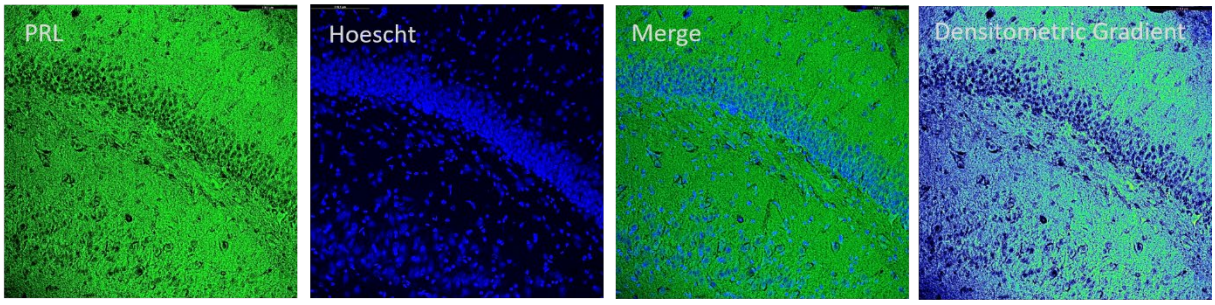
Old Females



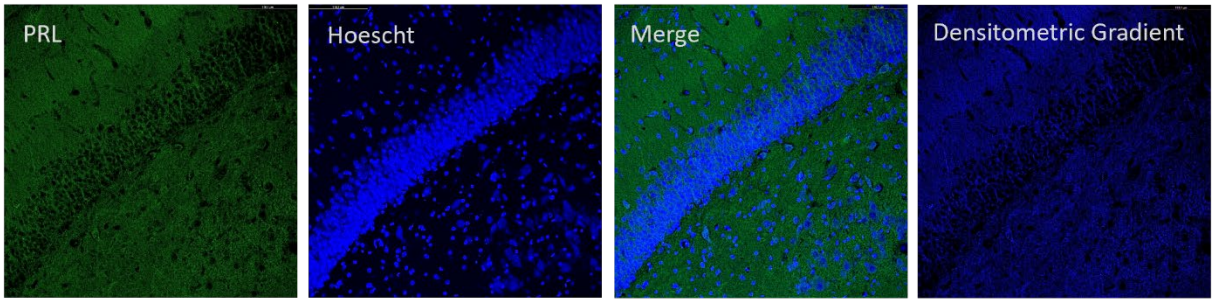
Prepubertal Males



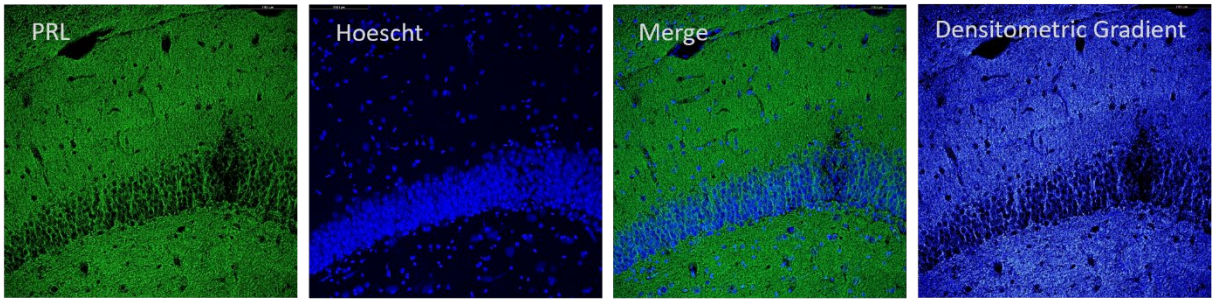
Postpubertal Males



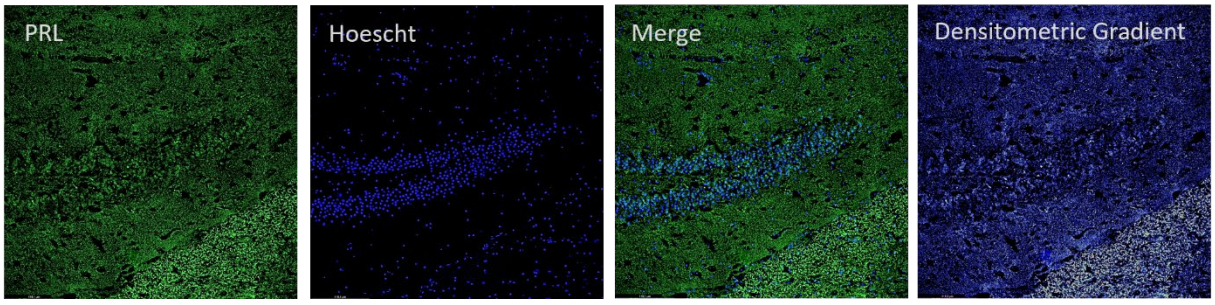
Young Adult Males



Adult Males



Old Males

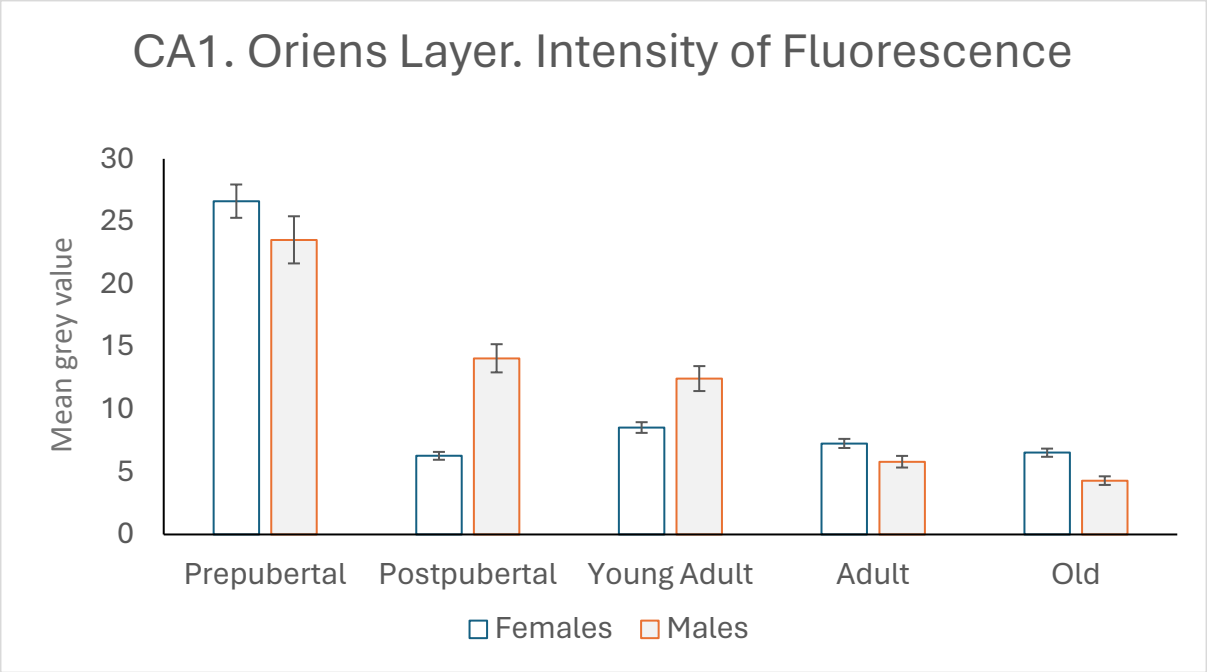


Prolactin Receptor S1-Synthesizing Cells. In situ Hybridization Study

Box1 expression in the CA1 region of the Amon's horn was significantly higher in prepubertal females than in the other female groups ($p<0.01$). From the postpubertal stage, there was a significant decrease ($p<0.01$), increasing slightly in young adult females in the oriens and radiated layers ($p<0.01$), maintaining similar values in the pyramidal layer, remaining in the pyramidal layer at the adult stage, and beginning to descend in the other two layers ($p<0.01$). Aging caused decreases in the radiated and pyramidal layers ($p<0.01$) but not in the oriens layer. The expression of box1 in the oriens layer of the CA1 region in males gradually decreased from the prepubertal stage to aging ($p<0.01$). In the pyramidal and radiated layers, prepubertal values were maintained in the postpubertal stage and decreased gradually and significantly from the young adult stage until aging ($p<0.01$). The differences observed between males and females were always statistically significant ($p<0.01$).

In the CA3 of female animals, the highest mean grey values in the three layers studied were found in the prepubertal stage ($p<0.01$, with respect to the other groups of females). In the oriens layer, they decreased in the postpubertal layer ($p<0.01$), increased again in young adults ($p<0.01$) without reaching prepubertal values ($p<0.01$), and decreased slightly in adulthood and aging ($p<0.01$). In the pyramidal layer, the values decreased from the postpubertal stage ($p<0.01$) and remained at similar values without significant differences until aging was reached, when they increased slightly ($p<0.01$). In the radiated layer, expression decreased greatly in the postpubertal stage, increasing significantly in young adults ($p<0.01$), increasing slightly in adults, and decreasing with aging ($p<0.01$). In the oriens layer of males, a gradual decrease was observed from the postpubertal stage to the adult stage ($p<0.01$), maintaining the values in the old ones. Similar results were observed in the radiate layer, although the expression was lower in old animals than in adults ($p<0.01$). In the pyramidal layer, an increase was observed in the postpubertal stage ($p<0.01$), which then gradually and significantly decreased with age ($p<0.01$). Except for the differences found in the pyramidal layer in adult animals, the rest of the differences found between males and females were statistically significant ($p<0.01$).

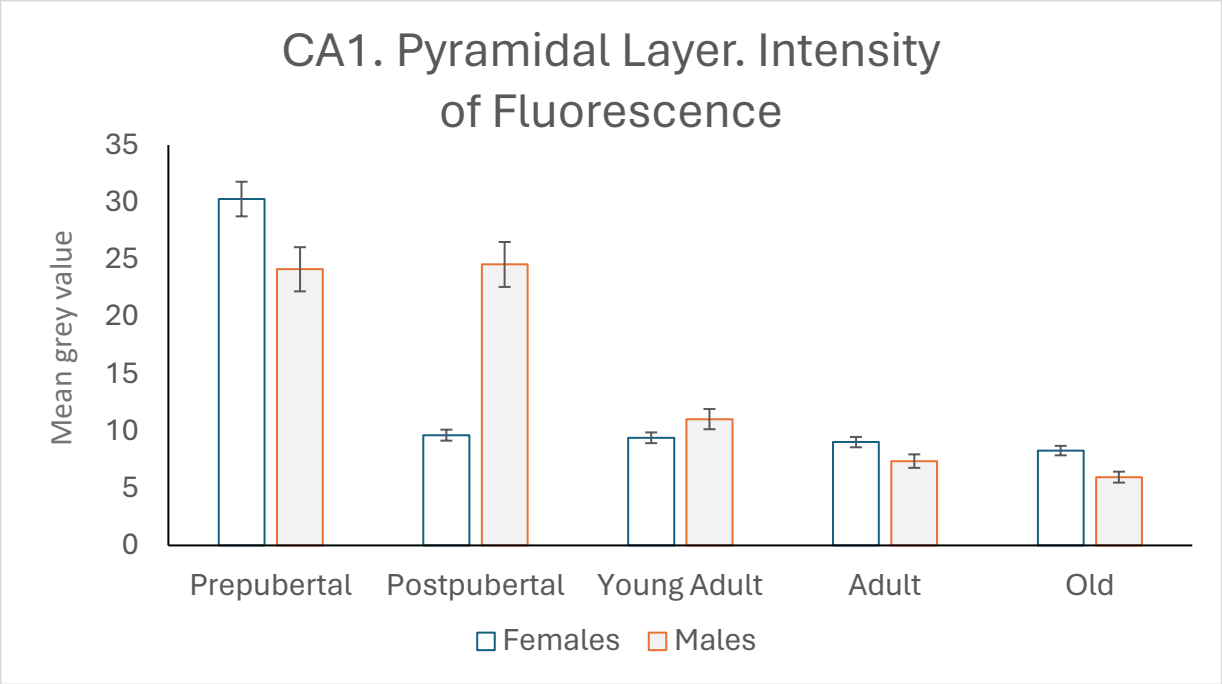
The granular layer and polymorphic layer showed a similar pattern of medium gray: the highest values were the prepubertal ones; they decreased considerably in the postpubertal stage to increase gradually and slightly until adulthood and decreased in the old ones. All the observed differences were statistically significant ($p<0.01$). In the molecular layer, prepubertal values decreased significantly in postpubertal values ($p<0.01$) and remained very similar until aging. In males, the three layers showed a gradual and significant decrease ($p<0.01$) from the postpubertal stage to aging. When comparing prepubertal and postpubertal males, the mean gray levels in the molecular layer were similar, increasing from pre- to postpubertal in the granular layer and decreasing from pre- to postpubertal in the polymorphic layer. In the three layers studied, in the prepubertal, postpubertal, and young adult stages, the values of medium gray were higher in males than in females, whereas the opposite was true in adult and old animals, all of which were significant ($p<0.01$).



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**<0.0001**	Postpubertal F		
Young Adult F	**<0.0001**	**<0.0001**	Young Adult F	
Adult F	**<0.0001**	**<0.0001**	**<0.0001**	Adult F
Old F	**<0.0001**	**0.7828, NS**	**<0.0001**	**0.0020, NS**
P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	**<0.0001**	**<0.0001**	Young Adult M	
Adult M	**<0.0001**	**<0.0001**	**<0.0001**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

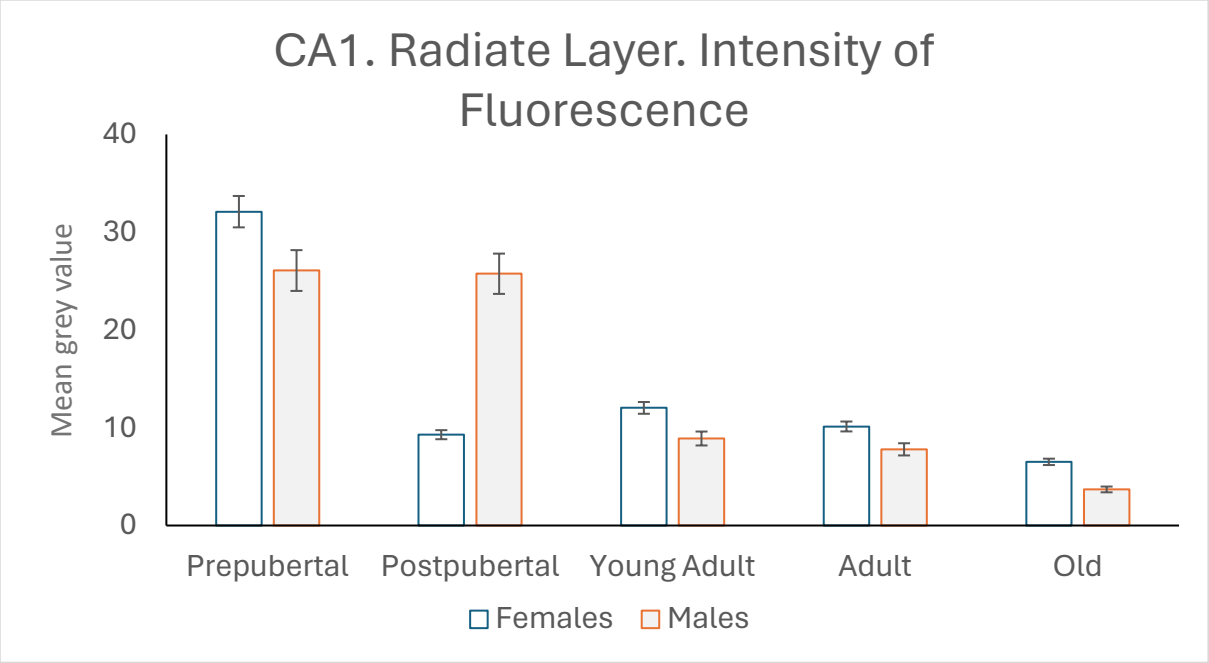
	P value	
Prepubertal F	**<0.0001**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**<0.0001**	Adult M
Old F	**<0.0001**	Old M



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**<0.0004**	Postpubertal F		
Young Adult F	**<0.0001**	**0.8495, NS**	Young Adult F	
Adult F	**<0.0001**	**0.0139**	**0.2854, NS**	Adult F
Old F	**<0.0001**	**<0.0001**	**<0.0001**	**0.0020**
P value	Prepubertal M			
Postpubertal M	**0.1826, NS**	Postpubertal M		
Young Adult M	**<0.0001**	**<0.0001**	Young Adult M	
Adult M	**<0.0001**	**<0.0001**	**<0.0001**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

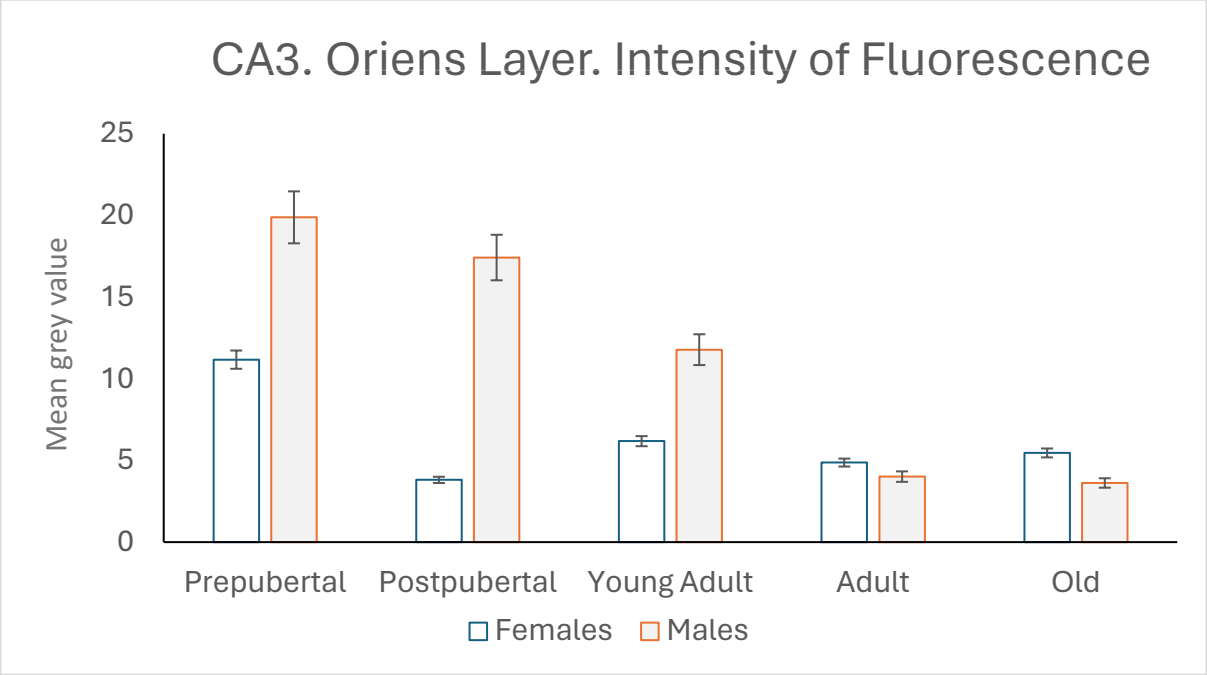
	P value	
Prepubertal F	**<0.0001**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**<0.0001**	Adult M
Old F	**<0.0001**	Old M



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**<0.0001**	Postpubertal F		
Young Adult F	**<0.0001**	**<0.0001**	Young Adult F	
Adult F	**<0.0001**	**0.0005**	**<0.0001**	Adult F
Old F	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
P value	Prepubertal M			
Postpubertal M	**0.4606, NS**	Postpubertal M		
Young Adult M	**<0.0001**	**<0.0001**	Young Adult M	
Adult M	**<0.0001**	**<0.0001**	**<0.0001**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

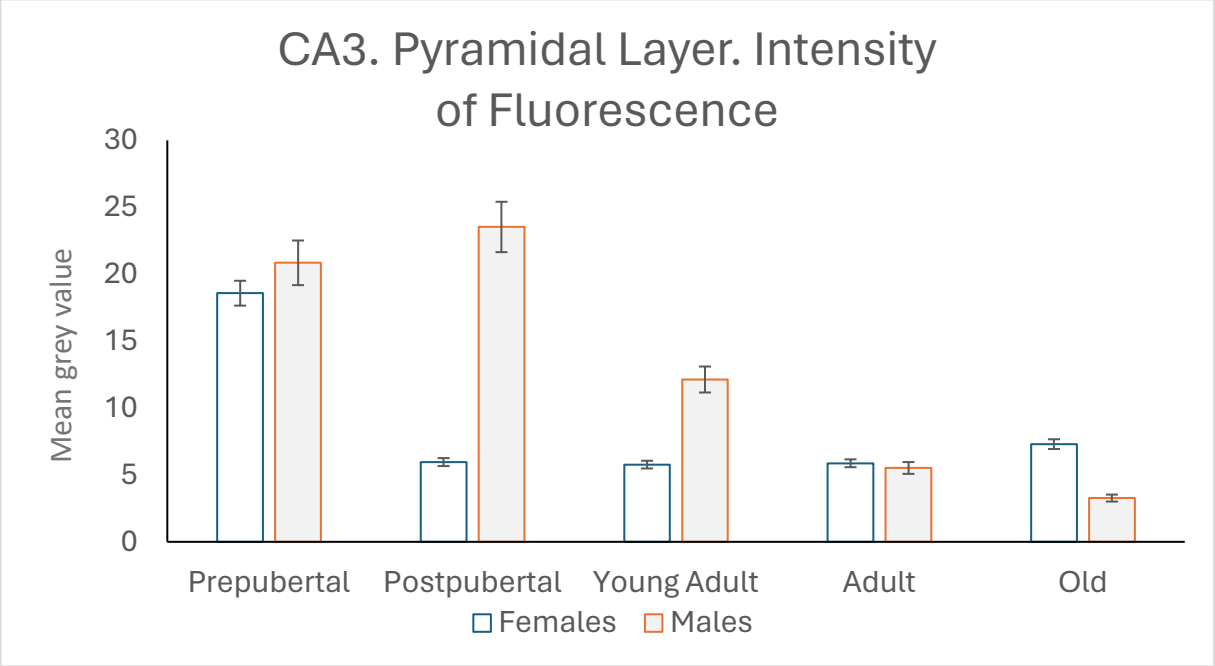
	P value	
Prepubertal F	**<0.0001**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**<0.0001**	Adult M
Old F	**<0.0001**	Old M



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**<0.0001**	Postpubertal F		
Young Adult F	**<0.0001**	**<0.0001**	Young Adult F	
Adult F	**<0.0001**	**<0.0001**	**<0.0001**	Adult F
Old F	**<0.0001**	**<0.0001**	**0.0007**	**0.0063**
P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	**<0.0001**	**<0.0001**	Young Adult M	
Adult M	**<0.0001**	**<0.0001**	**<0.0001**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**0.1458, NS**

	P value	
Prepubertal F	**<0.0001**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**<0.0001**	Adult M
Old F	**<0.0001**	Old M

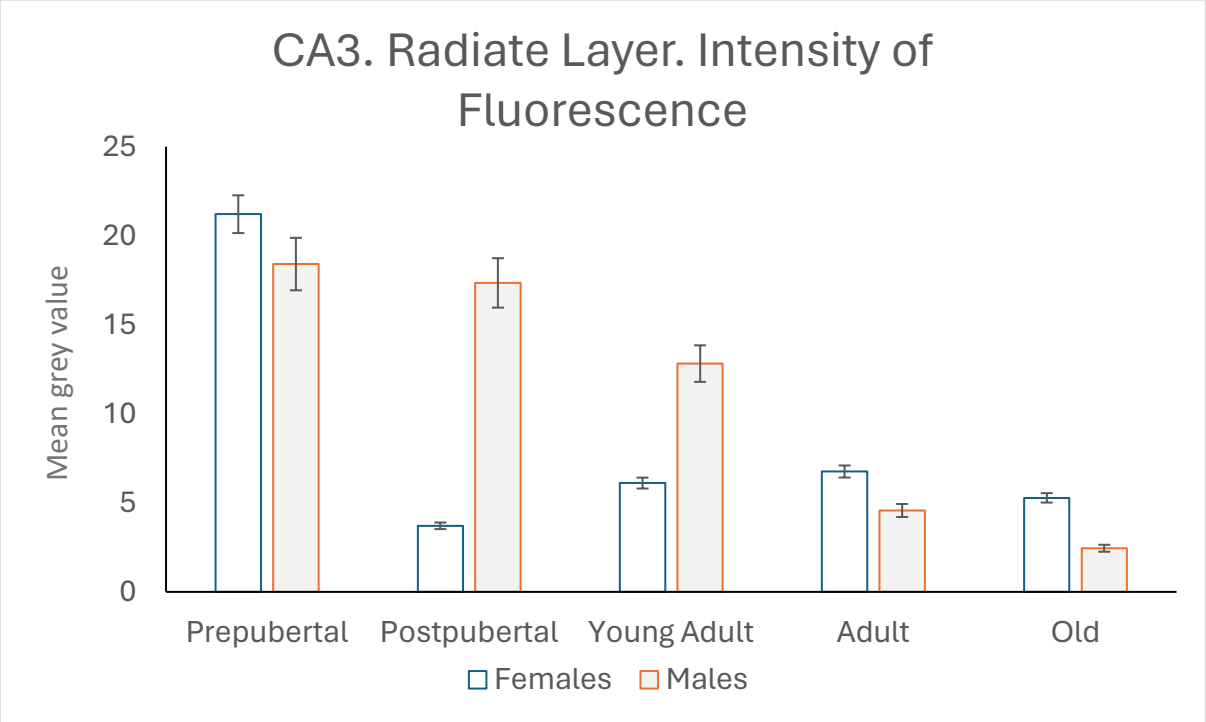


The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**<0.0001**	Postpubertal F		
Young Adult F	**<0.0001**	**0.8946, NS**	Young Adult F	
Adult F	**<0.0001**	**0.9993, NS**	**0.9984, NS**	Adult F
Old F	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	**<0.0001**	**<0.0001**	Young Adult M	
Adult M	**<0.0001**	**<0.0001**	**<0.0001**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

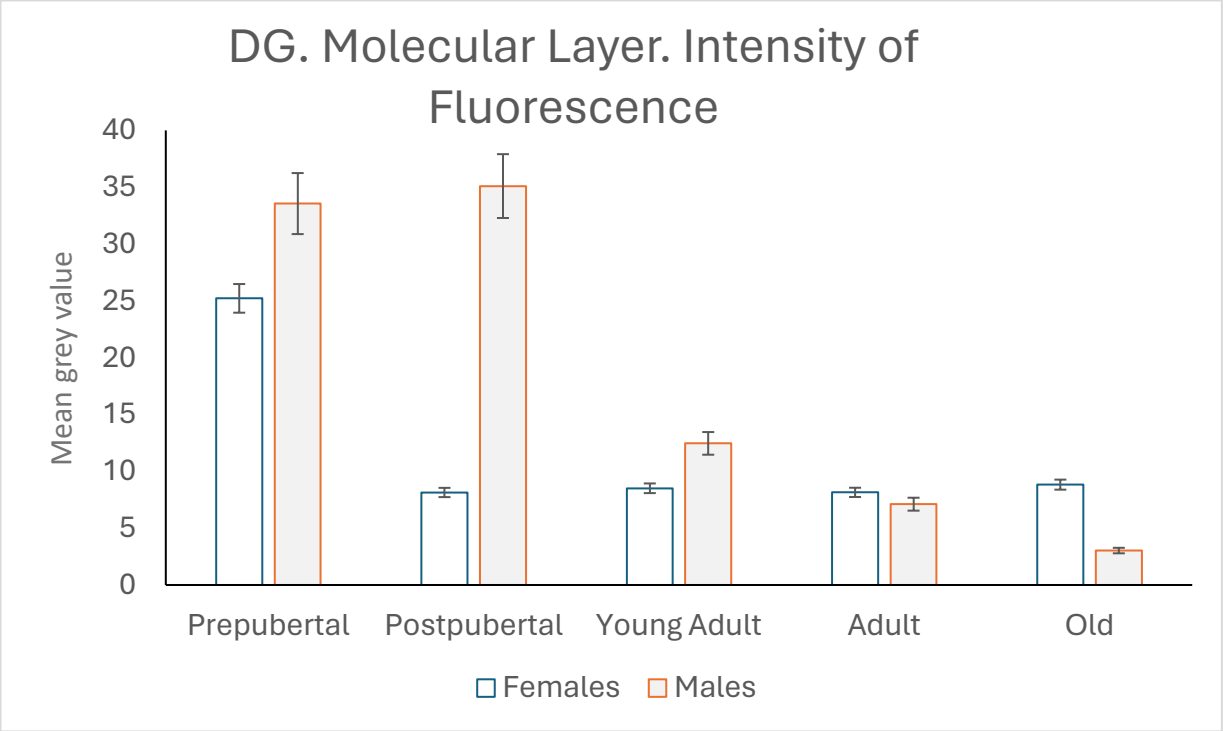
	P value	
Prepubertal F	**<0.0001**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**0.2462, NS**	Adult M
Old F	**<0.00001**	Old M



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**<0.0001**	Postpubertal F		
Young Adult F	**<0.0001**	**<0.0001**	Young Adult F	
Adult F	**<0.0001**	**<0.0001**	**0.0023**	Adult F
Old F	**<0.0001**	**<0.0001**	**0.0001**	**<0.0001**
P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	**<0.0001**	**<0.0001**	Young Adult M	
Adult M	**<0.0001**	**<0.0001**	**<0.0001**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

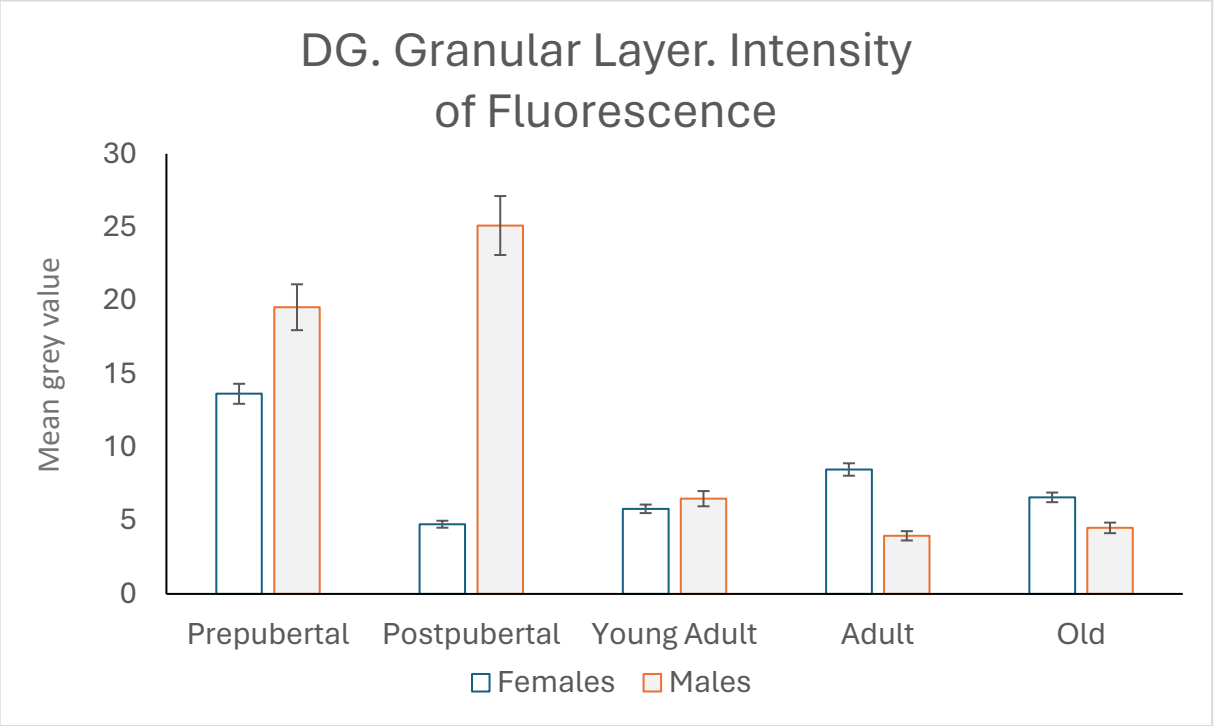
	P value	
Prepubertal F	**<0.0001**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**<0.0001**	Adult M
Old F	**<0.0001**	Old M



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**<0.0001**	Postpubertal F		
Young Adult F	**<0.0001**	**0.1908, NS**	Young Adult F	
Adult F	**<0.0001**	**>0.9999, NS**	**0.2171, NS**	Adult F
Old F	**<0.0001**	**<0.0012**	**0.3497, NS**	**<0.0014**
P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	**<0.0001**	**<0.0001**	Young Adult M	
Adult M	**<0.0001**	**<0.0001**	**<0.0001**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

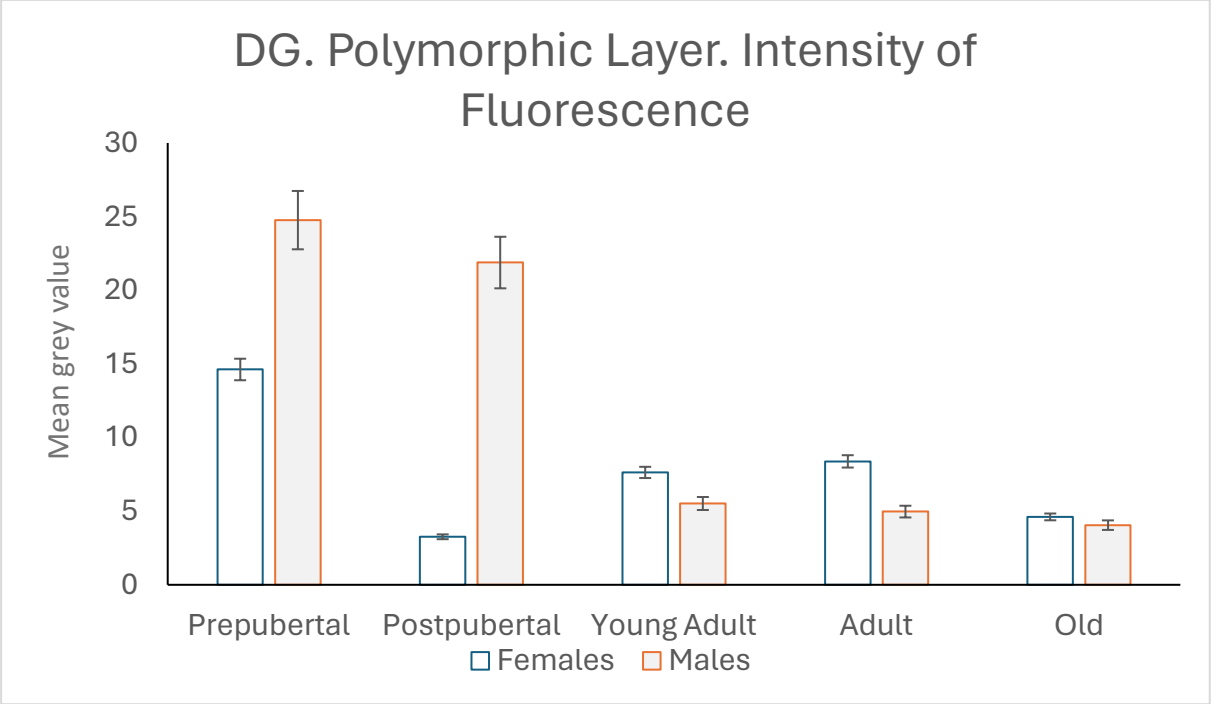
	P value	
Prepubertal F	**<0.0001**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**<0.0001**	Adult M
Old F	**<0.0001**	Old M



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**<0.0001**	Postpubertal F		
Young Adult F	**<0.0001**	**<0.0001**	Young Adult F	
Adult F	**<0.0001**	**<0.0001**	**<0.0001**	Adult F
Old F	**<0.0001**	**<0.0001**	**0.0003**	**<0.0001**
P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	**<0.0001**	**<0.0001**	Young Adult M	
Adult M	**<0.0001**	**<0.0001**	**<0.0001**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**0.0144**

	P value	
Prepubertal F	**<0.0001**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**0.0012**	Young Adult M
Adult F	**<0.0001**	Adult M
Old F	**<0.0001**	Old M



The following tables show the results found following One-way ANOVA test, Tukey test (F: Female, M: Male, NS: No significant).

P value	Prepubertal F			
Postpubertal F	**<0.0001**	Postpubertal F		
Young Adult F	**<0.0001**	**0.0004**	Young Adult F	
Adult F	**<0.0001**	**<0.0001**	**<0.0001**	Adult F
Old F	**<0.0001**	**<0.0001**	**<0.0048**	**<0.0001**
P value	Prepubertal M			
Postpubertal M	**<0.0001**	Postpubertal M		
Young Adult M	**<0.0001**	**<0.0001**	Young Adult M	
Adult M	**<0.0001**	**<0.0001**	**0.0122**	Adult M
Old M	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

	P value	
Prepubertal F	**<0.0001**	Prepubertal M
Postpubertal F	**<0.0001**	Postpubertal M
Young Adult F	**<0.0001**	Young Adult M
Adult F	**<0.0001**	Adult M
Old F	**0.0104**	Old M

Morphological Features of Box1 mRNA-Positive-Cells

CA1 Region

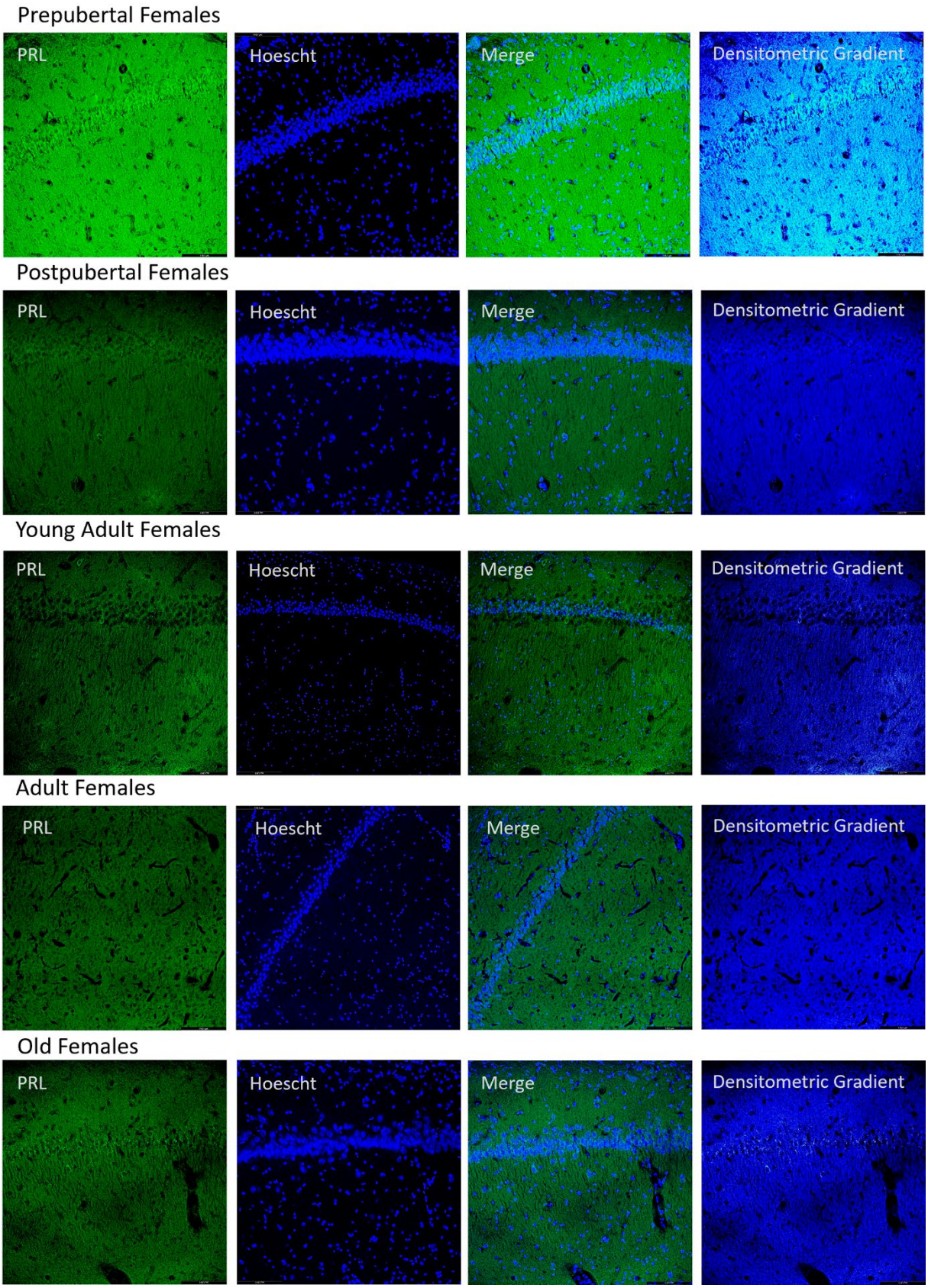
In female rats, the strongest intensity was observed in prepubertal females, with a strong intensity in the neurons of the pyramidal layer. Postpubertal females and young adults showed a similar reaction pattern, with isolated pyramidal neurons being intensely reactive and the rest not very reactive. Isolated secondary neurons were observed in both the age groups. As was the case with box2, adult females showed hardly any positivity in any of the three layers, whereas in the old females, an intense punctiform reaction was observed in the pyramidal neurons. In pre- and post-pubertal males, an intense signal appeared in all three layers studied, with strongly positive secondary neurons. The largest difference between these two age groups can be seen in the densitometric gradient: in prepubertal males, the dendrites of the radiated layer were more positive than in the postpubertal males, and in these males, the pyramidal neurons were more positive than in the prepubertal males. Young adult males had some intensely positive pyramidal neurons, although with a punctiform reaction, and positivity extended to areas adjacent to the pyramidal layer of the oriens and radiate layers. In adult males, the reaction predominated in a punctiform form in the neurons of the pyramidal layer. Old males had intensely reactive pyramidal neurons and well-reactive oriens layer fibers.

CA3 Region

Patterns like those described for CA1 were observed in the CA3 region of pre- and postpubertal females. However, young adult females only exhibited a punctiform reaction in neurons isolated from the pyramidal layer. Adult females only showed weak positivity in some isolated pyramidal neurons, and old females showed a reaction like that of young adults, although with a higher number of positive pyramidal neurons. There were no significant differences in the distribution of positivity and its intensity with respect to those described for the CA1 region in the different groups of males studied. However, the intense positivity of some pyramidal and secondary neurons of the oriens and radiated layers in postpubertal males stands out.

Dentate Gyrus

The dentate gyrus of prepubertal females showed greater positivity than that of the other groups, affecting the three layers but was more intense in the granular and molecular layers. Postpubertal females showed positive granular neurons, and the greatest reaction appeared in the marginal zone of the molecular layer. Young adult and adult females had weakly positive granular neurons along with many non-positive neurons, with the reaction predominating in the molecular and polymorphous layers. Old females had a reaction like that of the postpubertal females. In prepubertal and postpubertal males, an intense reaction affecting the entire dentate gyrus was observed. All granule cells and many secondary neurons were positive. Positivity was also found in all three layers of the dentate gyrus of young adult males, although at a lower intensity. Adult males had positive and negative cells, and the highest positivity was observed in the marginal area of the molecular layer. Old males showed strong positivity in the granular layer and part of the polymorphous layer. Subgranular-positive cells were observed mainly in the postpubertal males. Moreover, prepubertal males and females showed some positive cells.


Prepubertal Females
PRL
Hoescht
Merge
Densitometric Gradient
Postpubertal Females
PRL
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Merge
Densitometric Gradient
Young Adult Females
PRL
Hoescht
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Adult Females
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Old Females
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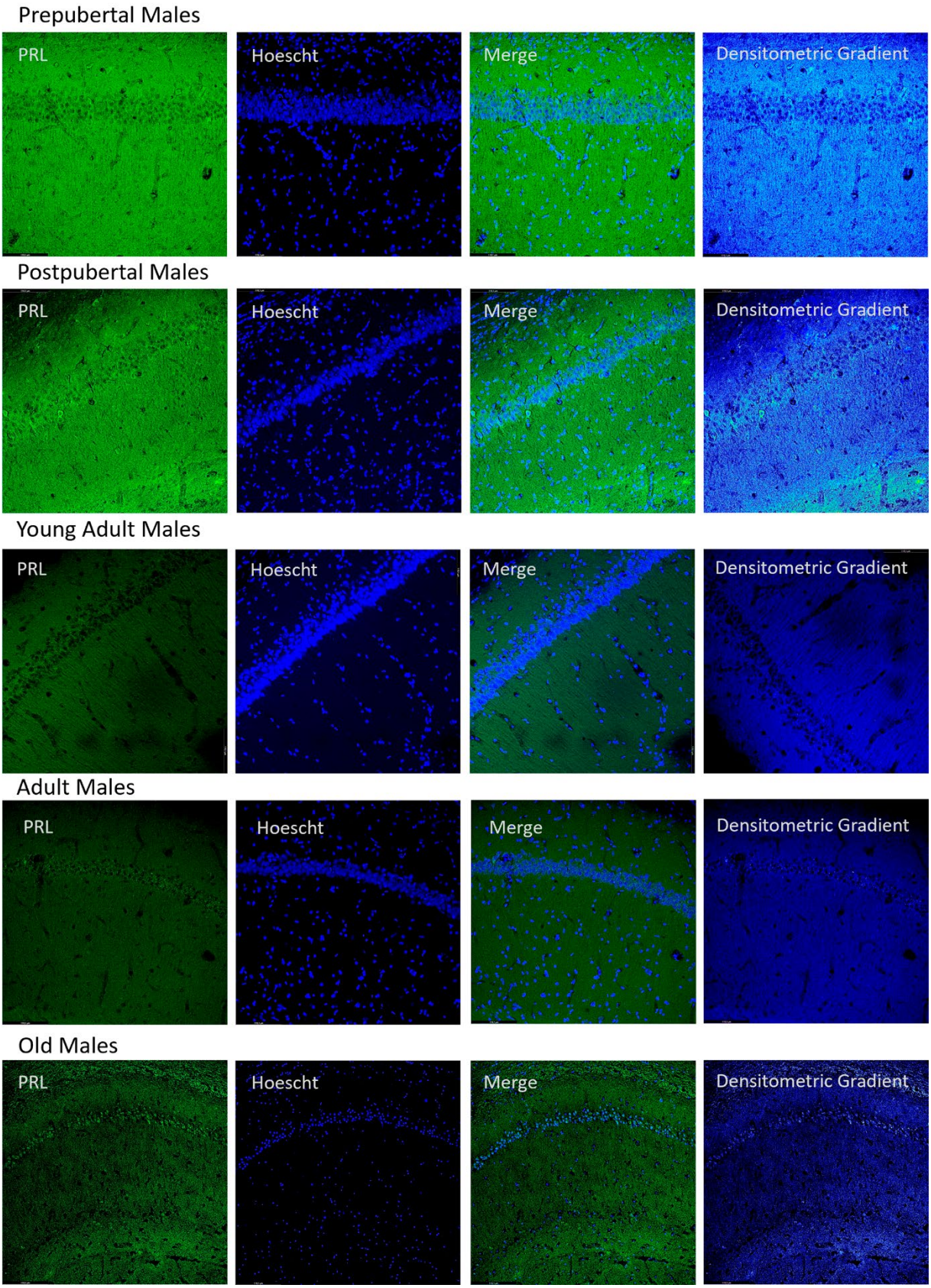
Densitometric Gradient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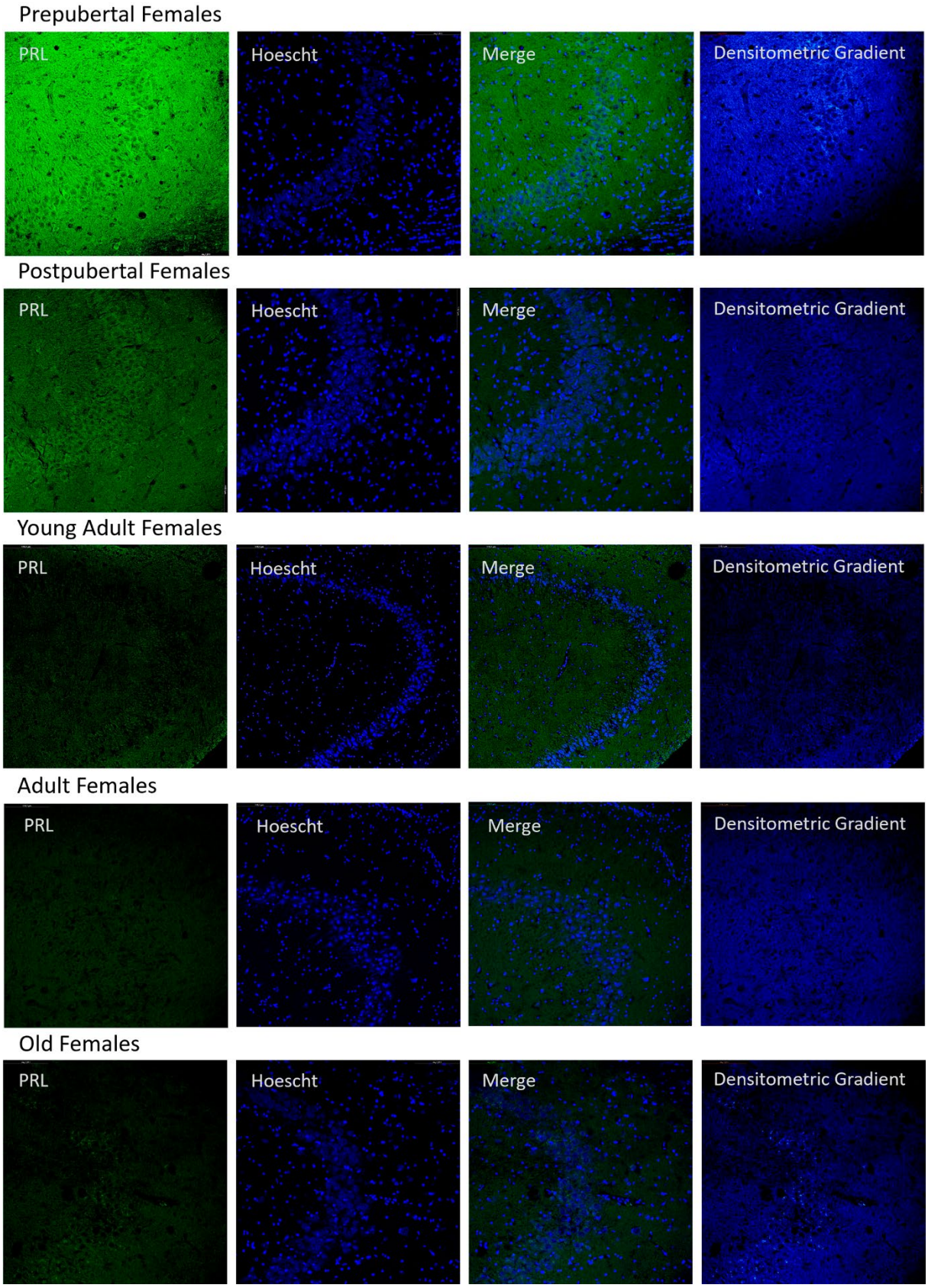
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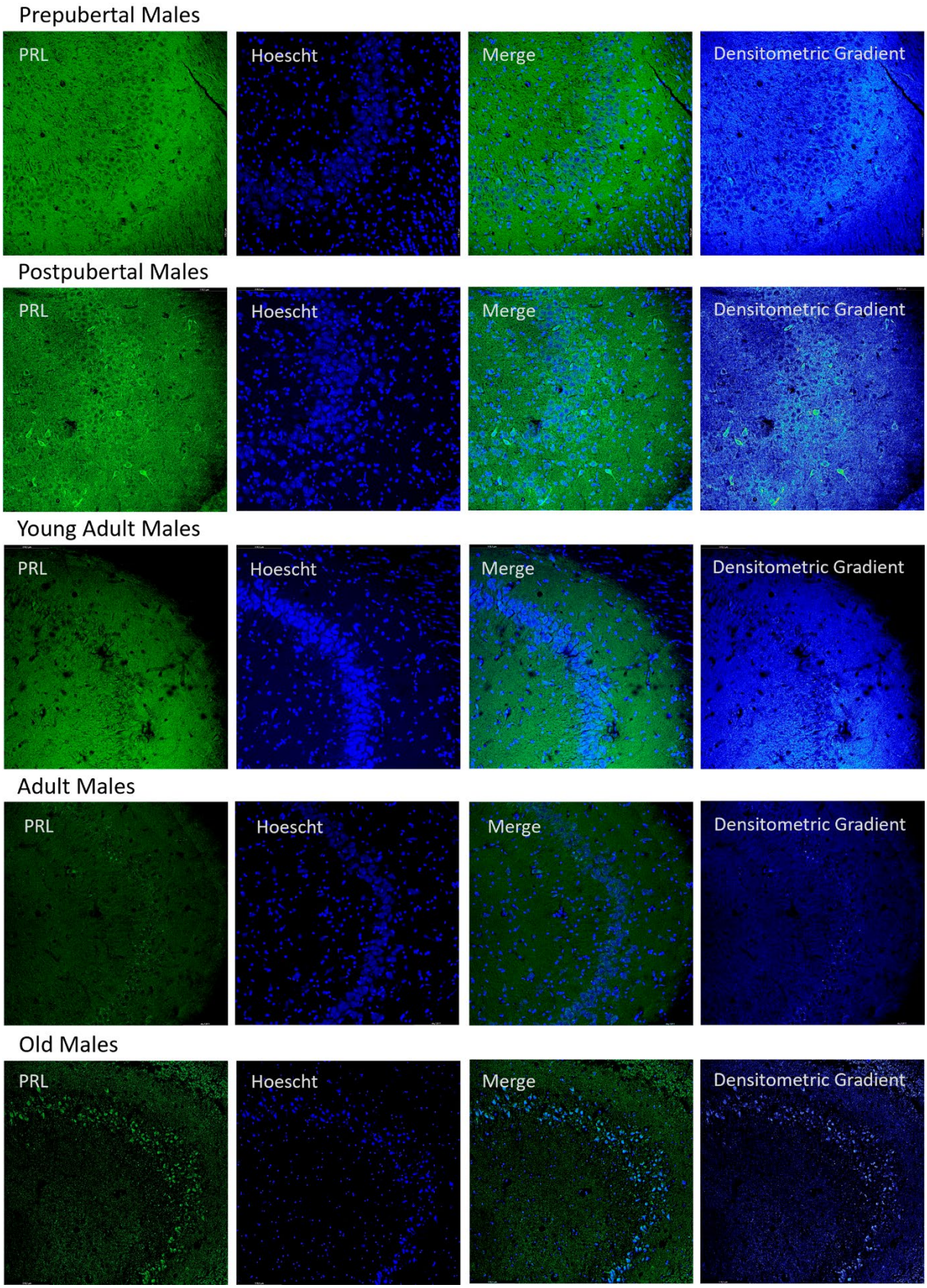
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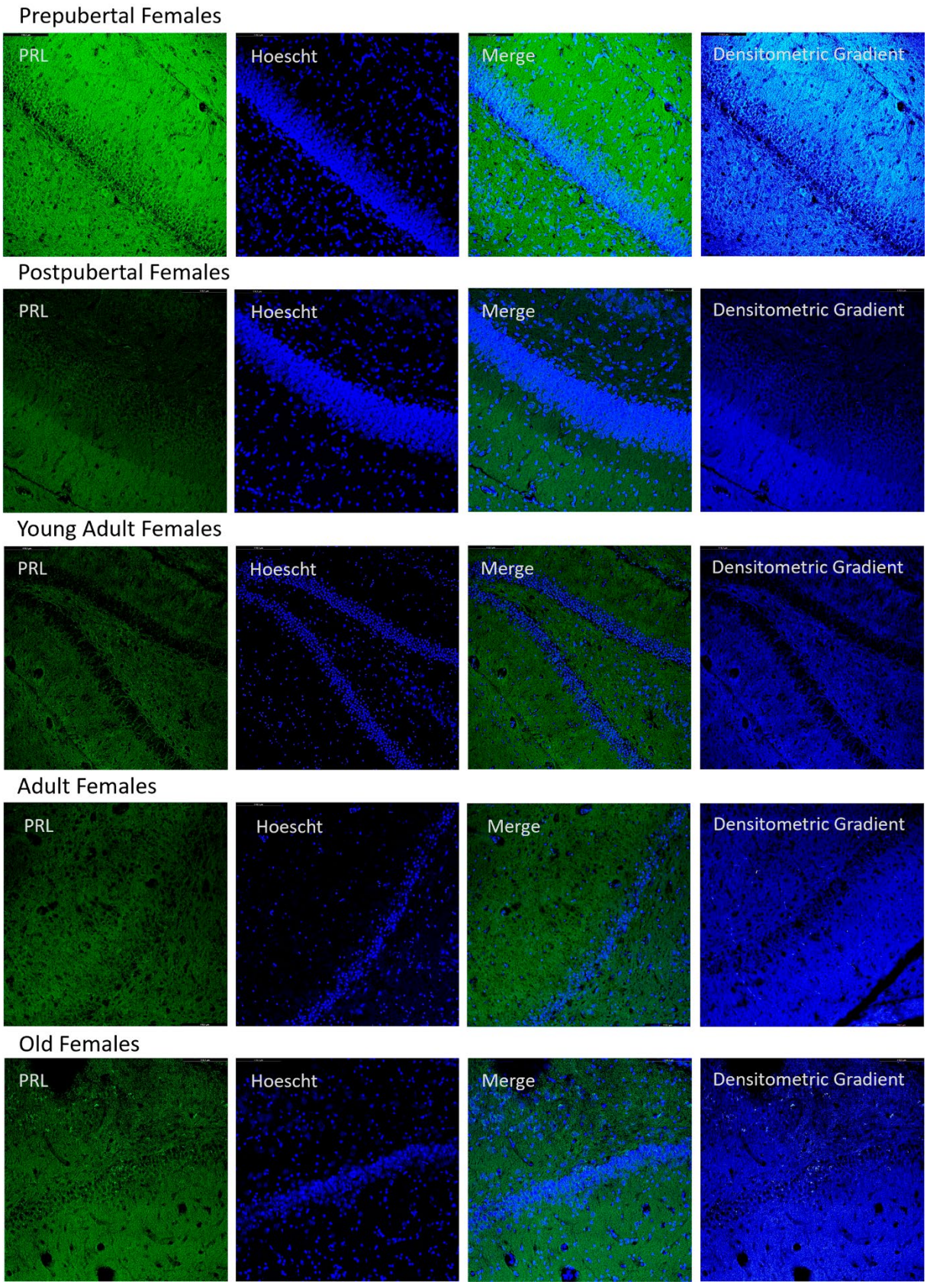
Prepubertal Females
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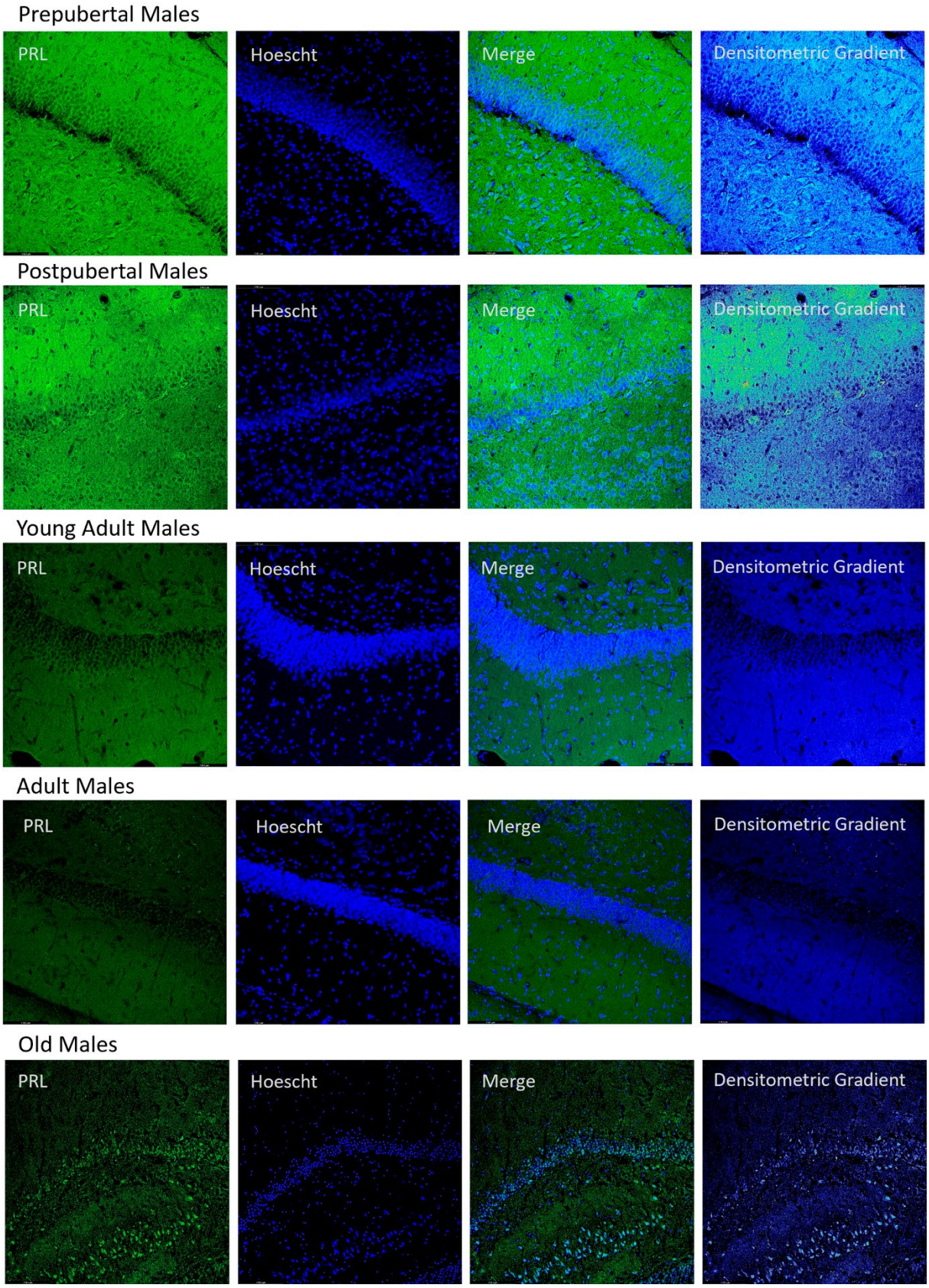
Prepubertal Males
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Hoescht
Merge
Densitometric Gradient
Postpubertal Males
PRL
Hoescht
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Densitometric Gradient
Young Adult Males
PRL
Hoescht
Merge
Densitometric Gradient
Adult Males
PRL
Hoescht
Merge
Densitometric Gradient
Old Males
PRL
Hoescht
Merge
Densitometric Gradient

Discussion

This is the first experimental study in rats to analyze the hippocampal prolactin system across the lifespan by combining hormonal determinations by ELISA, characterization of hippocampal prolactin with western blotting and immunohistochemistry, hippocampal hormone synthesis by qPCR and in situ hybridization, and expression of long and short isoforms of the prolactin receptor by qPCR and in situ hybridization. The use of all this methodology and the analysis of the results obtained has allowed us to achieve the objectives set for this Doctoral Thesis work. Then going on to contrast the results obtained with what has been partially described by other authors.

Serum Prolactin Levels and Aging

It is well known that the characteristic parameters of biological rhythms can change with ageing (Touitou, 1995). Controversial studies on the effects of aging on serum prolactin levels can be found in the scientific literature, especially in humans (Corona et al., 2014; Yamaji et al., 1976).

The findings reported in rats differ from one study to another, mainly related to the variability in the age periods studied. Translating the results of other authors to the age periods of our study, there is sufficient evidence that in male rats there is an increase in prolactin between puberty and adulthood (Esquifino et al., 2004; Goya et al., 1991), decreases in old males which could be related to a decrease in pituitary lactotroph cell activity in older males (van Putten et al., 1988) and that, in the female rat, serum prolactin increases with aging (Goya et al., 1991; van Putten et al., 1988).

In general, the findings of our study are in line with those described by other authors, mainly regarding higher serum prolactin levels in females than in males (Carretero et al., 1989; Yamaji et al., 1976)*. It is also important to note that in the animals in our study, the possibility of prolactinomas that are known to develop spontaneously with aging* (Carretero et al., 2002, 2013; García-Barrado et al., 2017) *was ruled out.*

Hippocampal Prolactin Levels and Aging

There are no articles in the scientific literature that determine prolactin levels in hippocampal lysates using ELISA or RIA. There are approximations in studies performed using western blotting (Duc Nguyen et al., 2022), although these are related to the possible participation of diabetes in the pathogenesis of Alzheimer's disease.

Our study is the first to analyze age-related changes in hippocampal prolactin levels in hippocampal lysates using ELISA. An analysis of the results obtained shows that prolactin levels were higher in females than in males in all age groups studied, suggesting sexual dimorphism that could be related to sex steroid levels, mainly estrogens.

Another fact that can be clearly deduced from our results is that r-value was negative in females and, although the r-value in the correlation study was positive in males, it did not reach statistical significance, indicating that serum prolactin and hippocampal

concentrations are not directly related. This suggests that without ruling out the possibility of circulating prolactin reaching the hippocampus, because the results obtained with ELISA and qPCR showed some differences, the totality of hippocampal prolactin does not originate from the bloodstream.

Origin of hippocampal prolactin

The precise mechanism by which biologically active prolactin may access the brain remains unclear.

There have been numerous reports on both prolactin and its mRNA expression in brain tissue. However, there are many controversies from one study to another that mainly deal with the origin of detectable prolactin in the brain and the specificity of what was found.

Some laboratories have questioned the immunoreactivity of prolactin in the brain since the antibody used could cross-react with opiomelanocortin (Harlan et al., 1989), which might account for some of the observations.

In contrast, in this study, pre-absorption of the primary antibody used in immunohistochemistry with prolactin was performed, no cells appeared positive, and with ACTH, the reaction was not modified. In addition, since the pars intermedia is very rich in opiomelanocortin, the pattern of the reaction between the two positive pituitary controls, one for prolactin and the other for ACTH in the pars intermedia, has been contrasted, resulting in these patterns being totally different.

Most studies analyzing the presence of prolactin in the brain by immunocytochemistry have been conducted by studying the hypothalamus or choroid plexuses. The original observation of immunoreactive-prolactin in the hypothalamus of rats (Fuxe et al., 1977) has been supported by several groups (DeVito, 1988; DeVito et al., 1987; Emanuele et al., 1986; Paut-Pagano et al., 1993; Shivers et al., 1989; S. Suzuki & Handa, 2005). Prolactin has also been detected by push-pull perfusions within the brain, with changing levels associated with conditions such as lactation (Torner et al., 2002, 2004).

One explanation for this might be that these studies detected pituitary prolactin after transport from the periphery.

However, in at least one of these studies (DeVito, 1988), prolactin-like immunoreactivity was not eliminated by hypophysectomy, suggesting that it was unlikely to be of pituitary origin.

In our study, to verify that the prolactin present in the hippocampus was not of pituitary origin, an immunohistochemical protocol was performed in the hippocampus of hypophysectomized young adult rats, verifying the presence of the reaction to prolactin in a similar way to that which occurred in non-hypophysectomized rats. This suggests that without ruling out the possibility of circulating prolactin reaching the hippocampus, hippocampal prolactin does not originate from the pituitary gland.

Because prolactin is a large protein (M.W.: 23 kDa) that can dimerize several times, it would be expected to be excluded from the brain by the existence of specialized tight junctions between vascular endothelial cells in the brain: the blood-brain barrier (Banks, 2012). However, circulating hormones less than 40 kDa in size can pass from the bloodstream to the brain in places where fenestrated capillaries exist, such as the median eminence and the ventromedial portion of the arcuate capillary nucleus (Ciofi, 2011; Norsted et al., 2008).

Several studies have reported that systemic administration of prolactin can rapidly activate prolactin receptors in all regions, using phosphorylation of signal transducer and activator of transcription 5 (STAT5) as a marker of prolactin action (Brown et al., 2010, 2011; Furigo et al., 2014, 2017; Oláh et al., 2018; Salais-López et al., 2017; Sapsford et al., 2012). These data are consistent with the model proposed in that prolactin is transported into the brain in a dispersed manner from the cerebral microvasculature, simultaneously activating neurons at a wide range of target sites.

Part of the blood-brain barrier is composed of the choroid plexuses. It is known that the choroid plexuses are one of the places in the brain where there are more receptors for prolactin (R. Augustine et al., 2003; Clemens & Sawyer, 1974; Ferraris et al., 2013; Hirai et al., 2013; Larsen & Grattan, 2012; Nogami et al., 2007; Patil et al., 2014; Tejadilla et al., 2010), and that cerebrospinal fluid contains prolactin. Several studies have reported that prolactin levels in the cerebrospinal fluid are parallel to those observed in the vascular system (Grattan & Averill, 1991; Login & MacLeod, 1977; Nicholson et al., 1980; Rubin & Bridges, 1989), suggesting that transport from the circulation is a primary mechanism for prolactin entry into the brain. It is hypothesized that prolactin transport occurs by internalization of the prolactin-prolactin receptor complex and posterior secretion as part of the protein component of the cerebrospinal fluid.

However, not all studies have demonstrated this, and the physiological importance of this system has been questioned. The concentrations of prolactin in the cerebrospinal fluid do not always match peripheral prolactin levels, and the biological activity of PRL is not consistently aligned with immunoreactive prolactin concentrations (Barbanel et al., 1986; Bridges et al., 1996).

Brown et al. (Brown et al., 2016) demonstrate that there was no change in the rate of labelled prolactin transport into the cerebrospinal fluid in mice lacking the prolactin receptor compared to control subjects and concluded that prolactin transport into the brain involves another unidentified transporter molecule apart from the prolactin receptor. Moreover, after intravenous administration of prolactin, it appeared only slowly in the cerebrospinal fluid, indicating that the cerebrospinal fluid is not the major route of prolactin access to the brain.

The current hypothesis is that transport occurs at the cerebral vasculature level via an unidentified carrier, allowing access throughout the brain. It likely ends up in the cerebrospinal fluid later, as part of the clearance from the brain (Bridges et al., 1996).

In our laboratory, in a complementary study to this thesis, we have sufficient evidence obtained by qPCR and in situ hybridization that prolactin mRNA is present in the ependymal epithelium of the choroid plexuses, so the presence of prolactin in the cerebrospinal fluid could be the result of its synthesis in the choroid plexuses and not necessarily of brain clearance (Carretero J et al., 2024).

Therefore, although there is evidence of systemic prolactin being transported into the brain, as well as prolactin receptor expression throughout the brain, the possibility that central prolactin receptors are influenced by prolactin of brain origin should also be considered.

Prolactin expression in the brain

In the central nervous system, although with discrepancies among authors, the possibility of synthesizing prolactin that would act locally has also been described (Ben-Jonathan et al. 1996). The possibility of prolactin production in the brain started when the hormone could still be detected in the brains of animals with the pituitary gland removed (DeVito, 1988).

Subsequently, using qPCR, prolactin mRNA was detected in several regions of the brain, such as the pons-medulla, hypothalamus, amygdala, thalamus, and choroid plexus in rodents (Clapp et al., 1994; Costa-Brito et al., 2021; DeVito, Avakian, et al., 1992; Emanuele et al., 1992; Jakob et al., 2014; Torner et al., 2002, 2004).

Using western blotting and in situ hybridization, the presence of prolactin and its mRNA in the brain of sheep was confirmed and was localized in the medial preoptic area, periventricular preoptic nucleus, bed nucleus of the stria terminals, and ventral paraventricular nuclei (Roselli et al., 2008). Most of these studies were performed on female animals during diestrus, pregnancy, lactation, and perinatal pups. There has been no confirmation using qPCR or in situ hybridization for the synthesis of prolactin in the hippocampus in the past.

This could be important because the extreme sensitivity of RT-PCR means there is potential for prolactin mRNA to be detected from contamination by lactotrophs in the tuberal region of the pituitary stalk (Ordronneau & Petrusz, 1980), or from circulating immune cells present within the tissue that may express prolactin (Horiguchi et al., 2004; Montgomery, 2001).

The possibility of contamination from the pituitary pars tuberalis can be accepted when the structure to be studied is the hypothalamus, as it is very common for the glandular portion to be attached through the meninges to the basal face of the median eminence of the hypothalamus.

In our study, this possibility does not occur because, as explained in the chapter on material and methods, a careful dissection is made under a microscope of the rat hippocampus, which, on the other hand, is too far from the pituitary gland and the median eminence to leave pituitary cells adherent.

In hippocampal dissection, once the lateral ventricle is accessed, the first step is to remove the choroid plexus. Although there may be hematic rests in intrahippocampal vessels, they

are not enough to suggest that there is contamination by immune cells that falsify the results obtained with qPCR, which, on the other hand, have been confirmed by in situ hybridization.

In our study, analyses have been carried out using different primers, of sequences published by other authors (Costa-Brito et al., 2021; Vergara-Castañeda et al., 2016) *for the prolactin mRNA and comparing the results, it was concluded that the primers used in the study were the ones that gave the most reliable results.*

Taken together with the lack of correlation between serum and hippocampal prolactin levels, western blotting, immunohistochemistry, and in situ hybridization, our results suggest that prolactin is synthesized in the hippocampus and dentate gyrus, which occurs in neurons and is modified in relation to age and sex.

This fact is relevant because in certain circumstances such as brain development (Walker et al., 2012), in the brain's response to injury (Dunkley et al., 2004; Hirano, 1986; Hodson et al., 2012; Möderscheim et al., 2007; Vergara-Castañeda et al., 2016), in vitro studies of hypothalamic fragments or glial cells in culture (DeVito et al., 1987, 1991; DeVito, Okulicz, et al., 1992), or in learning and spatial memory consolidation (Sánchez, 2018), prolactin production has been demonstrated.

In our study, using specific probes for prolactin mRNA, we were able to demonstrate the presence of hippocampal prolactin mRNA. Except for a previous study carried out in our laboratory (Sánchez, 2018)*, this is the first evidence of these mRNA using in situ hybridization in rats.*

Because animals were perfused and hematic rests were retired, the results obtained in our study cannot be attributed to hematic contamination.

An important finding is the modifications in prolactin mRNA expression in the hippocampus and dentate gyrus during aging. Usually, the lowest signal was found in adults and old animals and compared to pre- and/or postpubertal animals, the signal was almost non-existent in some layers of some regions. This could explain the controversy that prolactin mRNA by in situ hybridization has not been described previously, since false negatives could be obtained if the studies were carried out in animals older than 3 months.

The knowledge gap leads us to formulate other questions "how hippocampal and dentate gyrus expression of prolactin mRNA is regulated" and "what accounts for the observed pre- and postpubertal changes, as well as the decrease observed in adult and old animals*". These questions raised by our results represents a new challenge that needs to be studied in the future at our laboratory.*

Hippocampal prolactin expression and its possible physiological roles

More than 300 different biological functions have been described for prolactin, some of which are related to the central nervous system, including the stimulation of neurogenesis, modulation of stress responses, reduction of anxiety, transport of calcium, and regulation of the immune system (Ferraris et al., 2013; Larsen & Grattan, 2012; Patil et al., 2014).

Prolactin has been linked to similar neurogenic processes in adults, especially linked to subventricular zone (Larsen & Grattan, 2012) and hippocampal subgranular zone (Cabrera et al., 2009), where it has also been shown to produce neuroprotection (Cabrera et al., 2009; Morales et al., 2014; Reyes-Mendoza & Morales, 2016; Rivero-Segura et al., 2017; Tejadilla et al., 2010; Torner et al., 2009; Vergara-Castañeda et al., 2016). However, most of these studies were carried out in states of hyperprolactinemia or after exogenous administration of the hormone, which makes it difficult to determine whether these results are physiological or pharmacological.

The presence of prolactin and its mRNA in the brain and hippocampus has been described by RIA, immunocytochemistry, RT-PCR, and RT-qPCR (Cabrera-Reyes et al., 2015; DeVito, 1988; Emanuele et al., 1992; Fields et al., 1993; Roselli et al., 2008) and has been associated with neurodevelopment (Pardo et al., 2016), neurogenesis, cell proliferation, and neuroprotection (Morales et al., 2014; Reyes-Mendoza & Morales, 2016; Rivero-Segura et al., 2017), neuroplasticity, and dendritic remodeling during pregnancy and postpartum period (Brusco et al., 2008; Kinsley et al., 2006). In the hippocampus of adult mice, prolactin may be necessary for learning and memory (Walker et al., 2012).

However, the literature includes different studies that relate prolactin to maternal behavior, lactation, and pregnancy (R. A. Augustine et al., 2017; Brown et al., 2017; Mitani et al., 2018; Sairenji et al., 2017; Zhang et al., 2017; Zingler et al., 2017).

Prolactin is associated with neurogenesis in the dentate gyrus (Larsen et al., 2008; Pathipati et al., 2011; Torner et al., 2009; Wagner et al., 2009; Walker et al., 2012). The subgranular zone of dentate gyrus maintain proliferation capacity in adulthood (Torner et al., 2009; Walker et al., 2012).

> *The presence of positivity to prolactin and its mRNA, as well as to the mRNA of the long and short isoforms of the prolactin receptor in the subgranular cells of the dentate gyrus that we observed in our study, suggests that prolactin could have a neurogenic paracrine effect, mainly from the prepubertal stage to the young adult stage.*

The neurogenesis in the dentate gyrus has been directly related to learning, memory, and spatial orientation (Lévy et al., 2011). Results obtained from our laboratory (Sánchez-Robledo, 2018) in relation to learning and spatial memory and its consolidation demonstrated changes in immunoreactive prolactin, by western blotting and immunohistochemistry, and in prolactin mRNA expression, by qPCR and in situ hybridization, suggesting an enhancer and protective

effect of prolactin in memory consolidation, as was described posteriorly by other authors (Mellado et al., 2022; Moreno-Ruiz et al., 2021; Zamora-Moratalla & Martín, 2021).

The synthesis of de novo prolactin in the hippocampal regions studied by us, obtained by qPCR and in situ hybridization, suggests the possibility that prolactin could play an autoparacrine regulatory role in the hippocampus, which undergoes variations in the aging of the animal, and it is sexually dimorphic.

In our study, variations in prolactin mRNA expression observed by qPCR and in situ hybridization in the different age stages and sex analyzed are concordant with the findings obtained by western blotting and immunocytochemistry, so that the decrease in the hybridization signal, as well as the decrease in the expression of prolactin mRNA found after qPCR in the same periods, could justify the decrease observed when the protein was analyzed.

In this study, the presence of prolactin in the dentate gyrus and hippocampus –CA1 and CA3– was demonstrated by means of immunocytochemistry, ELISA and western blotting, in a similar way as previously described by other authors (DeVito, 1988; Freeman et al., 2000).

The presence of several western blotting bands for prolactin was observed in all groups, which was expected because there are different variants of prolactin because this hormone can form dimers, polymers, and aggregates that become macroprolactin, as reported previously (Cohen et al., 1994; Fahie-Wilson et al., 2005; Nyberg et al., 1981; Sherazi et al., 2018).

An unexpected band at approximately 60kDa was found by western blotting. After pre-absorption with prolactin, all bands except the 60kDa band disappeared, suggesting that this band was not prolactin. Thus, we did not consider this in the densitometric studies of western blotting to obtain signal profiles.

The molecular weight of 60kDa does not correspond to the molecular weight of other proteins that could presumably cross-react, such as opiomelanocortin or growth hormone, or to other proteins that could bind to the hippocampal prolactin receptor, such as interleukins. The possibility that it could be prolactin linked to a carrier or a receptor is remote since, in principle, the antibody would detect the hormone; therefore, after the preabsorption test, the band should disappear.

Costa-Brito and coworkers (Costa-Brito et al., 2021) postulated a new type of prolactin synthetized in the choroid plexus weighing 63 kDa. Their reasoning for such claims is that after incubation of the WB membrane with prolactin receptor peptide, this band could not be detected with an antiprolactin antibody, making this 63 kDa protein able to bind to the prolactin receptor.

Although the band that we discarded could be the same as that found by this group, our pre-absorption test contradicted that this could be a prolactin variant.

Rat prolactin has two different transcript variants, Ensembl ENSRNOT00000023412.5 Prl-201 and ENSRNOT00000100172.1 Prl-202, which resulting proteins are of 233 and 227 amino acids, respectively. This implies that the same cell can produce different variants of prolactin with slightly different molecular weights between 23 and 25 kDa.

The most logical explanation as to why we detected double bands in WB for prolactin is that we detected both variants of the hormone from different transcripts.

Prolactin receptor expression in the brain

Prolactin is a hormone with a specific receptor and develop its biological roles acting on these receptors.

There is evidence for expression of the prolactin receptor throughout several brain regions, using binding assays (Crumeyrolle-Arias et al., 1993), immunohistochemistry (Pi & Grattan, 1998, 1999; Roky et al., 1996), and in situ hybridization (Bakowska & Morrell, 1997, 2003; Brown et al., 2010; Chiu & Wise, 1994). Description of the prolactin receptor expression in the brain using a novel transgenic reporter mouse strain has been reported (Kokay et al., 2018). These studies have provided the widespread distribution of PRL receptors in the brain: in the medial periventricular hypothalamic nuclei, from the preoptic region through to the caudal arcuate nucleus, and in several extra-hypothalamic regions, such as the bed nucleus of the stria terminalis and the posterodorsal medial amygdala.

The expression of the prolactin receptor in the brain has been widely described in literature. In 1996, it was reported that the expression of the receptor changes depending on the hormonal status of female rats, with them being in different stages of the estrous cycle, pregnancy, or lactation (Sugiyama et al., 1996). The expression of the isoforms differed between the short and long forms, with the short form being more stable and less sensitive to hormonal changes in the serum.

Even during pregnancy, overexpression of the long isoform occurs earlier than that of the short isoform. These differences have been reported in the choroid plexus but not in the hypothalamic nuclei (R. Augustine et al., 2003). The short isoform seems to be the most common in the choroid plexus, whereas the long isoform is practically undetectable (Nogami et al., 2007).

The long isoform is also more highly expressed in the hippocampus and in certain types of cancer, which could be related to cell proliferation (Harvey et al., 2015; Kang et al., 2014; Tejadilla et al., 2010; Torner et al., 2009).

Our qPCR results support the published literature, indicating that the long form is more highly expressed than the short form. Considering that the long form has a higher affinity for STAT5 phosphorylation, it could be hypothesized that this intracellular pathway is favoured over others.

Overexpression of the prolactin receptor has been associated with maternal behavior, such as licking and nursing the pups (R. Augustine et al., 2003; Freeman et al., 2000) and stressful

situations (Torner et al., 2009); however, other studies claim that maternal rats express fewer receptors than virgin diestrus rats (de Moura et al., 2015).

Long and short isoforms of the prolactin receptor have been described in the hippocampus ((Cabrera-Reyes et al., 2015; Emanuele et al., 1992; Möderscheim et al., 2007; Tian et al., 2019; Torner et al., 2009)). Even at low levels, prolactin receptor gene expression in the hippocampus has been confirmed by qPCR in rats (Cabrera-Reyes et al., 2015; Vergara-Castañeda et al., 2016), supporting the idea that prolactin plays a role in this brain region mediated by its receptor.

> *The fact that we could detect the receptor in all age groups implies that prolactin plays a role in the hippocampus throughout life, and not exclusively in mating, maternal, and perinatal periods. The presence of receptor mRNA was confirmed by in situ hybridization, and it was localized in all regions of the hippocampus and dentate gyrus. Although with predominance in pre- and postpubertal animals and with differences related with age and sex for every isoform.*

Estradiol-17β treatment in menopausal rats also seemed to increase the overexpression of prolactin receptors in the hippocampus (Sárvári et al., 2015)); however, this was not the case in perinatal female rats that were also treated with estradiol-17β (Govindaraj et al., 2018).

> *The possibility that prolactin synthesis is mediated by its receptor in a paracrine system in the hippocampus remains to be elucidated. In our findings, male rats that reached puberty had a strong peak of synthesis for the receptor of both isoforms, but this was not reflected in hormone synthesis, making this kind of regulation unlikely. Further studies on hippocampal estrogen and its dynamics with prolactin synthesis and its correlation with prolactin receptor synthesis are necessary.*

Conclusions

After a critical analysis of the results obtained in our study and contrasting them with those existing in the scientific literature, we have reached the following conclusions:

First. - The hippocampus (Amon's horn and dentate gyrus) of the rat contains prolactin, as shown by the findings obtained by ELISA, western blotting, and immunohistochemistry, which allows us to speak of a hippocampal prolactin system.

Second. - The results obtained with ELISA show that there is no correlation between serum and hippocampal levels of prolactin, suggesting that, without being able to rule out that prolactin can pass into the hippocampus from the bloodstream, there is another source of prolactin in the hippocampus.

Third. - The hippocampus synthesizes prolactin, as shown by the results obtained with qPCR and in situ hybridization. The postpubertal stage in females and young adult in males are the times when prolactin synthesis is greatest, suggesting the possibility that hippocampal prolactin may develop paracrine effects.

Fourth. - The hippocampal prolactin system undergoes changes throughout life, with greater expression around puberty and in the early stages of adulthood, decreasing with aging.

Fifth. - In the rat hippocampus, throughout life, there are sex-related differences in the synthesis and presence of prolactin, mainly in the stages before and after puberty, suggesting the possibility that could be a sexual dimorphism.

Sixth. - The stages in which the presence and expression of prolactin is greatest in the hippocampus, prolactin is expressed in the subgranular cells of the dentate gyrus that are considered neuronal stem cells, suggesting a possible neurogenic role for the hormone.

Seventh. - The rat hippocampus synthesizes the two isoforms of the prolactin receptor, with marked differences between the ages studied and from one sex to the other. As evidenced by the results obtained by qPCR and in situ hybridization. The greatest expression of box1 and box2 occurs in age periods close to puberty. Box2 is expressed more in postpubertal animals, both females and males, while the highest expression of box1 occurs in prepubertal females and in pre- and postpubertal males, suggesting that prolactin have relevant physiological actions on hippocampus in these ages.

Eighth. - The presence of subgranular cells positives to prolactin, prolactin mRNA, box1 mRNA and box2 mRNA, mainly in pre- and postpubertal ages, suggest that the hormone could act as a neurogenic paracrine factor in the dentate gyrus.

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