The Contribution of Cell Imaging to the Study of Anterior Pituitary Function and Its Regulation

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Abstract

Advances in the knowledge of the neuroendocrine system are closely related to the development of cellular imaging and labeling techniques. This synergy ranges from the staining techniques that allowed the first characterizations of the anterior pituitary gland, its relationship with the hypothalamus, and the birth of neuroendocrinology; through the development of fluorescence microscopy applications, specific labeling strategies, transgenic systems, and intracellular calcium sensors that enabled the study of processes and dynamics at the cellular and tissue level; until the advent of super-resolution microscopy, miniscopes, optogenetics, fiber photometry, and other imaging methods that allowed high spatiotemporal resolution and long-term three-dimensional cellular activity recordings in living systems in a conscious and freely moving condition. In this review, we briefly summarize the main contributions of cellular imaging techniques that have allowed relevant advances in the field of neuroendocrinology and paradigm shifts that have im-

proved our understanding of the function of the hypothalamic-pituitary axes. The development of these methods and equipment is the result of the integration of knowledge achieved by the integration of several disciplines and effort to solve scientific questions and problems of high impact on health and society that this system entails.

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Introduction

For hundreds of years, attempts have been made to discover and understand the secrets of the complex neuroendocrine system. The relevance of its study is unquestionable, given the evolutionary importance and the profound implications that its alterations have worldwide on public health and the quality of life of a large number of people of all ages and socioeconomic groups.

The earliest formal approaches to the endocrine system date back to the times of Aristotle and Galen, when the term *pituuta* (from the Latin *pituuta*, “mucus”) was introduced to refer to a protrusion believed to secrete the mucus from the nose, later renamed hypophysis (from the Latin *hypo* “below” and *physis* “grow”). Progress in

the physiological understanding of the endocrine glands accelerated once the concept of internal secretion matured. By the early 20th century, there was already the idea that the endocrine system regulates, correlates, and integrates all local cellular metabolisms [1]. More than 60 years ago it was described how the brain can influence endocrine function through the portal system, which laid the groundwork for studies on the different mechanisms of pituitary control [2]. These investigations revolutionized the existing knowledge at the time and laid the foundations of neuroendocrinology. However, other scientific areas, such as physics, mathematics, and chemistry, have contributed greatly to the achievements and advances in the study of the pituitary gland function and regulation, especially those closely related to the development of cellular imaging. This led to a deeper understanding of biological systems, and the great “boom” of neuroendocrinology in the second half of the 20th century.

Historical Development of Imaging

The invention of the microscope more than four centuries ago was a milestone for biological research. Improvements in conventional light microscopy and the interest in the detailed characterization and visualization of tissues and individual cells led to the development of staining techniques and dyes for histological samples preparation, included Golgi staining, Indian ink staining, and biological colorations such as trypan blue and those using hematoxylin and/or eosin. These techniques allowed, for example, the first characterizations of the vasculature, the hypothalamic-pituitary connection [3], and the first classification of pituitary cells into three categories: chromophobe cells, acidophilic cells, and basophilic cells [4].

The interest in the problem of differential staining cellular types or components was solved with the advent of immune labeling (1941), nowadays one of the most widespread labeling techniques. Subsequently, finer and more specific labeling strategies have been developed, such as the genetic addition of small-molecule fluorophores [5, 6] and naturally or chimera fluorescent proteins, like genetically encoded green fluorescent protein (GFP) [7–10]. The latter undoubtedly revolutionized cellular imaging [11], leading to the development of several fluorescent proteins in a wide range of wavelengths and applications [12], which paved the way for biological research to advance into a new field of understanding processes and dynamics at the cellular and tissue level through fluorescence microscopy (FM) [13].

In the second half of the 20th century, a diversity of FM microscopes and applications were improved, such as the confocal microscope or multiphoton/two-photon microscopy [14, 15] and the development of chimeras and new and enhanced fluorophores that allowed the advent of advanced techniques, like fluorescence recovery after photobleaching [15–17], fluorescence resonance energy transfer [18, 19], and total internal reflection FM (TIR-FM) [15, 20]. Thus, one of the major questions resolved with this advance has been the understanding of the mechanism of transport and exocytosis of vesicles in the cell, from the trans-Golgi network to the plasma membrane [21], as observed with the use of a GFP chimera (VSVG3-GFP) coupled to the vesicles and real-time imaging [22].

Moreover, the limited spatial resolution of FM resulting from light diffraction (200 nm, laterally) has been overcome by the advent of super-resolution microscopy (SRM) or nanoscopy, a powerful tool for imaging biomolecules and cellular structures at the nanometer scale (typically 10–70 nm, down to 1 nm), even in live-cells. SRM groups several types or approaches, such as super-resolution by interference pattern, single-molecule localization microscopy, stimulated emission depletion microscopy, reversible saturable/switchable optical linear (fluorescence) transitions microscopy, photo-activated localization microscopy, stochastic optical reconstruction microscopy, minimal photon fluxes, AiryScan, lattice light-sheet microscopy, and interferometric approaches to increase axial resolution, such as 4Pi, among others [23–27].

Before SRM, the limit of the optical resolution had been surpassed with the invention of the electron microscope (EM) [28], achieving high power resolution (<0.5 nm), which made it the tool of choice for ultrastructural examinations [29]. There are different modalities of EM, either by irradiating through a sample, as in transmission electron microscopy (TEM) or by collecting back-scattered or secondary electrons on the surface of a sample, scanning electron microscopy (SEM) [30]. Before EM, it was not possible to resolve particular features of endocrine cells, such as the size of the secretory granules which have dimensions between 100 and 600 nm [31]. Recently, volume electron microscopy has been developed, which encompasses a set of EM techniques for three-dimensional (3D) analysis and can be performed by TEM or SEM [32]. These techniques include the serial section TEM or SEM (serial section SEM), electron tomography, serial block-face SEM, and focused ion beam SEM [33].

Fig. 1. First representations of the pituitary portal vascular system: Cajal drawing of the sagittal section of the pituitary of a mouse stained with the Golgi method (A: nerve lobe, B: glandular lobe, C: infundibulum) (a); first photograph of the pituitary and hypothalamus cleared in xylol of a newborn child from the front, Popa showed the hypophysio-portal system in the stalk of the pituitary (b); SEM photomicrograph of a monkey pituitary (c); in the pituitary gland, changes in blood flow in the portal circulation alter the pattern of exposure of pituitary cells to neurohormones and nutrient supply to facilitate secretion (d). Images reproduced and modified from Cajal 1909 [34] (a) Popa 1930 [178] (b) Page 1977 [37] (c) Le Tissier 2017 [55] (d).

Imaging the Hypothalamic-Pituitary Portal System

One of the early depictions of the connection between the hypothalamus and the pituitary was drawn by Ramón y Cajal based on the observation of Golgi-stain cells [34] (shown in Fig. 1a). Although Cajal points out the nerve connection, it was until 1930 that Popa and Fielding acquired the first photograph of the pituitary portal system and described a relationship between the hypothalamus and the gland via the pituitary stalk (shown in Fig. 1b). However, they erroneously assumed that blood flow went up the stem toward the hypothalamus [35]. This idea was reformulated in the following years when India ink and other acid markers, such as trypan blue, were used to state that the physiological supply goes from the hypothalamus to the pituitary, establishing the relationship between the median eminence (ME) and the anterior lobe of the pituitary [36, 37].

In the posterior lobe, axons of central nervous system neurons reach the peripheral blood vessels niche, an interface that is facilitated by pituicytes. In both cases, the study of the establishment and the 3D structure of these vascular niches had not been possible given the technical limitations presented by histological sections. Recently, using *in vivo* fluorescence techniques and the fish model,

it was concluded that in the posterior lobe of the pituitary gland the progenitor cells of hypothalamic neurons gradually acquire their neural phenotype and extend their processes toward the pituitary posterior lobe capillary (CAP) plexus which is formed by sprouting and extension of existing vessels under the stimulus of oxytocin, released by the hypothalamic neurons themselves [38–40].

Nowadays, the general consensus is that there are no direct neural connections between the hypothalamus and the anterior lobe, but a complex and unusual network of capillaries [41]. Since the 1970s, it was described that the axons of hypothalamic neurons reach the CAP plexus in the ME [42], we now know that this structure is very dynamic and that glial cells and tanycytes in the ME engulf the axonal terminals and interpose their cellular processes between the axonal terminals and capillaries, for example, when hormonal demand is low [43]. Several factors, such as noradrenaline or ATP, shape this dynamic process [43]. Hypothalamic tanycytes play an important role in the selective permeabilization of molecules to the brain. Recently, using cell tracing and intracellular calcium activity recording, it has been demonstrated that these cells respond to leptin by activating Ca^{2+} waves, controlling the entry of this hormone into the brain, inducing not only increased food intake and lipogenesis but

also glucose intolerance by a decrease in insulin secretion by pancreatic β cells [44]. Tanycytes also convert brain glucose into lactate which they transmit to the arcuate proopiomelanocortin neurons [45].

The CAP plexus in the hypothalamus and pituitary is complex and dynamic. The ME is not just the site where neuronal axon terminals release neurohormones and molecules into the portal CAP system, in recent years it has been demonstrated that it is also a passage of the signals that are in the blood to the brain, as is the case of ghrelin [46, 47]. Moreover, employing opto and chemogenetic manipulation, it was recently confirmed that melanin-concentrating hormone expressing neurons, key regulators of energy and glucose homeostasis, change ME permeability linking signals of the energy state of the organism with circadian rhythms [48]. Study of the 3D conformation of the vascular network in the anterior pituitary and its relationship with endocrine cell organization and hormonal secretion has recently begun [49]; establishing that capillaries are organized in a complex network forming inner loops and that they are highly correlated with endocrine cells, determining in many cases their morphology and activity [50]. In addition, it has also been possible to observe changes in vascular morphology (number, diameter, and shape) in glands, which can result in dramatic alteration in pituitary tumors [51].

One of the fundamental elements that make the hypothalamic-pituitary portal system functional is the presence of perivascular cells, which differ from smooth muscle, and whose function is not fully understood. It was not until 1989 that SEM images clearly evidenced their presence in the pituitary CAP network [52] (shown in Fig. 1c). These works define pericytes as a type of wall cell that is found throughout the system and were the first to suggest that the pituitary portal system should not be considered as veins, but as fenestrated capillaries covered by pericytes with highly branched cytoplasmic processes [52] that also contribute to the matrix formation [53]. Recently, using SEM, different shapes of pericytes (oval, elongate, triangular, and multiangular) have been observed whose proportion, except for oval pericytes, is higher with physiological changes such as castration [54].

Advances in imaging techniques have made it possible to demonstrate that the pituitary vascular system is highly dynamic [55] and that, contrary to its original description, it not only serves to provide nutrients and oxygen to cells but is also key to generate hormonal pulses [56, 57] (shown in Fig. 1d). In more recent studies, it was possible to measure the blood flow velocity in the pituitary

capillaries of growth hormone (GH)-EGFP transgenic mice, using a fluorescence microscope with an objective with a long working distance, 2.8 cm [56]. A detailed review of these was performed by Schaeffer et al. [58]. However, the relevance of the vasculature in the pituitary and its role in the generation of hormonal pulses has not been fully understood. It is now known that the CAP bed influences gland morphology and network formation [59, 60] and that the endothelium-pericyte-endocrine cell unit probably acts in a coordinated way to generate the pulses.

The Pituitary Endocrine Cells Type

Initially, most of the morphological correlations between the type of pituitary hormone contained in the secretory granules and endocrine cell type were made by observing the cellular changes derived from the ablation of an organ, from a lesion, or in a certain physiological condition [61]. Later, when histochemical techniques were developed and the antibodies could be coupled to enzymes that generated a color, the hormones contained in the cells were identified [62]. Although at that time these were still somewhat difficult and imprecise techniques [63], allow to establish the presence of five endocrine cell types: somatotrophs, lactotrophs, thyrotrophs, gonadotrophs, and corticotrophs [64]. However, in order to identify each of these cells *in vivo* and be able to study their function, it was necessary to use other techniques such as gene targeting in embryonic stem cells, to generate mice in which Cre recombinase is coexpressed with specific proteins, for example, gonadotropin-releasing hormone (GnRH) receptor [65], glycoprotein hormone alpha-subunit [66], and thyroid-stimulating hormone beta-subunit [67] or by transgenesis to express hormone fragments fused in frame with a fluorescent protein GH [68], prolactin (PRL [69]), luteinizing hormone (LH [70]), and proopiomelanocortin [71].

For a long time, the pituitary was considered to be a mass of randomly arranged secretory cells. During the late 20th century, it became possible to reproducibly and reliably study both characteristics of each cell type as well as the interactions and relationships between cells and their dynamic changes (reviewed in [72]). The development of fluorescent techniques [73] enabled the modification of the original pituitary concept to show that there is a highly interdigitated organization of cellular networks with homotypic and heterotypic interactions [74], such that the absence of one cell type affects the network

operation of another cell type [75]; for instance, the absence of corticotrophs modifies the structure and organization of gonadotrophs [70], the absence of gonadotrophs affects lactotrophs [76], and the deficiency of somatotrophs can affect more than one network [77].

Cellular networks are established from embryonic development. The corticotrophs are the first endocrine cell type to form homotypic interactions, probably influencing the organization of other cell networks such as the gonadotrophs that appear at e17.5 [78]. Similar to corticotrophs, GH cells appear as small isolated cells and by e18.5 they have formed strands [78]. In adults, the cellular organization is modified according to physiological conditions, such as lactation and puberty [79], wherein a very robust change in the organization of lactotrophs has been described being remarkable that the network is able to structurally memorize previous challenges [57, 79]; or somatotroph that has a sexually dimorphic network (higher proportion of responding cells in males than in females) regulated by gonadal steroids, whose activity allows a 1,000-fold rise of GH levels over basal levels in vivo [80–82]. These cellular networks can be remodeled through a mechanism of cellular process extension and cell mobility described in mammals and fishes in gonadotrophs [83–85] and somatotrophs cells [82].

In recent years, interest in reconstructing in vitro 3D microenvironments close to in vivo structures has increased [86]; however, the pituitary has not been as well explored in this regard as other organs [87]. Diverse techniques like cell aggregates and hanging drops have been used for the study of paracrine signaling and its influence on rat pituitary cells [88, 89]. Thereby, Denef's group demonstrates that factors such as T3, GHRH, glucocorticoids, among others, regulate the expression of different genes and the relationship in the cellular populations of the aggregates [90–93]. With some limitations, recent work has shown that this type of tool can be an option for the controlled study of cell morphology and interaction in the pituitary, with confocal microscopy and SEM suggesting a similar cell morphology as in vivo with the in vitro 3D experiment [94].

Cell-cell interaction is highly relevant for an appropriate pituitary tissue organization and hormonal secretion [72]. Although there is abundant evidence of the role played by tight junctions, adherens junctions, desmosomes, and gap junctions regulating several processes such as proliferation, death, differentiation, migration, and secretion [95–98], so far we do not have a complete view of the complex and dynamic homo and heterotypic relationship of the anterior pituitary endocrine cells.

Hormone Production and Secretion by Imaging

Identification of the pituitary as a secretory gland arose in the 18th century [99]. Later, the first clinical observations were made based on the effect of chemical messengers (hormones) on the control of physiological functions such as growth or organ activity [100]. Even before imaging, it was known from biochemical and physiological studies that the anterior pituitary produced at least six hormones: GH, corticotropin, PRL, TSH, LH, and follicle-stimulating hormone.

Hormonal secretion depends on multiple factors and the integration of systemic and internal signals, which include autocrine and paracrine mechanisms and the relationship of hetero- and homotypic cellular networks and transcription factors that determine hormonal gene transcription. Recently, quantum dots (semiconductor nanoparticles [101]) have been used to investigate the intracellular localization of both pituitary hormones, GH and PRL, and their mRNA simultaneously in 3D arrays [102, 103]. Other dynamic fluorescent methods, such as the expression of luciferase or destabilized enhanced GFP drive by the PRL gene expression, have allowed researchers to establish that the synthesis of hormones (PRL) although it is observed stochastically in small groups, is a coordinated event throughout the gland [104, 105]. Likewise, multiple levels of transcription rates and dynamics were also identified between embryonic and adult pituitary glands; the embryonic tissue displayed shorter durations of high-expressing states than the adult, which in turn exhibited local transcriptional coordination between PRL-expressing cells [106].

The secretion of pituitary hormones is an event that depends on the intracellular increase in calcium. In studies carried out with the fluorescent probe FM 1–43 [107], it was observed that lactotrophs have at least two mechanisms of exocytosis that allow them to enhance their secretory response to stimuli: increase of the fusion points at the plasma membrane and increased compound exocytosis [108]. Other studies support this idea, using a fluorescent pH sensor [109] or other endocrine cells, such as the pancreas [110, 111]. The kinetics, transport, fusion, and exocytosis [112] of secretory granules have been studied in different cell types, such as adrenal medulla [113, 114] and pancreatic beta cells [115, 116] combining TIRFM and GFP expression. Calcium is not only required for vesicle fusion but also for vesicle mobilization from the internal reservoirs toward the plasma membrane [112]. In the pituitary [117, 118], this technical approach has been used to describe that the lack of exon 3 in the GH

Fig. 2. Deciphering pituitary ultrastructure and cells network through evolving imaging techniques. **a** Tissue staining with Attia's tetrachrome staining technique of adenohypophysis of a bitch in the anoestrus phase of the cycle: PRL cells: red (P); TSH cells: yellow to yellow-orange (S); ACTH cells: greyish violet (A); Gonadotrophic cells: greenish-blue (G). **b** FIB/SEM tomography reconstruction of the anterior pituitary gland, showing 3D view of blood vessels and endocrine cells together. **c** Electron microscopy image of human pituitary cells filled with secretory granules of differing sizes and distinctive electron-density. At least five secretory cell types can be seen, with CAP shown at the bottom, with fenestrated epithelium and well-developed PVS. **d** Gonadotroph and corticotroph cell network development, ventral view of double E18.5 POMC-EGFP (green) and LH-cerulean transgenic pituitary showing initial appearance of LH-cerulean cells in the rostromedial AL, in contrast to POMC-EGFP cells that are more prominent in the lateral wings: R; C. Images reproduced and modified from Attia 1978 [63] (**a**), Yoshitomi 2016 [119] (**b**), Bergland 1969 [177] (**c**), Budry 2011 [70] (**d**). FIB, focused ion beam; POMC, proopiomelanocortin; ACTH, corticotropin; PVS, perivascular space; CAP, capillary; R, Rostral; C, caudal.

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gene (autosomal dominant GH deficiency type II) produces few secretory vesicles but accumulated secretory products in amorphous cytoplasmic aggregates [118]. However, TIRFM is an underexplored imaging technique for the study of the pituitary.

The secretion event and the generation of hormonal pulses in the bloodstream is a complex phenomenon not yet understood. The full 3D cellular architecture of the anterior pituitary tissue [119] (shown in Fig. 2b) revealed that contrary to the idea conceived that endocrine cells are in apposition of the vessels to secrete their content, approximately 30% of these are isolated from the perivascular space of the tissue [119]. These observations raise the possibility of having different modes of secretion according to hormonal requirements; for instance, fast pulses can occur by the secretion of cells in apposition

with capillaries or slow pulses by those isolated in the parenchyma. This hypothesis has been revised with more recent techniques by Mollard's group [56], where the coordination between the circulation and the secretory bursts to generate a hormonal pulse was modeled in vivo by the elimination of fluorescent markers from the parenchyma of the gland toward the blood vessels (shown in Fig. 2d). They observed that significant amounts of the largest markers accumulated in a perivascular compartment, similar to a reserve or bank for later sudden release [56].

Just a few years ago, the hypothalamic-pituitary regulation was established by three types of regulation: the agnathan diffusional type, the teleost direct innervation type, and the vascular type seen in all other vertebrates [120]. However, Golan et al. [121] used transgenic zebrafish

models to demonstrate that for instance gonadotropin regulation by GnRH3 combines neuroglandular and neurovascular components, breaking the established dogma.

Imaging the Intracellular Calcium Activity

Calcium (Ca^{2+}) is a ubiquitous intracellular signaling molecule that controls numerous cellular processes, including fertilization, proliferation, development, learning and memory, contraction, gene expression, and secretion [122]. The magnitude, spatial and temporal aspects of signaling make this enormous range of processes possible [123]. The Ca^{2+} signaling system has a very large combination of molecular components (toolkit) that in each cell can be mixed to produce a particular response, not just with heterogeneity between cell types but also amongst homotypic cells [123]. Generally, cells at rest have an intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) of 100 nM but are activated when this level rises to roughly 1,000 nM. The toolkit is composed of elements that increase the $[\text{Ca}^{2+}]_i$ through release from internal stores or external medium, and pumps, exchangers, and binding proteins that modulate Ca^{2+} in the cytoplasm and restore the resting state [123].

As in other excitable cells, endocrine pituitary cells present rises in $[\text{Ca}^{2+}]_i$, either spontaneously or following a stimulus, these rises can be produced by the entrance of extracellular Ca^{2+} through voltage-gated calcium channels but also from by the exit of this ion from the intracellular reservoirs [124, 125]. Depending on the cell type, this activity can generate localized or global Ca^{2+} signals, reaching the threshold for hormone secretion [124]. Each adenohypophyseal cell type has a distinctive fingerprint in terms of its intracellular calcium response [126, 127]. Some other intracellular pathways are important for hormone synthesis and secretion, such as the activation of adenylate cyclase, leading to an increase in cAMP, which in turn leads to an increase in protein kinase A activity. However, fluorescent sensors for these molecules are not common, and their use in imaging is limited. For instance, some of the possibilities that could now be used are assays for protein kinases that utilize a variety of strategies to detect phosphorylation of peptide and protein substrates [128], as well as a red fluorescent cAMP indicator which is a fluorescence resonance energy transfer fluorophore [129] or the yellow fluorescent protein-based cAMP indicator [130].

For a long time, Ca^{2+} measurements and secretion were performed using the patch-clamp electrophysiology technique [124, 131, 132]. Since then it has been known

that secretory activity is related to intracellular Ca^{2+} activity [133]. However, this technique has the limitation of measuring one cell at a time and the difficulty of recognizing the registered cell type, although there are electrophysiological studies that use fluorescent sensors such as genetically encoded proteins [134] to easily identify the cells of interest as gonadotrophs [135]. At the end of the last century, the development of a series of fluorescent Ca^{2+} sensors and new microscopes, such as one- or two-photon confocal microscopes, allowed the study of the activity in time of several cells, simultaneously and with high spatiotemporal resolution [136] or even more, cellular activity in free-moving animals with the miniaturized microscopes, “miniscopes” [137]. These indicators can be coupled to an ester molecule and thus cross the cell membrane, changing their fluorescence intensity or wavelength as a function of Ca^{2+} concentration [138]. Since then, these sensors have improved and diversified greatly, to the point that some of them could be specifically expressed by a particular cell type [139].

In recent years, in order to understand the functioning of neural and neuroendocrine networks, the need arose to develop new imaging methods and tools that would allow in situ visualization of changes in intracellular Ca^{2+} activity, and thus the study of hormonal secretion in the pituitary. Consequently, genetically encoded calcium indicators [140] were created, the most widely used being a circular permutation of fluorescent proteins, such as GcAMPs and pericams, which are based on the alteration of the conformation of a fusion protein containing a calmodulin (which binds to Ca^{2+}) and a fluorophore, whose intensity varies as a function of Ca^{2+} concentration [141]. The most common form for the specific expression of the genetically encoded calcium indicators is based on the Cre/loxP system [142]. The transgenic system uses the expression of Cre (Cre recombinase; tyrosine site-specific recombinases) by a promoter that specifically targets the cell or tissue of interest. Second, this enzyme recognizes the specific DNA fragment sequences called loxP and mediates site-specific deletion of this sequence which flanked DNA containing, for example, the protein of interest to be expressed as the GFP, GcAMP, among others [143].

Calcium Activity and Endocrine Pituitary Cellular Networks

The intracellular Ca^{2+} activity of adenohypophyseal cells has been extensively evaluated in cell culture, so we will only refer to a few examples that illustrate how ad-

vances in imaging have allowed us to change some of the concepts described. Numerous studies have shown that gonadotrophs isolated in primary culture exhibit robust and stereotyped dose-dependent intracellular Ca^{2+} signals in response to different concentrations of GnRH [144, 145]. An increase in $[\text{Ca}^{2+}]_i$ triggers exocytosis and gonadotropin synthesis [146]. It has long been assumed that the different Ca^{2+} patterns (oscillatory, biphasic, and transient) observed with increasing doses of GnRH underlie just the dose-dependent increase in gonadotropin secretion. However, it has also been suggested that these patterns encode other cellular functions [146]; for example, Ca^{2+} biphasic responses were associated with LH secretion and oscillatory responses to LH β subunit synthesis [146]. The possibility of improving the techniques to measure the variations in $[\text{Ca}^{2+}]_i$ in slices, has allowed us to document important differences to what is observed in cells in culture. For instance, the different Ca^{2+} patterns observed as dose-dependent, are no longer so clear in tissue slices, in which the number of gonadotrophs that respond to a GnRH concentration gradient is not constant and 58% of cells do not have a sequence of these canonical Ca^{2+} patterns; even some cells exhibit an atypical (nonoscillatory) response, not seen in culture, regardless of stimulus concentration [147].

In somatotrophs, using a combination of single-cell techniques (Fura-2 with digital microscopy and hemolytic plaque assay) in late 1987, $[\text{Ca}^{2+}]_i$ and hormonal GH secretion were measured simultaneously. The authors reported spontaneous Ca^{2+} oscillations with different frequencies and amplitudes in specific cells and related these oscillations to GH secretion [148]. Many years later, it was also demonstrated that Ca^{2+} outflow from intracellular stores could not only enhance GH secretion but also increase RNA expression [149]. Using a combination of imaging techniques, it was shown that somatotrophs are organized in a continuum connected by adherent junctions that permeate the entire gland and exhibit the three primary characteristics of biological networks: robustness of architecture throughout life, modularity correlated with pituitary GH contents, and body growth, and connectivity with stereotyped motifs, especially cellular synchronization coordinating activity [80]. Furthermore, the *in vivo* responses of the somatotrophic cell network were shown to be sexually dimorphic, with a greater proportion of cells responding synchronously in males than in females, resulting in greater GH release [81]. These findings have been a turning point in the thinking about the structure and organization of somatotrophs in particular but will undoubtedly be applicable to other endocrine cells.

An important imaging tool developed in the last decade is the miniscope, a system that seeks to integrate the capabilities of a microscope into a small device (<2 g) [150]. The miniscope is based on technological developments such as CMOS [151] image sensors, LED diodes [152], and GRIN lenses; elements that make possible an extremely reduced optical path and whose portability facilitates incorporation into living systems without interfering with their free movement, in addition to reducing motion artifacts in the recordings. Furthermore, unlike fiber photometry, with the miniscope, it is possible to distinguish the Ca^{2+} activity of more than one cell individually. Its use in neuroendocrinology research has begun [153]. Recently, the use of the miniscope to record somatotrophs cell activity in freely moving animals has been published [154]. This work is an example of the use of a combination of the latest imaging techniques since it uses tissue transparency (iDisco) for morphological characterization, the injection of viral vectors for the expression of calcium sensors, the recording of intracellular calcium in anesthetized and head fixed miniscope recordings, the expression of opsins for the control of somatotroph cell activity, as well as the monitoring of hormonal secretion by ELISA [154]. This represents a significant advance in the generation of tools that allow not only observation of cellular activity *in situ*, but to do this under particular conditions and to perform longitudinal studies of events of different temporality, for example, short-duration processes (such as calcium signals that control hormonal exocytosis) or others of slow evolution, such as tissue remodeling in health and disease over a period of days to weeks.

It is clear that the magnitude of hormonal pulses in the systemic circulation is not just the product of the simple sum of individual endocrine activity, but of cellular interactions in the tissue as a whole. Thus, the development of powerful tools such as the miniscope encourages the exploration of new facets of the behavior of these neuroendocrine cellular networks under conditions in which the anatomical-functional environment of the tissue is preserved, from tissue slices to the pituitary of anesthetized or freely moving animals.

Study of Hypothalamic-Pituitary Axis Function by Imaging

The concept of neurosecretion was originally based on observations made with light microscopy and later reaffirmed with histological staining and EM [155]. Great ef-

forts have been made to record and manipulate the action of hypothalamic neurons, and thus to understand their impact on hormone secretion. The stereotaxic placement of fine electrodes for electrical stimulation in various parts of the hypothalamus showed that it was possible to modulate, stimulate or inhibit, the secretions of practically every pituitary hormone [36]. However, it will always be preferable to study cells *in vivo* and be able to see their activity, since many cell networks, factors, and other anatomical-functional conditions are essential. Recent studies seek to develop *in vitro* strategies that allow the reconstruction of a controlled microenvironment that most closely resembles reality with organs-on-a-chip, organoids, and tissue engineering [156]; alternatively, there is a stream of analysis that seeks to advance *ex vivo* and *in vivo* strategies. Campos et al. [157] published an extensive review of the new techniques used to study the hypothalamus.

Two of the great inventions that have revolutionized modern life are fiber optics and lasers (Light Amplification by Stimulated Emission of Radiation). Both are based on the discoveries and observations of scientists, mainly physicists, made over two centuries [158] until the development of solid-state lasers [159], and led to the recent development of optogenetics and fiber photometry [160]. These techniques have been used to examine the activity of neuronal populations in freely moving animals [161]. Thus, using fiber photometry and the GCaMP6 calcium sensor in free-moving mice, it was shown that arcuate nucleus kisspeptin (ARNKISS) neurons have episodes of synchronous activity with a frequency that can vary between 43 and 347 min [162], and these periods are shortened and became closer in castrated animals [163]. Moreover, the expression of designer receptors exclusively activated by designer drugs into hypothalamic astrocytes surrounding GnRH neurons has demonstrated that their activation increased GnRH neurons electrical activity augmenting LH levels *in vivo*, without affecting ARNKISS [164].

To control neuronal activity, the expression of algal opsins (photosensitive membrane proteins) has been used in mammalian cells [165, 166]. Examples are channelrhodopsins, which is a blue light-activated ion channel (480 nm) that generates depolarization at the plasma membrane, and halorhodopsin, which is a red light-activated chloride pump (570 nm) [167]. This technique has been combined with the recording of Ca^{2+} population activity with fiber optics in freely moving animals to demonstrate the role of ARNKISS neurons in the regulation of GnRH and LH secretion [163], demonstrating for the

first time directly that activation of ARNKISS neurons for 1 min generates LH pulses in the blood circulation in free-behaving mice, and their inhibition for 30 min suppresses it [163].

The generation of hormonal pulses requires not only meeting the oxygen and nutrient demands of thousands of active endocrine cells but also the precise input of stimuli from the hypothalamus and peripheral circulation and the coordinated release of hormones into the bloodstream. We are beginning to understand how this is achieved; endocrine cell activity has to be temporarily correlated with blood flow. To date, a considerable number of studies have been conducted to understand how hormonal secretion is regulated and based on this how it can be modified/restored to prevent or reverse common hormonal disorders. However, little is known of how this process occurs dynamically *in vivo*.

The development of new *in vivo* approaches to measure local blood flow has made it possible to quantify the velocity of blood flow [56], the oxygen partial pressure, and the cellular activity with single-cell resolution and secretion in the mouse pituitary. GHRH injection has been shown to stimulate somatotrophic network activity and GH secretion, with a temporal association with increased blood flow rate and oxygen supply [56]. This study has been a milestone for the knowledge of the hypothalamic-pituitary function and is the product of the integration of many knowledge and techniques developed so far.

New Insights

Scientific questions are complex, increasingly; all sciences will be required to engage in an interdisciplinary scope to develop conceptual and experimental strategies that allow us to understand systems as complex and vast as the neuroendocrine. The answers to the biological and clinical questions that lie ahead must necessarily start from the search for new ideas in physics, chemistry, mathematics, and biology. In this sense, the development of new *in vitro* culture techniques, and the interest of physicists, mathematicians, and biologists in understanding and modeling cellular interactions with the appropriate microenvironment, will allow the development of new organ-on-chip [168] or organoid culture [169] techniques, conditions that may allow simulation of cellular and physiological conditions in a more realistic way. The results obtained so far are encouraging [94, 170], but further developments will be necessary to include factors as

important as blood flow, incorporating new advances in microfluidics and microfabrication [171]. With these modifications, it will be possible to evaluate controlled stimulation-secretion experiments.

Concerning to the anatomical and morphological description, it is not difficult to think that in the coming years we will be able to make 3D reconstructions in which the anatomical-morphological relationships of the tissue in a given context can be observed instantly and reliably and that it will be possible to compare them with any change that may occur in it. Substantial progress has been made; examples of this are the advances in instrumentation and visualization methods that allow increasing the resolution in all optical-axes such as light-sheet microscopy [172], as well as methods of tissue treatment that match the refractive index of cells with the medium, making it transparent [173].

Undoubtedly, the advancement of the miniscope [150], the two-photon miniscope [174], and optogenetics [160], as well as their combination [175] and the development of 3D cell activity recordings in awake mice and in adult brain slices [176], will allow progress in the knowledge of cell activity and its control in conscious animals and deeper understanding of neuroendocrinology.

Over the years, the field of neuroendocrinology has undergone relevant paradigm shifts. The problem posed by the neuroendocrine system, like any complex system given the nature and organization of its constituent elements, can hardly be approached from the perspective of a single discipline. Important advances in the physical, chemical, and mathematical sciences have allowed the development of tools and techniques such as microscopy to explore, from the molecular and cellular level to the interactions between diverse systems, and to understand individual and collective functioning in a truly common

trans- or interdisciplinary effort that has allowed scientific progress in general in recent decades. Neuroendocrinology is, therefore, a fertile field of research in which multidisciplinary and interdisciplinary research is favored, and to a large extent of a translational nature, thus narrowing the bridge between basic research and the resolution of problems of high impact on health and society.

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Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Tatiana Fiordelisio was responsible for the conception and design of the general line of the work, while Edgar Jiménez-Díaz and Diana Del-Rio contributed to the literature search and significant knowledge input. All authors participated in the drafting of the manuscript, critical revision of the intellectual content, and final approval of the version to be published.

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