Overview Article

Cell Networks in Endocrine/Neuroendocrine Gland Function

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Running head: endocrine cell network-driven hormone secretion

Abstract

Reproduction, growth, stress and metabolism control are determined by endocrine/neuroendocrine systems that regulate circulating hormone concentrations. All these systems generate rhythms and changes in hormonal pulsatility observed in a variety of pathophysiological states. Thus, the output of endocrine/neuroendocrine systems must be regulated within a narrow window of effective hormone concentrations but must also maintain a capacity for plasticity to respond to changing physiological demands. Remarkably most endocrinologists still have a 'textbook' view of endocrine gland organization which had emanated from 20th century histological studies on thin 2D tissue sections. However, 21st century technological advances which include in-depth 3D imaging of specific cell types have vastly changed our knowledge. We now know that various levels of multi-cellular organisation can be found across different glands, that organisational motifs can vary between species and can be modified to enhance or decrease hormonal release in order to maintain homeostasis and respond to altered physiological status. This review focuses on how the organisation of cells regulates hormone output using three endocrine/neuroendocrine glands that present different levels of organisation and complexity: the adrenal medulla, with a single neuroendocrine cell type; the anterior pituitary, with multiple intermingled cell types; and the pancreas with multiple intermingled cell types organised into distinct functional units. We give an overview of recent methodologies that allow the study of the different components within endocrine systems, and particularly their temporal and spatial relationships. We believe the emerging findings about network organization, and its impact on hormonal secretion, are crucial to understanding how homeostatic regulation of endocrine axes is carried out within the output endocrine organs themselves, and will lead to advances in new differential diagnosis and management of hormone defects.

Didactic Synopsis

Major teaching points:

1. The rate of secretion of a hormone, the principal determinant of its circulating concentration, must be maintained within a range and with a pattern that ensures effective regulation of target tissues, with implications for health throughout life.

2. Hormone concentrations must remain within a tight window in order to be effective but must also maintain a capacity for plasticity to respond to changing physiological demands.

3. Endocrine glands were long thought to be a heterogeneous arrangement of cells, but we now know they possess organisational network motifs with different level of complexity.

4. Technical development (such as improvement in imaging techniques, ability to study entire glands, *in vivo* experimentation) have revealed the impact of the organisation of cells and networks on hormone secretion.

5. Endocrine glands and particularly pituitary networks can modify their organisation and store long-term memories of increased output and enhance function on repeated challenge.

6. Understanding the importance of endocrine gland cell organisation on the regulation of circulating hormone concentrations provides new understanding of a range of endocrine axes defects and targets for novel therapies.

Didactic legends

Figure 1: *Teaching points*: The adrenal medullary tissue, which is responsible for the secretion of catecholamines (epinephrine and norepinephrine), is mainly composed of chromaffin cells. These are organized in lobules and each lobule is delineated by a basal lamina. The mammalian anterior pituitary is an organised endocrine gland. It shows some large-scale organization where specific cell types occupy specific regions of the gland as well as distinct morphological relationships between different cell types. At a smaller scale, motif organization and changes in these motifs can be observed for several cell types (particularly somatotrophs and lactotrophs). The pancreas houses upwards of a million islets in humans. Each islet is comprised of multiple endorince cell types involved in the regulation of blood glucose levels.

Figure 2. *Teaching points:* In order to study secretory cells *in situ*, it is necessary to work on multicellular preparations (acute slices, organotypic cultures) that maintain the tissular organization of the secretory gland. For acute slice preparation, the pituitary gland is quickly removed and cut into thin sections using a vibrating microtome blade. Pituitary cells are then imaged using a 2-photon microscope to sequentially visualise several layers of fluorescent cells within a slice. The images collected within each slice can be assembled to obtain a 3D reconstruction of fluorescent cells in the pituitary tissue. Pituitary gland function and associated hormone secretion can also be studied in organotypic cultures. Pituitary explants are cultured for several weeks, thus making possible the examination of long-lasting regulatory mechanisms. Organotypic co-cultures of a tissue and its target (the spinal cord and the adrenal medulla for example) is also possible, offering the opportunity to investigate additional steps of regulation.

Figure 3. ***Teaching points:*** Endocrine/neuroendocrine glands can be studied as a whole, in *ex vivo* preparations. Islets of Langerhans can be isolated from mice and humans by injecting collagenase into the bile duct (clamped at the intestinal ampulla). Following digestion, the islets can be subjected to functional assessment, including for insulin secretion. For the adrenal glands, multi organ preparation including the spinal cord + the splanchnic nerve + the adrenal gland allows investigation of the spatial and activity-dependent release of catecholamines. Regarding the pituitary tissue, intact connections between the pituitary gland and the brain can be preserved from *ex vivo* tissue preparations in the fish.

Figure 4. ***Teaching points:*** *In vivo* monitoring of the activity of endocrine/neuroendocrine cells is possible in fluorescent protein-tagged transgenic mice with long-range microscopy. In terminally anesthetized mice, the ventral side of the pituitary gland can be exposed after removal of the palate bone, allowing study of the activity of pituitary cells expressing calcium indicators. Alternatively, calcium imaging in head-fixed animals is now possible using GRIN lenses which are thin needle-like lenses that can be chronically implanted in the brain and provide optical access to pituitary cells expressing activity indicators. In anaesthetized mice, a microsurgical approach allows combined monitoring of splanchnic nerve stimulation and catecholamine secretion in the adrenal venous blood. The surgery starts with a laparotomy to uncover the left adrenal gland. The renal vein and the conjunctive tissue containing the splanchnic nerve are isolated and the splanchnic nerve is placed on a bipolar stimulation electrode. The renal vein is ligated on both the kidney and cava vein sides and then cannulated with a heparinised catheter, to form a reservoir collecting the blood flowing from the left adrenal. A glass microelectrode is introduced in the adrenal medullary tissue to simultaneously record electrical events occuring in response to splanchnic nerve stimulation.

Figure 5. ***Teaching points:*** Monitoring hormone secretion is required in the study of endocrine/neuroendocrine cell function. At the single cell level, the secretory events can be monitored by real-time amperometry, a technique based on the detection of oxidized molecules at the surface of an electrode. In electrophysiology, amperometry is used to study release events using a carbon fibre electrode, which is brought in a very close vicinity of the cell. Upon vesicle release, under basal or stimulated conditions (nicotinic cholinergic stimulation in adrenal chromaffin cells for example), the redox reaction of catecholamines induces currents that are measured at the surface of the electrode. The amplitude, the duration and the frequency of currents are valuable parameters to estimate the amounts of hormone secreted. Time-lapse tracking of fluorescent secretory granules can also be used to assess real-time hormone release in an individual cell. In response to a stimulus, the fluorescence within the cell quickly decreases, reflecting granule exocytosis and subsequent hormone release. Note that high-resolution imaging techniques are required to reliably follow the exocytotic events.

Figure 6. ***Teaching points:*** The measure of capacitance, an electrophysiological parameter reflecting cell surface, is another technique allowing real-time monitoring of exocytosis in a single cell. The fusion of a secretory granule with the plasma membrane increases the cell membrane surface, and therefore the capacitance of the cell. This experimental approach allows, for example, investigation of exocytosis in response to a Ca^{2+} entry through voltage-gated Ca^{2+} channels, or through ligand-gated channels, as well as in response to secretagogue-evoked intracellular Ca^{2+} oscillations.

Figure 7. ***Teaching points:*** Real-time imaging of anesthetised rodents with long working-distance objectives and dedicated microsurgery has allowed visualisation of the fate of

fluorescently-labelled dextrans that mimic the size of hormones as well as fluorescent metabolites.

Figure 8. ***Teaching points:*** Light stimulation is a non-invasive process and provides a spatio-temporal control of channel activation, and therefore Ca^{2+} entry into the cell. By manipulating cytosolic Ca^{2+} concentration, one can manipulate hormone exocytosis. In the pituitary gland, optogenetic stimulation can be carried out in terminally anesthetized mice using an optical fibre acutely placed above the pituitary gland and connected to a laser source. Alternatively, it can be carried out in awake mouse in which an optical fibre has previously been chronically implanted directly above the target cells. Pituitary cells that have been activated can secrete hormones and this can be measured using frequent blood sampling. In the pancreatic islets, photopharmacology relies on the light-sensitive properties of the azobenzenes. When these structures are incorporated into a drug, they allow isomerisation in response to illumination. The changes in molecular conformation alter how the drug acts at its target site and has been used to control glucose homeostasis *via* insulin release. In the adrenal gland, several photopharmacological/chemical optogenetic approaches have been successfully used to modulate both the pattern of electrical activity and the secretory function of chromaffin cells. One of theses techniques uses photoactivable nanoswitches that allow a bi-directional control of cell electrical firing and associated catecholamine secretion.

Figure 9. ***Teaching points:*** The adrenal glands are innervated by neurons originating from the thoracic spinal cord and forming the splanchnic nerve. About 90% of this innervation is monosynaptic, that is without relay between the spinal cord and the adrenal target. After crossing the adrenal cortex, the innervation invades and distributes throughout the medulla. In

the medullary tissue, chromaffin cells are organized in lobules, which are delineated by a basal lamina.

Figure 10. ***Teaching points:*** Within a lobule, each chromaffin cell receives a synaptic innervation. At the splanchic nerve-chromaffin cell synapse, acetylcholine is the primary neurotransmitter. Chromaffin cells are also coupled by gap junctions, an intercellular communication route that mediates electrical coupling and diffusion of messengers between cells. Both cholinergic neurotransmission and gap junctional communication are involved in catecholamine secretion by chromaffin cells.

Figure 11. ***Teaching points:*** The building blocks of gap junctions are connexins (Cxs). Each connexin is identified by its molecular weight (in kDa), such as Cx36. Rodent chromaffin cells express Cx36 and Cx43. The presence of Cxs can be revealed by specific antibodies and immunoreactive Cxs form a punctate labelling. In the mouse adrenal tissue, Cx36 is expressed only in the medulla, not in the cortex. Gap junctions are permeant to small molecules (with a molecular weight <1kDa) and their activity can be observed by fluorescent tracers such as Lucifer yellow. After loading into a single cell, the dye passively diffuses to adjacent cells through gap junction channels, resulting in a widespread staining.

Figure 12. ***Teaching points:*** Gap junction-mediated electrical coupling implies the diffusion of ions between coupled cells. The resulting junctional current can be recorded using the dual patch-clamp electrophysiological technique. Each cell of a pair is patch-clampled by a glass electrode introduced into the plasma membrane. One cell is stimulated (by current (or voltage) injection and the resulting changes in membrane potential (or current) is monitored in the two cells. An electrical coupling is present when potential (or current) changes are found in the

non-stimulated cell. The amplitude of potential (or current) changes reflects the strength of the electrical coupling, which varies between cell pairs, ranging from weak (small amplitude changes) to robust (large amplitude changes) coupling.

Figure 13. ***Teaching points:*** Rat chromaffin cells express gap junctions and their physiological relevance is linked with their ability to propagate electrical signals and subsequent rises in intracellular calcium concentration ($[Ca^{2+}]i$) in response to a stimulus applied on a single cell (electrical stimulation or cholinergic agonists such as nicotine or acetylcholine, or other neurotransmitters/neuropeptides targeting chromaffin cells). The quasi-simultaneous responses of cells are a robust indicator of a contribution of gap junctions in the cell co-activation process.

Figure 14. ***Teaching points:*** In endocrine/neuroendocrine cells, an increase in $[Ca^{2+}]i$ is a prerequisite for hormone exocytosis, and by conducting simultaneous $[Ca^{2+}]i$ increases in coupled cells, gap junctions contribute to hormone secretion. Catecholamine exocytosis can be monitored by recording amperometric signals araising from chromaffin cells. The principle of this technique is based on measuring the current generated by the oxydo-reduction reaction of catecholamines at the surface of an electrode, whose potential is clamped at the value of the redox potential of catecholamines (650 to 800 mV). Under these experimental conditions, catecholamine exocytosis is visualized through outwardly directed deflections of the electrode current. Applied to chromaffin cells in tissue slices, this technique has allowed demonstration of a $[Ca^{2+}]i$ increase that is evoked in a cell in response to the stimulation of another cell, resulting in a trigger of catecholamine secretion in the non-stimulated cell. At a multicellular/tissue scale, this shows the crucial role of gap junctions in mediating coordination of exocytosis signals and more generally the secretory response of the entire gland.

Figure 15. ***Teaching points:*** The gap junctional communication between chromaffin cells is remodelled in response to stress episodes. To cope with stressful situations, chromaffin cells produce a huge catecholamine release. Many components of the stimulus-secretion coupling are involved, including gap junctions. In stressed rodents (cold exposure at 4°C for 5 days), Cxs expression in the adrenal medullary tissue is enhanced. The subsequent blockade of gap junctions reduces the ability of chromaffin cells to secrete, thus showing the importance of this adaptive remodeling for catecholamine secretion *in vivo*.

Figure 16. ***Teaching points:*** To appropriately respond to an organism's needs and demands, the adrenal medullary tissue needs to continuously adapt its secretory function, both in physiological and pathological conditions. Each component of the stimulus-secretion coupling (chromaffin cell excitability, cholinergic/peptidergic neurotransmission, gap junctional coupling) individually represent a lever to regulate hormone release. In addition, the combination of each component offers multiple regulatory possibilities.

Figure 17. ***Teaching points:*** The development of transgenic mice in which a pituitary cell type is fluorescent (as illustrated here with a GH-EGFP mouse) allows a new level of understanding of cell organization within the whole tissue (from embryonic to adult tissues). It becomes possible to identify and locate the 3D position of every identified GH cell (or another secretory cell type) in the pituitary gland. Regarding GH-secreting cells, 3D imaging shows organized strands of GH-EGFP cells at embryonic day E15.5. Then, a unique continuum of contacting GH cells forms within the parenchyma within one day, at E16.5, and extends until adulthood to *in fine* set up a geometrically connected homotypic network of cells.

Figure 18. ***Teaching points:*** Pituitary cells form homotypic networks with distinct organizational motifs and relationships with the vasculature, which alter with physiological status. In the adult pituitary, lactotrophs form a network of honeycomb motifs that enable the congregation of cells along the fine pituitary capillary network while somatotrophs are aligned with or surrounded by capillaries.

Figure 19. ***Teaching points:*** In cultures of pituitary slices with intact cell organisation both somatotrophs and lactotrophs show network-driven coordination of cell activity leading to an enhanced output in response to stimulation. In this example, GHRH stimulation of GH cells produces recurrent functional motifs between cells which are linked to changes in cell calcium activities.

Figure 20. ***Teaching points:*** Lactotrophs are organized into a three-dimensional network. Structural connectivity is low in nulliparas but increases in primiparas during lactation when the prolactin demand is higher. Following weaning, connectivity is maintained despite cessation of stimulus. The calcium activity of lactotrophs cells is spontaneous in nullipara lactotrophs but is highly increased in primiparas before returning to basal levels after weaning. The functional connectivity is at its highest during lactation and remains high after weaning.

Figure 21. ***Teaching points:*** The organisation of cell networks relative to the vasculature and the dynamics of capillary blood flow influences the delivery of both nutrients and hypothalamic regulatory factors, as well as the clearance of secreted hormone. *In vivo* imaging experiments in mice have shown that when secretagogues are delivered to the pituitary, the timing and dynamic of exposure and the oxygen required vary to meet the energetic demands of secretion.

INTRODUCTION 14
ORGANISATION OF ENDOCRINE CELLS AND TISSUE 16
METHODOLOGY FOR STUDY OF NETWORKS IN ENDOCRINE CELL AND ORGAN FUNCTION 18
ANALYSIS AT MULTIPLE SCALES, FROM ISOLATED CELLS TO WHOLE ORGAN *IN VIVO* 19
Isolated cells *19*
Cell reaggregates *20*
Tissue slices, organotypic cultures and organoids *20*
Whole organ ex vivo *21*
Whole organ in vivo *22*
ANALYSIS OF INDIVIDUAL CELL FUNCTION 23
Monitoring hormone release *24*
Monitoring stages of stimulus-secretion coupling *28*
Monitoring intracellular signalling *30*
Monitoring gene expression *30*
ANALYSIS OF CELL POPULATIONS 31
Static incubation *32*
Perifusion systems *33*
Luciferase constructs *33*
Monitoring dye diffusion/transport *33*
Genetically modified animals *34*
Virus transduction *38*
In vivo microscopy/optogenetics/DREADDS *39*
HOMOTYPIC CELL MODEL: THE ADRENAL MEDULLARY TISSUE 42
ANATOMY AND DEVELOPMENT 42
INNERVATION 45
VASCULARIZATION 47
THE CHROMAFFIN CELL: THE NEUROSECRETORY UNIT OF THE STIMULUS-SECRETION COUPLING 48
CHROMAFFIN CELL NETWORK PLASTICITY 49
Gap junctions between chromaffin cells: a contribution to catecholamine release *49*
Synaptic transmission and gap junctional coupling between chromaffin cell: a concerted crosstalk to continuously adapt catecholamine release to the need of the organism *50*
HETEROTYPIC CELL MODEL: THE ANTERIOR PITUITARY GLAND 52
ANATOMY AND DEVELOPMENT 52
POSTNATAL DYNAMICS OF CELL POPULATIONS 53
PLASTICITY 55
HOMOTYPIC CELL ORGANISATION 55
Somatotroph cells *55*
Lactotroph cells *57*
Other anterior pituitary cells *57*
HETEROTYPIC CELL ORGANISATION 58
ORGANISATION RELATIVE TO THE VASCULATURE 59
FUNCTIONAL ANALYSIS 60
Memory and plasticity *61*
Pituitary blood flow, stimulus delivery and output removal *62*
MULTI-UNIT, HETEROTYPIC CELL MODEL: THE PANCREATIC GLAND 62
ANATOMY AND DEVELOPMENT 62
GLUCOSE-STIMULATED INSULIN SECRETION 64
SYNCYTIAL BETA CELL FUNCTION 65
ISLET-ISLET CROSSTALK 68
ISLET COMPENSATION 68
CONCLUSION AND PERSPECTIVE: STUDYING ENDOCRINE CELL NETWORKS IN THE 21ST CENTURY 69

REFERENCES **75**
FIGURE LEGENDS **130**

Introduction

Regulation of circulating hormone concentration is an important feature of endocrine/neuroendocrine systems, with implications for health throughout life. Remarkable examples of the importance of this regulation are well-documented in humans, where clinical conditions leading to both deficiency and excess of pituitary growth hormone (GH) and hypothalamic-pituitary-adrenal (HPA) axis activity lead to an increased risk of mortality (375). Although mechanisms occur for modifying or gating hormone bioavailability and regulating levels by variable clearance rates (131, 263), the principal determinant of a hormone's concentration in the circulation is its rate of secretion. The regulation of target-tissue function is then dependent on the circulating concentration of bioavailable hormone up to a ceiling where hormone resistance occurs as a result of receptor saturation, downregulation through constant activation or adaption of intracellular signalling. Whilst this demonstrates that the output of endocrine/neuroendocrine systems must be regulated within a tight window of effective hormone concentrations, they must also maintain a capacity for plasticity to respond to changing physiological demand. Thus, study of the regulation of hormone output is fundamental to the understanding of both normal physiology and pathophysiology, with implications for clinical management.

An understanding of hormone output regulation clearly requires identification of the interacting components of the systems, however, this must also include analysis of temporal and spatial relationships. Pulsatile, oscillatory output is an important feature of a majority of endocrine systems, occuring at multiple scales, ranging from circannual to circadian and ultradian. This pulsatile secretion can overcome the resistance that would occur with constant high levels of hormone but also allows an encoding of hormone action by a combination of amplitude and frequency (436). A further component of these oscillatory patterns is the orderliness, or sub-pattern consistency, of oscillatory behaviour, reflecting feedback and/or

346 feedforward interactions within an axis (427), which is altered in specific physiological states,
347 such as puberty and aging (426). The significance of patterned secretion for normal endocrine
348 axes function has been shown by increased effectiveness of episodic compared with continuous
349 stimulation in systems with a range of pulse frequencies from minutes in the case of insulin
350 (369) to hours for GH (145). A more recent study has also suggested that orderliness can alter
351 the effect of pulsatile GH, with variable daily doses of GH being more effective than fixed
352 daily doses at stimulating growth in dwarf rats (445). Thus, measurement of the dynamics of
353 hormone output is important in endocrine studies, which must also be assessed during the
354 dramatic changes occurring in response to altered physiological demand. These also occur with
355 varying time scales and frequencies, examples of which are the single but sustained increase
356 in GH at puberty (344), the increase in prolactin (PRL) during lactation, which recurs at each
357 pregnancy (and this with a varying frequency) (150) and the rhythm of gonadotrophin secretion
358 during the reproductive cycle (132).

359 This review is focused on the role of the organisation of cells into networks within
360 endocrine/neuroendocrine glands in the regulation of hormone output and how this relates to
361 changes in secretion in response to altered physiological demands. In earlier studies of
362 endocrine systems, a contribution of cell organisation to regulation was suspected, but the
363 underlying mechanisms were difficult to fully appreciate without the technological advances
364 at our disposal today (imaging, transcriptomics, mass cytometry, ...). Indeed, stimulation,
365 stimulation of cultured dispersed cells results in a severe reduction in secretion compared with
366 the intact endocrine gland, for example, pituitary GH (360, 409) and PRL (182) or pancreatic
367 insulin (198). Furthermore, study of endocrine tissue *ex vivo*, whether in cultured cells or tissue
368 slices, cannot replicate the patterned delivery of regulating factors, clearance of secreted
369 products from the gland or the multiple levels of control from peripheral tissues. Recent studies
370 have focused on the use of techniques which allow the study of endocrine cell function in intact

tissues *ex vivo* and, increasingly, *in vivo*. In this review we will describe the role of cell organisation in the regulation of hormone output, using three endocrine/neuroendocrine glands with organisation at differing scales as exemplars: the adrenal medulla, with a single neuroendocrine cell type; the anterior pituitary, with multiple intermingled cell types; and the pancreas with multiple intermingled cell types organised into distinct functional islets.

Organisation of endocrine cells and tissue

In the past, endocrine organs such as the pituitary were considered to be a heterogeneous arrangement of cells, as described by Feyrter (70). This was largely due to the lack of apparent organisation in thin tissue sections, suggesting that studies of isolated and purified cells would represent their activity *in situ* and that the pattern of output resulted from a summation of individual cell secretion, which in turn was a simple reflection of external regulation (318). Endocrine/neuroendocrine organs have a range of complexities, from those which have a defined structure containing a single hormonal cell type, such as the parathyroid glands, those with several hormonal cell types organised into discrete functional units, such as the pancreas, and those with little obvious organisation, such as the gastrointestinal tract. The concept that these varying levels of organisation may have a role in the function of the gland, rather than simply being a consequence of the developmental patterning leading to their formation in the embryo, is supported by several evolutionary differences in this organisation. The three examples we have chosen in this review illustrate the differential complexity of organisation between endocrine/neuroendocrine glands.

In the adrenal medulla, neurosecretory chromaffin cells (E- and NE-secreting) are clustered and arranged in lobules (Figure 1A). The organisation of chromaffin cells in lobules is present before birth in both the rat (428) and mouse (194). No significant changes (related to gender, physiological/pathological conditions, aging, etc…) in the lobular organisation have

been reported until now. Depending on the species, E- and NE-containing chromaffin cells exhibit distinct localisations throughout the chromaffin tissue. In the hamster, NE-secreting cells preferentially segregate at the periphery of the medulla (152), while in the rat, E and NE cells are more randomly oriented (121).

The mammalian anterior pituitary presents some large-scale organization where lactotrophs occupy the rostral and caudal regions of the gland, and somatotrophs are predominantly localized to the anterolateral wings (361) (Figure 1B). Preferential morphological relationships can also be found between different cell types. For example, somatotrophs are closely associated with corticotrophs, thyrotrophs with somatotrophs, and gonadotrophs with lactotrophs (280, 288). At a smaller scale, motif organization and changes in these motifs have been reported. At the time of puberty, GH cells form clusters that disappear later in life (41), while lactotrophs arrange into honeycomb structures that intensify during lactation (180). These morphological features and their physiological roles will be discussed in this chapter. Within mammals there are species variations in the organization and ontogeny of different cell types, with lactotrophs concentrated near the intermediate lobe of the horse and organized in clusters (417), and monohormonal gonadotrophs being present in the developing human but not in the murine pituitary (320). On the other hand, fish pituitaries have a very distinct organisation with the different pituitary cell types organized in zones (452), and lactotrophs localized in the rostral and somatotrophs in the distal pars distalis.

Islets are diffusely scattered throughout the pancreatic parenchyma, with no apparent large-scale organization other than a denser aggregation near the gastric and tail regions in mice and humans, respectively (183, 437). Strikingly, measured across species, the maximum diameter of islets is 500-800 μm (*i.e.* whales have similar sized islets to mice (384)). However, the cell composition and arrangement of islets (Figure 1C) differs between species: for example, humans possess more alpha cells (~ 40 vs 20% in proportion) than rodents, which

instead of being present at the islet mantle, are randomly distributed. This, together with differences in gap junction coupling/signalling and paracrine relationships, may account for the different patterns of beta cell activity in human compared to mouse islets, the former displaying more stochastic responses to glucose albeit with regional coordination (442). Moreover, innervation patterns are different between the species, with humans being more reliant on acetylcholine (ACh) secreted from alpha cells for parasympathetic suppression of beta cell activity, whereas rodents possess neuronal inputs (272, 334). Finally, the complement of ion channels at the cell surface is also different between the species, for example L-type Ca^{2+} channels being the predominant source of Ca^{2+} fluxes in rodents, whereas humans require P/Q-type Ca^{2+} channels (47, 341).

Methodology for study of networks in endocrine cell and organ function

The output of an endocrine gland in response to stimulation will depend on several factors, including the number of responding cells, the amount of readily-releasable hormone, the level of cell response and the physical interaction between the cells and the vasculature, both for delivery of stimulus and transport of secreted factors from the gland. Since secretagogue stimulation can lead to modification of one, some or all of these components over multiple time-scales depending on age and physiological status, study of the role of network organisation in their regulation requires methodology to assess each of these components, specifically to determine:

- Output responses (gene expression, protein dynamics, secretion) of cells to stimulation at both individual and population levels
- Signalling pathway response of both isolated and organised cells to secretagogue stimulation

- Identification of individual cell types, their 3-D organisation, developmental origin and organisational dynamics
- The relationship of endocrine cells with the vasculature and monitoring of the dynamics of secretagogue delivery and removal of hormone

Whilst each of the three endocrine glands that we have chosen as exemplars for this review have distinct challenges in the methodology required for their study, below we describe those specialised methodologies which are fundamental to the study of endocrine gland function, as well as their limitations. To fully understand the function of endocrine systems requires study at various levels, from primary cultures of single cells *in vitro* to the intact gland *in vivo*.

Analysis at multiple scales, from isolated cells to whole organ *in vivo*

Addressing the role of network organisation in endocrine/neuroendocrine gland function requires an understanding of response to stimulation of isolated cells, cells *in situ* in slice culture and ultimately to the *in vivo* response of the intact gland. Each of these scales have advantages and limitations since they reflect interactive complexity of a tissue within its environment.

Isolated cells

For many studies of isolated cells, stable cell lines, primarily derived from rodent tumours have provided a valuable research tool. However, as the transformation process results in a wide range of changes to normal gene expression and cell behaviour (immortalised pituitary cell lines, for example, rapidly divide, unlike those in a normal adult tissue (233)), the relevance of their study to that of normal endocrine tissue is limited. They remain a valid tool for studies, however, particularly as they provide a homogenous cell population. Primary cells more closely reflect the behaviour of cells within their normal tissue environment, although the process of enzymatic dispersion is likely to remove membrane receptors and alter other aspects

of normal cell function. Since culture conditions can be readily controlled, factors such as paracrine interactions can be studied by alteration of perifusion and culture conditions. They also have the advantage of allowing isolation of homotypic cell types and their analysis, which would become complicated when mixed cell populations are used. For example, the secretion of IL-6 by different pituitary cell types could be demonstrated after their purification by Percoll gradient centrifugation (323) and the role of paracrine signalling by gonadotroph angiotensin II in GnRH stimulation of prolactin release was possible by separation of gonadotroph and lactotroph cell populations (95). In the past decades, new genetic tools have allowed the identification and study of specific cell types within primary pituitary cultures. For example, lentiviral constructs bearing the expression of calcium indicators under the control of a POMC promoter have been used to identify corticotophs and study their response to secretagogues using calcium activity monitoring (337).

Cell reaggregates

Whilst isolated cells have proven invaluable for studies of many aspects of endocrine cell function, they lack the cell-cell contact and organisation of the normal tissue architecture. Aspects of this limitation can be overcome by using cell reaggregation (97), especially since the distribution of endocrine cell types in the reaggregate reflects aspects of that in the intact gland, as well as recreating the morphological relationship between different endocrine cell types (10). Since the proportion of different endocrine cell types in the reaggreagates can be altered, the importance of interaction between different cell types and the actions of paracrine factors can be analysed with these cultures (424), particularly when compared with the function of dispersed isolated cells.

Tissue slices, organotypic cultures and organoids

The cellular organisation and interaction that can be analysed in cell reaggregates may partially recreate the normal endocrine tissue architecture but it is likely that these will fail to

recapitulate specific aspects, particularly the network relationships that are dependent on physiological status. see R1's comment (minor) This limitation can be overcome by the use of acute slice culture, where thick vibratome sections are cut after tissue is embedded in agarose, preserving tissue viability and structure, allowing analysis of tissue function for a period of hours (31, 125, 249) (Figure 2A). Since slice preparations require minimal manipulation of the tissue and analysis takes place within hours of gland removal, the effects of physiological status are mostly preserved. An alternative approach which also maintains the architectural organisation of native tissues and with the additional advantage to be suitable for long-lasting manipulations (pharmacological treatments, viral transduction, etc) is organotypic culture of endocrine/neuroendocrine tissues and organs (161, 222, 248) (figure 2B). Organotypic cultures are also suitable for establishing long-term co-cultures. Two different culture protocols, the roller tube technique developed by Gähwiler (140) and the culture on transparent and porous membrane developed by Stoppini and colleagues (391) were successfully applied to hypothalamus-pituitary co-cultures from rodents (25) or from fish (40). Long-term cultured pancreatic tissue slices have also been described where potentially important interactions with exocrine tissue are preserved (250, 260). Co-cultures of rodent thoracic spinal cord explants with hemi-sectioned adrenal glands have also been successfully trialed (Guérineau, unpublished data, Figure 2C). Very recently, organoids with recapitulation of 3-dimensional structure from fetal human adrenal gland (319) and adult mouse pituitary (87) have been developed, enabling the exploration of the physiological/pathological mechanisms underlying tissue remodeling and differentiation.

Whole organ ex vivo

The size of many endocrine glands precludes their viable use for studies of the intact gland *ex vivo*, however, for tissues such as the pancreas, the small size and number of islets (typically a few hundred microns; approximately 200 can be isolated from C57BL/6 mice) allows their

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culture and useful analysis. Following isolation, using collagenase digestion and gradient separation techniques, islets can be maintained for a number of weeks in culture and subjected to functional challenge, including static incubation and perifusion assays for hormone release (123, 235) (Figure 3A). Whilst sympathetic and parasympathetic neurons tend to die off in islets within 24 hrs (Hodson DJ, unpublished observation), making their analysis difficult, the ability to culture over prolonged periods allows the transduction of recombinant probes, *e.g.* for imaging of cAMP, Ca^{2+} and other important second messengers (179, 181, 377). An extensive islet transplantation programme exists and human islets for research can be shipped and cultured analogously to those from rodents, facilitating translational findings (245, 281). Regarding adrenal medullary tissue, an elegant *ex vivo* spinal-splanchnic-adrenal preparation has been recently developed in the rat, allowing addressing the spatial and activity-dependent release of catecholamines (451) (Figure 3B). Studying pituitary gland function by the use of whole organ preparation is currently a topical challenge. In mammals, both the location of the gland at the base of the posterior brain and the fragile blood and nerve connections with the hypothalamus precludes safely removal of the whole gland without cutting the connections with the brain. Intact connections between the pituitary gland and the brain can be preserved from *ex vivo* tissue preparations in the fish, as illustrated in figure 3Ca with the use of a hypothalamus-pituitary slices to monitor dye coupling between pituitary cells (232) and in figure 3Cb describing the use of a whole brain-pituitary preparation to investigate the role of the multisynaptic neuronal inputs as regulatory mechanisms of LH and FSH release (205).

Whole organ in vivo

Clearly, analysing the function of endocrine tissue *ex vivo* loses many aspects of the normal function and regulation of that *in vivo*. An excellent example of this is the vasculature-endocrine network relationship and the role that capillary blood flow has in hormone output (117, 221), how molecules diffuse to and from endocrine cells (221, 266), and how this

545 becomes perturbed during insult (266). Study of endocrine glands *in situ*, where they are
546 subject to peripheral inputs, including neural and endocrine interactions (118), presents a level
547 of technological challenge, which depends on their location and size. Even with the pituitary
548 gland, one of the least accessible endocrine organs, this has been shown to be possible, and
549 imaging in anaesthetised mice has been described for a period of hours (221) (Figure 4A).
550 More recently, technical developments and rigorous, step-wise method developments have
551 allowed experimenters to image the pituitary gland in awake head-restrained mice (176).
552 Regarding the adrenal gland, basal and splanchnic nerve stimulation-evoked electrical activity
553 of the chromaffin tissue can be assessed during *in vivo* recordings in anaesthetised mice (Figure
554 4B), and the simultaneous collection of the adrenal venous blood before and during nerve
555 stimulation allows further catecholamine assay (100) (Figure 4C). The techniques aimed at
556 monitoring cellular/tissular signals *in vivo* continuously develops, as recently shown by the
557 recording of electrophysiological signals collected in the adrenal cortex and medulla of
558 anaesthetised rats using chronically implanted flexible probes (396). This innovative technical
559 development allows monitoring of cellular electrical activities in response to various stress
560 stimuli. The right or the left gland, or both, can be implanted, and longitudinal studies can be
561 performed for several weeks. Further technical developments are required to explore cell
562 activities in the mouse adrenal tissue.
563
564 **Analysis of individual cell function**
565 As will be described in this review, there is considerable heterogeneity in the behaviour of
566 individual cells within homotypic endocrine cell types, a phenomena which was observed in
567 early studies of somatotrophs (189), thyrotrophs (231) and gonadotrophs (96). This makes
568 measurement of the response of individual cells important, as effects may not be apparent at
569 the population level, and essential in studies where correlation of different components of cell

activity is being studied. Development of methodology allowing detection of single cell output, such as those described below, is thus important, whatever the scale of cell interaction (from isolated cell to *in vivo* tissue).

Monitoring hormone release

Reverse haemolytic plaque assay. The reverse haemolytic plaque assay is based on the complement mediated lysis of antibody-bearing erythrocytes on antigen binding (273) and was first applied to detection of hormones by Neill and Frawley (286). Such assays have been applied to detect multi-hormonal cell types (43) as well as the amount of hormone secreted (46) and concentration-dependent relationships between secretagogue and proportion of responsive cells (230). An excellent example of its utility is the study by Mouihate *et al.* (278), where the method was used to identify each pituitary cell type, as well as their secretion of epidermal growth factor. An additional advantage of the method is that, as the detection of hormone does not result in death of secreting cells, it can be used to determine cell type prior to other studies of cell function, for example calcium activity or capacitance measurement (see below) (240). By its nature, it has the inherent disadvantage of only being applicable to the analysis of isolated cells.

Amperometry/cyclic voltammetry. These electrochemical techniques allow the detection of oxidized or reduced molecules that are released from single cells (55). The most common technique relies on the use of carbon fibre as detector. The carbon fibre electrode is brought in very close proximity to the cell and records currents resulting from an oxidizing reaction following the release of the content of a vesicle into the extracellular medium. Carbon fibre amperometry has been successfully applied to isolated chromaffin cells (447) or acute adrenal slices (31, 254) to measure catecholamines secretion (Figure 5A). The use of fast-scan cyclic voltammetry, a technique allowing discrimination between epinephrine- and norepinephrine-releasing sites, contributed to exploration of the hormonal content of basal and stimulated

release events in chromaffin cells (317). While basal release sites are composed of either epinephrine or norepinephrine, stimulated release sites are made of a mixture of adrenergic and noradrenergic signals showing the heterogeneity of the adrenal medullary tissue. Amperometry has been successfully applied to pituitary melanotrophs for the detection of α-melanocyte stimulating hormone (306). Amperometry, *via* insulin-sensitive or carbon electrodes, has also been used in islets to detect exocytosis of insulin as well as 5-HT from beta cells {Huang, 1995 #500;Aspinwall, 1999 #501. More recently, the use of carbon microfibres (tip diameter, 30 μm) implanted in the hypothalamic median eminence in freely-moving mice allowed monitoring of dopamine release events and how these occur in relation to the inhibitory control by dopamine of spontaneous prolactin release from the pituitary gland (337). It is noteworthy that the use of amperometry/voltammetry techniques is limited to the detection of molecules that are electroactive. Constant potential amperometry (electrode clamped at a constant potential) has an excellent temporal resolution and provides information on the kinetics of the fusion event and the total amount of the molecule released from a secretory granule. However, two major limitations should be noted: i) it gives little information on the chemical nature of the molecules detected, as any molecule that is electroactive at the recording potential will be detected (ex: detection of catecholamines without discriminating between epinephrine or norepinephrine), and ii) it does not measure the real concentration of the target molecule. By contrast, the cyclic voltammetry provides the chemical signature of the molecules detected and allows the measurement of the concentration of the target molecule. But it lacks the time resolution of amperometry. Ongoing developments aim at improving the detection devices to monitor simultaneous recordings from multiple cells {Huang, 2018 #566;Huang, 2021 #567}.

Probes for monitoring secretion. Although the reverse haemolytic plaque assay can determine the amount of hormone released by an individual cell, it is a poor method for monitoring the dynamics of hormone secretion from individual cells. Co-localisation of fluorescent or

luminescent molecules with hormones in secretory granules allows monitoring of secretion with optical imaging (37, 154) (Figure 5B). For example, fusion of the secretory signal peptide of hen egg white lysozyme with enhanced yellow fluorescent protein (eYFP), results in labelling of secretory vesicles, allowing monitoring of eYFP to act as a surrogate of stimulated granules exocytosis (291). Modification of fluorophores to increase their pH-sensitivity, resulting in increased fluorescent quenching in the acidic environment of secretory granules, has provided tools, which enhance the use of secretory-granule targeted fluorophores (267). SNAP-tag self-labelling techniques have allowed different pools of secretory granules to be conditionally labelled simply by applying cell-permeable fluorescence substrate (*i.e.* to SNAP-tagged insulin) (193), although the rodent construct was associated with impaired glucose intolerance (209). Most recently, a ratiometric insulin probe (RINS1) has been described consisting of a superfolder GFP-insulin and mCherry-C-peptide fusions (367). While the superfolder GFP-insulin was released in response to stimulation, the mCherry-proinsulin was unexpectedly trapped in the cell, allowing ratiometric measures (367). One issue with these tools for monitoring release of hormone from single cells is the amount of background and out-of-focus fluorescence, as well as the density of granules in proximity to the membrane in primary tissues. However, this can be resolved with the use of total internal fluorescent microscopy (TIRF; reviewed in (22)), which relies on the generation of an evanescent field to selectively excite and image fluorophores within 100 nm of a solid surface. This allows imaging of the movement of fluorescently tagged secretory granules vertical to the coverslip at high resolution. The application of TIRF microscopy to the study of endocrine cells (reviewed in (327, 385)) has allowed studies of the dynamics of insulin granule movement in both immortalised (294) and primary (265, 295) pancreatic beta cells, pathways regulating thyroid stimulating hormone release (7) and the disruptive effect on granule formation of a mutation found in the human growth hormone gene (256). Another issue with fluorescent

probes is that they can affect secretion itself depending upon fluorescent protein used, as well as the amino acid sequence used to link the fluorescent protein to the cargo of choice. Thus, careful controls are needed to exclude probe-dependent effects, as well as ensure that the secretory process is intact (264). While unable to directly visualise cargo release, two photon imaging using polar tracers such as sulforhodamine are able to monitor fusion pore opening and granule fate across many cells (242, 404). Another approach is to use membrane-resident Zn^{2+}-sensitive dyes, allowing simple epifluorescent microscope monitoring of hormone release with high temporal and spatial resolution (234, 305). Since insulin crystalises as a hexamer with two Zn^{2+} ions at the core, Zn^{2+} release can be used as a convenient proxy for insulin release. Zn^{2+} probes have the advantage of allowing measurement of insulin release from dozens of beta cell across the islets, providing information about regionalisation of response (234), although they cannot measure levels of the native hormone *per se*. A disadvantage of chemical Zn^{2+} probes is that they can be taken up non-specifically into endocytotic vesicles. More recent approaches largely obviate this by tethering the Zn^{2+} probe to the extracellular surface using SNAP-tagged GLP1R, allowing high concentrations of probe in the vicinity of insulin granule fusion sites. Granule targeted probes have been reported, which show improved signal-noise-ratios for Zn^{2+} detection, as well as allow Zn^{2+} concentrations in the granule to be measured. Lastly, super-resolution approaches, including rapid structured illumination microscopy techniques promise to provide detail regarding hormone exocytosis in three dimensions in the intact tissue.

While small molecule probes can be applied to most preparations, there can be issues introducing charged moieties across the membrane, necessitating the use of detergents such as pluronic acid (which can themselves interfere with cell function). By contrast, genetically encoded probes can be introduced using mouse genetics or viral vectors. An issue with viral vectors is penetration of the islet, although this should be considered in light of the fact that

most imaging techniques cannot easily penetrate past the first few islet layers (with the exception of two-photon excitation and selective plane illumination).

Monitoring stages of stimulus-secretion coupling

Patch-clamp. Many types of endocrine/neuroendocrine cells are electrically excitable and intracellular calcium levels and hormone secretion are regulated by complex interactions between voltage- and ligand-gated ion channels, gap junctions and cell signalling pathways regulated by extrinsic factors (390). Analysis of this is possible at the single cell level using patch-clamp techniques (165) in combination with pharmacological manipulation to investigate which channels are responsible for the changes in firing pattern and frequency which leads to secretion (84, 155, 274). Examples of the use of this technique to understand endocrine function include the role of intermediate conductance calcium-activated (SK4) potassium channels in corticotroph response to secretagogues (237), the mechanisms linking glucose metabolism to beta cell excitability (340-342) and the role of voltage-gated sodium and calcium channels as key regulators of the electrical firing pattern and catecholamine release in chromaffin cells (422, 423).

Calcium imaging. The relationship between intracellular calcium and secretion has long been recognised (346), and the calcium response of endocrine cells to stimulation can be used as a surrogate for hormone output in a number of endocrine organs where Ca^{2+} fluxes are closely associated with quantal secretory granule release such as pituitary cells (368, 389). Examples of studies utilizing calcium imaging to monitor endocrine cell activity are those showing the existence of multiple functional states of lactotrophs with the calcium indicator dye indo-1 (240), the complex concentration-dependent calcium response in gonadotropin-releasing hormone-stimulated individual gonadotrophs (158), the oscillatory activity in response to stimulation and inhibition of isolated somatotrophs with fura-2 (412) and the generation of coordinated population responses in pancreatic beta cells (348). While first phase insulin

secretion is Ca^{2+}-dependent, changes in second phase insulin secretion are not necessarily accompanied by changes in intracellular Ca^{2+} concentration (172), although studies with better spatiotemporal resolution are required to confirm this. Lastly, it should be noted that the majority of experiments looking at Ca^{2+} used buffers with supra-physiological levels of the ion.

Capacitance/patch amperometry. The surface area of a cell inevitably increases as a fusion pore is formed during the process of granule secretion, leading to an increase in cell membrane capacitance, as this is proportional to the area of cell membrane. This can be monitored with patch clamp techniques (reviewed in (354)) and allows the temporal and quantitative measurement of secretory events. This has been applied extensively to studies of pancreatic beta cell (339), chromaffin cell (285) and pituitary cell (330) function, and has provided information on the contribution of voltage-gated calcium channels (20) (Figure 6A), ligand-gated ion channels (275) (Figure 6B) and intracellular calcium pools (418) (Figure 6C) to hormone exocytosis. Disadvantageously, however, capacitance output is based upon exocytosis as well as endocytosis, square wave pulse stimulations do not reflect endogenous stimulus, the glucose and Ca^{2+} concentrations used are non-physiological, and other nutrients are disregarded. Capacitance measurement can be coupled with simultaneous amperometric recordings of hormone release. This technique called "patch amperometry" (99) requires to place the amperometric detector directly inside the patch pipette. It has been successfully applied to chromaffin cells for the detection of individual catecholamine-containing secretory granules, first in cell-attached patches (8) and extended to cell-free membrane patches in the inside-out configuration (98). The patch amperometry technique has been used to investigate the regulation of quantal size and granule size in chromaffin cells (148, 403) and to measure the concentration of the cytosolic pool of catecholamines (277).

Monitoring intracellular signalling

Monitoring intracellular signalling following stimulation by secretagogues requires the quantification of receptor binding, the intermediates of the signalling transduction pathway, or measurement of the outcome at the level of gene transcription. The methods that are most applicable to this at the level of the single cell are principally based on monitoring fluorescent reporters of either protein-protein interactions, signalling-intermediate activity or gene expression. Recombinant probes have been used to monitor glucose-induced ATP/ADP ratios (181, 236), cAMP levels (179, 307, 410), as well as PKA activation in primary mouse beta-cells (116) and ERK activation in a pituitary lactotroph cell line (456). Transcriptional reporters of cell signalling in endocrine cells have principally been used in zebrafish, where there are transgenic lines reporting pancreatic Notch (311), retinoic acid (184), TGFβ, Bmp and Shh (366) and pituitary oestrogen receptor (149) signalling. In the mouse, a β-galactosidase based Wnt/β-catenin signalling reporter has been used to monitor activation of the pathway in a pituitary tumour model (142).

Monitoring gene expression

The highly dynamic and stochastic nature of gene expression (325) makes single cell measurement a requirement for analysis of the temporal response of endocrine cells to stimulation, which may be lost in averaging of the response of a cell population (381). The use of unstable fluorescent or luminescent reporters of gene expression provides a method of monitoring these dynamics with high resolution (reviewed in (27)). These have been applied to monitor the dynamics of PRL gene expression in individual cells, allowing the monitoring of gene expression heterogeneity in isolated cells or tissue slices (127, 128), in addition to insulin promoter activity in beta cells (399).

Analysis of cell populations

Some of the methods used for single cell analysis can be applied to that of cell aggregates or monolayer cultures, tissue slices/organ culture simply by recording multiple measurements of individual cells (calcium imaging of slice cultures) or analysis of cell populations (gene expression analysis and cell signalling pathways). More generally, measuring hormone release requires ultra-sensitive and multiplexed hormone assays, which allow *in vivo* hormone profiling from the pituitary gland. First, radio-immunological assays, which were developed in the last century, monitored hormonal profiles in both humans and moderate-large mammals for periods of hours to several days. Using freely-moving animals which were dorsally cannulated, 'on-line' monitoring of GH pulsatility became achievable in both male and female animals (333, 408), thus allowing resolution of sex-dependent GH profiles (144) and thereby their functional significance for encoding differential liver responses (440). With the use of automated blood sampling (ABS) systems, where small blood samples collected *via* the vascular cannula are sent to a fraction collector where they are deposited into collection tubes, the blood sampling procedure can be carried out with minimal disturbance to the animal. These experiments are considered stress-free for the animals and have been used extensively to study the pulsatile secretion of ACTH and CORT and elucidate the regulation of the hypothalamic-pituitary-adrenal axis (435, 449). Moreover, a huge effort in mathematical analysis and modelling has been devoted to these hormonal profiles giving a classification of gender differences and hormone diseases with regard to GH profile parameters (e.g. pulse and nadir characteristics, approximate entropy inversely correlated to regularity, etc) (426), and providing theoretical regulatory loops of the hypothalamic-pituitary-adrenal axis (435) and hypothalamic-pituitary-gonadal axis (432). Second, recent developments of high-sensitive ELISA assays for murine pituitary hormones (GH, PRL and LH) combined with frequent tail-tip blood micro-sampling (a couple of μl every few minutes) now make pituitary hormone profiles in mice possible,

including with the use of the wide variety of genetically-modified mouse models available worldwide (162). Because of the size of the samples this technique results in minimal blood loss and has been shown to be very efficient in characterizing GH and gonadotropin profiles in mice (386, 387).

Multiple pituitary hormones can be regulated using the same signals and/or might interfere with each other, thus, there has been a growing need for techniques that would allow the measurements of several pituitary hormones within a single small sample and multiplex systems have been developed for this purpose. These utilise combinations of antibody-coupled colour-coated beads and fluorescent-tagged secondary antibodies that can be laser-sorted and identified in a flow-cytometer. Computer-based analysis of simultaneous fluorescent signals for each bead quantifies hormone concentration based on standard curves. Whilst these multiplex systems hold great promise and are able to detect the presence of different pituitary hormones, to our knowledge, assays able to measure more than one hormone reliably in a small volume of blood do not exist yet.

Static incubation

Release of hormone into supernatant can be measured following static incubation of pituitary, adrenal or pancreatic tissue with secretagogues. Many techniques have been developed for the determination of catecholamines released by the chromaffin tissue (reviewed in (313, 419)), including enzyme immunoassays (444), capillary electrophoresis (316), radioenzymatic assays (33, 210), but high-performance liquid chromatography (HPLC) remains the more routinely used method enabling the simultaneous assay of several catecholamines (epinephrine (E), norepinephrine (NE) and dopamine) and/or their metabolites ((174) and reviewed in (38)). Catecholamine release can be monitored in many biological samples (plasma, urine, cerebrospinal fluid, adrenal slice supernatant (94)). A static incubation of endocrine/neuroendocrine tissues provides an endpoint measure of hormones secreted, and can

be used to quickly and reliably detect defects in hormone secretion (289). However, dynamic information is lost, meaning that more subtle impairments, for example in first phase insulin exocytosis, may be missed.

Perifusion systems

Incubation of pituitary slices and islets under perifusion conditions allows supernatant fractions to be withdrawn as aliquots over a number of timepoints. Whilst reagent and tissue intensive, such experiments provide valuable detail on hormone release kinetics, which is important when considering insults that may target distinct events (*e.g.* first or second phase insulin secretion (289)).

Luciferase constructs

Gaussia luciferase expression can be driven downstream of hormone promoters, such as insulin (57, 203). Following hormone processing in the cytoplasm (*i.e.* cleavage of prohormone), luciferase is released into the supernatant, where it can be measured using high dynamic range bioluminescence assays following addition of the substrate coelenterazine. Luciferase-based approaches are amenable to high throughput screening, but are unable to report hormone dynamics. In addition, they are so far restricted to immortalized cell lines.

Monitoring dye diffusion/transport

One hallmark of the endocrine system is the peculiar role of the microvasculature. Due to the presence of diaphragmed fenestrations, the rich meshwork of capillaries wiring the endocrine parenchyma allows on one hand, extravasation of incoming blood-borne molecules, metabolites and oxygen, and on the other hand, intravasation of secreted hormones towards capillary lumen while a significant proportion of paracrine/autocrine factors only diffuse within the extracellular space (151, 398). Real-time imaging of anesthetized rodents with long working objectives and dedicated microsurgery has allowed measurement of the extravasation/intravasation rates of fluorescently-labelled dextrans that mimic the size of

817 hormones as well as fluorescent metabolites (221, 266) (Figure 7). Moreover, on-line
818 monitoring of partial oxygen pressure (resulting from the balance between blood oxygen
819 supply and cellular oxygen consumption) and parenchymal distribution of secreted molecules
820 is achievable using Clark-type oxygen microsensors (tip diameter, 4-6 μm) and
821 microiontophoretic injection, respectively (221).

822 ***Genetically modified animals***

823 With regards to the adrenal gland, transgenic mouse lines were generated by targeting one of
824 the three enzymes involved in catecholamine biosynthesis, that is tyrosine hydroxylase (TH),
825 dopamine beta hydroxylase (DbH) and phenylethanolamine-*N*-methyl transferase (PNMT), the
826 latter converting norepinephrine to epinephrine. The first transgenic mouse models were
827 developed late in the 1980s and early in the 1990s. Mouse chromaffin cells were genetically
828 modified by insertion of the human *Pnmt* gene fused with the Simian virus 40 (26), the human
829 *Dbh* gene fused to the reporter gene LacZ (261), the human *TH* gene (204), or the rat *TH* gene
830 fused to the bacterial chloramphenicol acetyl transferase gene (28). This opened the possibility
831 to selectively ablate E- or NE-secreting cells within the chromaffin tissue, by using a cell-
832 specific gene to drive the expression of a toxic gene product, as also reported in pancreatic
833 acinar cells (301). The fluorescent targeting of adrenal chromaffin cells came much later. A
834 reporter mouse strain in which the gene encoding the PNMT enzyme was fused to a nuclear-
835 localized EGFP (nEGFP) was generated (453), allowing the study the E-secreting cell
836 population of the adrenal medulla. The variability in nEGFP fluorescence between cells is an
837 interesting observation, suggesting that the functional activity of the medullary adrenergic cell
838 is not uniform. To date, the genetic constructs used to fuse the human *TH* gene to enhanced
839 GFP failed to label adrenal chromaffin cells, both in the mouse and in the rat (190, 213) and
840 no transgenic mouse line expressing a fluorescent DbH in the adrenal medullary tissue has been
841 reported. The development of knockout mice often informs the importance of the targeted gene.

While *Pnmt* knockout mice are viable, fertile and with no apparent developmental defects (29, 119, 395), the inactivation of *TH* or *Dbh* alleles results in mid-gestational lethality (216, 220)}. TH mutant mouse embryos can be rescued *in utero* by administrating L-DOPA to the pregnant females and by continuing the treatment after birth (457). Similarly, DbH mutants can be rescued by a prenatal treatment with the synthethic catecholamine precursor L-DOPS (220), demonstrating the extent that catecholamines are essential for fetal development and postnatal survival. Crossing knock-in mouse models with insertion of the Cre recombinase gene into *TH* (239), *Pnmt* (119) or *Dbh* locus (394) with mice expressing the gene of interest flanked by *LoxP* sequences allows specific manipulation of gene expression in a tissue- and/or cell-type specific manner, as demonstrated by the targeted knockout of *TH* in PNMT-containing cells, thus producing a mouse deficient in catecholamines only in adrenergic cells (395). The early development of the adrenal medullary tissue through following the distribution of chromaffin cells in embryos can also be addressed in genetically modified animal models. The crossing of a *PNMT-Cre* mouse with a ROSA26-beta galactosidase reporter mouse, enabling identification of both cells actively expressing PNMT and those that expressed PNMT sometime earlier in development, provided information on the distribution of adrenergic-derived cells throughout development (119).

Transgenic mouse, rat and fish models have been used to study various aspects of pituitary development and function. The pathophysiology of diseases caused by excess or insufficient pituitary hormone production have long been investigated but were impeded by the difficulty of dissecting the individual components. Thus, cell specific gene targeting is invaluable for studying the function of genes that have pleiotropic effects on development, function, or carcinogenesis of vital organs causing embryonic lethality or complications due to multi-organ effects. Pituitary cell-specific Cre driver lines have allowed the study of particular pituitary cell functions and cell lineage relationships. Cre-loxP technology provided

researchers with the ability to knockout or insert genes within specific pituitary cell-types by crossing hormone-specific Cre driver line with gene-specific floxed lines and provided an unprecedented opportunity to dissect out the regulation of specific hormones secretion (89). Several transgenic mouse lines have been developed to allow Cre expression in the 5 hormone-secreting cell types. The TSH-Cre mouse line targets Cre expression to thyrotrophs (65), GH-Cre to somatotropes (with limited expression in lactotropes) (282), LH-Cre to gonadotrophs (72), POMC-Cre to corticotrophs (225) and and PRL-Cre to lactotrophs (66). While using the hormone promoters to drive the expression of Cre in pituitary cells has been an evident and valuable choice for many studies, it is important to note that it does not necessarily lead to even expression across the cell population. An alternative approach has been to generate receptor-driven transgenics. Of particular interest GHRHR-Cre mice that express Cre under the control of the rat growth hormone releasing hormone receptor and lead to expression in somatotrophs, lactotrophs and thyrotrophs that derive from Pituitary Transcription Factor-1 (Pit-1) lineage (455). Similarly, the GnRHR-Cre mice that express Cre under the control of the rat gonadotrophin releasing hormone receptor, allows specific targeting of virtually all gonadotroph cells (441).

The five hormone-secreting pituitary cells arise from progenitors in Rathke's pouch, the embryonic primordial of the pituitary gland, in a temporal and spatial specific fashion during pituitary development. While the multiple extrinsic and intrinsic mechanisms that regulate progenitor cell proliferation, lineage commitment, and cell fate terminal differentiation will be discussed in a further section of this review it is worth noting that transgenic mouse line such as Hesx1-Cre (14), Prop1-Cre (91) and Pit1-Cre (296) have been key tools to study pituitary development and lineage. Alongside Cre-driver lines, reporter lines that express fluorescent proteins under the control of cell-specific promoters have been developed. These reporter lines have been extensively used for cell-type identification in *ex vivo*

electrophysiology or primary pituitary cell cultures (110, 111). If the hormone signal peptide is included in the transgene construct, the fluorophore is localised in the secretory granule and co-secreted with hormone (169). This can be exploited in studies of of the role of cell organisation using perfusion of acute slice preparations, monitoring fluorescent protein output in the eluate in real-time in response to secretagogue stimulation (357). In addition, transgenic animals with fluorescently identified cell types have become an invaluable tool to perform *in situ* experiments. For example, GH-eGFP transgenic mice were utilised to image *in vivo* somatotroph cells within the pituitary gland and revealed coordinated regulation of pituitary microcirculation and GH cell network function (221).

Early transgenesis resulted in mice harboring GFP under the influence of the mouse insulin 1 promoter (MIP) (166). These and similar animals provide the ability to identify individual beta cells *in situ*, allowing *in vivo* imaging approaches (292) and FACS-identification of subpopulations based on size, granularity and insulin secretion (207). The subsequent advent of Cre-Lox technology led to the production of a number of Cre driver lines for conditional beta cell expression. Initial efforts using the rat insulin promoter (RIP) (also known as the ancestral *Ins2* gene) displayed extra-pancreatic recombination in the hypothalamus and other central sites involved in food intake and glucose homeostasis (246, 446). Whilst these findings spurred on the generation of more specific lines, including MIP-Cre/ERT driven by the Ins1 promoter (446), RIP-Cre animals provided unexpected insight into the role of the arcuate nucleus -*via* stereotactic injection with virus harboring constructs with protein expression dependent on loxP recombination- in energy expenditure (217, 345). More recent knock-in lines including those possessing Cre recombinase under the Ins1 promoter display good beta cell specificity with little or no extra-pancreatic leakiness and excellent recombination efficiency (~ 90%) (411). In addition, knock-in models are free from the effects of the human growth hormone minigene, used as a transgenic enhancer, which binds the

prolactin receptor to induce a pregnancy-like phenotype in beta cells (298). Animals for conditional alpha (Glu-Cre, iGlu-Cre, etc) and delta (Sst-Cre) cell expression similarly exist, although concerns remain regarding specificity and efficiency (379, 380) of the former model, and somatostatin (Sst) by nature is also a neuronal marker (405). In any case, CRISPR-Cas9-assisted gene targeting promises to address many of the aforementioned issues by replacing the endogenous locus with Cre (2). It should be noted however that irrespective of the approach used, Glu-Cre driver lines lead to recombination in the gut and hindbrain, in line with proglucagon expression in GLP1-secreting enteroendocrine cells and neurons. For gene overexpression, a conditional Tet-ON system can be used, whereby doxycycline administration drives expression of genes under the regulatory control of the tetracycline-responsive promoter element. This becomes conditional when animals are crossed with a strain harboring a reverse tetracycline-controlled transactivator (rtTA) protein under the control of the insulin or glucagon promoter (86, 269).

Virus transduction

In the adrenal gland, viral transduction strategies are suitable to both investigate the neuronal network connecting the chromaffin tissue and the central nervous system and to manipulate adrenal medulla signalling. As such, the central autonomic circuits involved in top-down control of the adrenal medulla can be retrogradely labeled by viral transneuronal tracing techniques using intra-adrenal injections of pseudorabies or rabies virus. Interestingly, this approach not only provides an anatomical cartography (107-109, 415), but also enables envisionment of functional issues (107-109, 415). An *in vivo* gene transfer method using delivery of adenoviral vectors either directly into the medulla or through the suprarenal veins was successfully applied to the adrenal medullary tissue, allowing for example manipulation of G protein-coupled receptor kinase 2 activity (244).

Expression of recombinant probes can be induced in the endocrine pancreas using adenoviral or baculovirus vectors (376, 377). Adenovirus induces strong expression when coupled to U6 or CAG promoters and appears to be tropic for beta cells (181), largely obviating the requirement for complex promoter-linked constructs, and allows insertion of large genes compared to most adeno-associated virus serotypes, which also needs to be double-stranded for efficient infection (439). The exact reasons for this are unknown, but may reflect lowered protein translation in alpha *versus* beta cells (252). *In vivo* transduction of the pancreas can be achieved using bile duct injection of adeno-associated virus virus (usually AAV8) (5), although high titres have been shown to achieve similar results *via* the less invasive *i.p.* route (414). While introduction of adeno-associated virus *via* the tail vein leads primarily to hepatic uptake, pancreatic expression can be induced following bile duct delivery (199).

Harnessing the power of viruses to deliver genetic information into the pituitary gland so far been limited, mostly due to the location of the pituitary on the ventral side of the brain. Until recently, local delivery of specific genes by viral transduction had only been achievable to very large pituitary tumors by trans-auricular viral injection (329), but a 2019 study has shown that adeno-associated transfection of pituitary cells can be achieved using stereotaxic injection from the top of the skull, through the meninges, down to the pituitary gland (176).

In vivo microscopy/optogenetics/DREADDS

The pancreas is amenable to *in vivo* imaging approaches, and studies investigating the organ directly in the abdomen have provided important insight into the direction of blood flow (predominantly beta -> alpha cells) with relevance for paracrine interactions (293), effects of glucose on flow rates (292), and the impact of obesity on molecule access to the islets (266). Although longitudinal approaches are limited by the highly lytic milieu of the exocrine pancreas and risk of pancreatitis, relatively non-invasive transplantation of both rodent and human islets into the anterior chamber of the eye is possible, where vascular and neural supplies

re-wire (1, 59, 335). These elegant studies have shown that long term treatment with liraglutide, an anti-diabetic, is detrimental for human islet function (1) and that autonomic nervous system regulates insulin release directly from islets (335). They have also been translated to primates, facilitating translational findings relevant for islet transplantation (315). However, all these *in vivo* imaging approaches have been performed in anesthetized animals. Future developments are now needed to image endocrine glands in awake animals, thus avoiding the deleterious effects of anaesthetics on hormone release.

Optogenetic control of pituitary function has largely be related to light-controlled opsin manipulation in hypothalamic neurons at the level of hypophysiotropic neurons (e.g. GnRH neurons (60)) and their inputs (e.g. kisspeptin neurons controlling GnRH neurons (76). Combining selective optogenetic control of these hypothalamic neuronal subsets with tail-tip blood microsampling in live mouse models allowed to identify which firing patterns of hypothalamic neurons efficiently control hormone release from the pituitary cell networks (60). Direct optogenetic control of pituitary cell activities has been performed in larval zebrafish in which a Beggiatoa photo-activated adenylyl cyclase had been selectively expressed in pituitary corticotrophs (93) and more recently optogenetic stimulation of somatotrophs was used in GH-Cre x ROSA26-fl/fl-ChR2-dtTomato mice to induce GH secretion *in vivo* (176) (Figure 8A).

Optical tools have also been developed for better understanding the complexity of β-cell signalling and insulin release (reviewed in (134)). Optogenetic control of beta cell function and insulin release has been achieved using both channelrhodopsin and halorhodopsin, by crossing either RIP-Cre or Ins1Cre deleter strains with animals possessing a Cre-conditional optogene (48, 200, 328, 443). Along similar lines, islets have provided one of the major test beds for photopharmacology, also referred to as chemical optogenetics, which harnesses the spatiotemporal precision of light to modulate the activity of exogenously-applied compounds (50). This approach relies on the use of azobenzenes ('Azologization'), small molecular

switches that undergo isomerization following illumination (54). In general, low wavelengths of light favour *cis*-isomerization and higher wavelengths *trans*-isomerization. These subtle alterations in molecular motion/shape translate to the optical control of ligand conformation/binding, thus turning ion channels and receptors into endogenous photoswitches (50). To this end, azobenzene photoresponsive units have been incorporated into sulfonylureas (49, 53) and incretins (51, 52) to allow the optical control of ATP-sensitive potassium channels and glucagon-like peptide-1 receptor function with light. Similary, azo-logues of atrial natuiretic peptide, diacylglycerol and GW9508 (GPR40 agonist) have been produced, all with applicability for the interrogation of endocrine tissue (reviewed in (134)). Recent studies have shown the potential of photopharmacology for the *in vivo* interrogation of endocrine function. By treating mice with the light-activated sulfonylurea JB253, insulin release and glucose homeostasis could be controlled remotely *via* fibre optics placed in proximity to the pancreas (240) (Figure 8B).

Similar photopharmacological/chemical optogenetic approaches have been successfully used to modulate the pattern of electrical activity and neurosecretion of chromaffin cells. Calcium influx-mediated secretory granule exocytosis can be tightly controlled in bovine chromaffin cells expressing a light-gated glutamate receptor (LiGluR, (402)), a glutamate receptor engineered to covalently accept a photoswitchable tethered ligand termed maleimide-azobenzene-glutamate (195, 196). The wavelength dependency of LiGluR-activated currents allows a graded control of calcium influx, and can therefore be used as a modulator of the secretory rate of chromaffin cells. Similarly, light-controllable nicotinic acetylcholine receptors (LinAChRs), have been engineered with photoswitchable tethered agonists and antagonists (229, 413). This includes the heteromeric $\alpha 3\beta 4$ nAChR, the nAChR subtype expressed in adrenal chromaffin cells, thus opening future perspectives for study of adrenomedullary tissue. After being validated in recombinant cells, their use to control

nicotinic neurotransmission have been reported in brain slices (358) and *in vivo* in freely behaving mice (112). Synthetic photoactivable nanoswitches based on ruthenium-diimine complexes, such as RubpyC17, have been exploited to optochemically control membrane potential of pancreatic insulin-producing cells and mouse chromaffin cell electrical firing and associated catecholamine secretion (336). Once incorporated into the plasma membrane of cells, illumination of RubpyC17 allows manipulation of electrical activity in both directions (Figure 8C). The presence of ascorbate in the bathing medium triggers depolarization, while in ferricyanide-containing saline, cells hyperpolarize. Note that since the release of the article, no further publication reported the use of RubpyC17.

Homotypic cell model: the adrenal medullary tissue

Anatomy and development

The adrenal glands (also called suprarenal glands, in reference to its body location) encompass two secretory tissues, the cortex and the medulla (Figure 9A), with distinct embryonic origins. Whereas the cortex originates from the mesoderm, the medullary region evolves from the neuroectoderm. The cortical region is divided into three zones, each of them secreting different hormones, namely aldosterone for the *zona glomerulosa*, glucocorticoids for the *zona fasciculata* and gonadocorticoids for the *zona reticularis*. The adrenal medulla is a neuroendocrine tissue, which is involved in the early adaptive response to stress. Upon stressful situations, catecholamines (mainly E and NE) are among the first hormones to be released into the blood circulation and, *via* their peripheral actions on arterial blood pressure, heart rhythm, glucose production and bronchodilation, they significantly contribute to the 'fight or flight' response (371). Circulating E (90%) and to a lesser extend NE (7%) originate from the adrenal chromaffin cells which constitute the neurosecretory unit of the adrenal gland.

In rodents, the adrenal medulla primordium forms around E12.5, with the primary adrenal anlage appearing at E11.5 (reviewed in (187). It is currently generally accepted that the developmental origin of chromaffin cells stipulates that they derive from a migratory stream of neural crest cells, committing to a common sympathoadrenal lineage (12). This dogma on the sympathoadrenal origin of chromaffin cells has been recently challenged. By the use of sophisticated *in vivo* inducible genetic lineage-tracing approaches, Furlan and colleagues were able to show that peripheral glia stem cells (called Schwann cell precursors) are chromaffin cell ancestors (138). This provocative discovery reveals that the origin of chromaffin cells is still not fully elucidated, with wider implication for the field of neuroendocrine tissue differentiation in vertebrates (71).

The developmental program leading to chromaffin cell differentiation requires a specific transcriptional network with sequential activation/repression of transcription factors. The study of mice deficient for various transcription factors allowed their respective contribution to the chromaffin cell developmental program to be ascertained (reviewed in (185). One of the earliest expressed transcription factors is the homeobox gene *Phox2b*. This transcription factor is expressed under the control of local bone morphogenetic protein-driven signals, much before the formation of the adrenal medulla anlage and co-expressed with the neural crest marker *Sox10* in all sympathetic ganglionic cells at E10.5 (312). Revealing the master role of *Phox2b* in the development of noradrenergic phenotype in vertebrates, *TH* and *DbH* expression is fully absent in the adrenal medullary tissue of *Phox2b* knockout mice (312). Additionally, *Phox2b* deficiency prevents the maturation of presumptive chromaffin cells at an early developmental stage, leading to an absent centrally located medulla (188). Supporting an indispensable contribution to adrenal medulla development, *Phox2b* is also required for the induction of many components of the transcriptional network underlying chromaffin cell differentiation. Unlike *Phox2b*, the loss of the transcription factor *Mash1*, also expressed at an

early developmental stage and also leading to the absence of *TH* and *DbH* expression, does not cause a complete loss of chromaffin cell development (186). As mentioned before, the adrenal medullary tissue is envelopped by the adrenal cortex and as such an effect of cortical tissue on chromaffin cells may be expected. Surprisingly, cortical cues do not appear to be essential signals for the early determination of chromaffin cell fate, but rather are required at a later stage for chromaffin cell survival (309), colonization of the adrenal anlage (163), or growth of the adrenal medullary tissue (39).

The acquisition of an E or NE phenotype by chromaffin cells differs in time (297, 428), related to the sequential expression of the enzymes involved in NE and E synthesis (429). At E14-E16, adrenomedullary cells are only immunoreactive to DbH and contain high amounts of NE compared to E. Coinciding with the expression of phenylethanolamine N-methyltransferase, likely induced by glucocorticoids secreted by the fetal cortex (429), E synthesis and storage begin at E16-E18, in a population of 'mixed cells' able to produce both NE and E. Shortly after, E synthesis becomes predominant. The ultimate separation between E- and NE-containing chromaffin cells occurs just after birth (428).

Similar to the anterior pituitary, the secretory cells of the adrenal medulla are surrounded by 'supporting' glial-like cells, termed sustentacular cells, which were initially identified by their immunoreactivity to S100 antigen (78). Their small size, shape and intermingled distribution between chromaffin cells resemble those described for the anterior pituitary folliculostellate cells (see below). Very little is known about sustentacular cells, especially their functional role and how they impact chromaffin cell stimulus-secretion coupling. In the rat, sustentacular cells differ in their number, size and shape between neonate and adult (6). The stellate shape, associated with numerous slender and long branched cytoplasmic processes observed in the adult, suggests close appositions with chromaffin cells and subsequent functional interactions. Another example illustrating a role underlying

plasticity, at least anatomically, comes from the recent observation of an increased covering of chromaffin cells by sustentacular cell processes in stressed mice (300). Interestingly, sustentacular cells appear more frequently associated with NE- than E-containing chromaffin cells (397), suggesting a differential regulation of the two hormones.

Innervation

Anatomically, when compared to the anterior pituitary gland, the presence of numerous nerve fibres (sympathetic fibres) within the medulla (Figure 9C) confers an interesting peculiarity to the adrenal gland, which is functionally analogous to a sympathetic ganglion. As will be described below, the splanchnic nerve-chromaffin cell synapse is the privileged regulatory site of stimulus-secretion coupling, managing and regulating the secretion of catecholamines. Interestingly, the synaptic vesicles of nerve terminals innervating the medulla contain various compounds, including neurotransmitters (ACh) and neuropeptides (PACAP, etc), pointing to the composite cholinergic and non-cholinergic nature of the adrenomedullary neurotransmission (156). Taking the rat as an example, the innervation arises from the intermediolateral cell column of thoracic spinal segments (T1-L1 (212) with 50% of neurons located within T7-T10 (370) (Figure 9B). Of the sympathetic axons innervating the adrenal gland, 88% are preganglionic. The axons pass through the sympathetic chain, exit as the greater and the lesser splanchnic nerves (for fibres arising from T5-T9 and T10-T11, respectively), and directly synapse onto adrenal chromaffin cells. The remaining adrenal innervation originates from postganglionic axons arising from the sympathetic chain and the suprarenal ganglion (212).

Recently, direct injection of rabies virus in the adrenal medulla has revealed the cortical areas that are involved in the top-down control of the chromaffin tissue (107, 109), showing thus that i) the brain regions connecting to the adrenal medulla substantially overlap with those

included in depression networks and ii) the motor cortical network is the major source of descending influence over the adrenal medulla. Approximately 500-700 axons innervate the rat adrenal medulla (308). It is noteworthy that the innervation of the two glands is asymetrical. The left gland receives more cerebral inputs than the contralateral one, with a predominance in suprarenal innervation (416). Additionally, the left adrenal receives the greatest contribution from the spinal cord T8 segment while the right gland is mainly innervated by the T9 segment (370). Also, the greater splanchnic nerve bifurcates into an anterior and a posterior branche before innervating the gland (67). Whether the distinct innervation between the two glands and the contribution of the anterior and posterior divisions, are functionally relevant in terms of stimulus-secretion coupling (efficiency, robustness, etc) is still open but addressing this issue is complex. First, and as reported in a recent study performed in an elegant *ex vivo* spinal-splanchnic-adrenal preparation addressing the possible distinct contribution of the anterior and posterior branches innervating the medulla to catecholamine release (451), E seems to be preferentially released from the periphery and NE secreted from the center of the medulla. Second, the measurement of E and NE, both under basal and cholinergic stimulation, does not reveal significant differences between the right and the left gland (94).

Regarding the development of the adrenal gland innervation, synaptic contacts are already present in chromaffin cells at E15.5 (90). Granules start to be produced at E12.5 (120) with the first granules identifiable at E13.5. Their number, size and accumulation under the cytoplasmic membrane gradually increase at later developmental stages (90). Although all determinants involved in the adrenal stimulus-secretion coupling are morphologically present at the fetal stage, the innervation of the adrenal medullary tissue is not fully competent at birth, but matures during the first postnatal weeks, at least in rodents (194). Maturation is associated with the acquisition of the neurogenic control of catecholamine secretion and the postnatal period thus critically shapes functional stimulus-secretion coupling. Another crucial

determinant of stimulus-secretion coupling is the post-synaptic expression of cholinergic (nicotinic and muscarinic) receptors in chromaffin cells. The study of the ontogenetic maturation of nicotinic and muscarinic AChRs, as demonstrated by nicotinic or muscarinic agonist-induced $[Ca^{2+}]i$ changes, revealed a sequential expression of the two receptor types (297). The nicotinic receptor-mediated signalling pathway appears first in development (around E19.5), coinciding with the development of synaptogenesis and neurotransmission. The signalling pathway triggered by the activation of muscarinic receptors takes place later, around postnatal day 0.

Vascularization

Vascularization is another critical feature of endocrine/neuroendocrine tissues that have a dedicated function of release of hormones in the general blood circulation. The concept that the blood supply plays a critical role in the adrenal medulla secretory function has been long postulated. After pioneering work in the dog (133), in the cat (34) and in the mouse and rat (143), several studies in the 1950s-1980s have combined to describe the anatomy of the mammalian adrenal blood supply (85, 168, 215, 352). The adrenal blood supply depicts a centripetal circulation. The medulla receives blood both from the cortex through an arteriolar plexus and from a direct supply through medullary arteries (1-2 in the mouse, up to 4 in the rat) which arise as branches of a subcapsular arterial plexus, and pass through the adrenal cortex usually without branching (85). They penetrate the medulla and end in capillaries. The cortical blood is collected into radicles of the central vein at the cortex-medulla junction and it then flows in larger venous vessels to reach the main vein (85). Unlike the pituitary gland, although anastomoses between medullary capillaries and venous channels can be morphologically observed, no portal blood circulation has been functionally described in the adrenal gland (85, 215). In addition, E and NE-secreting chromaffin cells are not vascularized by selective blood

supply (85). As observed in the anterior pituitary gland, the endothelium of both cortex and medulla is fenestrated (352). It is noteworthy that the afferent and efferent blood supply is also distinguishable between the two glands. The left gland is vascularized from the renal artery while the right receives blood from the inferior phrenic artery. For both glands, the effluent blood is collected into a central vein, which projects into the renal vein for the left gland while the venous blood flowing from the right gland is directly drained into the inferior *vena cava*. Like innervation, whether the distinct vascular inputs and outputs between the right and the left gland impact catecholamine secretion remains to be investigated.

The chromaffin cell: the neurosecretory unit of the stimulus-secretion coupling

In situ, chromaffin cells are arranged in clusters or 'lobules' (Figure 1A) which are surrounded by connective tissue. The lobules have a round or slightly elongated shape, with a diameter of about 60-80 μm (202, 317). Each chromaffin cell receives 1-4 synaptic boutons (202). Although conventionally referred to as the unique 'chromaffin' designation, adrenal chromaffin cells can be morphologically partitioned in either E or NE-secreting cells, with a 4:1 ratio in the rat (428) and in mouse (194). The tissular distribution of both secretory cell types differs among species, from no preferential spatial location, as observed in the rat (121) to a clear segregation between E and NE cells, as observed in the hamster adrenal, in which NE-secreting cells preferentially position at the periphery of the chromaffin tissue (152). Catecholamine release is chiefly, but not exclusively, controlled by the cholinergic splanchnic nerve inputs synapsing onto chromaffin cells (104, 434) (Figure 10). A comprehensive review recently published by Carbone and colleagues (62) will elegantly supplement this introduction on chromaffin cells.

The resulting ACh-evoked chromaffin cell depolarization and subsequent cytosolic Ca^{2+} rise are key processes for catecholamine exocytosis. This traditional view of the adrenal

stimulus-secretion coupling prevailed for many decades, but has been revisited at the beginning of the 2000s. Indeed, although electrical discharges invading the splanchnic nerve endings are the major physiological stimuli triggering catecholamine release, compelling evidence from studies both in acute adrenal slices and *in vivo* in anaesthetised rodent indicates that a local communication mediated by gap junctions between chromaffin cells represents a functional route by which biological signals propagate between adjacent cells and subsequently contribute to catecholamine release (79, 81, 100, 157, 177, 254) (Figure 10).

Chromaffin cell network plasticity

At any time when required by the organism, the network of chromaffin cells must provide an appropriate secretory response. This requires highly dynamic mechanisms of plasticity which can mobilize various sites in the adrenal chromaffin tissue. Gap junction-mediated chromaffin cell intercellular communication and the neurotransmission at the chromaffin cell-splanchnic nerve synapse are two major targets for remodeling (156, 157, 159, 160).

Gap junctions between chromaffin cells: a contribution to catecholamine release

Gap junctions are connexin (Cx)-built intercellular channels, which allow propagation of ions and signalling molecules between adjacent cells. Taking the rodent adrenal medullary tissue as an example, gap junctional coupling can be demonstrated by various techniques, allowing assessment of both its presence (immunohistological detection, Figure 11A, western blot) and its functionality (permeability to fluorescent dye, Figure 11B or propagation of electrical signals between cell pairs, Figure 12).

After their early ultrastructural identification in the adrenal medulla of a variety of species (153), medullary gap junctions were later suspected to mediate electrical coupling between chromaffin cells (68, 130, 276, 283). The first Cx identified in the adrenal medullary tissue was Cx43, in the rat (259). Of the Cxs expressed in the adrenal medulla, reviewed in (81,

177), Cx43 and Cx36 are the two main Cxs expressed in chromaffin cells. It was only at the beginning of the 2000s and by using acute adrenal slices that gap junctions between chromaffin cells have been given unequivocally functional roles. Gap junctions support propagation of spontaneous and depolarization-evoked action potentials and ensuing increases in cytosolic Ca^{2+} ($[Ca^{2+}]i$) (254) (Figure 13A). Similarly, cholinergic stimulation of a single cell can evoke multicellular synchronized $[Ca^{2+}]i$ rises in neighboring cells (Figure 13B). Of physiological relevance is the finding that these multicellular $[Ca^{2+}]i$ rises (either evoked by action potentials or in response to a nicotine application) are sufficient to trigger catecholamine release (254) (Figure 14). To date, no evidence of gap junction-mediated coupling between heterotypic cells (endocrine and/or non-endocrine) has been established in the adrenal medulla. In particular, whether E- and NE-secreting chromaffin cells are coupled to each other *via* gap junctions is still an unsolved issue.

Synaptic transmission and gap junctional coupling between chromaffin cell: a concerted crosstalk to continuously adapt catecholamine release to the need of the organism

A rise in circulating catecholamine levels is a critical step triggered by the organism to cope with a stressful situation, and by releasing both E and NE, the adrenal medullary tissue crucially contributes to this response. To ensure an appropriate catecholamine secretion under all circumstances (basal release, stress-stimulated release), the adrenal medulla has elaborated a finely tuned dialogue between the synaptic inputs and gap junctions (160). The switch from electrical coupling to synaptic neurotransmission that occurs during adrenal gland postnatal development is a remarkable example of this crosstalk in a physiological situation (253). The transition to extrauterine life associated with birth is a critical period associated with hypoxia, which threatens organ homeostasis. A huge catecholamine rise is required to cope with this stressful situation. In neonates, and associated with the fact that the neurogenic control of catecholamine secretion is not yet competent, chromaffin cells are robustly coupled by gap

junctions. During the first posnatal week and coinciding with the acquisition of the neurogenic control of catecholamine secretion, cholinergic synapses mature and gap junction-mediated electrical coupling lessens (253). A similar dialogue between chemical and electrical synapses occurs when the cholinergic neurotransmission is impaired, as evidenced after unilateral splanchnectomy or after a pharmacological blockade of chromaffin cell nicotinic receptors (255).

Another remarkable example illustrating interactions between cholinergic transmission and gap junctional communication comes from studies performed in acute adrenal slices of stressed animals. Unlike in neonates in which catecholamine secretion is splanchnic nerve-independent, the cholinergic terminals synapsing onto chromaffin cells are the main stimulus triggering hormone release in the adult, and are therefore a primary stress-targeted component of the stimulus-secretion coupling. A stressful situation, such as a cold exposure, enhances the frequency of post-synaptic excitatory currents, indicative of the increased sympathetic activity innervating chromaffin cells (80). In parallel, gap junction-mediated communication in also enhanced (80) (Figure 15A), leading to the establishment of a robust electrical coupling between chromaffin cells (82). As such, in response to a stressor, synaptic activity and electrical communication act in a coordinated manner to stimulate catecholamine secretion, as required to appropriately manage the stress episode. The contribution of gap junctions to catecholamine secretion was unambiguously revealed by *in vivo* experiments performed on anesthetized mice, in which gap junctional communication was pharmacologically blocked (100) (Figure 15B). Collectively, all these finding designate gap junctions between chromaffin cells as an additional pathway relaying or contributing to synaptic transmission, thus enabling hormone release (Figure 16).

Beyond the beneficial effect of catecholamine secretion elicited by an acute stress, sustained and/or repetitive catecholamine secretion episodes can have deleterious outcomes

(407). They can contribute to the development of cardiac and vascular pathologies, such as chronic elevated blood pressure, which in turn impinges on the functioning of multiple organs and therefore harms body homeostasis. Although still unsolved, it is likely that, under pathological states, the adrenal medulla develops plasticity mechanisms of the stimulus-secretion coupling aiming at protecting the organism against damaging effects of redundant elevated catecholamine secretion and associated organ dysfunctions.

Heterotypic cell model: the anterior pituitary gland

Anatomy and development

The pituitary gland is located below the hypothalamus, close to the optic chiasm, in a recess of the sphenoid bone described as the sella turcica (Turkish saddle). It is divided into two anatomically distinct lobes which have differing embryonic origins: the anterior pituitary is derived from oral ectoderm, whilst the posterior pituitary and stalk joining the gland to the base of the hypothalamus develops from neural ectoderm located on the base of the forebrain (208). The posterior pituitary contains axonal projections from the hypothalamic neurons producing oxytocin and vasopressin and can be considered a completely distinct organ from the anterior pituitary (271). As such it will not be considered further here. The adult anterior pituitary is made up of five distinct hormonal cell types: gonadotrophs producing luteinizing hormone (LH) and follicle stimulating hormone (FSH); somatotrophs producing growth hormone (GH); thyrotrophs producing thyroid stimulating hormone (TSH); corticotrophs producing adrenocorticotropic hormone (ACTH); and lactotrophs producing prolactin (PRL) which are all derived from cells which express *Sox2* (331) and differentiate into specific lineages through expression of a cascade of transcription factors (92). Hormone-negative, Sox2 positive cells are also present in the adult pituitary, can differentiate into all anterior pituitary endocrine cell types and a small proportion of these have characteristics of adult stem cells (13, 74, 331).

Included in the fraction of Sox2-positive cells with stem cell characteristics are folliculostellate cells, glial-like cells which express the proteins S100 and GFAP (13). Finally, there is a rich plexus of vasculature within the gland, essential for both delivery of hypothalamic regulatory factors and distribution of secreted pituitary hormones to the periphery (221). The developmental origin of this vasculature and its regulation is not well defined; given its importance in the function of the gland, this an area of research requiring further detailed investigation.

Postnatal dynamics of cell populations

The cell number of all pituitary cell types continues to increase after birth, and in addition there is considerable cell turnover, particularly in the young adult, which slows in later adulthood (233). Common to all axes is the ability to increase pituitary cell number in response to altered hypothalamic regulation or peripheral organ feedback. Overexpression of growth hormone releasing hormone in transgenic mice, for example, leads to somatotroph hyperplasia (17) and a similar increase in the lactotroph population results from loss of dopamine inhibition (18, 353). Target-organ ablation leads to increased numbers of corticotrophs and gonadotrophs following removal of the adrenals and gonads, respectively (290). Dramatic and reversible changes in cell number have been described in normal physiology for the lactotroph cell population, with an increase in cell number associated with the increased demand for prolactin during lactation in both rats (164) and humans (147). This may be species specific, as studies in mice have shown either no increase in lactotroph cell number during lactation but an increase in cell volume (66, 180) or only a modest increase (175). These changes in cell number could result from transdifferentiation between cell types (430), which may be identified in a population of cells in the pituitary which express both growth hormone and prolactin (mammosomatotrophs) (135). Again, however, more recent studies in mice suggest that in this

species, at least, only a small proportion of lactotrophs arise by transdifferentiation of somatotrophs (243) and that there is no corresponding transdifferentiation reversal when the requirement for increased prolactin ceases at weaning (66). Other lineage tracing studies in mice has shown that transdifferentiation does not occur in other cell types (LH-Cre (72), TSH-Cre (65) and POMC-Cre (225)) and that increases in the number of differentiated cells must occur as a result of proliferation of differentiated (355) or progenitor cells (290) which then presumably differentiate by the same cascade of transcription factor expression as that described in the embryo.

In the end-organ ablation studies of pituitary cell proliferation described above (290), it was noted that the increase in cell number resulted from increased proliferation of hormone negative cells. This may indicate an important functional role for the Sox2-postive pituitary cell population with characteristics of stem cells (see above). Indeed, lineage tracing following adrenalectomy has revealed an increase in corticotrophs derived from Sox2-positive cells (332). It is noteworthy, however, that the increase in corticotrophs in response to adrenalectomy is limited and ceases within a few weeks, despite continued loss of end-organ feedback (290). This limited proliferative capacity of potential pituitary stem cells has also been demonstrated in models with inducible ablation of somatotrophs (44, 136, 137, 448). Thus, whilst the evidence for pituitary stem cells is strong, it is likely that they normally have limited regenerative capacity and have a minor role in normal pituitary cell turnover. This is supported by lineage tracing studies over extended time periods (13), by transgenic mouse models showing that ablation of the Sox2 population does not significantly alter normal pituitary physiology (338) and by studies showing that division of differentiated corticotroph cells replenishes this cell population during normal physiology (224).

Plasticity

A key feature of pituitary hormones is their ability to dramatically increase their output in response to physiological demand. For some of these, this plasticity goes beyond the normal feedback regulation of endocrine axes that maintains homeostasis, or even allostasis, and can lead to dramatic changes in physiological function. Examples of this include the change in growth hormone output that occurs at puberty in a range of species (173) and seasonal variation and reproductive variation in prolactin output, supporting successful pregnancy and rearing of offspring and other associated changes such as horn growth and pelage shedding in sheep (238). Plasticity also includes differences that could exist between males and females. The effect of female physiology on the physiological control of reproduction, pregnancy and growth by the pituitary are well characterised (180, 357, 364) but further characterisation of the stress and thyroid axes are required (141, 310).

Homotypic cell organisation

Despite *in vitro* evidence that cell-cell contact may affect basal and stimulated hormone release (described above) pituitary cells have classically been described as essentially randomly organised, although some large-scale differences in the density of various cell types had been noted (361). This concept, that there is an essentially random distribution of each pituitary cell type, was largely a result of their apparent distribution in immunostaining of thin tissue sections. With the advent of techniques allowing imaging of thick tissue sections in depth, however, it has become apparent that there is a large-scale 3-dimensional organisation of cells (described in more detail below), which is distinct for each cell-type.

Somatotroph cells

Somatotroph cells comprise 25-40% of all pituitary cells dependent on gender and age and are first detected in the developing pituitary at embryonic day E15.5. Their organisation is

currently the best characterised of the anterior pituitary cell types, in part because the development of a transgenic mouse with fluorescently labelled somatotrophs preceded those labelling other differentiated pituitary cells (247). Imaging of these fluorescently labelled cells not only allows their ready identification in thick sections or intact glands but also allows monitoring of cell movement in pituitary slices (364). Imaging of embryonic glands reveals that somatotrophs initially appear as single cells (41) but within a day, at E16.5, contact each other to form a continuum, thus one day before the gland begins to release GH (Figure 17). Whilst this would be expected if the cells divide and remain attached to their neighbouring cells, monitoring of cell dynamics has shown that cell division at this stage is minimal (92) and cell movement extensive (364), indicative of an active process of homotypic cell network formation. After birth, as rapid differentiated cell proliferation expands the somatotroph population (61, 406), the network organisation of somatotroph into a continuum is maintained, with clusters of somatotrophs linked with strands of single cells (Figure 17). The dependence of this clustering on the hypothalamic regulator growth hormone releasing hormone (GHRH) is demonstrated by a comparison of transgenic mice with reduced somatotroph numbers resulting from their ablation or reduction from loss of GHRH: similarly low numbers of somatrophs cluster in animals with intact GHRH stimulation but are isolated as single cells in its absence (433). The organisation is sexually dimorphic, with a more robust network organisation and increased clustering in males in comparison to females, and modifiable by altering sex steroid exposure (357, 363). This demonstrates that the organisation is not hard-wired in development but instead has a degree of plasticity, which allows modification in response to altered physiological challenge. Indeed, there is augmented clustering in males coincident with the post-pubertal increase in pulsatile GH secretion, and this is later reversed when the demand for pulsatile GH is reduced (41) (Figure 17).

Lactotroph cells

Lactotroph cells comprise approximately 35% of anterior pituitary cells in females, but only around 20% in males, consistent with the relative differences in physiological requirement for the hormone in each sex. They are detected from E17.5 but in very small number before birth (126)and then the population increases rapidly from birth until 5-8 weeks of age (191). The generation of transgenic mice with DsRed fluorescent protein expressed in lactotrophs has allowed similar studies of their organisation to that somatotrophs cells; imaging of thick section of pituitary in 3D reveals a similar but distinct network organisation, with a honeycomb structure formed of rings of a single layer of lactotrophs (180). During lactation, this honeycomb structure is more complete, with a decrease in gaps within the structure and increased contact between cells. This is consistent with a role for endocrine networks in mediating high levels of hormone output, as the network modification only forms if there is a high demand for prolactin secretion. Remarkably, when high demand has driven network modification, this persists for months after weaning, despite prolactin secretion being low throughout this period. Thus, there is a contrast with the somatotroph network reorganisation post-puberty, which is reversible.

Other anterior pituitary cells

The homotypic organisation of other anterior pituitary endocrine cell types has been studied in less detail than that of somatotrophs and lactotrophs, and in the case of thyrotrophs there is no detailed published description. Immunostaining of rat pituitaries has shown that some thyrotrophs form isolated clusters (32) but since thin sections were used in this study any extensive 3D organisation would not have been apparent.

Transgenic mice with fluorescent labelling of gonadotrophs ((56) has shown that these appear as isolated cells at E17.5 but within one day form clusters. In the adult, gonadotrophs are organised as strings of cells but are divided into two populations- whilst a network of cells

located at the ventral surface of the gland have projections towards the centre of the gland, another network, which is also organised as strings of cells, remain close to the surface and mainly arise during the first two weeks post-natally. Altered network organisation has not been described in differing physiological states, however, there are large scale changes in the distribution of gonadotrophs at puberty and clustering of cells in lactating animals (9).

Corticotrophs are the first pituitary cell type to terminally differentiate, with fluorescently labelled cells in POMC-GFP transgenic mice first appearing as isolated cells at E13.5 (56). Like gonadotrophs, these rapidly cluster and then become strands of cells that extend into the centre of the gland, an organisation which is maintained throughout life. In contrast to the other pituitary cell types, however, these cells have long cytoplasmic projections, or cytonemes, which maintain contact between apparently isolated corticotrophs and may facilitate communication within this homotypic cell population. Note that corticotroph somas are located far away from fenestrated capillaries and send cytonemes containing ACTH granules towards perivascular spaces.

The glial-like folliculostellate (FS) cells found throughout the anterior pituitary gland are also organised into an anatomical network (431), however, a lack of specific markers for this cell type early in development (304) has precluded study of its initial formation and organisation. Since this population of cells is likely to include the potential stem cells of the pituitary (see above) their organisation suggests that this network of cells may form a niche, which has been shown to be required for stem cell maintenance in a number of systems (201).

Heterotypic cell organisation

A key feature of the homotypic networks described above is that they are interspersed, a feature which allows the generation of the varying distinct motifs. Examples of this are the intermeshed networks of lactotrophs and somatotrophs and whilst this could occur as a consequence of each

pituitary cell type developing from a common progenitor, with multiple foci of homotypic cell differentiation and proliferation, the zonal organisation of cell types in fish (452) and large-scale movement of cells observed during development suggests that this organisation is an active process with functional consequences (227). Indeed, there may be a role for early differentiating cells providing a scaffold in directing the differentiation, proliferation and organisation of other cell types, since loss of corticotrophs in TPit knockout mice leads to increased proliferation and mislocalisation of gonadotroph cells, which are also reduced in size (56). This is not a unique relationship between corticotrophs and gonadotrophs, since gonadotroph ablation leads to a specific alteration in both the organisation of lactotrophs (374) and prolactin gene expression (425). There is also strong evidence for an active interplay in the organisation of differing pituitary cell types beyond that found in development from *in vitro* studies of cell re-aggregation following cell dispersion. Pituitary cells rapidly form clumps, which coalesce (97) and recreate aspects of normal pituitary organisation and topographical affinities (10, 302, 303). Functionally, the organisation of heterotypic cells would be expected to play an important role in axes crosstalk. The juxtaposition of different cell types will allow increased paracrine signalling, especially of those factors released at low levels or which are labile, as well as gap junction signalling, since gap junctions are common between heterotypic cell types (180).

Organisation relative to the vasculature

An important feature of each of the homotypic anterior pituitary cell networks is their relationship with the rich capillary plexus that pervades the gland (299). Differential organisation of homotypic cell networks with the vasculature are exemplified by the contrast between the cell types which are most closely developmentally related, somatotrophs and lactotrophs. In the adult both clusters and strands of somatotrophs are aligned with or

surrounded by capillaries (221), whilst fragments of the honeycomb lactotroph network are both closely and distantly associated with capillaries (Figure 18). Differential organisation of corticotrophs, with long processes (cytonemes) from distant cell bodies maintaining contacting with the vasculature (56), and gonadotrophs have a similar close association to that of somatotrophs.

Functional analysis

The consequences of network organisation for altered pituitary endocrine cell function have been characterised in greatest detail in the somatotroph and lactotroph populations. Increased basal and reduced regulated release of both growth hormone and prolactin have been shown *in vitro* with loss of anterior pituitary homotypic cell contact (182, 360, 409). However, imaging the calcium activity mediating stimulus secretion coupling (105, 106, 368) has yielded the most revealing information of the functional role of networks in mediating enhanced regulation of hormone output. In cultures of pituitary slices with intact cell organisation both somatotrophs and lactotrophs show network-driven coordination of cell activity leading to an enhanced output in response to stimulation (41, 180) (Figure 19). Additionally, the enhanced network organisation of both somatotrophs and lactotrophs associated with times of increased demand described above, leads to a corresponding increase in network activity (180, 357). At all times, the network coordination is mediated, in part at least, by gap junctions since blocking this mode of communication leads to reduced cell-cell coordination. Since gap junctions are found between heterotypic cell types, and this may change during times of altered demand (180), it is possible that intermeshed networks, and in particular those involving FS cells, may mediate part of this coordination. However, disruption of somatotroph network organisation by cell ablation in transgenic models leads to loss of this coordination (433), demonstrating at least a permissive requirement for homotypic cell contact.

Whilst stimulus-secretion coupled release of secretory granule stores is clearly the principal regulator of hormone output, the rates of hormone gene transcription is also an important factor, particularly where chronic high demand results in depletion of stores. Prolactin gene transcription, for example, may determine lactotroph output in lactation (64) and a role for cell-cell communication in its regulation has been suggested in studies with prolactin gene transcription monitored with single cell resolution in luciferase and destabilised GFP reporter transgenic rats (167, 372). Furthermore, elegant mathematical modelling to determine rates of transcription has shown increased coordination in adjacent lactotrophs, which is lost following network disruption (127). This is unlikely to be unique to lactotrophs, particularly as TSH gene expression has been shown to be dependent on cell-cell contact (32).

Memory and plasticity

While the GH network undergoes plasticity in response to development or gonadal steroid input, the PRL network displays even more remarkable plasticity during pregnancy and lactation. Thus, in response to lactation, the PRL network increases in density, primarily due to increased cell size and not number, leading to increase physical connectivity (180) (Figure 20A). In terms of function, this presents with an increase in coordinated cell behaviour whose topology is characterized by the appearance of a minority of cells that host the majority of connections (Figure 20B), analogous to beta cell hubs that have subsequently been described in the islet (200). Following weaning, physical lactotroph-lactotroph connectivity is maintained with specific highly connected cells coordinating network activity still predominating, albeit more sparsely wired. In response to a second pregnancy and lactation, an increase in coordinated lactotroph activity again occurs, but this time displaying almost synchronous activity across the population, leading to an even larger upregulation in PRL secretion (~ 2-fold higher than in multiparas *vs* primiparas). Mechanistically, this network 'memory' is driven by demand, since it can be disrupted by reducing the number of pups during the first lactation,

and is associated with upregulated gap junction signalling. While this is functionally reminiscent to hub cells in the pancreas, including the ability of well-connected lactotrophs to entrain activity in distant cells, it is unlikely that hubs are hard-wired. Instead, they may alter as a consequence of ion channel expression levels, shown to possess turnover times in the hours range (88).

Pituitary blood flow, stimulus delivery and output removal

The organisation of cell networks relative to the vasculature and the dynamics of capillary blood flow will influence the delivery of both nutrients and hypothalamic regulatory factors, as well as the clearance of secreted hormone. *In vivo* imaging in anaesthetised mice has demonstrated that delivery of small molecules to pituitary regions is not homogeneous (Figure 21). Thus, the timing of exposure of pituitary cells to both a pulse of hypothalamic secretagogue and the oxygen required to meet the energetic demands of secretion varies, with a dynamic which itself may be modulated by stimulation with secretagogue (221). Whether this heterogeneous exposure has a beneficial functional consequence in regulating the function of pituitary axes is unclear, however, it does suggest a requirement for coordination of responses, which can be provided by functional networks. Similarly, the exit of secreted hormone has been modelled and again suggests that vascular organisation and blood flow has an important influence on the dynamics of pituitary hormone to the periphery, as well as intrapituitary exposure to paracrine and autocrine factors (221, 226).

Multi-unit, heterotypic cell model: the pancreatic gland

Anatomy and development

The pancreas is a large and diffuse organ located between the spleen, duodenum and stomach, and develops from the fusion of dorsal and ventral endodermal diverticula. While the exocrine pancreas is largely tasked with digestive functions, the endocrine portion regulates glucose

homeostasis through the secretion of hormones. Rather than forming two discrete parts, the endocrine pancreas is organized into oval or egg-like structures termed the islets of Langerhans (reviewed (102)). In humans and rodents, approximately 1 million and 10,000 islets, respectively, are diffusely scattered throughout the pancreatic parenchyma, with denser aggregations located close to the gastric portion. This is an unusual anatomical structure, which is highly conserved between mammals including humans, rats/mice, hyenas and camels, and even whales (384). Each islet comprises ~100-1000 cells including beta cells (insulin), alpha cells (glucagon), delta cells (somatostatin), PP cells (pancreatic polypeptide) and G cells (ghrelin) (102). While beta and alpha cell function is well-characterized, less is known about delta cell, PP cell and G cell function. Delta cells exert tonic inhibitory tone on beta and alpha cells and are likely to act as a "brake" excessive insulin and glucagon secretion (343, 420). On the other hand, PP cells primarily regulate exocrine pancreas secretion, while G cells secrete ghrelin to suppress delta cell function (4, 101). Endocrine cells originate from multipotent pancreatic precursors, with the differential expression of key transcription factors such as Pdx1, Nkx6.1, NeuroD, MafA (beta cells) (279, 365) and Arx (alpha cells) (86), determining fate specification *via* a Ngn3-positive progenitor pool (279). There appears to be an anatomical limit to islet size, with increases in hormone quantity reflected by increases in islet number rather than diameter (*e.g.* whales have the similar sized islets to humans (384)). While the mechanisms are not characterised, it is worth noting that the pancreatic vasculature is instructive for beta cell development (77). While islet size might be similar across species, the ratios and organisation of the different cell types shows marked species divergence: whereas rodent islets comprise a beta cell core (~80%) with an alpha cell mantle (~20%), human islets house more alpha cells (~50%) that are interspersed with beta cells (~50%) (58). Assessment of islet development has shown that human islets are in fact similar to rodent islets during embryogenesis, however, there is a tertiary folding step that leads to a concertina effect and the

apparent random alpha cell-beta cell arrangement (45). Indeed, a number of studies now suggest that human and rodent islet cytoarchitecture might be more similar than first envisaged (42, 114). While a role for the islet microvasculature in determining islet cytoarchitecture/size has long been assumed, this has been questioned by recent studies showing that the islet capillaries are continuous with those in the exocrine pancreas (115). In general, there is a need to re-assess islet composition/architecture aross species using high-resolution three-dimensional imaging approaches, similar to those employed to map the pituitary (114).

Glucose-stimulated insulin secretion

Single beta cells respond only to high glucose concentration due to expression of low sensitivity glucose transporters (GLUT2 in rodents, GLUT1 in humans). In the canonical pathway, glucose is taken up into the cell and phosphorylated by glucokinase to glucose-6-phosphate, before generation of pyruvate (glycolysis), which then enters the TCA cycle in the mitochondrial matrix (351). Alternative fates for glucose (*e.g.* lactate) are suppressed by suppression of genes such as *Ldha* (322). The ensuing increase in ATP/ADP ratios (211) leads to closure of K_{ATP} channels due to interactions with pore-forming Kir6.2 subunits, which together with SUR1, comprise the octameric channel through which K^+ ions pass (19, 83). The increase in membrane potential, in part driven by influx of cations such as Na^+, leads to opening of voltage-dependent Ca^{2+} channels (VDCC) (340, 341). Wheareas L-type VDCC ($Ca_v1.3$) contribute most conductance in rodent beta cells, there is increased dependency on P/Q-type VDCC ($Ca_v2.1$) in human beta cells, as well as contributions from voltage-gated Na^+ channels ($Na_v1.6$/$Na_v1.7$) (341). Extracellular Ca^{2+} floods into the cell and through interactions with SNARE proteins, drives granule fusion and release of insulin cargo (388). Ca^{2+} is also released from intracellullar stores, including the endoplasmic reticulum, Golgi apparatus, granule and lysosome, through still poorly characterised mechanisms such as Ca^{2+}-induced Ca^{2+} release,

IP3 signalling and NAADP (350). A range of other fuel sources are also important for beta cell metabolism and hence signalling, including amino acids (leucine, ketoisocaproate) and fatty acids, the latter *via* beta oxidation to acetyl CoA (321). In parallel to the canonical pathway, a range of other inputs can influence beta cell metabolism and/or signalling, including activation of GLP1R and GIPR (cAMP/PKA), as well as free fatty acid receptors (PLC/DAG) (192, 257). Of note, when K_{ATP}-channels are opened using diazoxide, glucose is still able to stimulate insulin secretion, pointing to the existence of K_{ATP}-channel independent or amplifying pathways, including cAMP, glutamate and Acyl CoA (171, 172).

Syncytial beta cell function

The islet context provides an important gain-of-function for beta cell function. Indeed, beta cells respond in a highly coordinated manner to glucose, which is thought to be critical for appropriate insulin release (reviewed in (35)). In islets, homotypic gap junction coupling *via* connexin36 is well characterised between beta cells (170, 373). Heterotypic gap junction coupling is less well characterised in the islet, although recent studies suggest that optogenetic stimulation of beta cells leads to rapid activation of delta cells, an effect blocked by the gap junction inhibitor carbenoxolone (48). Mechanistically, electrical coupling through gap junctions plays a major role in driving cell-cell coordination (258), since silencing of connexin 36 both *in vitro* and *in vivo* leads to more stochastic population activity and impaired insulin secretion (170, 326). However, in all these studies, connexin 36 is deleted in the early postnatal period and as such gap junction-independent effects on cell development and ergo function cannot be easily discriminated. Future studies using inducible knockout or connexin 36 rescue are required. Other contributing factors to islet communication may include paracrine factors such as glutamate and somatostatin (16), and cilia that connect a subset of beta cells (reviewed in (59, 348, 349).

The mechanistic origins of coordinated islet function are widely accepted to involve electrical coupling (with the above caveats noted). However, numerous studies have shown that Ca^{2+} signals within the islets obey a power-law distribution, *i.e.* a hub and node setup (200, 219, 251, 356, 392, 393), which goes against the established electrophysiological theory that all cells are similar by virtue of their coupling, despite known heterogeneity in cell traits and even gap junction permeability itself (36, 124). For example, recent optogenetic/photopharmacological studies have shown that a small subpopulations of beta cells might also contribute to coordinated islet responses to glucose (200, 443). One of the subpopulations, termed hubs/leaders possess lowered levels of the beta cell identity markers, Pdx1 and Nkx6.1, high levels of glucokinase, the rate-limiting enzyme in glucose metabolism, and low levels of insulin and the ER Ca^{2+} ATPase, SERCA2 (200). These characteristics configure hubs to respond early to rising glucose levels by increasing mitochondrial potential, without biosynthesising insulin, which would otherwise be promoted by lowered SERCA2 expression (63, 401). Hubs/leaders were subsequently shown to exist *in vivo* in islets transplanted into the anterior eye chamber, as well as in zebrafish where photoablation of hubs/leaders reduced coordinated islet activity (356). Most recently, studies used viral transduction approaches to clamp maturity across the islet showed a reduction in hub cell number and a decrease in coordinated activity, thus confirming the validity of the previous studies using an unbiased approach and extending the concept to the entire immature beta cell subclass (284). Similar beta cell subpopulations have been described that are capable of disproportionate control of islet function, including first responder cells (219), as well as ChR2.0 cells (443), and it is likely that there is some overlap between such populations and hubs/leaders. Pertinently, FLIM imaging supports the existence of hub-like cells that metabolically recruite other cells into oxidative phosphorylation (438).

Commented [MOU2]: Nathalie to David: should we define "ChR2.0 cells"?

While the role of beta cell subpopulations such as hubs in driving islet coordination is not debated, their mechanistic basis and functional relevance is more controversial (362). A number of modelling studies have shown that perturbing cells with hub-like characteristics influences beta cell dynamics in response to glucose (113, 228, 241), although removal of hubs from a coupled islet model does not perturb Ca^{2+} oscillations thus questioning their relevance (113). Moreover, experiments with multi-electrode arrays suggest that electrical coupling leads to large clusters of functional cells, in contrast to findings using Ca^{2+} imaging (197).

Very recent studies have however reconciled electrophysiological and imaging standpoints, by showing that at different glucose concentration, hubs/leaders dynamically emerge according to cell-cell communication, but that all hubs are identical (upholding electrophysiological theory) (218). Moreover, electrical coupling between beta cells cannot explain all observations in the islet, Ca^{2+} is itself an important transcriptional signal (382), and other modes of communication need to be considered when examining syncytial responses, *i.e.* paracrine, ciliary and even delta cell-beta cell coupling (48, 348). Nonetheless, it is clear that islet syncytial function is more complicated than meets the eye, conflicts with established electrophysiological principles, and as such further mechanistic evidence is required to unequivocally demonstrate the role of beta cell subpopulations in insulin secretion.

In contrast to mouse islets, human tissue does not display such sharply coordinated responses to glucose. Rather, coordination is regionalised into discrete beta cell clusters, which may reflect the unique cytoarchitecture of human islets (324, 442). However, incretins released from enteroendocrine cells following meal ingestion are able to coax globally coordinated responses in human islets, which are dependent on gap junction (Cx36) expression (178). This incretin-regulated connectivity is targeted by fatty acids and fails in obese individuals (178).

Islet-islet crosstalk

The hundreds of islets across the pancreas must presumably be able to communicate their activities to release pulses of insulin. How this might occur is largely unknown due to the technical difficulties in monitoring responses across such distances, as well as the fact that most experiments involve isolated islets. Autonomic neurons, present in the rodent pancreas, provide one likely candidate. Studies showed that pulsed delivery of muscarinic agonist is able to entrain responses between isolated islets (3), and modelling studies point to a role of neurons in synchronizing islet-islet activity *in situ* (129). Moreover, recent 3D imaging studies of cleared, whole pancreas have shown the presence of long distance NF200-positive neurons that form a network densely innervating islets in both mouse and human tissue (11). However, the chances of preserving long-range neural interactions in acute pancreatic slices (260, 393), or even detecting two islets in the same preparation, are low. As such, *in vivo* imaging approaches applied directly to the pancreas are ideally required (266), combined with optogenetics/photopharmacology/DREADDS or other modalities for the specific manipulation of single islet or neuronal function. Alternatively, islet transplantation into the anterior eye chamber may be useful, particularly when studying xenografted human tissue. Another possibility is that nutrient feedback loops might entrain islets; one model postulates that the liver senses insulin, modulates blood glucose levels and by virtue of all islets receiving blood supply, entrainment occurs (314).

Islet compensation

Expansion of functional beta cell mass occurs in the early stages of insulin resistance in addition to normal physiological states including pregnancy. The source of such adaptation is still debated and may be secondary to increased beta cell size, number, transdifferentiation, insulin content, function or combinations thereof (69, 262, 268, 400). Whilst a decrease in the

insulin immunopositive area seems to be a universal feature, recent studies in *Pax6*-null animals, as well as tissue from donors with type 1 diabetes have shown the continued presence of islets, as demarcated using chromogranin or low (but detectable) insulin levels (223, 270). This raises the possibility that beta cells remain during type 1 diabetes, but with reduced identity, and that restoration of beta cell mass may require reinstitution of insulin biosynthesis, as well as regeneration of beta cells from various cell types (progenitor, alpha cell, gamma cell, stomach, ductal cell) (15, 86, 139, 454). Interestingly, longitudinal imaging studies have demonstrated that reductions in beta cell/islet function parallel and outweigh any changes in mass (75). Investigating whether such defects/adaptations occur throughout the islet population within the intact pancreas, or just in a subpopulation, is paramount to understanding whether insulin secretory capacity can be restored during diabetes. Indeed, beta cell adaptation in duodenal, gastric and splenic regions is topologically heterogeneous when assessed using morphometry of immunostained sections (122), and regional differences are seen during beta cell loss (437).

Conclusion and Perspective: Studying endocrine cell networks in the 21st century

It is clear that the adrenal gland, pituitary gland and pancreatic islets are all highly-organized structures. By encompassing multiple levels of regulation, spanning cell networks through to neural input, these endocrine/neuroendocrine tissues are able to robustly, repeatedly and appropriately secrete hormones in response to demand throughout the lifecourse. In addition, all three structures must undergo considerable plasticity at different timepoints, often converging on top of one another. Throughout this chapter, we have highlighted the differences in organization and function of these networks, but common mechanisms that regulate

hormone secretion are present in all of them. Heterogeneity in cell responses to secretagogues indicates that some cells of the network will respond to specific stimuli while others will not. Providing a further level of regulation, plasticity of intercellular coupling through gap junctions and/or other modes of cell-cell communication allows incoming signals to be processed and propagated from cell to cell in a specific manner, and is relevant for the coordination and/or synchronization of hormone release from endocrine and neuroendocrine tissues (177, 226).

The 'hypercommunication' present within the endocrine/neuroendocrine gland networks can also become a disadvantage when it comes to chronic challenge. Indeed, it is likely maladaptive if all cells respond synchronously to a chronic stimulus, since other mechanism must also increase their output to maintain homeostasis. The pancreas and its exposure to metabolic stress is a good example of this. Islet cell populations are highly heterogeneous (21, 36). Thus, beta (and other) cell states exist, characterized by their maturity, secretory capacity, proliferation, function, senescence and vulnerability to immune attack. Following metabolic stress, this heterogeneity changes and as such might become constrained, limiting system plasticity and predisposing to failure. Highlighting the importance of heterogeneity, immature beta cells tend to be proliferative and poorly functional (24, 103, 378, 421) (reviewed in (36)). However, these cells are well placed to ramp up de novo protein system when their more mature highly-secretory counterparts need a break. If this switch cannot happen, the latter state would become prone to ER stress and failure. Alongside this, beta cell subpopulations capable of disproportionate control (200, 443) are metabolically adapted and as such might be vulnerable to metabolic stress, leading to heightened generation of reactive oxygen species.

Another way to counterbalance the danger that limiting plasticity of each communication system might imply is to interconnect them, offering thus additional possibility for the organ/tissue to adapt to a challenging dysregulation of its homeostasis. In this context,

the adrenal medulla is examplary, with a continuous and reciprocal crosstalk between the cholinergic synaptic neurotransmision and the gap junction coupling (81, 157, 255). Although this dialogue is primarily engaged in 'physiological' conditions (*i.e.* leading to adjust catecholamine secretion to body needs), one can reasonably envision its involvement in more detrimental situations, such as chronic stress challenges or overshoots of epinephrine secretion.

Another fundamental aspect of cell networking within endocrine tissues deals with the origin of the cells that initiate cell-cell communication. A remarkable example of the importance of understanding the origin of these initiating cells is the network-driven coordination of signal-transduction that depends on a hard-wired, blue-printed information transfer from specialized "hub", "leader" or "first responder" cells towards cell followers in pancreatic islets (200, 219, 356). The network system is highly functional in homeostatic conditions but is impaired when specific cells fail in the face of cytokine or fatty acid challenge (200). While plasticity is inherent in pancreatic cell networks, their logic is not adapted to the uncontrolled metabolic demands that we have encountered during the past decades. More flexible trajectories exist in other endocrine/neuroendocrine networks which control basic body functions such as growth (146, 226, 357).

While our understanding of endocrine/neuroendocrine networks in animal models has dramatically improved, it is now necessary to study the function of endocrine/neuroendocrine glands in humans to determine translational relevance from model species, especially given some of the reported species differences. The challenge in humans is also to be able to study and visualize endocrine functions in healthy adult volunteers. Indeed, most of our knowledge has been learnt from samples that were either collected postmortem, sometimes from foetuses, or from sick patients. It is therefore necessary to develop non-invasive techniques that allow

anatomical and functional investigation of endocrine/neuroendocrine functions in healthy patients.

Functional magnetic resonance imaging (fMRI) is a powerful tool to safely and non-invasively study brain processes. fMRI studies rely on a measure called blood-oxygenation-level dependent (BOLD) contrast, which is based on the different magnetic properties of oxygenated and deoxygenated blood. Increased neuronal activity in the brain elicits a local haemodynamic response, which causes an increase in blood flow that results in local magnetic field disturbances that can be detected on a T2-weighted imaging protocol. While BOLD-MRI has been mainly used for brain research and as a diagnostic aid, it has proven equally useful for the study of kidney disease (287), as well as pancreatic function (73). Resting-state fMRI (similar to BOLD-fMRI except that the patient is not required to perform any task or are not subjected to any drug challenge) are used to study the functional connectivity between different anatomical structures. This technique would be typically used to study the functionnal connectivity between different brain areas but it can be adapted to study the connectivity of the pituitary (214) and can also be used to visualize circadian variations of brain-pituitary connectivity within the same individual. Optoacoustic imaging, also termed photoacoustic imaging, is an alternative method for visualizing endocrine physiology and disease at different scales of detail: microscopic, mesoscopic and macroscopic. Similar to MRI contrast, optoacoustic contrast arises from endogenous light absorbers, such as oxygenated and deoxygenated haemoglobin, lipids and water, or exogenous contrast agents, and reveals tissue vasculature, perfusion, oxygenation, metabolic activity and inflammation on the basis of their absorption spectra. Multi-spectral optoacoustic tomography (MSOT) allows high resolution imaging and has now been used in both mice and humans to assess a number of different conditions associated with endocrinology including oxidative metabolism, muscle oxygenation and hypoxia, cardiovascular imaging, muscular dystrophy, inflammation, and vascularization

and total blood volume ((206) for review). This technique only allows a tissue penetration of 2-4cm at high resolution, therefore imaging of the pancreas and the adrenals for example remains a challenge in humans. However, MSOT provides non-invasive, label-free imaging of contrasts that relate to tissue function and metabolism, offers portability (hand-held device) and good level of safety so that it can be used for frequent and longitudinal studies. It can also operate concurrently with ultrasonography, using the same ultrasound detector, enabling the simultaneous collection of complementary information (206). Finally, we can imagine that the signal-transduction signature of endocrine cell networks, which is in most cases is monitored with microscopic imaging of calcium signals can also be studied with mesoscopic functional ultrasound imaging (fUS) which allows to detect rapid changes in blood flow related calcium signals in cell neighbours (23). Since fUS allows ultrafast imaging in depth *via* the cortex, and even deeper in infants *via* the fontanelle cleft (30), we can propose that such techniques would allow detection of the changes in pituitary blood flow (221) as non-invasive, on-line reporters of the blood supply of oxygen and metabolites that dynamically changes during the highly coordinated hormone pulses delivered by the pituitary gland. Such approaches may be essential if the potential for the use of pituitary stem cell therapy for the treatment of hypopituitarism is to be realised. The transsphenoidal access to the human pituitary that allows surgery for pituitary tumours would also provide a potential route for delivery of stem cells but monitoring of tissue regeneration, its vascualrisation and restoration of endocrine function would be facilitated by fUS. Development of such approaches would then easily be applicable to other glands with functional cell networks like the adrenal gland.

An important feature of endocrine/neuroendocrine glands is that they not only need to remodel and adapt their activity to respond to momentary demands, but they also need to work with one another to trigger a coordinated response to ensure an effective response to homeostatic disruption. An excellent example of this is the response to acute stress, with the

amygdala, responsible for processing fear, arousal, and emotional stimuli, signalling to the hypothalamus, activating the sympathetic nervous system and the adrenal medulla, which in turn releases a surge of catecholamines. This is followed by activation of the HPA axis, increasing levels of circulating glucocorticoids (GCs) from the adrenal cortex, resulting in increase supply of blood glucose to the brain. The increased gluconeogenesis, decreased glycogen synthesis, decreased glucose uptake and consumption, and increase protein degradation driven by GCs is augmented by cortisol action on the pancreas to decrease insulin secretion and increase glucagon release. The overall net effect is an increase in available glucose thus supplying an immediate energy source to the brain and the large muscles. This sequence of events is simplistic but illustrates the fact that the reponse to a single acute stressor involves a wide modification of the physiology of the individual. Superimposed on this is the impact of patterned hormone secretion, which in the case of GCs has been shown to be a result of dynamics of feedforward and feedback regulation of the HPA axis (435) in the absence of stress, and its important impact on target cell function (383). Given that the effect of stress on a number of sequelae is dependent on the timing of the stressor relative to the phase of a GC pulse (359, 450), these interactions are clearly complex and the effect on multiple axes are not easily predictable.

The powerful interaction between axes also has important implications when it comes to chronic endocrine/neuroendocrine diseases. In the stress axis example above, for example, following protracted stress and sustained GC secretion, the hypothalamic control of the HPA axis changes (reduced sensibility to GC feedback, changes in CRH/vasopressin balance), leading to changes in the secretory activity of corticotrophs. At the same time, the insulin resistance provoked by excess GC leads to insulin hypersecretion from pancreatic beta cells, and their functional adaptation. However, the newly imposed demands for insulin secretion leave beta cells vulnerable to endoplasmic reticulum stress and apoptosis (type 2 diabetes).

These effects or 'side-effects' of chronic axis activation are debilitating for patients, they could represent an opportinity to predict the state of the system or provide an indirect window into endocrine/neuroendocrine gland function. A greater understanding of the interactions between endocrine systems may provide an opportunity both for discerning aspects of dysfunction, inform therapy and provides an impetus to develop methodologies for dynamic hormone replacement (*e.g.* (347)) or stem cell therapy.

Overall, in the past decade, our understanding of endocrine/neuroendocrine functions in health and diseases has dramatically evolved. New investigation techniques and animal models have shown that endocrine glands are highly organized into functional networks and that chronic demands are able to disturb these networks leading to body-wide pathological repercussions. Challenges for the years to come will be to find ways to reset these networks and broaden our knowledge of human physiology in order to offer a more comprehensive clinical management of endocrine/neuroendocrine disorders.

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Figure legends

Figure 1: Compared anatomical organization of the endocrine/neuroendocrine cells within the secretory tissue

(A) Lobular organisation of the chromaffin cell tissue within the rat adrenal medulla. Left picture, Bodian silver-stained nerve fibres in acute slices. Dashed lines delineate lobules. Adapted, with permission, from Kajiwara R, et al., 1997 (202). Right picture, clusters of TH immunoreactive chromaffin cells. (B) Organisation of the mammalian anterior pituitary. Schematic of a hemi-anterior pituitary showing the preferential organisation of the five hormone-secreting cell types. (C) The endocrine pancreas is a multi-unit structure, where upwards of a million islets are diffusely scattered throughout the parenchyma. Each human islet of Langerhans is a self-contained endocrine unit, comprising a number of cell types that secrete hormones involved in glucose homeostasis and other facets of metabolism. Images adapted from Servier Medical Art under a CC-BY licence (https://creativecommons.org/licenses/by/3.0/).

Figure 2: Slice tissue preparations for studying endocrine/neuroendocrine function

(A) Acute pituitary slice and imaging methods to study pituitary cells *in situ*. (Aa) Acute slice preparation. The pituitary gland is immobilized within a droplet of ultra-low temperature gelling agarose, before cutting at low temperature (1-4°C) with a vibrating microtome blade. (Ab) Imaging of pituitary cells *in situ*. Pituitary cells within an acute pituitary slice are imaged with two-photon excitation microscopy (horizontal red lines). (Ac) Image deconvolution and 3D reconstruction of image stacks help to view the three-dimension organisation of fluorescent cells in the pituitary tissue. Adapted, with permission, from Fauquier T, et al., 2002 (125). (B) Organotypic culture of a rat anterior pituitary gland, according to the roller tube technique described by Gahwiler (140). Measurement of prolactin secretion by radioimmunoassay in a 4

week-old culture treated either with forskolin to increase hormone release or with the dopaminergic agonist bromocriptine (CB-154) to inhibit PRL release. Adapted, with permission, from Guerineau NC, et al., 1997 (161). (C) Organotypic co-culture of mouse thoracic spinal cord and hemisectioned adrenal gland (roller tube technique, 42 days *in vitro*). Chromaffin cells still express the biosynthetic enzyme TH, exhibit spontaneous action potentials and spontaneous post-synaptic excitatory currents (patch-clamp recordings). The thoracic spinal cord also exhibits spontaneous synaptic currents.

Figure 3: *Ex vivo* tissue and whole organ preparations for studying endocrine/neuroendocrine function

(A) *Ex vivo* preparation of the pancreas. (Aa) Procedure for mouse pancreas perfusion and removal. Adapted, with permission, from Li DS, et al., 2009 (235). (Ab) Islets (~40-80) are perifused with buffer containing various glucose concentrations or generic depolarising stimulus (KCl). Insulin secreted into the perfusate is assayed for insulin over a number of timepoints, revealing first and second phase responses. Images adapted from (235) and from Servier Medical Art under a CC-BY licence (https://creativecommons.org/licenses/by/3.0/). (B) *Ex vivo* rat spinal cord-splanchnic nerve-adrenal gland preparation. (Ba) Ventral view of the thoracic section isolated from the animal. (Bb) Schematic representation helping to identify the various tissular components of the preparation. (Bc) Experimental protocol to assess catecholamine secretion. The splanchnic nerve is electrically stimulated by a cuff electrode (CE). Catecholamine release is monitored by cyclic voltammetry with a carbon fibre electrode (FE) positioned at proximity of the medullary tissue (AM), which was unmasked after gland hemisection. Adapted, with permission, from Wolf K, et al., 2016 (451). (C) *Ex vivo* tissue preparations preserving the connections between the pituitary gland and the brain in the fish. (Ca) Use of a hypothalamus-pituitary slice to monitor Lucifer yellow diffusion between

pituitary cells in the teleost fish *Oreochromis niloticus*. Adapted, with permission, from Levavi-Sivan B, et al., 2005 (232). (Cb) Gonadotropin-releasing hormone (GnRH)-triggered cytosolic calcium rises imaged in fura-2-loaded gonadotrophs from a whole brain-pituitary preparation of the teleost fish *Oryzias latipes*. Adapted, with permission, from Karigo T, et al., 2014 (205).

Figure 4: *In vivo* monitoring of the activity of endocrine/neuroendocrine cells

(A) Cellular *in vivo* imaging of the pituitary gland in fluorescent protein-tagged transgenic mice with long-range microscopy. (Aa) Schematics of the experimental arrangement. An air-transmission 20x magnification objective with 2.8 cm working distance is fitted on a fluorescent microscope, equipped with a variable light beam excitation and an EM-CDD camera acquisition setup. It allows real-time *in vivo* imaging with adaptive final magnification (8-800x) of the ventral side of the pituitary gland is exposed in an anesthetized mouse after removal of the palate bone. (Ab) Fluorescent image (λex 488 nm) of the open palate bone of a GH-eGFP mouse. Aa and Ab, adapted, with permission, from Lafont C, et al., 2010 (221). (Ac) Schematic shows the arrangement of calcium imaging in head-fixed animals injected with AAV5-CAG-GCAMP6s particles into the pituitary. (Ad) Field of GCAMP6s cells viewed from the dorsal pituitary side with the selection of cells as regions of interest (ROIs) shown in coloured circles. (Ae) Coordinated calcium spikes recorded in the cells shown in Ad. Ac-Ae, adapted, with permission, from Hoa O, et al., 2019 (176). (B) *In vivo* extracellular recording of spontaneous chromaffin cell electrical activity in an anaesthetized mouse, illustrated by the occurrence of both individual spikes (Ba, left inset) and bursting activities (Ba, right inset). (Bb) The electrical firing is reversibly abolished by a local application of the voltage-gated Na^+ channel blocker TTX on the splanchnic nerve. (Bc) Reversible decrease of the nerve stimulation-evoked electrical activity in the presence of the nicotinic acetylcholine receptor

antagonist hexamethonium. Adapted, with permission, from Desarmenien MG, et al., 2013 (100). (C). *In vivo* approach enabling simultaneous stimulation of the splanchnic nerve and adrenal venous blood collection in anaesthetised mouse. After laparotomy, the left adrenal gland is uncovered and the renal vein and the conjonctive tissue containing the splanchnic nerve are isolated. The splanchnic nerve is placed on a bipolar stimulation electrode. The renal vein is ligated on both the kidney and vena cava sides to form a reservoir in which a heparinised catheter is implanted. Adrenal venous blood samples are collected before and during splanchnic nerve stimulation. Assay of catecholamine by ELISA shows that E and NE are secreted in a stimulus frequency-dependent manner. Adapted, with permission, from Desarmenien MG, et al., 2013 (100).

Figure 5: Monitoring of hormone release in individual endocrine/neuroendocrine cells (A) Amperometry-based measurement of catecholamine secretion in rat acute adrenal slices. Catecholamine release is evoked by an iontophoretic application of the cholinergic agonist nicotine. (B) Use of the fluorescent probe FFN511 accumulated in secretory granules to track exocytosis in a mouse chromaffin cell. Individual granules are imaged by total internal reflection fluorescence microscopy. In response to high K^+-containing saline (dotted line), the fluorescence quickly decreases, reflecting granule exocytosis. Adapted, with permission, from Gubernator NG, et al., 2009 (154).

Figure 6: Both extracellular and intracellular Ca^{2+} sources contribute to hormone exocytosis

Simultaneous measurement of membrane capacitance and cytosolic Ca^{2+} concentration $[Ca^{2+}]i$ in isolated endocrine/neuroendocrine cells. Representative examples of membrane capacitance increase in response to (A) a Ca^{2+} entry through voltage-gated Ca^{2+} channels in a bovine

chromaffin cell. Adapted, with permission, from Augustine GJ and Neher E, 1992 (20), (B) a Ca^{2+} entry through ligand (nicotine)-gated ion channels in a bovine chromaffin cell. Adapted, with permission, from Mollard P, et al., 1995 (275) and (C) a Ca^{2+} release from internal stores in response to gonadotropin-releasing hormone in an isolated rat gonadotroph. Adapted, with permission, from Tse A, et al., 1993 (418). Note that the source of Ca^{2+} engaged in exocytosis (extracellular, intracellular or both) relies on the mode of action of each secretagogue. All mechanisms illustrated here can occur in all tissues, but unlikely to equal extent.

Figure 7: Monitoring dye diffusion/transport *in vivo*

Schematics of the experimental arrangement. An air-transmission 20x magnification objective with 2.8 cm working distance is fitted on a fluorescent microscope, equipped with a variable light beam excitation and an EM-CDD camera acquisition setup. This allows real-time *in vivo* imaging with adaptive final magnification (8x-800x) of the ventral side of the pituitary gland/pancreas surgically exposed in an anesthetized mouse. A jugular catheter is placed for *i.v.* injection and blood sampling and the pituitary/pancreas surfaces are continuously irrigated with saline through inlet and outlet tubes, respectively. Top panels: *In vivo* imaging of rhodamine dextran-labelled vasculature in the pancreas (left) and pituitary gland (right).

Figure 8: Optogenetic control of hormonal secretion in endocrine cells

(A) Optogenetic stimulation of endocrine cells in the pituitary cells expressing optogenes after viral transfection. (Aa) Laser light illumination of the ventral pituitary side can be carried out in terminally anesthetized mice using a fibre optic acutely placed above the pituitary gland and connected to a laser source. Blood is sampled simultaneously using a jugular catheter or tail-tip blood sampling. (Ab) Optogenetic stimulation of the pituitary cells can be carried out in awake mice in which a fibre optic has previously been chronically implanted directly above

the target cells. Mice are subjected to simultaneous tail-tip blood sampling. (Ac) GH pulses triggered by a train of laser blue light pulses (300-ms pulses at 1 Hz) *in vivo* in GH-Cre mice expressing ChR2 specifically in GH cells. Adapted, with permission, from Hoa O, et al., 2019 (161). (B) Photopharmacological modulation of insulin secretion *in vivo*. (Ba) Photopharmacology relies on endogenously expressed receptors and ion channels to optically control cell function. The fourth-generation light-activated sulfonylurea, JB253, converts ATP-sensitive K^+ (K_{ATP}) channels into molecular photoswitches, allowing reversible stimulation of beta cell function. The light-activated sulfonylurea JB253 thus allows optical control of K_{ATP} channel activity, voltage-dependent Ca^{2+} channel activity and insulin release. (Bb) Schematic showing the experimental protocol for optical control of glucose homeostasis using JB253. (Bc) JB253 (50 mg/kg, administered *per os*) significantly lowers glycemia in animals exposed to blue light. Adapted, with permission, from Mehta ZB, et al., 2017 (240). Images adapted from Servier Medical Art under a CC-BY licence (https://creativecommons.org/licenses/by/3.0/). (C) Optical modulation of stimulus-secretion coupling in adrenal chromaffin cells. (Ca) Modulation of stimulus-secretion coupling using a synthetic chemistry approach based on ruthenium diimine complexes in a RubpyC17-loaded mouse chromaffin cells (right picture). (Cb) Light illumination electronically excites RubpyC17 and generates a photoexcited complex that is either an electron donor or an electron acceptor. The presence of a reductant substrate in the extracellular medium allows electron transfer from the donor to RubpyC17, generating a negative surface potential at the cell membrane, which is detected by the cell as a depolarization. Optically driven changes in the electrical activity (Cc) and catecholamine secretion (Cd) in a RubpyC17-loaded mouse chromaffin cell. Adapted, with permission, from Rohan JG, et al., 2013 (311).

Figure 9: Macroscopic anatomy and innervation of rat the adrenal gland

(A) Double immunofluorescent staining of laminin (in green) which labels both the cortex and the medulla and tyrosine hydroxylase (in red) which stains the neurosecretory chromaffin cell tissue. Note the chromaffin cell organization in clusters. (B) Schematic representation of the spinal-adrenal innervation. The preganglionic innervation synapsing onto chromaffin cells extensively occurs through a direct pathway without relaying the sympathetic chain or the suprarenal ganglion. (C) Immunofluorescent detection of nerve fibres invading the medulla with an anti-neurofilament antibody.

Figure 10: Scheme of the adrenal medulla stimulus-secretion coupling

Two interconnected pathways underlie adrenal catecholamine secretion from chromaffin cells. The master stimulus comes from ACh release by splanchnic nerve terminals synapsing onto chromaffin cells. In addition, and reinforcing or even supplanting the cholinergic synaptic neurotransmission, chromaffin cell intercellular communication mediated by gap junctions contributes to hormone release.

Figure 11: Gap junctions in the rodent chromaffin cell tissue

(A) Immunohistofluorescent detection of Cx36 in the mouse medulla. Note the punctiform labelling, representative of gap junctional plaques. (B) Lucifer yellow diffusion between gap junction-coupled rat chromaffin cells in acute adrenal slices. The fluorescent dye was introduced into a single chromaffin cell through a patch pipette, and diffused into coupled cells within a couple of minutes.

Figure 12: Monitoring functional gap junction-mediated intercellular coupling by electrophysiological recordings of cell pairs

(A) Schematic representation of the dual patch-clamp technique, used to monitor junctional currents in a chromaffin cell pair. Two adjacent cells (cell 1 and cell 2) are patch-clamped and recorded in the current-clamp mode. A depolarizing current step is applied to cell 1 (cell 1*) and the subsequent membrane potential changes are simultaneously monitored in both cell 1 and cell 2. (B) Cell 1 depolarization does not evoke potential changes in cell 2, indicating that the two cells are not functionally coupled by gap junctions. (C and D) Propagation of the depolarization evoked in cell 1, resulting either in a small depolarization in cell 2 (= weak electrical coupling) or in the triggering of action potentials (= robust electrical coupling). Regarding subsequent hormone secretion, the latter case is particularly relevant, because of the $[Ca^{2+}]i$ transients ensuing each action potentials.

Figure 13: Functional gap junctional coupling between chromaffin cells

Rat acute adrenal slices were loaded with a calcium-sensitive fluorescent probe and $[Ca^{2+}]i$ were simultaneously imaged in neighboring chromaffin cells. (A) A single cell was electrically stimulated, leading to a burst of action potentials. A $[Ca^{2+}]i$ increase is recorded in the stimulated cell, as expected, but also in an adjacent chromaffin cell. Adapted, with permission, from Martin AO, et al., 2001 (254). (B) Similar simultaneous $[Ca^{2+}]i$ increases in several adjacent cells in response to a iontophoretic application of nicotine.

Figure 14: Catecholamine exocytosis in gap junctions-coupled chromaffin cells

Secretory events from individual chromaffin cells were detected by amperometry in acute slices. (A) A single chromaffin cell is stimulated by iontophoretically-applied nicotine and the ensuing rise in $[Ca^{2+}]i$ is imaged in the stimulated cell (cell 1*) and in two adjacent cells (cell 2 and cell 3). Cell 2 responded to cell 1 stimulation by an increase in $[Ca^{2+}]i$, while no $[Ca^{2+}]i$ change occurred in cell 3. (Ba and Bb) catecholamine exocytosis recorded in either cell 2 (Ba)

or cell (Bb), in response to the nicotinic stimulation of cell 1. Secretory events, observed as outward current deflections, were detected in cell 2, which exhibited $[Ca^{2+}]i$ rise upon cell 1 stimulation, but not in cell 3. Adapted, with permission, from Martin AO, et al., 2001 (254).

Figure 15: Adrenal medullary gap junctions contribute to catecholamine secretion *in vivo*

(A) Up-regulation of Cx36 immunoreactivity in the adrenal medullary tissue of stressed rats (cold exposure at 4°C, 5 days). (B) Involvement of gap junctions in hormone secretion was assessed by intraperitoneal injection of the uncoupling agent carbenoxolone (CBX), an uncoupling agent, or its inactive structural analog glycyrrhizic acid (GZA) in anaesthetised control or cold stressed mice. In control animals, CBX significantly reduces NE but not E secretion evoked by a high frequency (4 Hz) splanchnic nerve stimulation. In stressed mice in which Cx36 gap junction expression is enhanced, both splanchnic nerve stimulation-triggered NE and E release is dampered by CBX. Adapted, with permission, from Desarmenien MG, et al., 2013 (100).

Figure 16: Summary of the adaptive mechanisms of chromaffin tissue to cope with physiological stressful situations (birth, acute stress)

The data were collected from experiments carried out on both males and females, as specified. ND, not determined.

Figure 17: Somatotroph cell network development and organisation

GH cells develop to form a cell continuum across the mouse's lifespan. Surface rendering of GH-GFP cell contours from two-photon image stacks of GH-GFP pituitaries. Animal ages are indicated on the right of 3D representations of GH cell positioning (E, embryos; P, postnatal). Adapted, with permission, from Bonnefont X, et al., 2005 (41).

Figure 18: Relationships between endocrine cell networks and the pituitary vasculature

(A) Typical cell network motifs imaged in 3D using 2-photon microscopy. Top panel*:* interconnected clusters of somatotrophs in 2-month-old male GH-GFP (green) mice. Bottom panel*:* Honeycomb units of the PRL cell network in female PRL-DsRed (red) mice. (B) Hemi-pituitary-scale 2-photon imaging in a double transgenic GH-GFP (green)/PRL-DsRed (red) female mouse. The pituitary is orientated with the top and bottom of the image representing the dorsal and ventral sides of the pituitary, respectively. (C) and (D) Schematic representation of the anatomical relationship of pituitary fenestrated capillaries (grey) with the GH cell (C, cells in green), PRL cell (D, cells in red) networks. Adapted, with permission, from Le Tissier PR, et al., 2012 (227).

Figure 19: Connectivity and calcium activity of somatotrophs cells

GHRH triggers recurrent motifs of GH cell connectivity in the lateral zones. (A) Field of GH cells labeled with EGFP in the lateral zone. Only cells circled with a dashed line were also loaded with the fluorescent calcium dye fura-2. The yellow and green lines illustrate the potent cell pairs for two recorded GH cells. (B) Representative traces of calcium spikes due to electrical activity before and after GHRH (10 nM) application. (C) Linear correlation (Pearson R) between calcium recordings among all cell pairs (C1-C2, C1-C3, ... C N -1-C N) taken every 5 min. Although some cell pairs displayed high R values, no large-scale cell connectivity ($P<0.001$) was observed during spontaneous calcium spiking but recurrent motifs of connectivity among large cell ensembles were observed in lateral regions ($P<0.001$) after GHRH application. (D) Distribution of numbers of connected cell pairs vs. time of calcium recording. GHRH triggered a delayed, cycling increase in connected cell pairs. Adapted, with permission, from Bonnefont X, et al., 2005 (41).

Figure 20: Lactotroph organization and plasticity

(A) Lactotrophs are organized into a three-dimensional network. Structural connectivity is low in nulliparas (left panel) but increases in primiparas (middle panel). Following weaning, connectivity is maintained despite cessation of stimulus (right panel). Insets are typical cell profiles in prolactin-DsRed transgenic mice. (B) Top panels show representative cytosolic Ca^{2+} traces in nullipara lactotrophs (AU, arbitrary units), primiparas (middle panel) and following weaning (right panel). Bottom panel shows a functional connectivity map depicting the location of significantly correlated cell pairs. Adapted with permission from Hodson DJ, et al., 2012 (180).

Figure 21: Pituitary microcirculation and molecule diffusion

In vivo imaging of incoming molecules through the microvasculature, fate of products released into the extracellular space, and *in situ* oxygen response to GHRH. (A) Time-lapse imaging of 4-kDa rhodamine dextran in pituitary vessels at high magnification. Values indicate the time delay after *i.v.* dye injection. (B) Decay times for fluorescence clearance in blood vessels following iontophoretic injections of 4-kDa and 20-kDa fluorescent dyes, respectively. The red line represents a simulation of the 4-kDa dye clearance from the vessel as its measured blood flow. (C) *In vivo* measurements of heart rate (top trace), pituitary O2 levels (middle traces) and red blood cell velocity (bottom traces) in anesthetized GH-eGFP mice before (left) and after (right) *i.v.* GHRH injection. Adapted, with permission, from Lafont C, et al., 2010 (221).