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# Bioinformatics analyses of cell-cell interactions in the pituitary gland

**Linda Emilia Madeleine Laiho**

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**Declaration**

I declare that this dissertation has been solely composed by myself and except where stated otherwise the work presented is entirely my own. Any part of this dissertation has not been submitted for a previous degree.

Linda Laiho

Date: 31 March 2022


*The social lives of pituitary cells*

**ABSTRACT**

The anterior pituitary gland is integral to the endocrine system, regulating the activities of several peripheral organs whose secretions help maintain homeostasis. Due to its intermediate position between the brain and the periphery, the gland is often considered a passive relayer of hypothalamic regulation. However, mounting evidence suggests that the pituitary also actively coordinates and modifies its response to extracellular signals through cell-cell interactions within the gland. The studies herein aim to improve our understanding of this intercellular communication by identifying candidate molecules involved in both cell contact-based and ligand-based communication between pituitary cells. Through analysis of pituitary transcriptome data, candidate molecules mediating selective homotypic adhesion between endocrine cells were generated and subsequent examination of their expression patterns gave evidence of how adhesion specificity might be achieved in the pituitary. The role of intercellular communication during a physiological challenge was investigated by inferring paracrine responses of pituitary cells to chronic stress based on changes in gene expression. This approach revealed both broad shifts to communication patterns between cells and individual ligand-receptor interactions that may underlie these changes. These candidate molecules provide a valuable starting point for future studies aiming to understand the internal regulation of the pituitary.

**LAY SUMMARY**

The anterior pituitary gland is located under the brain and instructs different organs in the body to secrete hormones that are needed to survive based on signals coming from the brain. The pituitary can adjust how it responds to these signals through communication between the different cells in the gland. This communication can be either through physical contact between cells or through molecules that move between cells. The aim of this study was to identify factors involved in either type of communication. Using information about which genes were active in specific pituitary cells, I could predict which cells communicate with each other. First, I identified molecules that could allow cells of the same type to adhere to each other. This is important since pituitary cells of the same type are known to form networks that let them coordinate their responses. Next, I investigated how pituitary cells change their communication when an organism is under chronic stress. I found that the cells both change the number of signals they pass between each other, and I identified the specific molecules that are changed. These findings help scientists understand how the pituitary can adapt its functions to match the needs of the body.

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# ABBREVIATIONS

| | |
|---|---|
| **Ackr3** | Atypical chemokine receptor 3 |
| **ACTH** | Adrenocorticotrophic hormone |
| **Adgrl1** | Adhesion G protein-coupled receptor L1 |
| **Agt** | Angiotensinogen (serpin peptidase inhibitor, clade A, member 8) |
| **Alcam** | Activated leukocyte cell adhesion molecule |
| **Angpt1** | Angiopoietin |
| **ANP** | Atrial natriuretic peptide |
| **AT1b** | Angiotensin II type 1b |
| **ATII** | Angiotensin II |
| **ATP** | Adenosine triphosphate |
| **AVP** | Arginine vasopressin |
| **Axl** | AXL receptor tyrosine kinase |
| **β-END** | β-endorphin |
| **BBN** | Bombesin |
| **Bdnf** | Brain derived neurotrophic factor |
| **BNP** | Brain natriuretic peptide |
| **Cadm1** | Cell adhesion molecule 1 |
| **Calca** | Calcitonin/calcitonin-related polypeptide, alpha |
| **CAM** | Cell adhesion molecule |
| **cAMP** | Cyclic adenosine monophosphate |
| **CART** | Cocaine and amphetamine regulated transcript |
| **Cartpt** | CART prepropeptide |
| **CCK** | Cholecystokinin |
| **Cd44** | CD44 antigen |
| **Cd6** | CD6 antigen |
| **Cdh** | Cadherin |
| **cDNA** | Complementary DNA |
| **Ceacam1** | Carcinoembryonic antigen-related cell adhesion molecule 1 |
| **CGRP** | Calcitonin gene-related peptide |
| **CNP** | C-type natriuretic peptide |
| **Cntn1** | Contactin 1 |
| **Cntnap4** | Contactin associated protein-like 4 |
| **Cntnap5a** | Contactin associated protein-like 5a |
| **Cntnap5b** | Contactin associated protein-like 5b |
| **Cntnap5c** | Contactin associated protein-like 5c |
| **Col1a1** | Collagen, type I, alpha 1 |
| **Cort** | Corticotrophs |
| **CRH** | Corticotrophin releasing hormone |
| **CRHR1** | Corticotrophin releasing hormone receptor 1 |
| **Cspg4** | Chondroitin sulfate proteoglycan 4 |
| **CXCL** | C-X-C motif chemokine ligand |
| **CXCR** | C-X-C motif chemokine receptor |
| **DA** | Dopamine |

| | |
|---|---|
| **Dcc** | Deleted in colorectal carcinoma |
| **DEG** | Differentially expressed gene |
| **Dlg4** | Discs large MAGUK scaffold protein 4 |
| **Dscam** | DS cell adhesion molecule |
| **DSIP** | Delta sleep-inducing peptide |
| **$E_2$** | Oestradiol |
| **ECM** | Extracellular matrix |
| **Efna5** | Ephrin A5 |
| **Efnb1** | Ephrin B1 |
| **Efnb2** | Ephrin B2 |
| **Egf/EGF** | Epidermal growth factor |
| **eGFP** | Enhanced green fluorescent protein |
| **Egfr** | Epidermal growth factor receptor |
| **End/endoth** | Endothelial cells |
| **Enho** | Energy homeostasis associated |
| **Epha5** | Eph receptor A5 |
| **Epha6** | Eph receptor A6 |
| **Ephb2** | Eph receptor B2 |
| **Erbb4** | Erb-b2 receptor tyrosine kinase 4 |
| **ET** | Endothelin |
| **F11r** | F11 receptor |
| **Fgf/FGF** | Fibroblast growth factor |
| **Fgf1** | Fibroblast growth factor 1 |
| **Fgf9** | Fibroblast growth factor 9 |
| **Fgfr1** | Fibroblast growth factor receptor 1 |
| **Fgfr3** | Fibroblast growth factor receptor 3 |
| **Fib/fibrobl** | Fibroblasts |
| **FS** | Folliculostellate |
| **FSH** | Follicle stimulating hormone |
| **Fshb** | Follicle stimulating hormone beta |
| **Fzd1** | Frizzled class receptor 1 |
| **Fzd2** | Frizzled class receptor 2 |
| **Fzd3** | Frizzled class receptor 3 |
| **Fzd6** | Frizzled class receptor 6 |
| **Fzd8** | Frizzled class receptor 8 |
| **GABA** | γ-aminobutyric acid |
| **Gal** | Galanin and GMAP prepropeptide |
| **GAL** | Galanin |
| **Gas6** | Growth arrest specific 6 |
| **GCs** | Glucocorticoids |
| **GH** | Growth hormone |
| **Ghr** | Growth hormone receptor |
| **GHRH** | Growth hormone releasing hormone |
| **GnRH** | Gonadotrophin releasing hormone |
| **Gon** | Gonadotrophs |
| **GPCR** | G protein-coupled receptor |
| **Gpr19** | G protein-coupled receptor 19 |
| **Grp/GRP** | Gastrin releasing peptide |
| **Hbb-bt** | Hemoglobin, beta adult t chain |
| **Hbegf** | Heparin binding EGF-like growth factor |
| **HPA** | Hypothalamic-pituitary-adrenal |

| | |
|---|---|
| **HPG** | Hypothalamic-pituitary-gonadal |
| **i.c.v.** | Intracerebroventricular |
| **i.p.** | Intraperitoneal |
| **i.v.** | Intravenous |
| **Icam1** | Intercellular adhesion molecule 1 |
| **IFN** | Interferon |
| **Igdcc4** | Immunoglobulin superfamily, DCC subclass, member 4 |
| **IGF** | Insulin-like growth factor |
| **IL** | Interleukin |
| **Il1r1** | Interleukin 1 receptor, type I |
| **Il1rapl2** | Interleukin 1 receptor accessory protein-like 2 |
| **Insr** | Insulin receptor |
| **Itga6** | Integrin alpha 6 |
| **Jam2** | Junction adhesion molecule 2 |
| **Kit** | KIT proto-oncogene receptor tyrosine kinase |
| **KO** | Knockout |
| **Lac** | Lactotrophs |
| **Lgr4** | Leucine-rich repeat-containing G protein-coupled receptor 4 |
| **Lgr6** | Leucine-rich repeat-containing G protein-coupled receptor 6 |
| **LH** | Luteinising hormone |
| **Lhb** | Luteinising hormone beta |
| **LIF** | Leukaemia inhibitory factor |
| **LPS** | Lipopolysaccharide |
| **Lrp5** | Low density lipoprotein receptor-related protein 5 |
| **Lrp6** | Low density lipoprotein receptor-related protein 6 |
| **Lrrn1** | Leucine rich repeat protein 1, neuronal |
| **Lsr** | Lipolysis stimulated lipoprotein receptor |
| **Macroph** | Macrophages |
| **Mel** | Melanotrophs |
| **Mif** | Macrophage migration inhibitory factor (glycosylation-inhibiting factor) |
| **Mki67** | Antigen identified by monoclonal antibody Ki 67 |
| **Mki67+** | Mki67+ve cells |
| **mRNA** | Messenger RNA |
| **MT** | Melatonin |
| **Nampt** | Nicotinamide phosphoribosyltransferase |
| **Ncam1** | Neural cell adhesion molecule 1 |
| **Ncam2** | Neural cell adhesion molecule 2 |
| **Neto2** | Neuropilin (NRP) and tolloid (TLL)-like 2 |
| **NGF** | Nerve growth factor |
| **Nkx2-1** | NK2 homeobox 1 |
| **Nmb** | Neuromedin B |
| **Nmu** | Neuromedin U |
| **NO** | Nitric oxide |
| **Notch1** | Notch 1 |
| **NPK** | Neuropeptide K |
| **Nppc** | Natriuretic peptide type C |
| **NPY** | Neuropeptide Y |

| | |
|---|---|
| **NPγ** | Neuropeptide γ |
| **Nrg1** | Neuregulin 1 |
| **Nrp1** | Neuropilin 1 |
| **Nrp2** | Neuropilin 2 |
| **NT** | Neurotensin |
| **Ntrk2** | Neurotrophic tyrosine kinase |
| **OT** | Oxytocin |
| **OVX** | Ovariectomy |
| **P2rx4** | Purinergic receptor P2X, ligand-gated ion channel 4 |
| **$P_4$** | Progesterone |
| **PACAP** | Pituitary adenylate cyclase activating polypeptide |
| **PAMP** | Proadrenomedullin N-terminal 20 peptide |
| **Pax7** | Paired box 7 |
| **Pcdh** | Protocadherin |
| **Pcdh10** | Protocadherin 10 |
| **Pcdh9** | Protocadherin 9 |
| **Pcdhb2** | Protocadherin beta 2 |
| **Pdyn** | Prodynorphin |
| **Pecam1** | Platelet/endothelial cell adhesion molecule 1 |
| **Per** | Pericytes |
| **Pomc** | Proopiomelanocortin |
| **Post** | Posterior pituitary cells |
| **PP** | Pancreatic polypeptide |
| **PRL** | Prolactin |
| **Prlr** | Prolactin receptor |
| **Pros1** | Protein S (alpha) |
| **Ptprc** | Protein tyrosine phosphatase, receptor type, C |
| **Ptprj** | Protein tyrosine phosphatase, receptor type, J |
| **Ptpro** | Protein tyrosine phosphatase, receptor type, O |
| **Ptprz1** | Protein tyrosine phosphatase, receptor type Z, polypeptide 1 |
| **RBCs** | Red blood cells |
| **RNA-seq** | RNA-sequencing |
| **Rspo3** | R-spondin 3 |
| **s.c.** | Subcutaneous |
| **scRNA-seq** | Single cell RNA-sequencing |
| **Slitrk1** | SLIT and NTRK-like family, member 1 |
| **Slitrk6** | SLIT and NTRK-like family, member 6 |
| **Smo** | Smoothened, frizzled class receptor |
| **Som** | Somatotrophs |
| **Sort1** | Sortilin 1 |
| **Sox2** | SRY (sex determining region Y)-box 2 |
| **Sox2+** | Sox2+ve cells |
| **SP** | Substance P |
| **SST** | Somatostatin |
| **St6gal1** | Beta galactoside alpha 2,6 sialyltransferase 1 |
| **T** | Testosterone |
| **$T_3$** | Triiodothyronine |
| **$T_4$** | Thyroxine |
| **Tac1** | Tachykinin 1 |
| **Tenm3** | Teneurin transmembrane protein 3 |

| | |
|---|---|
| **Tenm4** | Teneurin transmembrane protein 4 |
| **TGF** | Transforming growth factor |
| **Thyr** | Thyrotrophs |
| **TNF** | Tumour necrosis factor |
| **Tpit** | T-box transcription factor |
| **TRH** | Thyrotrophin releasing hormone |
| **TSH** | Thyroid stimulating hormone |
| **Tshb** | Thyroid stimulating hormone subunit beta |
| **UCN2** | Urocortin 2 |
| **UMAP** | Uniform manifold approximation and projection |
| **UTP** | Uridine triphosphate |
| **Vegfa/VEGFA** | Vascular endothelial growth factor A |
| **VIP** | Vasoactive intestinal peptide |
| **WBCs** | White blood cells |
| **Wnt4** | Wingless-type MMTV integration site family, member 4 |

# INTRODUCTION

The anterior pituitary gland is a cornerstone of the endocrine system. Based on instructions from the hypothalamus, it controls the functions of several peripheral organs that together maintain homeostasis in an organism. The hypothalamus, anterior pituitary and peripheral organs are linked into five separate neuroendocrine axes, each with their own principal function: reproduction, growth, metabolism, lactation or the stress response.

Based on its intermediate position in these axes, the pituitary is generally considered a *passive* transmitter that relays the signals generated by the hypothalamus to the rest of the body where the instructions are converted into physiological effects. The axes are considered as separate from each other and are self-regulated through negative and/or positive feedback from hormones secreted by the end organs. In this conventional “pipeline” model (Figure 1), the pituitary is merely a container through which information flows in independent axes.

This dissertation examines the pituitary from an alternative perspective: one where the pituitary is made up of cells that interact with and influence each other to together create an *active*, whole-gland response to external signals (Figure 2). This view is based on research from recent decades that supports the presence of communication systems within the pituitary that regulate the response of the gland to hypothalamic and peripheral signals. Evidence for both contact-dependent as well as ligand-mediated communication has been presented, but the molecular mediators involved are far from understood. That incomplete understanding is the motivation behind the studies presented in this dissertation. Using bioinformatics analyses of transcriptomics data on the pituitary, I will identify candidate molecules that may mediate interactions between pituitary cells and consider the potential significance of these interactions for pituitary function.

The subsequent sections of the introduction will present the basic structure and functions of the pituitary gland, discuss the evidence for internal regulation of the pituitary and propose a conceptual model of the pituitary as a social gland consisting of multiple overlapping communication networks. It is hoped that the “collectivistic” perspective adopted in this dissertation will complement the more individualistic, single-axis, studies usually conducted on the pituitary gland.

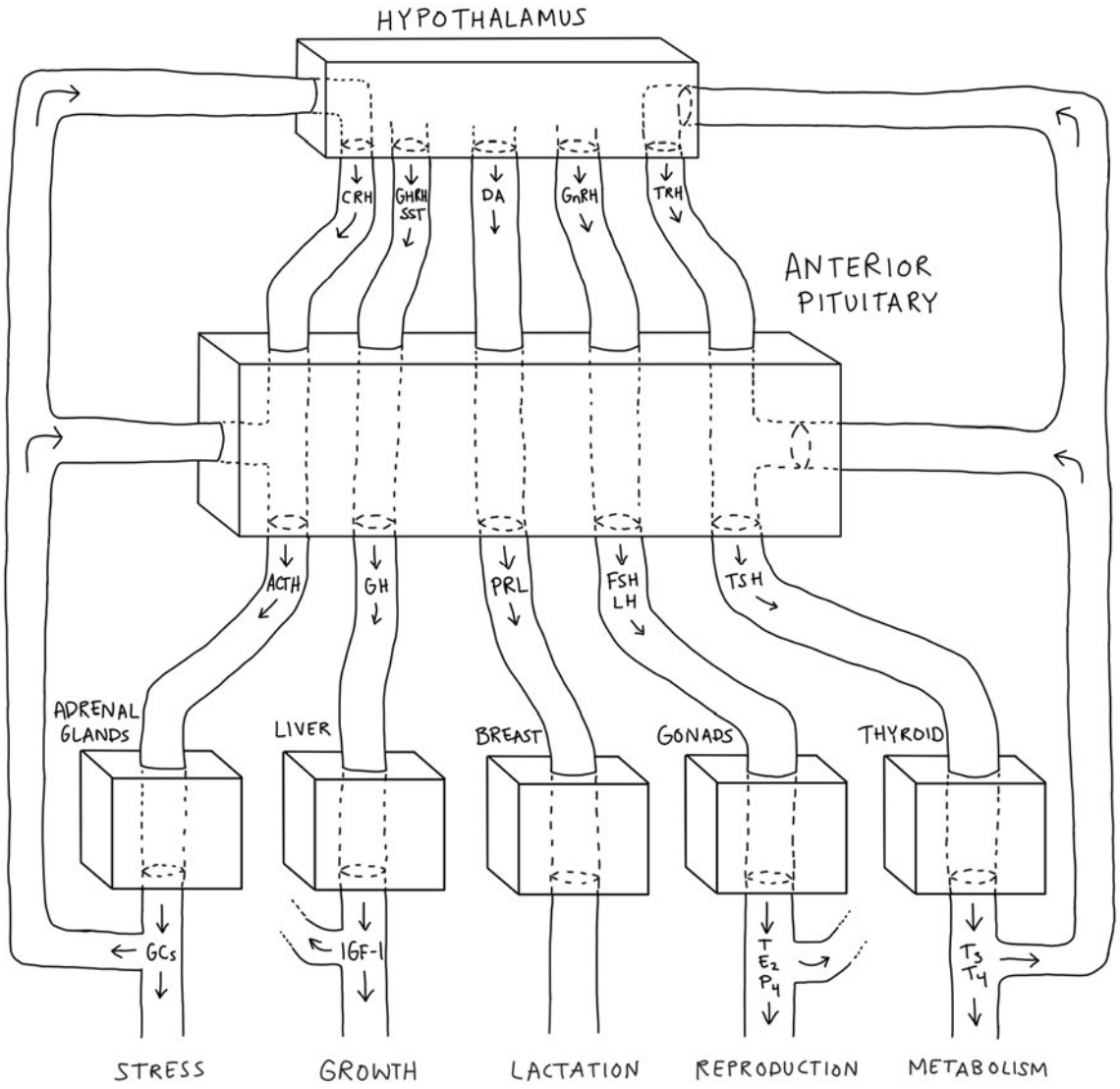


**Figure 1: The traditional "pipeline" model of the 5 neuroendocrine axes.** Factors released from the hypothalamus act on separate cell types in the anterior pituitary to stimulate or inhibit hormone release. The pituitary hormones in turn act on peripheral tissues to induce the release of effector hormones which regulate important physiological functions and provide feedback at the level of the hypothalamus and pituitary. Corticotrophin releasing hormone (CRH), growth hormone releasing hormone (GHRH), gonadotrophin releasing hormone (GnRH) and thyrotrophin releasing hormone (TRH) stimulate anterior pituitary hormone secretion whereas somatostatin (SST) and dopamine (DA) inhibit pituitary hormone secretion. Adrenocorticotrophic hormone (ACTH) induces the release of glucocorticoids (GCs) from the adrenal glands to regulate the stress response. Growth hormone (GH) acts directly and via insulin-like growth factor 1 (IGF-1) to promote growth and regulate metabolism. The principal role of prolactin (PRL) is to induce lactation. Follicle stimulating hormone (FSH) and luteinising hormone (LH) regulate gonadal function and induce release of testosterone (T), oestradiol ($E_2$) and progesterone ($P_4$). Thyroid stimulating hormone (TSH) stimulates triiodothyronine ($T_3$) and thyroxine ($T_4$) secretion from the thyroid gland to regulate metabolism. GH, IGF-1, PRL, T, $E_2$ and $P_4$ also feed back to the hypothalamus and pituitary to regulate axis activity.

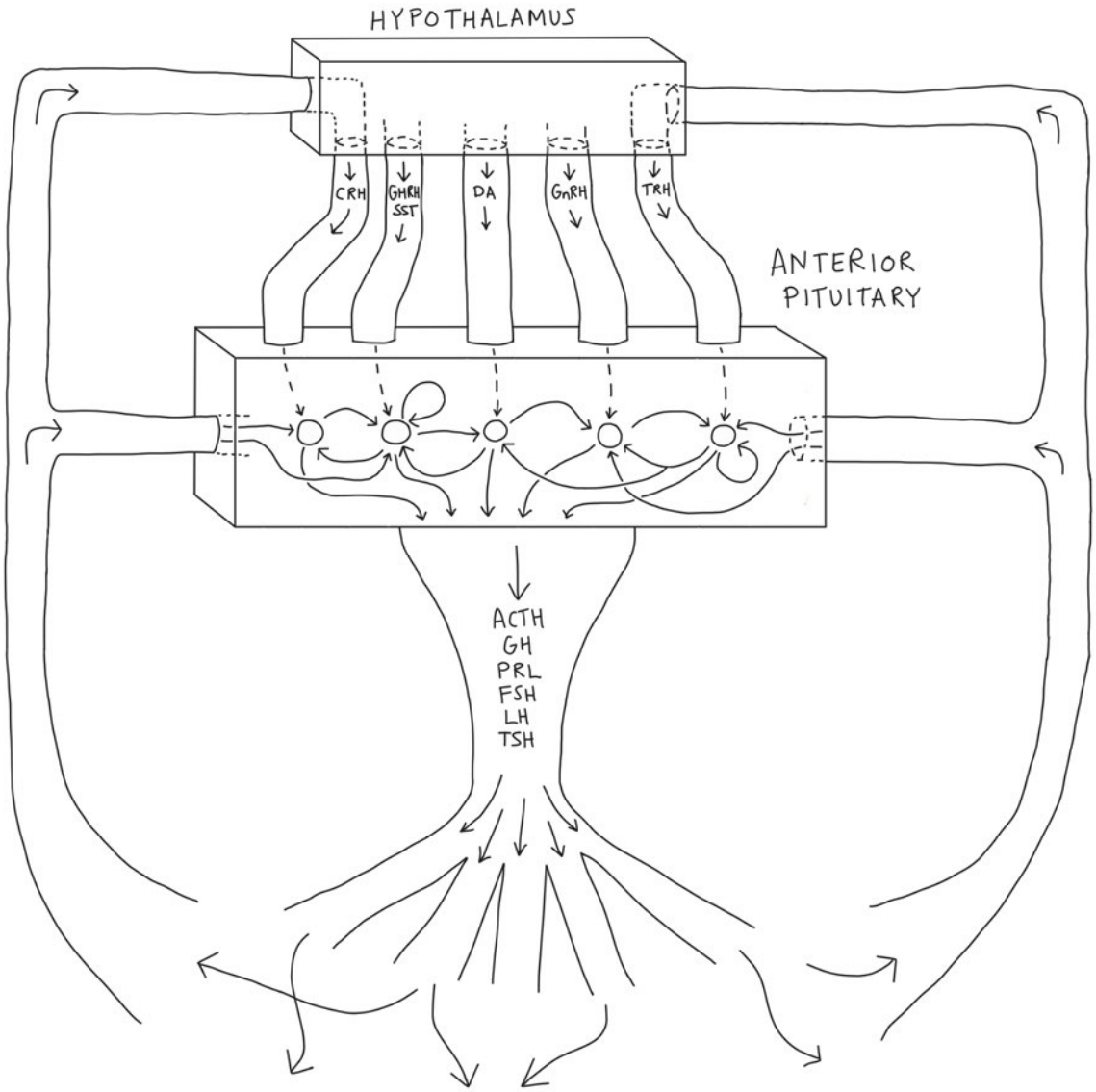


**Figure 2: A model of interconnected neuroendocrine axes where interactions between pituitary cells play an important role in determining hormone output.** Abbreviations as in the legend to Figure 1.

# 1 An overview of the structure and functions of the pituitary

## *1.1 The anatomical and functional relationship between the hypothalamus and the pituitary*

The pituitary gland is located underneath the hypothalamus in a small cavity of the sphenoid bone called the sella turcica (Holt et al. 2022). The gland is divided into an anterior and a posterior lobe, which are separated by an intermediate lobe (Figure 3). The anterior part is derived from Rathke's pouch, an outgrowth of the oral ectoderm, whereas the posterior part develops from the neuroectoderm. The anatomical link between the hypothalamus and the pituitary is provided by the pituitary stalk.

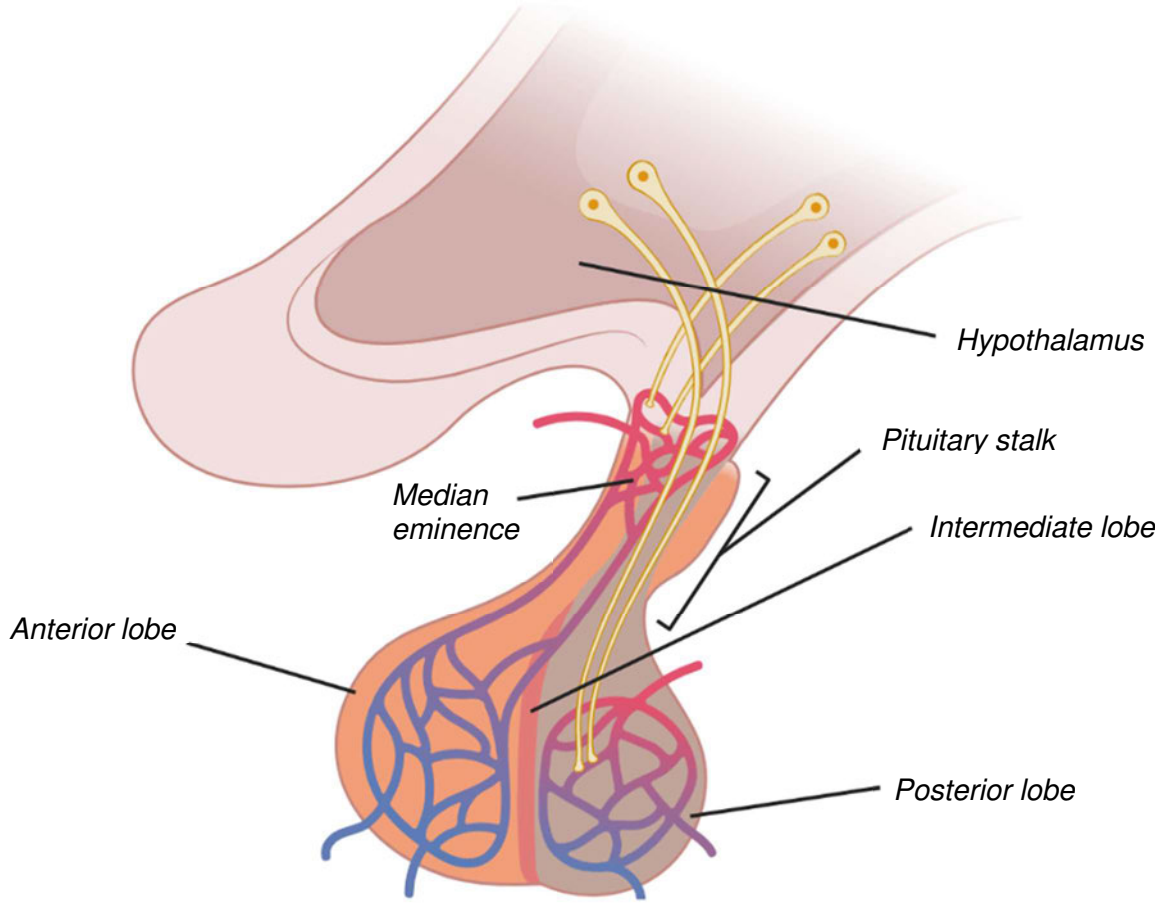


**Figure 3: Basic anatomy of the hypothalamus and pituitary gland.** Made with BioRender.com.

The cells of the posterior and intermediate lobes are under direct neural control from the hypothalamus. The pituitary stalk contains axons of neuroendocrine neurons that pass oxytocin and vasopressin synthesised in the hypothalamus to the posterior lobe to be released into the blood. The intermediate lobe is innervated by hypothalamic and extra-hypothalamic neurons that regulate secretion of melanocyte stimulating hormone (MSH) from melanotrophs in the gland (Saland 2001).

In contrast, the anterior lobe is controlled by hypothalamic releasing hormones that travel to the pituitary via a specialised vascular structure, the hypophyseal portal system. The median eminence at the base of the hypothalamus collects the releasing hormones secreted by hypothalamic neurons and delivers them into portal veins in the pituitary stalk that drain into the anterior pituitary capillary bed. The releasing hormones then stimulate or inhibit anterior pituitary cells to secrete hormones that in turn regulate peripheral tissues.

### *1.2 The composition of the anterior pituitary gland*

The anterior pituitary consists of several types of parenchymal and stromal cells that vary in relative abundance depending on factors such as sex, age and hormonal status (Ben-Shlomo and Melmed 2017). Of the hormone producing parenchymal cells, growth hormone (GH) producing somatotrophs are the most abundant, making up 35-50% of anterior pituitary cells (Shimon 2014; Ben-Shlomo and Melmed 2017). Between 15 and 35% of anterior pituitary cells are prolactin-secreting lactotrophs, which are more abundant in females (Nolan and Levy 2009; Shimon 2014). Corticotrophs, secreting adrenocorticotrophic hormone (ACTH), and gonadotrophs, secreting follicle stimulating hormone (FSH) and luteinising hormone (LH), both make up 10-20% of the cells in the gland (Ben-Shlomo and Melmed 2017). Thyroid stimulating hormone (TSH) secreting thyrotrophs are the least abundant, making up only 5% of cells in the gland (Ben-Shlomo and Melmed 2017). All pituitary hormones are released in a pulsatile manner in patterns that are unique for each hormone (Veldhuis 2008).

The anterior pituitary also contains agranular folliculostellate (FS) cells (5-10% of cells) which have a variety of functions related to the support of endocrine cells in the gland (Devnath and Inoue 2008). How they relate to a population of Sox2-expressing stem cells that have also been identified in the gland (Vankelecom and Chen 2014) is a matter of debate (Le Tissier and Mollard 2021). The anterior pituitary is highly vascularised to facilitate hormone transport into the systemic circulation. Endothelial cells and pericytes (Fletcher et al. 2019; Mayran et al. 2019; Ruf-Zamojski et al. 2021) regulate transport of substances

between the parenchyma and the blood. A small population of resident immune cells, largely macrophages (Mayran et al. 2019; Lopez et al. 2021), are also present in the gland.

### *1.3 Anterior pituitary hormones regulate a range of physiological functions directly and indirectly via actions on peripheral organs*

GH has a primary role in growth, acting both directly on tissues and via stimulation of insulin-line growth factor 1 (IGF-1) release from the liver to regulate aspects of bone growth, body composition, muscle mass and cardiovascular function (Bonert and Melmed 2017). In females, prolactin facilitates lactation by inducing milk production and may also influence metabolism in both sexes (Binart 2017). ACTH is critical for the stress response, inducing the production and secretion of glucocorticoids from the adrenal gland. The gonads are target glands for FSH and LH. In males, FSH is essential for spermatogenesis, whereas LH stimulates the production of testosterone. The female functions of FSH include regulation of oestrogen production and facilitating the later stages of ovarian follicle development. A rise in LH triggers ovulation in females and the hormone is also important for ovarian steroid synthesis. TSH acts on the thyroid gland to stimulate the production of thyroid hormone, which has a wide range of metabolism-promoting effects.

## 2 Evidence for the pituitary as a gland containing interdependent cell types that actively regulate the pituitary response

Hypothalamic and peripheral hormone inputs are undoubtedly the major determinants of pituitary hormone output. However, the pituitary is far from a passive gland. It has the ability to regulate its own response to the external inputs and act autonomously to control hormone secretion. Moreover, the different cell types of the pituitary do not respond to hypothalamic stimulation in isolation but are interconnected and influence each other's functions. Evidence to support both these assertions is discussed below.

### *2.1 A disconnect between hypothalamic and pituitary output*

The pulsatile release of pituitary hormones is conventionally thought to be determined by the pulsatile release of hypothalamic releasing hormones with additional tuning provided by peripheral hormone feedback (Veldhuis 2008). Yet there are examples of the absence of pituitary response to hypothalamic input and pituitary hormone release that does not mirror hypothalamic input. As an example of the first, a mismatch between gonadotrophin releasing hormone (GnRH) and LH release has been detected in 1-week-old male mice (Glanowska et al. 2014). Despite high-frequency pulses of GnRH measured at the median eminence, LH

release from the pituitary was undetectable even though both GnRH receptor and LH beta subunits were expressed in the gland (Glanowska et al. 2014). In contrast, multiple lines of evidence suggest that the pituitary is capable of generating ACTH pulses without hypothalamic corticotrophin releasing hormone (CRH). Pituitary stalk-transected sheep still produced ultradian and stress-induced pulses of ACTH (Engler et al. 1990), human pituitary cells independently released ACTH in a pulsatile manner *in vitro* (Gambacciani et al. 1987) and mathematical modelling has suggested that the origin of hypothalamic-pituitary-adrenal (HPA) axis pulsatility lies within the pituitary and adrenal glands (Spiga et al. 2015). Pulsatile release of ACTH (Walker et al. 2012) and TSH (Samuels et al. 1993) was also maintained despite constant instead of pulsatile CRH and thyrotrophin releasing hormone (TRH) inputs. Altogether this evidence suggests that the patterns of pituitary hormone release are also intrinsically regulated by the gland.

### *2.2 The pituitary in vitro does not respond like the gland in vivo*

Given the difficulty of studying the pituitary *in vivo* due to its small size and buried location, the gland is usually studied *in vitro*. However, the secretory response of the pituitary *in vitro* where the gland architecture has been disrupted does not mirror the response *in vivo*. Administration of a growth hormone releasing hormone (GHRH) analogue induced GH secretion 1,000-fold above basal in male rats (Painson and Tannenbaum 1991), whereas *in vitro* administration produced at most a 25-fold increase from dispersed rat pituitary cells (Sugimoto et al. 1991). GHRH potentiated the effect of TRH on TSH release *in vivo* but when the cells were dispersed in culture the effect of GHRH disappeared (Looij et al. 1995).

### *2.3 Pituitary hormone secretion is regulated by cellular proximity*

The density of pituitary cells in culture has a significant influence on basal hormone secretion and the secretory response of the cells to hypothalamic secretagogues and paracrine factors (Perez et al. 1995). Basal release of GH (Sugimoto et al. 1991), prolactin (Oosterom et al. 1983) and ACTH (Perez et al. 1995) decreased with increasing plating density of rat pituitary cells. In human pituitary adenomas, LH secretion decreased while FSH secretion increased as plating density was increased (Atkin et al. 1998). Reduced access to nutrients could explain the lower secretion at high cellular densities, however, GH secretion remained low in high-density cultures placed in perifusion where any unequal distribution of nutrients should be minimised (Sugimoto et al. 1991; Perez et al. 1995).

GHRH-stimulated GH release from rat pituitary cells was increased at high plating densities (Sugimoto et al. 1991), as was GnRH-stimulated gonadotropin release from rainbow trout

pituitary cells (Weil et al. 1986). The inhibitory effects of dopamine on prolactin secretion was also increased at higher plating densities of rat pituitary cells (Hoefer et al. 1984). In contrast, rat corticotrophs plated at low concentration secreted more ACTH in response to CRH (Jia et al. 1992). Less is known regarding the effects of cellular proximity on TSH secretion, however, TRH increased thyroid stimulating hormone subunit beta (*Tshb*) expression in rat pituitary fragments but not when the cells were dispersed (Bargi-Souza et al. 2015). Plating density also altered the response to paracrine factors: cholinergic agonist carbachol inhibited prolactin secretion only when cells were in close contact with each other (Carmeliet et al. 1989) and dissociated rat pituitary cells abolished the ability of β-adrenergic agonists to enhance prolactin secretion (Perkins et al. 1985) and the ability of angiotensin II to influence GH secretion (Robberecht and Denef 1988) compared to when the cells were reaggregated. In contrast to the previous results, activin A-stimulated FSH secretion was diminished at higher cell densities (Kitaoka et al. 1989).

Cellular proximity also regulates global processes such as protein synthesis. Rat pituitary cells in close proximity in culture exhibited protein synthesis rates that were 30% lower compared to cells positioned further apart (Perez et al. 1995). Cellular contents of cyclic adenosine monophosphate (cAMP) also increased in cultured rat pituitary cells as plating density decreased (Sugimoto et al. 1991).

### *2.4 Interdependence of pituitary cell types*

Pituitary cell types are dependent on each other for normal development. Ablation of gonadotrophs during development reduced the number and size of lactotrophs at postnatal day 1 in mice (Seuntjens et al. 1999) and reduced the expression of prolactin mRNA (Vankelecom et al. 2003). Pituitaries in T-box transcription factor (Tpit) deficient mice that lack terminally differentiated corticotrophs and melanotrophs contained more and smaller gonadotrophs, particularly in the lateral wings of the gland (Pulichino et al. 2003; Budry et al. 2011). Somatotroph deletion in mice reduced hormone contents of all pituitary cell types, an effect that was dependent on the extent of somatotroph ablation (Waite et al. 2010).

Functional co-dependence of pituitary cell types during adulthood is supported by evidence showing that the secretory response of a particular cell type is dependent on the presence of another pituitary cell type(s) in culture. GnRH stimulated prolactin release from rat pituitary cell aggregates but only when significant numbers of gonadotrophs were present (Denef and Andries 1983). Interferon γ inhibited CRH-stimulated ACTH secretion from rat pituitary cell aggregates (Vankelecom et al. 1990) but when FS cells were depleted from culture, the response to interferon γ disappeared (Vankelecom et al. 1992). Angiotensin II induced

robust release of prolactin from rat pituitary cells in culture but had only a weak effect when the cultures were depleted of other cell types than gonadotrophs and lactotrophs (De Paul et al. 2000). Co-incubation of purified mouse gonadotrophs with other pituitary cell types produced an 8-fold increase in basal FSH expression compared to when gonadotrophs were present alone (Wu et al. 2004).

Finally, it has been suggested based on evolutionary evidence that anterior pituitary cells were under paracrine control before they came under haemal hypothalamic influence during the water-to-land transition (Trudeau and Somoza 2020). The importance of interactions between pituitary cell types can be derived from evidence that pituitary hormones in more primitive organisms not only influence peripheral functions but also have important regulatory roles within the gland. For example, studies performed on grass carp pituitaries have shown that GH, LH (Zhou et al. 2004, 2005) and prolactin (Lin et al. 2015) act as autocrine/paracrine factors that regulate secretion of other pituitary hormones. The relationships between the different pituitary cell types may therefore have a long evolutionary past.

## 3 Physical networks and paracrine interactions in the pituitary

The evidence presented in the previous section indicates that cell-cell interactions play an important role in regulating pituitary output. However, it is not clear whether the gland architecture or paracrine mechanisms are responsible for the observed effects. Both components have been the focus of research for the past few decades and will be discussed next.

### *3.1 Pituitary cells form physical networks*

Evidence against the random organisation of pituitary cells was suggested early on by the observation of preferential juxtaposition between corticotrophs and somatotrophs and between gonadotrophs and lactotrophs above what would be expected from cell type abundance (Nakane 1970; Siperstein and Miller 1970). These selective associations were later confirmed quantitatively (Noda et al. 2001). The importance of gland architecture for pituitary cell function was also suggested by studies showing that lactotrophs (Boockfor and Frawley 1987; Mukherjee et al. 1991) and somatotrophs (Perez and Hymer 1990) obtained from different parts of the pituitary exhibited strikingly different hormone secretion profiles and responses to peripheral hormones.

In the past two decades, 3D imaging in fish and mammals has revealed that pituitary cells form gland-wide homotypic networks where cells of the same type are in contact with each other (Le Tissier et al. 2012). So far, homotypic network arrangements of somatotrophs (Bonnefont et al. 2005), lactotrophs (Hodson et al. 2012), corticotrophs and gonadotrophs (Budry et al. 2011; Golan et al. 2016) as well as FS cells (Fauquier et al. 2001) have been described.

The functional relevance of the mammalian homotypic networks is beginning to be elucidated, so far with a focus on mouse somatotrophs. The male mouse somatotroph network structure varies across lifespan with denser clusters appearing in lateral parts of the pituitary at puberty, coinciding with the increase in GH secretion and growth rate of the animals (Bonnefont et al. 2005). A role for the somatotroph network in supporting GH secretion is also indicated by the lack of growth phenotype observed despite low circulating GH as long as the network structure is maintained (Waite et al. 2010). The sex differences in somatotroph calcium responses to GHRH observed using GH-enhanced green fluorescent protein (eGFP) labelling in intact glands disappeared when responses were measured in isolated somatotrophs, suggesting that somatotroph organisation in the pituitary is needed for sexually dimorphic GH secretion patterns (Sanchez-Cardenas et al. 2010). Network connectivity in terms of coordinated calcium activity also appears to be regulated by GHRH (Bonnefont et al. 2005).

In fish, research has focused on the gonadotroph homotypic network. Tilapia and zebrafish gonadotrophs form networks where LH-expressing cells are more tightly connected than FSH-expressing cells, the latter which sometimes form attachments via cytoplasmic extensions (Golan et al. 2016). Gap junction blockers inhibited the 7-fold increase in LH secretion in response to GnRH in tilapia pituitary fragments (Golan et al. 2016), suggesting that the homotypic network mediates functional coordination of the gonadotroph population. The mammalian homotypic networks also appear to be coupled by gap junctions (Fauquier et al. 2001; Hodson et al. 2012).

Together the functional evidence has led to the proposal that the networks facilitate dynamic coordination of pituitary cellular responses, thereby promoting plasticity in response to changing physiological demands (Le Tissier et al. 2012). Despite this importance of the networks in regulating pituitary function, the molecular basis for homotypic adhesion in the pituitary is largely unknown. Identification of cell adhesion molecules (CAMs) that may facilitate homotypic adhesion between pituitary cells will therefore form one of the aims of this dissertation.

### *3.3 Paracrine regulation of pituitary cells*

Paracrine signalling allows nearby cells to communicate using secreted ligands that act on receptors on the target cell. This is usually distinguished from autocrine signalling in which the same cell is the source and target of secreted signals. However, given the difficulty of experimentally differentiating between the two, paracrine communication will henceforth be used to refer to both autocrine and paracrine signalling.

Early support for paracrine regulation of pituitary cell function came from studies showing that the effects of one pituitary cell type on another could be mimicked by medium conditioned with the first cell type. For example, GnRH-induced prolactin secretion from rat pituitary cell aggregates was absent if gonadotrophs were depleted from culture but the addition of gonadotroph-conditioned medium reinstated the effect (Denef and Andries 1983). Similarly, ultrafiltrated material from gonadotroph-conditioned medium could reproduce the effects of gonadotrophs on lactotroph proliferation *in vitro* (Tilemans et al. 1994). The effects on lactotrophs therefore appear to be mediated by paracrine factors secreted by gonadotrophs into the culture medium.

In subsequent years, research has focused on the identification of paracrine factors capable of altering pituitary function, culminating in the discovery of over 100 paracrine ligands locally produced in the gland (Denef 2008). The potential for paracrine communication in the pituitary is also indicated by the diversity of G protein-coupled receptor (GPCR) expression, which exceeds that of 29 other human tissues (Marti-Solano et al. 2020).

Interest in the paracrine regulation of the pituitary peaked around the turn of the millennium and has since diminished (Figure 4). The paracrine ligands identified to date have been shown to alter diverse processes in the pituitary *in vitro* including basal and stimulated pituitary hormone secretion, protein synthesis, gene transcription, cellular proliferation, differentiation and migration (Houben and Denef 1994; Schwartz 2000; Denef 2008). Despite the capacity of these ligands to influence the pituitary, the contribution of paracrine signalling to physiological states that require altered secretion of pituitary hormones remains largely unexplored. Thus, the second aim of this dissertation will be to examine how paracrine communication between corticotrophs and other pituitary cell types is altered during chronic stress.

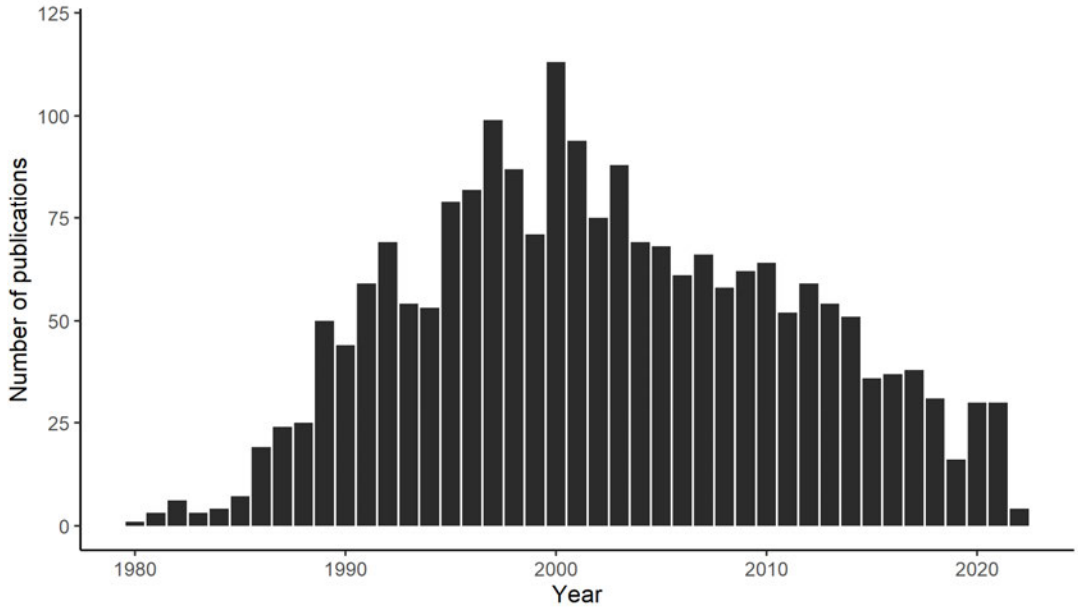


**Figure 4: Number of publications on paracrine regulation of the pituitary 1980-2022**. Data source: PubMed search with the query ("pituitary" OR "hypophysis" OR "adenohypophysis") AND ("paracrine" OR "autocrine").

### *3.3 A model of the pituitary as a multi-layered social network of cells*

Far from being just morphological and physiological units, pituitary cells are therefore social units as well (Reynolds 2018). In contrast to studies on isolated cell types, this dissertation examines the pituitary gland from a social perspective that emphasises the interactions that connect the cells in the gland. The structural and paracrine networks discussed form two separate layers of a complex web of communication utilised by the gland (Figure 5). Together, they may allow pituitary cells to exhibit collective behaviours with emergent properties or provide modularity that facilitates adaptation to diverse physiological states (Ratzke and Gore 2015). The structural and functional/paracrine organisation of pituitary cells is likely complementary and contributes to the orchestration of pituitary responses to hypothalamic and peripheral inputs as well as to the optimal secretion of pituitary hormones.

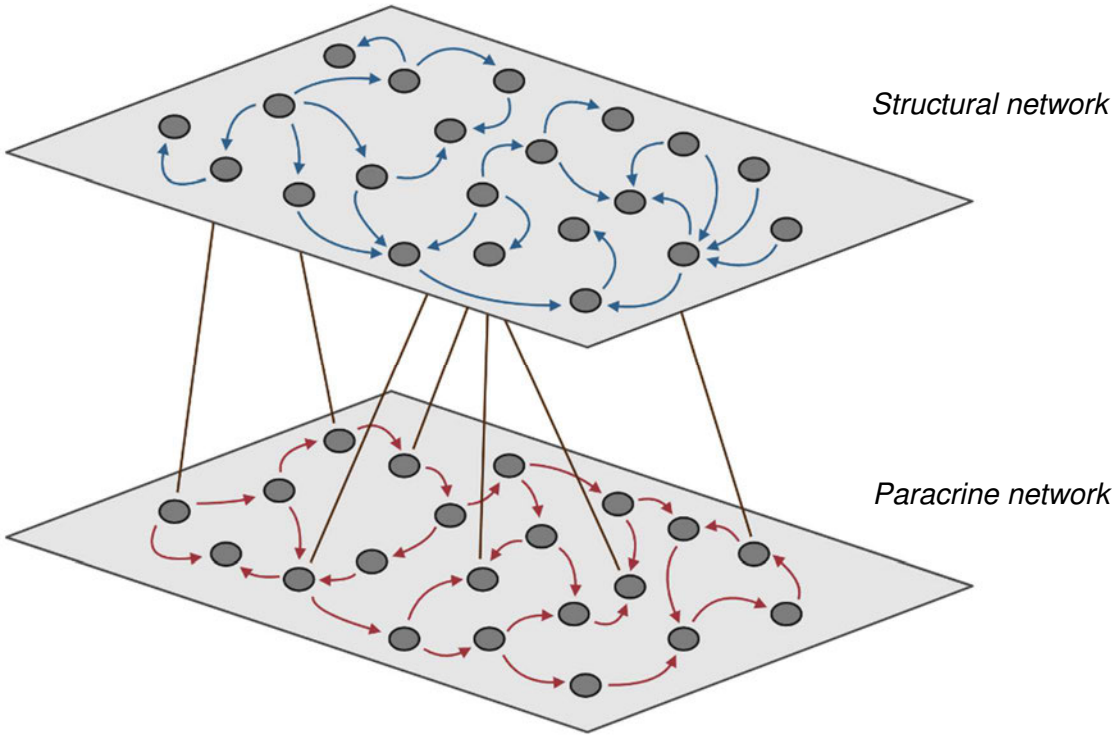


**Figure 5: Structural and paracrine networks are two overlapping layers of the pituitary cell interactome.** Figure made with BioRender.com.

## 4 Using bioinformatics to identify candidate molecules involved in intrapituitary interactions

Bioinformatics approaches are increasingly integrated with traditional experimental tools to understand tissue function, including pituitary biology. Genome/exome analyses have identified mutations contributing to the aetiology of pituitary tumours (Song et al. 2016; Bi et al. 2017) and proteomics has helped elucidate perturbed pathways involved in pituitary tumorigenesis (Zhan and Desiderio 2010). Transcriptomics techniques, both microarray and high-throughput RNA and single cell RNA sequencing, have provided valuable insights into fundamental parts of pituitary biology. Transcriptomics studies performed on the pituitary have revealed novel cell type markers (Cheung et al. 2018), cell type heterogeneity (Fletcher et al. 2021), sex differences (Fletcher et al. 2019), genes regulating cellular differentiation (Mayran et al. 2019) and human pituitary development (Zhang et al. 2020b) as well as local factors mobilised in response to a physiological or pathological challenge (Lopez et al. 2021; Vennekens et al. 2021). Gene expression datasets have also helped identify clinical targets for hypopituitarism (Davis et al. 2009) and pituitary adenomas (Ghatnatti et al. 2020). These

publicly available pituitary datasets represent a valuable resource for discovering new aspects of pituitary biology.

Recently, bioinformatics tools have been developed that allow the prediction of cell-cell interactions from single cell RNA-sequencing (scRNA-seq) data (Armingol et al. 2020). The tools analyse expression of known ligand-receptor pairs in cell types pre-identified in scRNA-seq and as such predict cell-cell communication in the tissue of interest. Supported by later experimental validation, these tools have already been successfully applied to for example identify a role for apolipoprotein E signalling during brain development (Sheikh et al. 2019), the contribution of tumour necrosis factor and interleukin 1 to monocyte recruitment in the liver (Bonnardel et al. 2019) and the involvement of bone morphogenetic protein signalling in lung alveolar growth (Zepp et al. 2017). These large-scale bioinformatics analyses therefore have the potential to generate candidate molecules that can then be subjected to experimental tests to ascertain any contribution to cell-cell communication.

### *4.1 Aims*

This dissertation is aimed at harnessing bioinformatics approaches and available transcriptomics datasets to identify candidate molecules involved in pituitary cell-cell interactions. In Chapter 1, available murine pituitary scRNA-seq data are integrated to create a large dataset that can be explored for expression of molecules involved in intrapituitary interactions. In Chapter 2, this dataset is combined with pituitary microarray data to infer candidate CAMs involved in the formation of homotypic networks. Chapter 3 is an investigation into chronic stress and how it alters paracrine communication between corticotrophs and other pituitary cell types based on changes in gene expression. The final chapter is a discussion of the findings in relation to the literature and provides a perspective on future studies into intrapituitary regulation.

# CHAPTER 1: INTEGRATION AND EVALUATION OF PITUITARY SINGLE CELL RNA-SEQUENCING DATA

## 1 INTRODUCTION

The overarching aim of this dissertation is to identify candidate mediators of cell-cell interactions between pituitary cells from scRNA-seq data. Inferring cell-cell communication from gene expression data has recently risen in popularity with the decreasing cost of single cell transcriptomics and the growth of computational tools that allow prediction of potential ligand-receptor interactions between cell types (Armingol et al. 2020). Despite a number of successes where this approach has been applied to identify novel paracrine signalling (Camp et al. 2017; Bonnardel et al. 2019; Sheikh et al. 2019; Molenaar et al. 2021), the limitations inherent in scRNA-seq data may prevent detection of some interactions operating between cells. These limitations include the dropout effect where lowly expressed genes are not always detected by scRNA-seq technology and the differences in scRNA-seq expression profiles obtained for the same tissue depending on the dataset (Crow et al. 2018; Hicks et al. 2018). One way to reduce the impact of these limitations is to combine different scRNA-seq datasets together into one large resource, generally referred to as dataset integration. Integrating cells from different datasets has been suggested to both increase the power to detect gene expression due to the larger number of cells included (Haghverdi et al. 2018; Stuart and Satija 2019) and to reduce the impact of dataset dependent expression signatures on downstream analyses because cells from different animals are included (Tung et al. 2017).

To maximise the chances of detecting relevant cell-cell interactions from pituitary scRNA-seq data, the goal of the present chapter was to create an integrated mouse pituitary scRNA-seq dataset. Comparisons to individual scRNA-seq datasets and data obtained by other transcriptomics technologies were then performed to evaluate the effects of integration. Finally, to evaluate the predictive power of pituitary scRNA-seq data in terms of protein expression, the distribution of intrapituitary factors at mRNA level in scRNA-seq data and protein level in the literature was examined.

## 2 METHODS

### 2.1 Analysis of pituitary scRNA-seq data

All analyses were performed in R (version 4.0.3) using Seurat (version 4.0.0) (Hao et al. 2020) following recommended best practices (Luecken and Theis 2019).

### *2.1.1 Data preprocessing and quality control*

Publicly available mouse pituitary scRNA-seq data associated with 4 publications (Cheung et al. 2018; Mayran et al. 2019; Ho et al. 2020; Lopez et al. 2021) were downloaded from the Gene Expression Omnibus (Barrett et al. 2013) or author-maintained GitHub repositories (Table 1). Only the control samples were included from the Lopez et al. dataset and only male samples from the Ho et al. dataset. For the dataset generated by Mayran et al., the raw reads were aligned to the mm10 mouse reference genome using 10X Genomics software Cell Ranger (version 5.0.0), for all other datasets author-supplied aligned files were used.

| Characteristic | Cheung 2018 | Ho 2020 | Lopez 2021 | Mayran 2019 |
|---|---|---|---|---|
| **Mouse strain** | C57BL/6 | CD1 | C57BL/6N | C57BL/6 |
| **Mouse age** | 7 weeks | 8 weeks | 10 weeks | 4 months |
| **Library preparation platform** | 10x Genomics v2 | 10x Genomics v2 | 10x Genomics v2 | 10x Genomics v2 |
| **Data source** | GSE120410 | https://github.com/wulabupenn/mPit | GSM4914024 | GSM3579942 |
| **Number of cells after quality control** | 13,286 | 1,614 | 4,918 | 8,930 |
| **Mean number of distinct genes per cells (mean ± SEM)** | 2,171 ± 6 | 602 ± 10 | 2,214 ± 11 | 1,865 ± 8 |
| **Mean number of reads per cell (mean ± SEM)** | 11,115 ± 47 | 1,459 ± 37 | 9,923 ± 64 | 6,120 ± 32 |

**Table 1. Characteristics of the 4 mouse pituitary scRNA-seq datasets used** (Cheung et al. 2018; Mayran et al. 2019; Ho et al. 2020; Lopez et al. 2021).

Separate Seurat objects were created for each dataset and quality metrics were assessed by examining plots of the total number of genes detected per cell and the proportion of reads mapping to the mitochondrial genome for each cell. Cells with a low number of expressed genes and a high proportion of reads mapping to the mitochondrial genome likely reflect damaged cells which leak nuclear genome-derived mRNAs through a perforated membrane, whereas mitochondria-derived mRNAs remain within the mitochondria and therefore within the cell (Ilicic et al. 2016). The thresholds used to filter out low quality cells in each dataset are presented in Table 2.

| Metric | Threshold | | | | | |
|---|---|---|---|---|---|---|
| | Cheung 2018 | Ho 2020 | Lopez 2021 | Mayran 2019 | Fletcher 2019 | Cheung unpublished |
| **Number of unique genes detected per cell** | - | >200 & <3,000 | - | >500 | >1,200 | >900 |
| **Maximum proportion of mitochondrial reads (%)** | 10 | 10 | 10 | 10 | 10 | 15 |

**Table 2. Thresholds used to filter out low-quality cells from pituitary scRNA-seq datasets.**

### *2.1.2 Cell type identification*

Regularised negative binomial regression (sctransform (version 0.3.2) (Hafemeister and Satija 2019)) was applied to normalise the data for sequencing depth. The 3000 most variable genes identified by sctransform were subsequently selected for downstream analyses. For dimensionality reduction, principal component analysis using RunPCA was performed to compute 50 principal components for each dataset. The number of principal components that account for most of the variation in the data was estimated using the elbow method (Satija Lab 2021), and uniform manifold approximation and projection (UMAP) plots were produced based on this number of principal components.

All datasets were clustered on the number of principal components identified in the previous step using a graph-based approach. First, the FindNeighbors function in Seurat was applied to identify the nearest neighbours for each cell. A Louvain community detection algorithm was then applied to the nearest neighbours graph to find clusters using the FindClusters function in Seurat. Cell types were identified based on marker genes for endocrine and non-endocrine pituitary cells (Table 3). A whole cluster was assigned to a cell type if most cells in the cluster expressed marker gene(s) for that cell type.

### *2.1.3 Dataset integration*

Mouse pituitary scRNA-seq datasets from 3 publications (Cheung et al. 2018; Mayran et al. 2019; Lopez et al. 2021) were integrated using Harmony (version 1.0) (Korsunsky et al. 2019). First, all cells were combined into a single Seurat object and the top 2,000 variable genes were identified using FindVariableFeatures. The data were scaled using ScaleData and the top 20 principal components were calculated using RunPCA. The RunHarmony

function was then applied to the combined data with default parameters to factor out the influence of the dataset-of-origin on gene expression values. Finally, clustering and cell type identification was performed as outlined in section 2.1.2.

| Cell type | Marker gene(s) |
|---|---|
| Corticotrophs | *Pomc,* absence of *Pax7* |
| Endothelial cells | *Pecam1* |
| Fibroblasts | *Col1a1* |
| Gonadotrophs | *Fshb, Lhb* |
| Lactotrophs | *Prl* |
| Melanotrophs | *Pomc, Pax7* |
| Mki67+ve cells | *Mki67* |
| Pericytes | *Cspg4* |
| Posterior pituitary cells | *Nkx2-1* |
| Red blood cells | *Hbb-bt* |
| Somatotrophs | *Gh* |
| Sox2+ve cells | *Sox2* |
| Thyrotrophs | *Tshb* |
| White blood cells | *Ptprc* |

**Table 3: Marker genes used to identify cell types from clusters in pituitary scRNA-seq data.**

### *2.1.4 Downstream analyses*

To statistically compare the number of genes and number of reads expressed per cell between the scRNA-seq datasets, a negative binomial generalised linear model was fit to the data to account for overdispersion. The glm.nb function of the MASS R package (Venables and Ripley 2013) was used for this purpose with post-hoc comparisons of the estimated marginal means between datasets performed using the emmeans function in R package emmeans (Lenth et al. 2022). To compare the relative abundance of cell types in each dataset, chi-squared tests were performed analysing whether the number of cells belonging to a certain cell type compared to the number of cells belonging to all other cell types differed between datasets. Post-hoc tests were applied using the function pairwiseNominalIndependence from the rcompanion package (Mangiafico 2022) to perform pairwise comparisons.

To calculate the correlation between the ranks of genes expressed in a cell type between datasets, log-normalised expression values were used. Random subsampling of integrated

corticotrophs was performed by the sample function in base R. To estimate a plateau value for the number of genes expressed in random subsamples of corticotrophs, a quadratic plateau model (Cerrato and Blackmer 1990) was fit to the data. This model follows a quadratic function until a join point after which it becomes linear and was defined as follows in R:

```
qp ← function(x, a, b, jp) {
  c  ← -0.5 * b / jp
  if_else(condition = x < jp,
          true  = a + (b * x) + (c * x * x),
          false = a + (b * jp) + (c * jp * jp))
}
```

where a is the intercept, b is the slope, c is the quadratic term and jp is the join point. Starting values for x and y were calculated by the self-starter function SSquadp3xs from R package nlraa (version 1.00) (Miguez et al. 2022). The final model was fit using the nls function from base R.

Differentially expressed genes between cell types were determined by the FindMarkers function in Seurat using default parameters (differentially expressed genes have to be expressed in at least 10% of one cell type and a $\log_2$-fold cut-off of 0.25). In addition, only genes meeting a threshold of 0.05 for p values adjusted with the bonferroni correction were included.

To create a list of genes that code for molecules likely involved in cell-cell interactions, the genes associated with the Gene Ontology Biological Process term cell adhesion (GO:0007155, 1,459 genes) and cell-cell signalling (GO:0007267, 1,751 genes) were downloaded from the Mouse Genome Informatics database (database update 02/01/2022) (Blake et al. 2021).

### 2.2 Analysis of corticotroph RNA-sequencing data

The animal experiments and RNA-sequencing were performed by Prof Mike Shipston's group. POMC-eGFP C56BL6/J mice (Pinto et al. 2004) were kept at 22°C and fed standard chow supplemented with sunflower seeds. At an age of 14 weeks, the mice were killed by cervical dislocation and the anterior pituitaries were extracted and dissociated as previously described (Duncan et al. 2014). Pituitary cells were sorted by fluorescent activated cell sorting (FACS) to obtain cells positive for GFP. Subsequent RNA isolation, library preparation and sequencing were performed by GENEWIZ, LLC. (South Painfield, New

Jersey, USA) according to their standard procedures. Demultiplexed FASTQ files were aligned to the GRCm38 genome, release 98, using STAR 2.7.1a by Dr Nicola Romanò.

Aligned count data were then analysed using R package DESeq2 (version 1.32.0) (Love et al. 2014). To count genes detected in corticotrophs, any genes expressed > 0 in at least one sample were included. Variance stabilising transformation was applied to normalise the count data with the vst function of DESeq2.

### 2.3 Analysis of microarray data

The microarray data were kindly provided by Dr Paul Le Tissier and Dr Jacques Drouin (McGill University). Data on corticotrophs and gonadotrophs were obtained from male mice on a C57BL6/J background whereas data on somatotrophs and lactotrophs were obtained from female mice on a C57BL6/J/CBA/Ca background. The mice were transgenic for fluorescent reporters identifying corticotrophs (Lavoie et al. 2008), gonadotrophs (Budry et al. 2011), somatotrophs (Magoulas et al. 2000) and lactotrophs (He et al. 2011), allowing separation of the relevant cell type using FACS. All pituitaries were obtained from mice 3 or 8-10 weeks old. Following FACS purification, RNA was extracted and hybridised on an AffyMetrix GeneChip® Mouse Gene 1.0 ST. Affymetrix array software was used to analyse signal intensity.

### 2.4 Comparison of paracrine ligand expression at mRNA and protein level in pituitary cell types

A list of 49 paracrine ligands known to play a role in pituitary function was obtained from a comprehensive review on the topic (Denef 2008). To examine the distribution of ligand mRNA expression in different cell types, the integrated mouse pituitary scRNA-seq dataset was used. scRNA-seq data from adult male and female rats (Fletcher et al. 2019) were downloaded from the Gene Expression Omnibus with accession number GSE132224 and processed in the same way as the individual mouse datasets described in section 2.1. The dropout effect in scRNA-seq data (Kharchenko et al. 2014) makes it difficult to establish the presence or absence of expression for genes with low expression values. For this reason, cell type distribution was determined for the 13 ligands expressed in at least 10% of at least one pituitary cell type in mice or rats. To find experimental evidence regarding cell type expression of the 13 ligands at protein level, PubMed was searched for the term “pituitary” and the name of the ligand. Publications with evidence of ligand expression in at least one pituitary cell type from mice or rats were included. For each paper, expression of a ligand

was scored as “high” if expression was predominant in a cell type and “low” if expression was described as markedly lower compared to another cell type. Absence of expression in a cell type, if experimentally determined, was also noted.

### 2.5 Data visualisation

The ligand distribution tables were made using flextable (version 0.6.7) (Gohel et al. 2021) in R. Figures were made with R packages ggplot2 (version 3.3.3) (Wickham 2016), VennDiagram (version 1.7.1) (Chen 2021) and Seurat (functions VlnPlot, DimPlot and FeaturePlot) (version 4.0.0) (Hao et al. 2020).

## 3 RESULTS

### 3.1 Dataset-dependent differences in the scRNA-seq profiles of healthy male mouse pituitaries

Considerable differences have been identified between the cell types recovered and their expression profiles in different scRNA-seq datasets on the same tissues (Macosko et al. 2015; Crow et al. 2018; Sun et al. 2021). To determine whether pituitary scRNA-seq data also exhibit such dataset-dependence, 4 published datasets on the healthy male mouse pituitary were compared (Cheung et al. 2018; Mayran et al. 2019; Ho et al. 2020; Lopez et al. 2021). The pituitaries in the studies were obtained from C57BL/6 or CD1 mice 7 weeks to 4 months of age and 10X Genomics technology was used to prepare the libraries in all studies (Table 1). To remove the influence that the clustering approach used has on the results (Vieth et al. 2019), each dataset was reanalysed from the aligned read files with one uniform analysis pipeline (see Methods section 2.1). A negative binomial generalised linear model with log link was fitted to the count data to test whether the number of genes or reads per cell differed significantly between datasets. The negative binomial distribution is best fitted to account for the overdispersion of the data (Figure 6). The results indicated that all datasets differed significantly from each other in terms of the total number of genes and total number of reads expressed per cell (adjusted p value < 0.001 for all pairwise comparisons of the estimated marginal means, Figure 6). However, the Ho 2020 dataset exhibited clearly lower expression values compared to the other 3 datasets (Table 1). All typical anterior pituitary endocrine cell types were identified in each dataset (Figures 7A-D). Statistical comparisons between the number of cells belonging to each cell type compared to the number of cells belonging to other cell types between datasets indicated that the cell type

abundance differed between datasets for all cell types ($X^2$ test, $p<1x10^{-5}$ for all cell types, Figure 7E). The greatest differences were observed for somatotrophs (which ranged from 34.7 to 67.7% of all cells between the datasets) and lactotrophs (ranging from 9.1% to 26.9% of cells per dataset) (Figure 7E). The proportion of non-endocrine cells was low in all datasets (Figure 7E). The Ho 2020 dataset differed clearly from the other 3 datasets in terms of the lower total number of cells (Table 1), the presence of a multihormonal cell cluster and an absence of endothelial cells and Sox2+ve cells (Figures 7D and E).

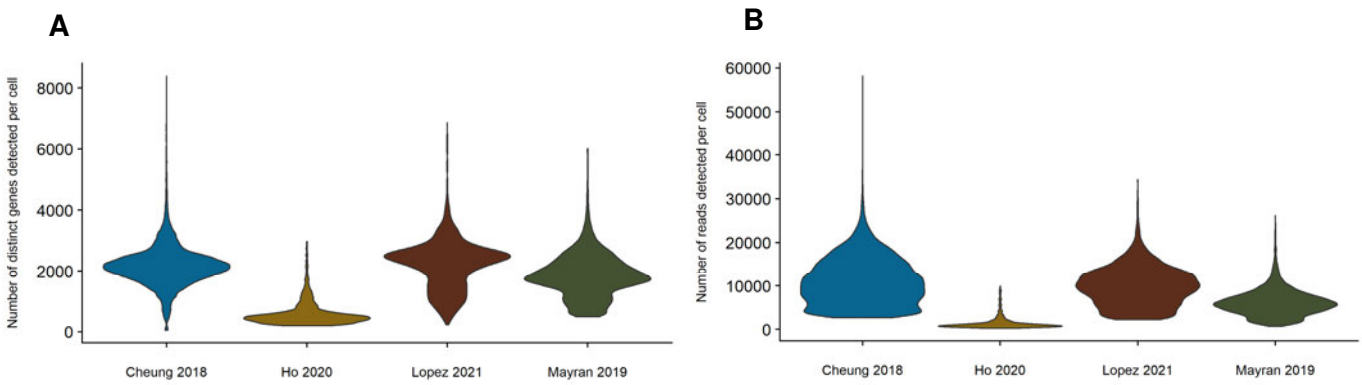


**Figure 6. The distribution of (A) the number of genes per cell and (B) the number of reads per cell in the datasets.** All datasets differed significantly from each other in terms of genes per cell and reads per cell (negative binomial generalised linear model with log link on raw count data). Genes per cell, estimated coefficients (Ho: 0.2775, 95% confidence interval [0.2721, 0.2831], Lopez: 1.0202 [1.0076, 1.0329], Mayran: 0.8593 [0.8506, 0.8681]) and test statistics (z) (Ho: $5x10^{-56}$, Lopez: 0.232, Mayran: $3x10^{-13}$). Reads per cell, estimated coefficients (Ho: 0.1324 [0.1278, 0.1348], Lopez: 0.8928 [0.8778, 0.9081], Mayran: 0.5507 [0.5431, 0.5583]) and test statistics (z) (Ho: $2x10^{-65}$, Lopez: $2x10^{-6}$, Mayran: $2x10^{-37}$]). All model p values < 0.001. Post-hoc comparisons of the estimated marginal means indicated that all datasets differed significantly from each other ($p < 0.001$).

To assess whether the datasets differed aside from cell type proportion, gene expression profiles of the endocrine cell types were compared. Genes expressed in common by each cell type in all datasets were ranked based on their mean expression in each dataset and their rank correlation was determined. Gene expression rank was highly correlated in Cheung 2018, Lopez 2021 and Mayran 2019 for all endocrine cell types (Spearman's rank correlation coefficient $\rho=0.87$-$0.97$, $p<0.001$ for each comparison, Table 4), whereas Ho 2020 differed from all the other datasets based on gene ranks ($\rho=0.47$-$0.72$ for all cell types, $p<0.001$) (Table 4). The Cheung 2018, Lopez 2021 and Mayran 2019 datasets therefore shared gene expression profiles but differed in the cell types recovered. The Ho 2020 dataset was clearly different from the other 3 datasets on all accounts.

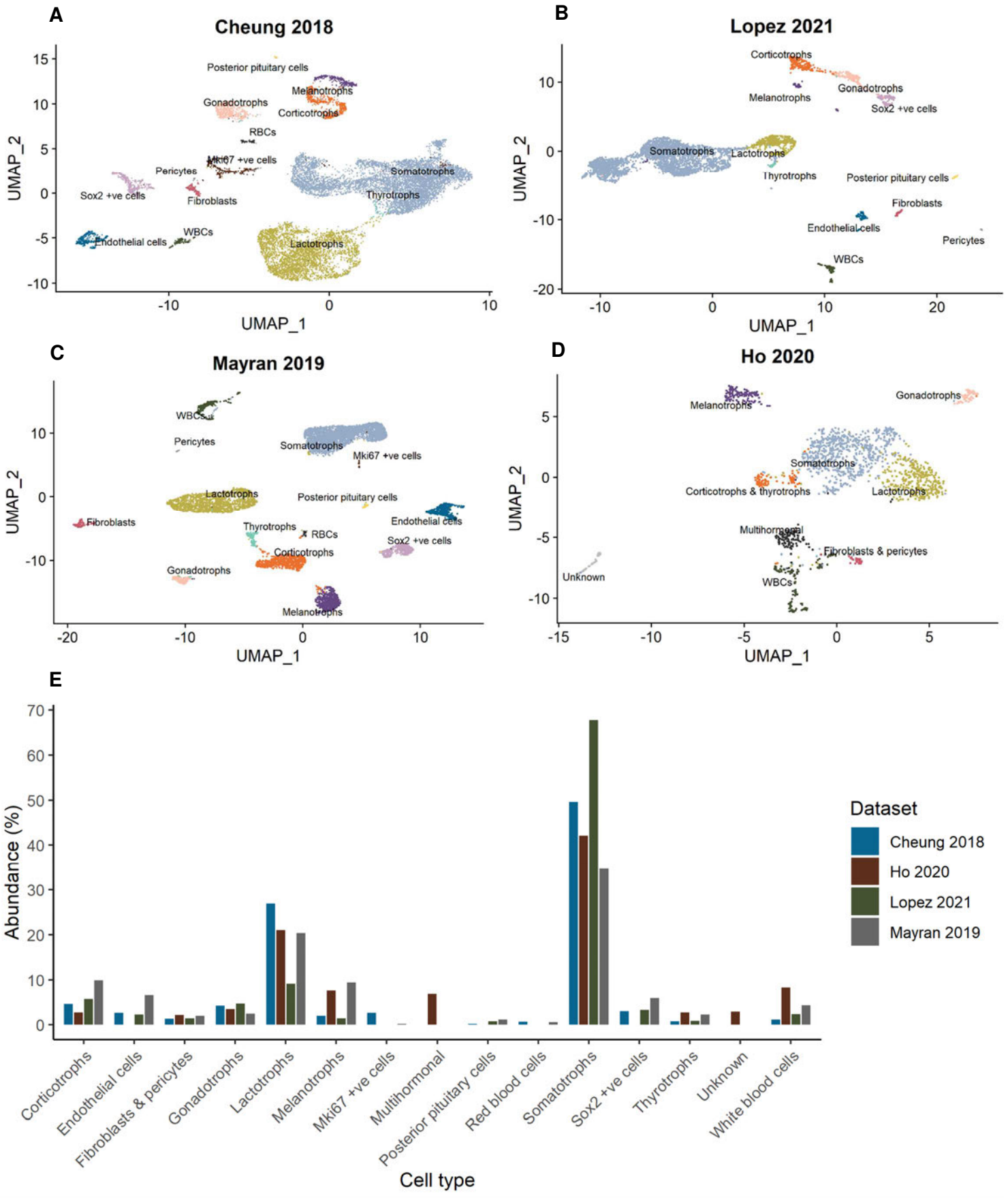
A
Cheung 2018
Posterior pituitary cells
Melanotrophs
Gonadotrophs
Corticotrophs
RBCs
Mki67 +ve cells
Pericytes
Somatotrophs
Sox2 +ve cells
Fibroblasts
Thyrotrophs
WBCs
Endothelial cells
Lactotrophs
UMAP_2
UMAP_1
B
Lopez 2021
Corticotrophs
Melanotrophs
Gonadotrophs
Sox2 +ve cells
Somatotrophs
Lactotrophs
Thyrotrophs
Posterior pituitary cells
Fibroblasts
Endothelial cells
Pericytes
WBCs
UMAP_2
UMAP_1
C
Mayran 2019
WBCs
Pericytes
Somatotrophs
Mki67 +ve cells
Lactotrophs
Posterior pituitary cells
Fibroblasts
Endothelial cells
Thyrotrophs
RBCs
Sox2 +ve cells
Corticotrophs
Gonadotrophs
Melanotrophs
UMAP_2
UMAP_1
D
Ho 2020
Melanotrophs
Gonadotrophs
Somatotrophs
Corticotrophs & thyrotrophs
Lactotrophs
Multihormonal
Fibroblasts & pericytes
Unknown
WBCs
UMAP_2
UMAP_1
E
Abundance (%)
Corticotrophs
Endothelial cells
Fibroblasts & pericytes
Gonadotrophs
Lactotrophs
Melanotrophs
Mki67 +ve cells
Multihormonal
Posterior pituitary cells
Red blood cells
Somatotrophs
Sox2 +ve cells
Thyrotrophs
Unknown
White blood cells
Cell type
Dataset
Cheung 2018
Ho 2020
Lopez 2021
Mayran 2019

**Figure 7. Cell type-identified clusters in 4 mouse pituitary scRNA-seq datasets.** UMAP plots of clusters matched to cell types based on marker gene expression distribution (A-D) and the abundance of each cell type per dataset (E). Statistical comparisons between the number of cells belonging to each cell type compared to the number of cells belonging to other cell types between datasets indicated that the cell type abundance differed between datasets for all cell types ($X^2$ test, $p<1x10^{-5}$ for all). All post-hoc pairwise comparisons were significant at adjusted p value < 0.05 except: corticotrophs (Lopez vs Ho), endothelial cells (Cheung vs Lopez), fibroblasts & pericytes (Cheung vs Lopez and Mayran vs Ho), gonadotrophs (Cheung vs Lopez, Cheung vs Ho and Lopez vs Ho), melanotrophs (Mayran vs Ho), Mki67+ve cells (Mayran vs Ho), posterior pituitary cells (Cheung vs Ho and Lopez vs Mayran), red blood cells (Cheung vs Mayran), Sox2+ve cells (Cheung vs Lopez), thyrotrophs (Cheung vs Lopez).

| Comparison | Spearman's rank correlation coefficient (ρ) | | | | |
|---|---|---|---|---|---|
| | Corticotrophs | Gonadotrophs | Lactotrophs | Somatotrophs | Thyrotrophs |
| **Cheung 2018 – Ho 2020** | - | 0.47 | 0.63 | 0.69 | - |
| **Cheung 2018 – Lopez 2021** | 0.95 | 0.96 | 0.97 | 0.97 | 0.87 |
| **Cheung 2018 – Mayran 2019** | 0.95 | 0.94 | 0.97 | 0.96 | 0.90 |
| **Ho 2020 – Lopez 2021** | - | 0.50 | 0.65 | 0.72 | - |
| **Ho 2020 – Mayran 2019** | - | 0.49 | 0.62 | 0.72 | - |
| **Lopez 2021 – Mayran 2019** | 0.96 | 0.94 | 0.96 | 0.97 | 0.88 |

**Table 4: Correlation between the ranks of shared genes expressed in each cell type between different pituitary scRNA-seq datasets.** Corticotrophs and thyrotrophs could not be distinguished from each other in the Ho 2020 dataset, hence comparison was not performed for these cell types in the dataset.

## 3.2 Creation of an integrated scRNA-seq dataset

Given the differences revealed between the datasets, relying on a single dataset may provide a biased representation of gene expression in pituitary cells. One way to reduce the impact of this bias is to merge the different datasets into one (Butler et al. 2018; Forcato et al. 2021). To create a large scRNA-seq resource on the pituitary, the Cheung 2018, Lopez 2021 and Mayran 2019 datasets were combined, merging a total of 27,134 pituitary cells. The Ho 2020 dataset was excluded based on the results indicating that it diverged clearly from the other 3 datasets (Tables 1 and 4, Figure 7).

### *3.2.1 Uncorrected combined datasets exhibit dataset-dependent clustering*

A common problem with combining cells from scRNA-seq datasets is the tendency of the cells to cluster together based on their dataset of origin instead of their cell type identity (Butler et al. 2018). This is due to the strong effects that different protocols and laboratory environments have on the genes expressed by cells (Tung et al. 2017; Haghverdi et al. 2018; Luecken and Theis 2019). To assess whether this problem applied to the present combined dataset, the cells were first clustered naively without any correction for their dataset of origin. An unbiased clustering approach showed that the clusters of cells separated by dataset (Figures 8A and C); only 7 out of 15 clusters were composed of cells from all 3 datasets (Figure 8E). Cells from the Cheung 2018 and Lopez 2021 datasets showed greater co-clustering (all clusters containing cells from Lopez 2021 also contained cells from Cheung 2018, and all but 1 cluster containing cells from Cheung 2018 also contained cells from Lopez 2021), whereas cells from the Mayran 2019 dataset clustered more with each other (4 clusters were made up of cells from the Mayran 2019 dataset only) (Figure 8E). These results indicate that the influence of the dataset of origin must be removed before shared cell types can be found between the datasets.

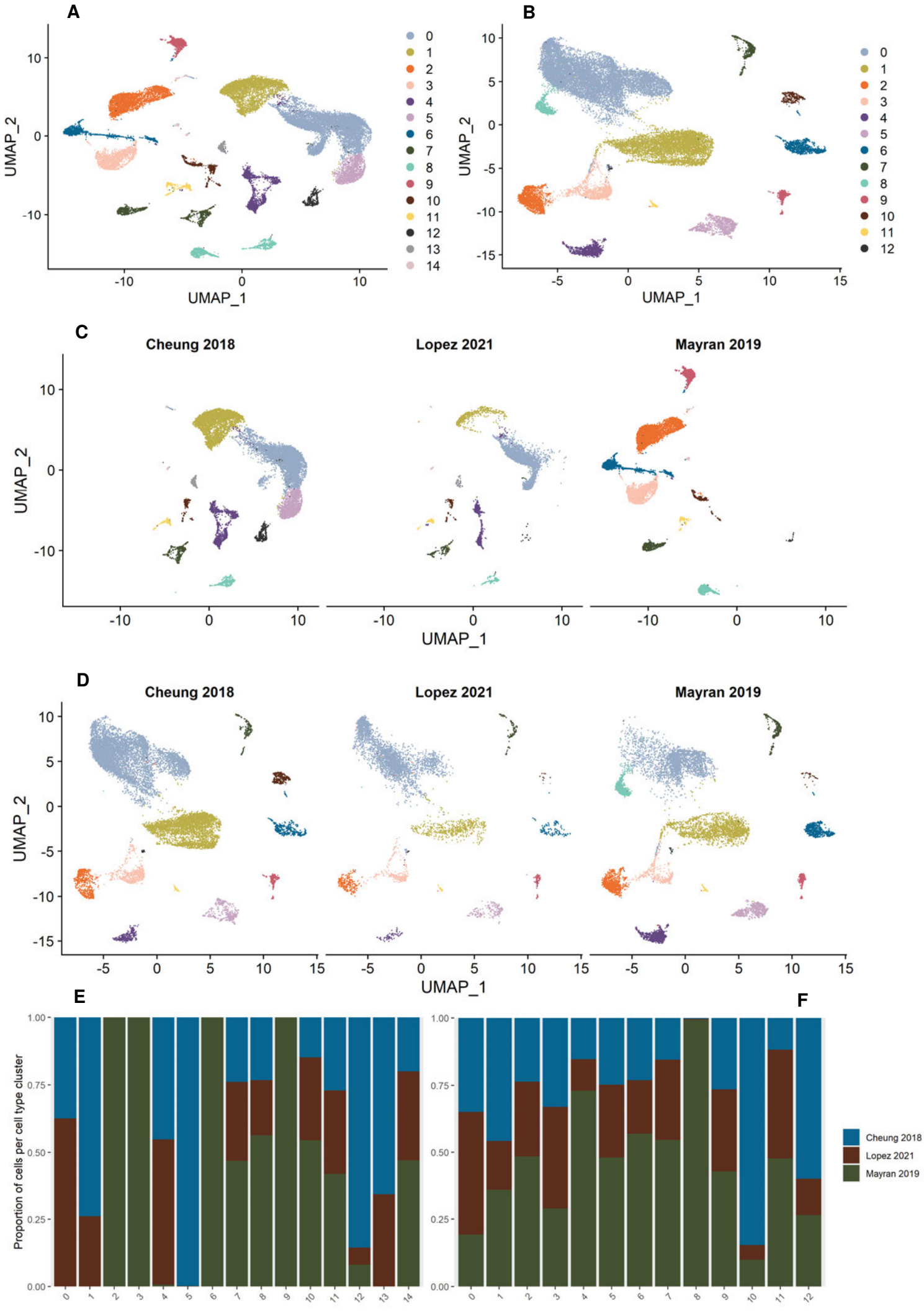
A
B
C
D
E
F
UMAP_1
UMAP_2
Cheung 2018
Lopez 2021
Mayran 2019
Proportion of cells per cell type cluster
Cheung 2018
Lopez 2021
Mayran 2019

**Figure 8. Loss of dataset-specific clustering with data integration.** Clusters on a UMAP plot identified at resolution 0.1 with log-normalised expression values uncorrected for dataset-of-origin (A). Clusters on a UMAP plot identified at resolution 0.1 following dataset integration to account for influence of dataset on expression values (B). Uncorrected (C) and integrated (D) clusters on a UMAP plot split by dataset. Proportion of cells per cluster in the uncorrected (E) and integrated (F) dataset split by dataset of origin.

### *3.2.2 Integration allows alignment of cell type identities across datasets*

Harmony (Korsunsky et al. 2019) is an algorithm that removes dataset-specific influences from combined scRNA-seq data using an iterative approach that biases against the formation of clusters with cells from a single dataset. Unbiased clustering following data integration by Harmony produced clusters that were composed of cells from all datasets (except a single cluster containing cells from the Mayran 2019 dataset only, Figures 8B, D and F). To identify cell types among the clusters, the expression distribution of common marker genes for pituitary cell types was examined (Figure 9A). In total, 14 different cell types were identified based on marker gene expression (Figures 9A and B). Aside from potential ambient contamination of hormone transcripts, expression of each marker gene was restricted to a specific cluster/cell type (Figure 9B). All endocrine cell types of the pituitary (corticotrophs, gonadotrophs, lactotrophs, somatotrophs, and thyrotrophs) were represented in the dataset, with somatotrophs and lactotrophs making up a large majority of cells (Figure 9D). A cluster of cells characterised by proliferation marker Mki67 also expressed growth hormone and some prolactin above ambient expression of the hormones in all cell types, indicating that the cluster is likely made up of proliferating somatotrophs and lactotrophs (Figure 9B). A range of non-endocrine cell types: endothelial cells, fibroblasts, pericytes, red blood cells, Sox2+ve cells, and white blood cells were also identified. A small cluster of posterior pituitary cells was present, as well as a cluster of intermediate lobe melanotrophs.

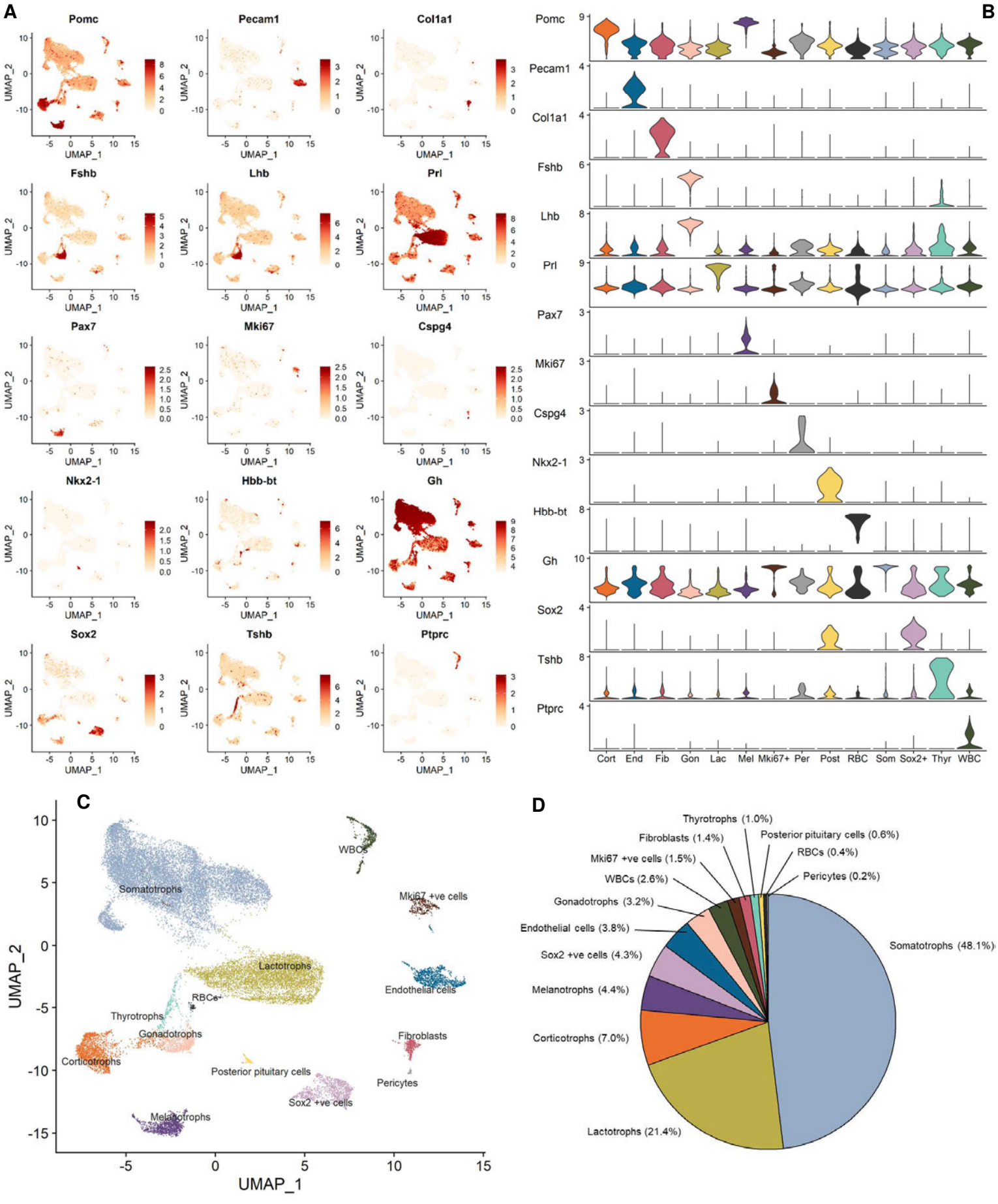
A
Pomc
Pecam1
Col1a1
Fshb
Lhb
Prl
Pax7
Mki67
Cspg4
Nkx2-1
Hbb-bt
Gh
Sox2
Tshb
Ptprc
UMAP_1
UMAP_2
B
Cort
End
Fib
Gon
Lac
Mel
Mki67+
Per
Post
RBC
Som
Sox2+
Thyr
WBC
C
Somatotrophs
Lactotrophs
Thyrotrophs
Gonadotrophs
Corticotrophs
Melanotrophs
RBCs
WBCs
Mki67 +ve cells
Endothelial cells
Fibroblasts
Pericytes
Posterior pituitary cells
Sox2 +ve cells
D
Somatotrophs (48.1%)
Lactotrophs (21.4%)
Corticotrophs (7.0%)
Melanotrophs (4.4%)
Sox2 +ve cells (4.3%)
Endothelial cells (3.8%)
Gonadotrophs (3.2%)
WBCs (2.6%)
Mki67 +ve cells (1.5%)
Fibroblasts (1.4%)
Thyrotrophs (1.0%)
Posterior pituitary cells (0.6%)
RBCs (0.4%)
Pericytes (0.2%)

**Figure 9. Cell type identities of clusters in the integrated dataset.** Expression distribution of pituitary cell type-specific markers among clusters (A) and identified cell types (B). Corticotrophs (Pomc), endothelial cells (Pecam1), fibroblasts (Col1a1), gonadotrophs (Fshb, Lhb), lactotrophs (Prl), melanotrophs (Pomc, Pax7), Mki67 +ve proliferating cells (Mki67), pericytes (Cspg4), posterior pituitary cells (Nkx2-1), red blood cells (Hbb-bt), somatotrophs (Gh), Sox2+ve cells (Sox2), thyrotrophs (Tshb), white blood cells (Ptprc). UMAP plot showing 14 distinct clusters of male murine pituitary gland cells integrated from 3 different datasets (C). Percent of each cell type in the integrated dataset (D). Cell type abbreviations: cort = corticotrophs, end = endothelial cells, fib = fibroblasts, gon = gonadotrophs, lac = lactotrophs, mel = melanotrophs, Mki67+ = Mki67 +ve cells, per = pericytes, post = posterior pituitary cells, RBCs = red blood cells, som = somatotrophs, Sox2+ = Sox2+ve cells, thyr = thyrotrophs, WBCs = white blood cells.

## 3.3 Assessing the dropout effect in pituitary scRNA-seq data

### *3.3.1 Does integration increase the power to detect expression of genes?*

A major limitation of scRNA-seq data is the dropout effect, in which expression values for a large number of genes are missing or very low (Kharchenko et al. 2014). Integration of scRNA-seq datasets can increase the power to detect gene expression by combining expression data from a greater number of cells (Haghverdi et al. 2018; Stuart and Satija 2019). A case study of corticotrophs was undertaken to determine whether the integrated pituitary scRNA-seq dataset provided richer gene expression data compared to a single dataset. The number of corticotrophs identified in the integrated dataset (1,893 cells) was slightly larger than the sum of corticotrophs in the 3 different datasets (Cheung 2018: 620 cells, Lopez 2021: 284 cells, Mayran 2019: 878 cells, total: 1,782 cells), indicating that cells not categorised as corticotrophs in the individual datasets were assigned as corticotrophs in the integrated dataset. The total number of genes detected in the integrated dataset was only marginally greater than the number detected in individual datasets (Figure 10A). This indicated that (a) the genes expressed in corticotrophs from the different datasets overlapped to a great extent and (b) that there is a limit beyond which including more corticotrophs does not increase the breadth of gene expression in terms of the number of genes detected. To determine where this limit may occur, the number of genes expressed in random subsamples of integrated corticotrophs was calculated (Figure 10B). Fitting a quadratic plateau model to the resulting data suggested that including any more than approximately 630 cells from the different datasets no longer diversified the genes expressed in the overall cell type population (Figure 10B).

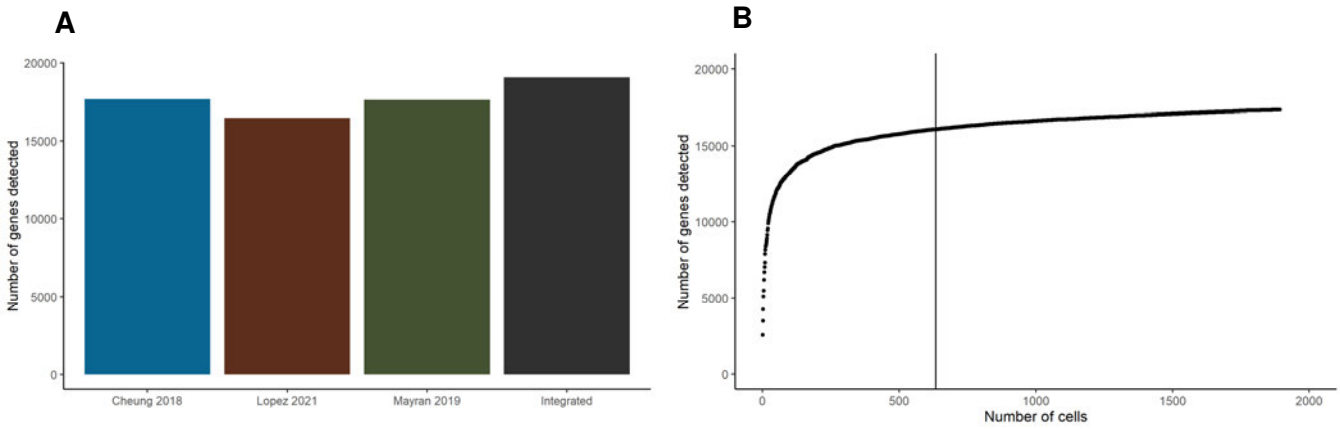


**Figure 10. The effect of dataset integration on the number of genes detected in corticotrophs.** (A) The number of genes detected in corticotrophs identified in the integrated and individual scRNA-seq datasets. (B) The number of genes detected in increasing numbers of random cells from the integrated corticotroph population. The vertical line at 630 cells indicates the number of cells after which the number of genes detected plateaus, as determined by fitting a quadratic plateau model to the data.

To assess whether integration increases the power to detect differentially expressed genes (DEGs), genes distinguishing corticotrophs from gonadotrophs and somatotrophs from lactotrophs were found in the individual datasets and the integrated dataset. The pairs of cell types were chosen to compare populations with as similar numbers of cells as possible. The number of markers found for corticotrophs was higher in the integrated dataset compared to any of the single datasets, however, more markers were found in the individual Lopez 2021 and Mayran 2019 datasets than in the integrated dataset for gonadotrophs (Table 5). For somatotrophs, more markers were found in the Lopez 2021 and Mayran 2019 datasets compared to the integrated dataset, whereas for lactotrophs more markers were found in the Mayran 2019 compared to the integrated dataset (Table 5).

| Cell type | Number of differentially expressed genes | | | |
|---|---|---|---|---|
| | Cheung 2018 | Lopez 2021 | Mayran 2019 | Integrated |
| **Corticotrophs** | 437 | 252 | 391 | 589 |
| **Gonadotrophs** | 457 | 716 | 603 | 468 |
| **Somatotrophs** | 56 | 101 | 126 | 69 |
| **Lactotrophs** | 1,516 | 1,188 | 1,784 | 1,685 |

**Table 5. The number of differentially expressed genes distinguishing corticotrophs from gonadotrophs and somatotrophs from lactotrophs in integrated and individual scRNA-seq datasets.**

### *3.3.2 Extent and impact of the dropout effect in pituitary scRNA-seq data*

Widely different estimates for the dropout rate (here defined as the percent of genes not detected by scRNA-seq data but detected by other technologies) and its effects on downstream analyses have been reported in the literature (Hicks et al. 2018; Qiu 2020; Cain et al. 2020; Xu et al. 2020). To estimate the extent of the dropout effect in pituitary scRNA-seq data and its impact on the ability to use scRNA-seq data to predict molecules involved in cell-cell interactions, gene expression profiles of corticotrophs from available scRNA-seq, bulk RNA-seq and microarray data were compared.

First, taking an unbiased approach where any gene expressed above zero was counted as expressed, the total number of genes detected in corticotrophs was highest in bulk RNA-seq data (29,280 genes), followed by microarray (24,405 genes) and scRNA-seq data (17,345 genes) (Figure 11A). Of the 36,576 unique genes, 39% were detected by all 3 methods, 25% were detected by bulk RNA-seq only, 16% by microarray only, and 2.9% by scRNA-seq only (Figure 11B). The overall dropout rate in corticotrophs was therefore 47% (Figure 11B). Expression of the genes common to all datasets was correlated to a similar extent between the different methods ($\rho$=0.78-0.81, gene rank comparison, Figures 11C-E). Plotting the dropout rate as a function of gene expression level in bulk RNA-seq and microarray data showed that the dropout rate is higher for lowly expressed genes (Figures 11F and G).

To try to exclude genes that were only detected due to technical noise, a more biased approach was taken where the genes expressed were selected based on density distributions of the expression values (Figures 12A-C). Using expression thresholds (Figures 12A-C) that would filter out abundant, lowly expressed transcripts based on the distributions, the highest number of genes was still detected in bulk RNA-seq data (15,240 genes), followed by microarray (9,938 genes) and scRNA-seq data (6,723 genes) (Figure 12D). The overall dropout rate based on this set of genes was 60%, a greater percentage compared to the previous estimate due to a greater proportion of genes uniquely detected by bulk RNA-seq (33% vs 25%) and by both microarray and bulk RNA-seq (21% vs 11%) (Figure 12E).

Next, the impact of dropout on the ability to detect expression of genes relevant to pituitary cell-cell interactions in scRNA-seq data was examined. Of 2,948 genes annotated to Gene Ontology terms ‘cell adhesion’ and ‘cell-cell signalling’, 2,768 were detected in microarray, 2,583 in bulk RNA-seq and 2,212 in scRNA-seq data from corticotrophs (Figure 12F). Most genes (75%) relevant to cell-cell interactions were detected by all techniques and the dropout rate for these genes was 23% in scRNA-seq data (Figure 12G).

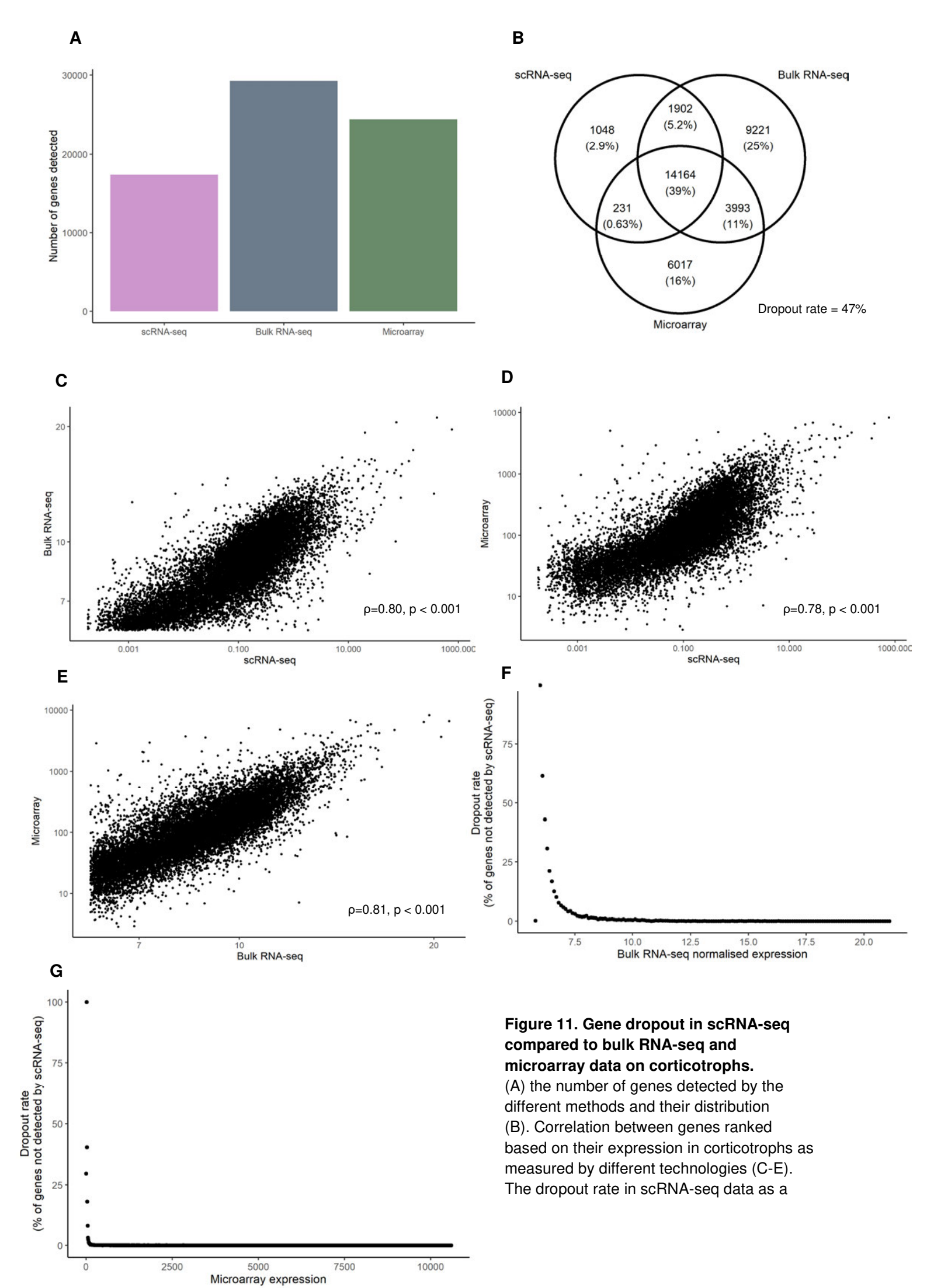


**Figure 11. Gene dropout in scRNA-seq compared to bulk RNA-seq and microarray data on corticotrophs.**
(A) the number of genes detected by the different methods and their distribution (B). Correlation between genes ranked based on their expression in corticotrophs as measured by different technologies (C-E). The dropout rate in scRNA-seq data as a

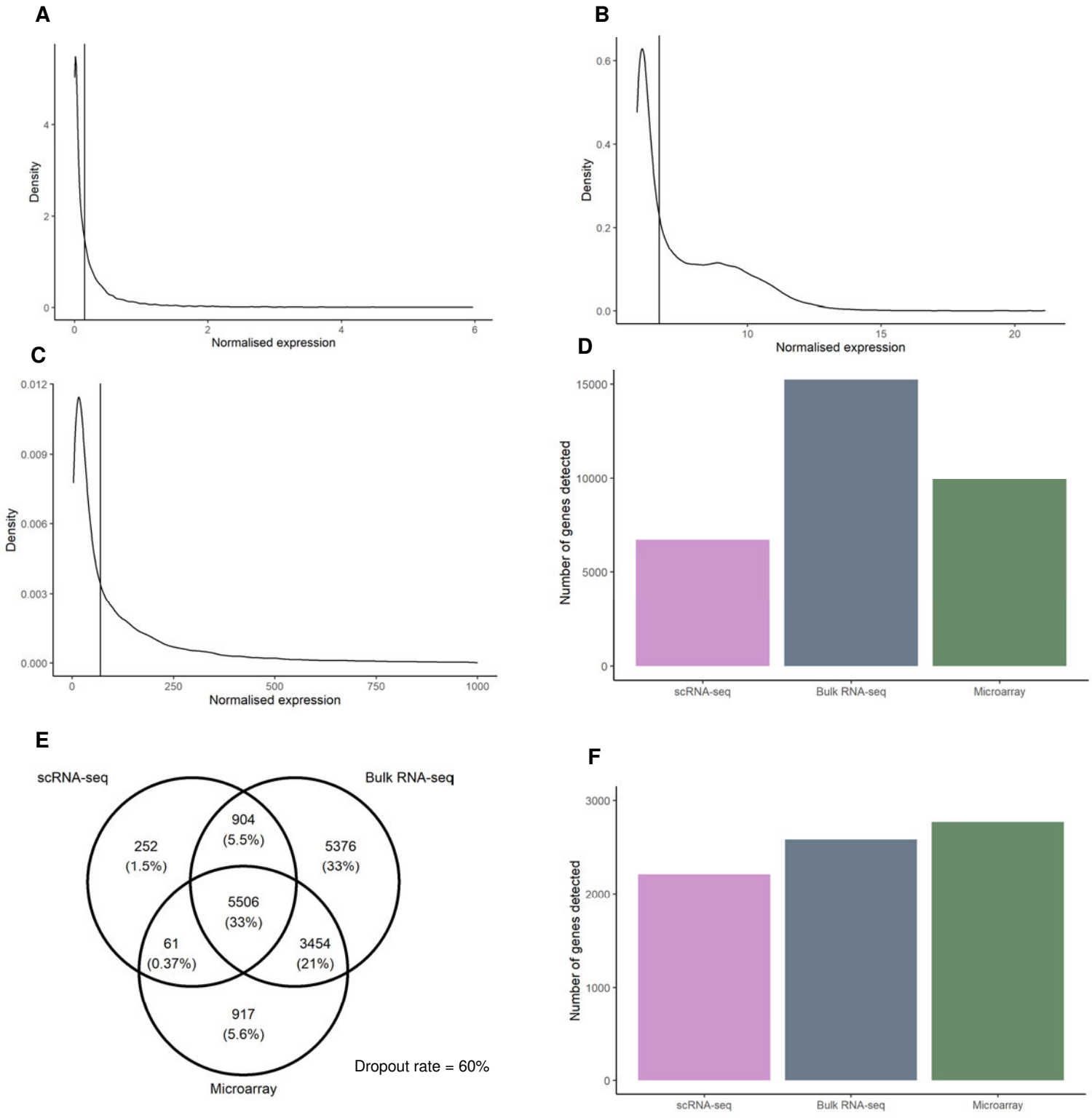


**Figure 12. Gene dropout for subsets of genes expressed in corticotrophs.** Density distributions of normalised gene expression values in corticotrophs measured by scRNA-seq (A), bulk RNA-seq (B) and microarray (C) and the thresholds selected (scRNA-seq: 0.15, bulk: 6.7, microarray: 70). (D) The number of genes expressed in corticotrophs based on the thresholds and (E) their distribution between methods. (F) Number of genes relevant for cell-cell interactions detected in corticotrophs and (G) their distribution between methods.

## 3.4 Correlation between the pituitary cell types expressing mRNA and protein for paracrine ligands

Prediction of bona fide cell-cell interactions from transcriptomics data relies on the assumption that there is a strong correlation between mRNA and protein expression for the molecules involved. To investigate if this assumption holds true for molecules involved in cell-cell interactions in the pituitary, the distribution of different ligands with established paracrine functions in pituitary cell types at protein level in the published literature was compared with their mRNA distribution in pituitary scRNA-seq data. The comparison was restricted to ligands with paracrine functions given the lack of molecules known to mediate homotypic adhesion between pituitary cells.

### *3.4.1 Heterogeneity between studies, species and sex for expression of pituitary paracrine ligands*

There was great heterogeneity between studies regarding the cell type(s) found to express each ligand, even for the same sex and species (Table 6). For example, Morel *et al.* found expression of substance P in gonadotrophs and lactotrophs and no expression in somatotrophs or thyrotrophs of male and female rats, whereas Brown *et al.* found expression in somatotrophs and thyrotrophs but no expression in gonadotrophs or lactotrophs, also in male and female rats (Morel et al. 1982b; Brown et al. 1991).

Comparison between ligand mRNA expression in male mouse and male and female rat pituitaries revealed remarkable species and/or sex differences (Table 7). Neuromedin U was expressed in 29.7% of gonadotrophs and less than 2% of all other endocrine cell types in the male mouse, whereas it was expressed in 57.6% of thyrotrophs and over 15% of all other endocrine cell types except lactotrophs in male and female rats (Table 7). Expression of prodynorphin was low in all endocrine cell types in the male mouse pituitary, whereas 46.3% of melanotrophs and 33.7% of gonadotrophs expressed the gene in male and female rat pituitaries (Table 7).

| | | | | Cell type | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Gene | Protein | Sex and species | Reference | Corticotrophs | Folliculostellate cells | Gonadotrophs | Melanotrophs | Lactotrophs | Somatotrophs | Thyrotrophs |
| *Agt* | Angiotensin II | Male rat | Deschepper et al., 1986 | | | High | | | | |
| | | Male rat | McKenzie et al., 1985 | | | High | | No | No | |
| | | Male rat | Steele et al., 1982 | | | High | | High | | |
| | | Male rat | Thomas and Sernia, 1990 | | No | High | No | No | | |
| | | Male rat | Naruse et al., 1986 | | | High | | No | | |
| | Angiotensinogen | Male rat | Thomas and Sernia, 1990 | | No | High | Low | No | | |
| | | Male rat | Deschepper and Ganong, 1991 | No | | No | | No | No | No |
| *Angpt1* | Angiopoietin 1 | Male rat | Nag et al., 2005 | No | | High | No | | | No |
| *Calca* | Calcitonin gene related peptide | Male and female rat | Gon et al., 1990 | | | High | Low | | Low | Low |
| *Cartpt* | Cocaine and amphetamine regulated transcript | Male rat | Kuriyama et al., 2004 | No | No | High | | No | No | No |
| | | Male and female rat | Smith et al., 2006 | Low | | No | No | High | Low | No |
| | | Mouse | Mortensen and Camper, 2016 | No | | No | | No | High | No |
| *Gal* | Galanin | Male and female rat | Steel et al., 1989 | | | | | High | High | High |
| | | Male rat | Hyde et al., 1991 | | | | | | High | |
| | | Male mouse | Moore et al., 1999 | No | | No | | Low | High | Low |
| | | Male and female rat | Cimini et al., 1993 | High | | | | High | | |
| *Grp* | Gastrin releasing peptide | Male rat | Houben and Denef, 1991 | High | | Low | | High | Low | Low |
| *Nmb* | Neuromedin B | Female rat | Steel et al., 1988 | | | | No | | | High |
| | | Female mouse | Steel et al., 1988 | | | | No | | | High |
| *Nmu* | Neuromedin U | Female rat | Steel et al., 1988 | High | | | High | | | |
| | | Female mouse | Steel et al., 1988 | High | | | High | | | |
| | | Male and female rat | Cimini et al., 1993 | High | | No | | No | No | High |
| *Nppc* | C-type natriuretic peptide | Male rat | McArdle et al., 1994 | No | | High | | No | No | No |
| | | Female rat | Thompson et al., 2009 | | | High | | | | |
| *Nrg1* | Neuregulin 1 | Female rat | Zhao and Ren, 2011 | No | No | High | | Low | No | |
| *Pdyn* | Dynorphin | Female rat | Knepel et al., 1986 | | | High | | | | |
| | | Male rat | Schwenk et al., 1987 | | | High | | | | |
| *Tac1* | Substance P | Male and female rat | Morel et al., 1982 | No | | High | | High | No | No |
| | | Male and female rat | Brown et al., 1991 | No | | No | | No | High | Low |
| *Vegfa* | Vascular endothelial growth factor A | Female rat | Alfer et al., 2015 | | | | | High | | |
| | | Male rat | Mukdsi et al., 2005 | | High | High | | High | High | |

**Table 6. Cell type distribution of paracrine ligand expression in the pituitary based on published protein immunostaining data.** "High" indicates predominant expression in a cell type, "Low" indicates low expression in a cell type, "No" indicates that expression was not measured but not detected in a cell type and an empty cell indicates that expression of the ligand was not studied in the cell type. All studies were performed on adult animals and distribution of protein expression was determined using antiserum or purified antibodies to the ligand specified and to markers of pituitary endocrine cell types. Gene abbreviations: *Agt* (Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)), *Angpt1* (Angiopoietin 1), *Calca* (Calcitonin/calcitonin-related polypeptide, alpha), *Cartpt* (CART prepropeptide), *Gal* (Galanin and GMAP prepropeptide), *Grp* (Gastrin releasing peptide), *Nmb* (Neuromedin B), *Nmu* (Neuromedin U), *Nppc* (Natriuretic peptide type C), *Nrg1* (Neuregulin 1), *Pdyn* (Prodynorphin), *Tac1* (Tachykinin 1), *Vegfa* (Vascular endothelial growth factor A).

### *3.4.2 Inconsistent distribution of mRNA and protein of paracrine ligands in pituitary cell types*

Considering the ligands with highest consensus regarding their cell type expression at protein level, there was still a discrepancy between the cell types expressing ligand mRNA and protein (Tables 6 and 7). C-type natriuretic peptide protein was highly expressed in male and female rat gonadotrophs in two studies, with no expression in corticotrophs, lactotrophs, somatotrophs or thyrotrophs also found in one of the studies (Table 1) (McArdle et al. 1994; Thompson et al. 2009). Yet in male and female rat scRNA-seq data, expression of the ligand was highest in corticotrophs and low in gonadotrophs (Table 7). Neuromedin U was consistently expressed in both mouse and rat corticotrophs (Steel et al. 1988; Cimini et al. 1993). However, in mouse scRNA-seq data, expression was low in corticotrophs and instead higher in gonadotrophs (Table 7). In rat scRNA-seq data, neuromedin U was expressed in over 15% of all endocrine cell types except melanotrophs (Table 7). Yet, no neuromedin U protein was found in gonadotrophs, lactotrophs or somatotrophs in rat pituitaries (Cimini et al. 1993). mRNA for vascular endothelial growth factor A (VEGFA) was high in gonadotrophs of both mice and rats and low in other cell types (Table 7). At protein level, two studies found high VEGFA expression in rat lactotrophs (Mukdsi et al. 2005; Alfer et al. 2015) and one of them also detected VEGFA in FS cells, gonadotrophs and somatotrophs (Mukdsi et al. 2005). Thus, protein expression of paracrine ligands in different pituitary cell types does not always reflect mRNA expression of the ligands in scRNA-seq data.

| | Percent of cell type expressing each gene | | | | | |
|---|---|---|---|---|---|---|
| **Gene** | **Corticotrophs** | **Gonadotrophs** | **Lactotrophs** | **Melanotrophs** | **Somatotrophs** | **Thyrotrophs** |
| | | | **Mouse** | | | |
| *Agt* | 0.6 | 13.6 | 0.1 | 0.0 | 0.1 | 1.4 |
| *Angpt1* | 1.7 | 0.9 | 37.3 | 1.8 | 1.3 | 1.8 |
| *Calca* | 5.3 | 1.9 | 0.8 | 0.3 | 0.6 | 0.0 |
| *Cartpt* | 5.2 | 2.6 | 6.5 | 0.6 | 6.6 | 10.4 |
| *Gal* | 6.3 | 22.0 | 28.2 | 7.6 | 5.5 | 9.0 |
| *Grp* | 1.7 | 18.7 | 0.2 | 0.3 | 0.1 | 0.4 |
| *Nmb* | 17.1 | 14.4 | 2.6 | 11.0 | 1.8 | 4.7 |
| *Nmu* | 0.7 | 29.7 | 0.1 | 0.6 | 0.5 | 1.1 |
| *Nppc* | 3.7 | 10.1 | 0.6 | 0.7 | 0.3 | 2.9 |
| *Nrg1* | 8.6 | 27.0 | 5.5 | 2.1 | 8.0 | 6.8 |
| *Pdyn* | 3.6 | 2.5 | 2.4 | 8.1 | 4.3 | 0.4 |
| *Tac1* | 0.4 | 0.3 | 0.2 | 0.3 | 0.6 | 0.0 |
| *Vegfa* | 1.5 | 40.9 | 1.3 | 0.5 | 1.0 | 2.2 |
| | | | **Rat** | | | |
| *Agt* | 0.3 | 0.4 | 0.8 | 0.0 | 0.7 | 0.0 |
| *Angpt1* | 20.6 | 4.2 | 14.3 | 3.7 | 23.1 | 14.4 |
| *Calca* | 3.8 | 8.0 | 1.7 | 5.6 | 13.1 | 6.5 |
| *Cartpt* | 4.5 | 4.6 | 10.8 | 11.1 | 4.9 | 9.4 |
| *Gal* | 2.2 | 2.7 | 33.8 | 0.0 | 24.7 | 7.2 |
| *Grp* | 1.6 | 0.4 | 0.0 | 0.0 | 0.0 | 0.0 |
| *Nmb* | 27.4 | 18.0 | 13.9 | 11.1 | 14.5 | 24.5 |
| *Nmu* | 29.7 | 37.2 | 6.6 | 18.5 | 23.2 | 57.6 |
| *Nppc* | 18.9 | 5.0 | 2.7 | 1.9 | 2.0 | 5.8 |
| *Nrg1* | 30.1 | 32.2 | 15.8 | 3.7 | 25.7 | 32.4 |
| *Pdyn* | 5.6 | 33.7 | 2.3 | 46.3 | 2.8 | 0.7 |
| *Tac1* | 2.0 | 0.4 | 1.5 | 1.9 | 21.6 | 7.9 |
| *Vegfa* | 2.0 | 55.9 | 3.5 | 0.0 | 4.5 | 11.5 |

**Table 7: Expression of paracrine ligands in endocrine cell types of mouse and rat pituitary single cell RNA-sequencing data.** The mouse data are based on the integrated mouse pituitary single cell RNA-sequencing dataset, whereas the rat data are from a publication where single cell RNA-sequencing was performed on a mix of male and female adult rat pituitaries (Fletcher et al. 2019). For gene abbreviations, see Table 6 legend.

# 4 DISCUSSION

This chapter showed that computational approaches can be applied to merge cells from murine pituitary scRNA-seq datasets that differ from each other in terms of their cell type representation and gene expression profiles. A comparison of corticotroph scRNA-seq data with data obtained by bulk RNA-seq or microarray indicated that scRNA-seq data has reduced ability to detect low abundance transcripts but nevertheless a large majority of genes relevant to cell-cell interactions are represented in scRNA-seq data. Examining the cell type distribution of pituitary paracrine ligands in the literature and scRNA-seq data in turn revealed a lack of overlap between protein and mRNA for particular ligands. This pituitary-specific assessment of the impacts of scRNA-seq data integration will guide the approaches taken in the following chapters and help interpret the cell-cell interactions found from the scRNA-seq dataset.

## 4.1 How well do scRNA-seq datasets represent pituitary biology?

scRNA-seq datasets are often presented as unbiased atlases or transcriptome landscapes that offer comprehensive coverage of a tissue (Carter et al. 2018; Zhang et al. 2020a). However, there is growing evidence that the scRNA-seq datasets produced by different laboratories can differ considerably in terms of the clusters of cells identified and the gene expression profiles of these clusters for the same tissue (Crow et al. 2018; Haghverdi et al. 2018). These differences are likely to be a consequence of a variety of biological and technical factors. One significant factor is the low number of biological replicates typically included in transcriptomics studies, which increases the likelihood that the samples reflect the transient, fluctuating nature of gene expression in the specific set of samples instead of the tissue at large. Technical factors, such as differences in library preparation (Luo and Zhang 2018) dissociation procedure and tissue storage (Denisenko et al. 2020; Engelbrecht et al. 2021), and sequencing technology (Mereu et al. 2020) also affect the cells and transcripts recovered in an experiment. Thus, scRNA-seq datasets are products of the specific experimental conditions the cells were exposed to during the experiment.

To increase the probability of detecting dominant biological features of the pituitary instead of some of these dataset-specific characteristics, pituitary scRNA-seq datasets were combined. A comparison between the proportion of endocrine cell types represented in this integrated dataset to the actual anterior pituitary gland can indicate the presence of any obvious biases in the cells recovered. While the comparison is complicated by the variable proportions of cell types reported for the male pituitary, the proportions of somatotrophs (48.1%), lactotrophs (21.4%) and corticotrophs (7.0%) appear consistent with the literature

(Shimon 2014). However, gonadotrophs (3.2%) and thyrotrophs (1.0%) appear to be underrepresented compared to the 10-20% and 5%, respectively, typically present in the pituitary (Ben-Shlomo and Melmed 2011; Shimon 2014). Selective loss of gonadotrophs and thyrotrophs has been reported from passing cells through a flow cytometer, attributed to their larger size compared to other pituitary cell types (Hatfield and Hymer 1986). Gonadotrophs and thyrotrophs also disappear quickly when present in culture (Baker et al. 1974), as does expression of thyroid stimulating hormone beta (Bargi-Souza et al. 2015). However, even if the proportions of cell types in scRNA-seq data and *in vivo* were consistent, it does not exclude that scRNA-seq might selectively recover specific subpopulations of the cell types.

The reasons behind the heterogeneity between the individual pituitary scRNA-seq studies (Cheung et al. 2018; Mayran et al. 2019; Ho et al. 2020; Lopez et al. 2021) also remain unclear. Do the differences reflect true biological differences in the proportions of cell types in the pituitaries subjected to analysis or are the methods of analysis themselves responsible for the differences? There was a potential correlation between the extent of the biological differences between the animals used in the studies and the extent of the differences between the datasets that may support the presence of biology-driven differences. The pituitaries from the Ho 2020 dataset, which was clearly different from the other 3 datasets (Table 4, Figures 6 and 7), were from CD1 mice, whereas all the other studies sequenced pituitaries from C57BL/6 mice (Table 1). Mouse strain can significantly influence the expression of a variety of pituitary factors (Crabbe et al. 1981; Kappeler et al. 2006) and cells from other tissues have been shown to cluster by strain (Kumar et al. 2018; Yang et al. 2021). Moreover, the cells in the Mayran 2019 dataset, which clustered more separately from to the Cheung 2018 and Lopez 2021 datasets (Figures 8C and E), came from older pituitaries (4 months vs 7-10 weeks), which differ both in gene expression and cell type proportion from younger pituitaries (Siperstein et al. 1954; Birge et al. 1967; Marzban et al. 2002). However, any influence of the different processing methods employed to prepare the pituitaries and the influence of the distinct environmental conditions of the laboratories might also contribute to the observed differences.

### 4.2 Benefits of integrating pituitary scRNA-seq data

Integrated datasets composed of cells from many different studies have been shown to increase both the diversity and depth of total gene expression detected due to the increased number of cells (Haghverdi et al. 2018). However, integration of pituitary scRNA-seq datasets did not increase the number of genes expressed in corticotrophs (Figure 10A), suggesting that the corticotrophs sequenced by the different studies expressed similar genes

or that the scRNA-seq technology used detected similar transcripts from the corticotrophs in the different studies. A relatively high correlation between the ranks of genes expressed in corticotrophs of the different datasets (Table 4) also supports the similarity of the corticotrophs. It therefore appears that a limit to the diversity of corticotroph gene expression that can be attained using the current methodology is reached already at a few hundred cells (Figure 10B). Part of this may be because such a large proportion of transcripts in pituitary endocrine cells are hormone transcripts. Integration also did not consistently increase the number of DEGs between pairs of endocrine cell types (Table 5). In fact, the number of DEGs was higher in an individual dataset than in the integrated dataset for gonadotrophs, somatotrophs and lactotrophs (Table 5). This result may indicate that integration reduced the false positive rate for differential expression compared to the individual datasets.

### 4.3 Gene dropout in pituitary scRNA-seq data

Most genes in a cell analysed by scRNA-seq have expression values of zero (Hicks et al. 2018), a phenomenon often referred to as gene dropout (Kharchenko et al. 2014). This is in contrast to data obtained by bulk RNA-sequencing or microarray which have a much lower proportion of zeroes (Bacher and Kendziorski 2016). Whether these zeroes arise for biological and/or for technical reasons has been a point of contention for the last few years (Hicks et al. 2018; Svensson 2020). The proponents of “biological zeroes” argue that the zeroes represent true absence of gene expression in a cell caused by quick turnover of mRNA (Svensson 2020), whereas the proponents of “technical zeroes” argue that experimental limitations (low capture efficiency of cDNA from mRNA and low sequencing depth of the cDNA available) lie behind the abundant zeroes (Hicks et al. 2018).

Assessing the extent of the dropout effect in scRNA-seq data is challenging since the “ground truth” for genes truly expressed in a tissue is unknown. Instead, the dropout rate is often estimated by comparing gene expression data obtained by scRNA-seq to data obtained by other transcriptomics technologies on the same tissue, an approach fraught with the biased transcript recovery and false positives inherent in these technologies (Zheng et al. 2011; Li et al. 2014; Mandelboum et al. 2019; Davies et al. 2021). An attempt to estimate the dropout rate in pituitary scRNA-seq data was nevertheless made by comparing available gene expression data from corticotrophs obtained by scRNA-seq, bulk RNA-seq and microarray, which hopefully gives an indication of dropout in the pituitary at large. The dropout rate (percent of genes detected as expressed by other technologies but not by scRNA-seq) was estimated as 47% when considering all genes detected, with more than half of these missed genes detected by bulk RNA-seq only (Figure 11B). However, counting

any gene detected as expressed in the different datasets likely overestimates the number of genes expressed due to contamination and ambient expression characteristic of single cell and bulk RNA-seq data (Young and Behjati 2020; Moutsopoulos et al. 2021) and the baseline signal present for all genes in microarray data. For this reason, the dropout rate was also estimated using user-selected thresholds for gene expression based on density distributions (Figures 12A-C), which produced an even higher dropout rate (60%, Figure 12E). This was unexpected since lowly expressed genes accounted for most of the dropouts when considering the larger set of genes (Figures 11F and G), a finding consistent with the literature (Kharchenko et al. 2014). It therefore appears that the genes expressed at higher levels in the different datasets did not overlap as well. Whether this reflects biological differences in the pituitaries analysed or technical biases is unclear. Despite the high dropout rate for all genes, the rate was lower (23%) for genes relevant to cell-cell interactions, a positive finding with regards to detecting cell-cell interaction molecules from pituitary scRNA-seq data.

## 4.4 mRNA expression is uncoupled from protein expression for many pituitary paracrine ligands

The lack of correlation between mRNA and protein abundance (Maier et al. 2011; Fortelny et al. 2017; Wang et al. 2019) is an elephant in the room for biological research. Less than 50% of variation in protein expression can be explained by variation in mRNA (de Sousa Abreu et al. 2009), yet due to its low cost, transcriptome profiling is routinely used to infer perturbed biological pathways, build cellular interaction networks and identify new subtypes of cells. Part of this is justified by the axiom that although expression of mRNA and protein might be variable, as long as the mRNA is there in a cell at reasonable levels, then the protein will also be there (Vogel and Marcotte 2012) i.e. the presence of detectable mRNA is predictive of the presence of protein. However, the results in this chapter do not support that assumption.

Examining the expression patterns of pituitary paracrine ligands reveals both “missing” mRNA (protein detected without mRNA) and “missing” protein (mRNA detected but no protein). For example, angiotensinogen mRNA is detected in less than 1% of rat gonadotrophs, whereas angiotensin II is consistently highly expressed in the cell type in rats (Tables 6 and 7). Conversely, over 25% of rat corticotrophs and somatotrophs express neuregulin 1 mRNA, but no neuregulin 1 protein was found in the cell types in the rat (Tables 6 and 7). In part, this could be explained by technical factors. For example, staining for cocaine and amphetamine regulated transcript (CART) protein in the pituitary varied from no

staining to staining throughout the pituitary depending on the antiserum used (Koylu et al. 1997), consistent with the known issues surrounding the specificity of antibodies and antisera (Saper 2009). Furthermore, many of the pituitary paracrine ligands have a number of different protein isoforms for which detection might differ depending on the antibody used (Falls 2003; Stein et al. 2006; Harper and Bates 2008). This might help explain the different protein localisation observed between studies for the same ligand (Table 6). scRNA-seq data in turn suffer from the dropout effect (Kharchenko et al. 2014). It is therefore possible that “missing” mRNA is present in a cell but is too low to be picked up during library preparation. However, this still leaves the conundrum of how cells with such low quantities of mRNA produce an abundance of the protein.

Biological reasons are also likely to contribute to the discrepancies. High mRNA expression without expression of the protein has previously been observed in several tissues, particularly in the testis where 478 proteins were undetected despite expression of mRNA (Wang et al. 2019). These “missing” proteins included GPCRs and cytokines (Wang et al. 2019), all important components of cell-cell communication. It has been proposed that maintaining high mRNA expression might allow a cell to quickly produce protein when the need arises (Perl et al. 2017). It is therefore conceivable that the pituitary ligands with high mRNA-protein correlation might have more of a constant housekeeping function during baseline conditions, whereas ligands with high mRNA and low protein might be required in response to a stimulus. Why and how some proteins are highly expressed despite a low transcript abundance is not clear, but likely at least involves high translation efficiency. mRNA expression can vary considerably between species, whereas expression of protein is better conserved (Laurent et al. 2010), possibly due to much higher selection pressure at the level of proteins (Khan et al. 2013). Despite limited data for protein expression, this pattern also appeared to apply to pituitary paracrine ligands, as the cell types expressing mRNA for a paracrine ligand was generally more inconsistent compared to expression of the protein in mice and rats (see e.g. neuromedin B and neuromedin U, Tables 6 and 7). The fact that discordant gene expression and protein storage and/or secretion has also been shown for pituitary hormones (Scarbrough et al. 1991; Noel and Mains 1991; Castano et al. 1997) may further support that the mRNA-protein disparity of paracrine ligands reflects a true biological phenomenon.

Finally, there are some issues regarding the interpretation of data to consider. First, there is evidence that expression of paracrine ligands can be highly context dependent. For example, expression of CART in the rat pituitary varies widely depending on strain, sex and stage of the oestrus cycle (Kappeler et al. 2006). The mRNA-protein disparity may therefore partly arise from determining mRNA and protein expression in animals that are inherently

different in some important way or have different exposure to certain contextual factors. Furthermore, it should be noted that paracrine ligands are secreted, yet the protein is measured intracellularly. mRNA and protein have been found to correlate poorly for certain secreted factors (Meissner et al. 2013), potentially due to a high rate of secretion which depletes intracellular stores of protein and therefore precludes detection of the secreted factor in a cell. It is also possible that unfolded precursor peptides not detectable by antibodies to the mature form are secreted from the cell type making the mRNA and is converted into the mature form in another cell type, a mechanism proposed for angiotensin II in the pituitary (Ganong 1993). In summary, technical, biological and interpretational issues probably all contribute to the poor correlation between the cell types that express mRNA and protein of pituitary paracrine ligands.

## 4.5 How will these results guide the approaches taken in the following chapters?

The present results have clarified the extent of the limitations of pituitary scRNA-seq datasets. By exploring these limitations before starting any further analyses, it was possible to adjust analysis approaches to try to account for the limitations in the following ways.

### *4.5.1 Prediction of pituitary cell adhesion molecules*

First, the results suggest that relying on a single dataset may provide a biased view of gene expression (Figure 7). For this reason, the integrated dataset will be used to predict pituitary CAMs. Secondly, the extent of the dropout effect and the differences in gene expression profiles obtained by different transcriptomics technologies (Figures 11 and 12) suggest that some CAMs may be missed by relying solely on scRNA-seq data. To mitigate this, microarray data available from different pituitary cell types will be incorporated into the analysis to increase the confidence in the predictions.

### *4.5.2 Prediction of pituitary paracrine ligand-receptor interactions*

The disparity between expression of mRNA and protein for pituitary paracrine factors poses a challenge to predicting paracrine communication from pituitary scRNA-seq data. To try to increase confidence in the predictions, two aspects of the approach were altered. First, instead of investigating paracrine communication during baseline conditions, communication altered by chronic stress was studied instead given evidence that a higher mRNA-protein correlation has been shown for genes differentially expressed during a challenge (Koussounadis et al. 2015). Furthermore, instead of just predicting paracrine interactions based on expression of ligands and receptors, another computational algorithm was

incorporated that predicts functional paracrine interactions based on gene expression changes downstream of receptor activation (Browaeys et al. 2020).

## 4.6 Conclusion

Bioinformatics predictions can only be as good as the underlying data. For this reason, pituitary scRNA-seq data was thoroughly explored in this chapter before setting out on any further analyses. The present results show how scRNA-seq dataset integration can bring together divergent datasets into a single resource but also indicate the limits of integration in terms of increasing the power to detect changes in gene expression, specifically in the pituitary. The dropout rate in pituitary scRNA-seq data varied considerably depending on the subset of genes included in the analysis, highlighting how difficult it is to determine what genes truly are expressed in a cell or tissue. The pituitary cell types expressing paracrine factors in scRNA-seq data frequently differed from the cell types expressing the factors in studies of protein localisation, a bewildering phenomenon that warrants further investigation. Together, these results provide a valuable perspective on the suitability of pituitary scRNA-seq data for bioinformatics analyses of cell-cell interactions.

# CHAPTER 2: IDENTIFYING CANDIDATE CELL ADHESION MOLECULES MEDIATING HOMOTYPIC CELL ADHESION IN THE PITUITARY GLAND

## 1 INTRODUCTION

### 1.1 Pituitary cells form homotypic and heterotypic networks

The longstanding view of the anterior pituitary as a mere static responder to hypothalamic factors has changed in recent decades as growing evidence suggests that the gland architecture plays an active role in modulating cellular responses to extrapituitary factors (Mollard et al. 2012). GH secretion in response to GHRH *in vivo* is several fold higher compared to secretion from dispersed cells *in vitro* (Painson and Tannenbaum 1991; Sugimoto et al. 1991) and studies of pituitary cells in culture have shown that cellular proximity regulates the extent of basal pituitary hormone secretion and secretion in response to hypothalamic secretagogues and paracrine agents (Perez et al. 1995). An important step forward in elucidating the underlying mechanisms was provided by 3D imaging studies that revealed the presence of extensive homotypic cellular networks in the pituitary where cells of the same type formed connections with each other (Le Tissier et al. 2012). The cellular connectivity facilitated communication across the gland as shown by coordinated calcium activity (Fauquier et al. 2001; Bonnefont et al. 2005; Hodson et al. 2012). So far, the homotypic network arrangements of somatotrophs (Bonnefont et al. 2005), lactotrophs (Hodson et al. 2012), corticotrophs and gonadotrophs (Budry et al. 2011; Golan et al. 2016), as well as the non-endocrine FS cells (Fauquier et al. 2001) have been described. The homotypic networks are intermingled with heterotypic networks and together they might allow cellular coordination to ensure optimal pituitary output (Mollard et al. 2012).

### 1.2 Differential cell adhesion could explain the network organisation of the pituitary gland

When pituitary cells are separated and allowed to reaggregate in culture, they spontaneously arrange themselves so that cells of the same type are close to one another (Pals et al. 2006, 2008) (Figure 13). This selective sorting behaviour suggests that the cells have an intrinsic ability to recognise and adhere to other cells homotypically as they do in the pituitary even in the absence of their *in vivo* developmental milieu.

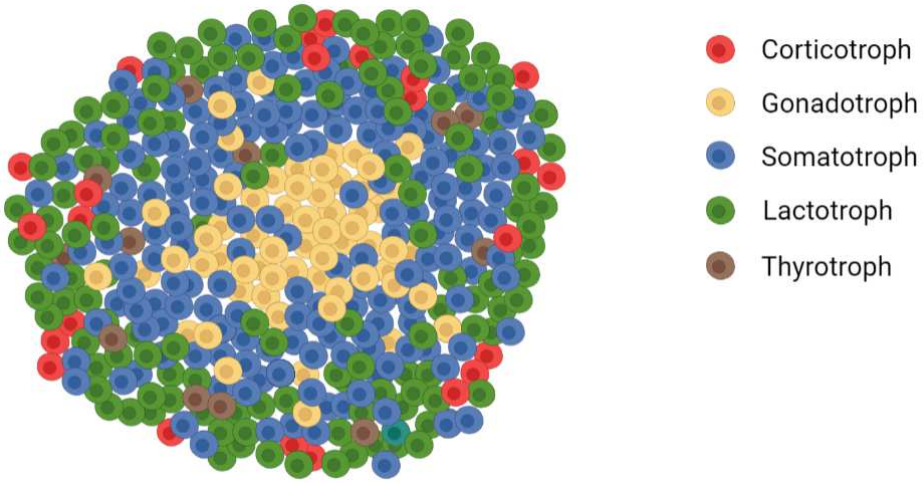


**Figure 13: Cell type distribution in reaggregates of dispersed pituitary cells.** The figure is a representation of the data presented in two publications where pituitaries from 14-day-old rats were dissociated and the cells were allowed to reaggregate in culture (Pals et al. 2006, 2008). Sections of the aggregates were immunolabelled for the different pituitary hormones to identify the cell types present.

Adhesion between cells is mediated by CAMs which are most commonly single-pass transmembrane proteins expressed at the cell surface (Földy et al. 2016). Differential expression of CAMs between cell types has been shown to underly the architecture of tissues such as the pancreas (Rouiller et al. 1991; Jia et al. 2007) and it might therefore also help explain the formation of pituitary networks.

### 1.3 Identification of potential CAMs involved in homotypic networks

Despite the growing interest in pituitary homotypic networks and their functional significance in coordinating pulsatile hormone release (Le Tissier et al. 2012), little has been done to characterise the molecular basis for homotypic adhesion. If differential expression of CAMs is responsible, each pituitary cell type would be expected to exhibit a distinct profile of homotypic adhesion factors to prevent the complete intermixing of cells.

Expression of members of several common CAM families, including cadherins (Chauvet et al. 2009), claudins (García-Godínez et al. 2014) and integrins (Horacek et al. 1994; Kikuchi et al. 2005) has already been described in the pituitary, but definition of their expression patterns has not been sufficient to explain the assembly of pituitary cells into homotypic networks. With regard to junctional structures, different studies have described exclusively tight junctions (Krisch and Buchheim 1984), desmosomes (Saunders et al. 1982), a variety of intercellular junctions (Herbert 1979; Wilfinger et al. 1984) or the absence of any typical

junctions between endocrine cells in the pituitary (Kagayama 1965; Noda et al. 2003). These contradictory findings offer no further clue as to the family of CAMs involved, making the molecular underpinnings of the adhesion in homotypic pituitary cell networks still largely unknown.

To identify potential CAMs mediating homotypic adhesion in corticotrophs, gonadotrophs, somatotrophs and lactotrophs, I performed bioinformatics analyses of available microarray and scRNA-seq datasets of the pituitary gland. The aims of the study were threefold:

1. To create a comprehensive list of genes involved in cell adhesion to facilitate discovery of potential CAMs involved in homotypic networks

2. To generate an initial set of candidate homotypic CAM genes in each cell type

3. To evaluate the set of CAMs to rank candidates in each cell type.

## 2 METHODS

### 2.1 List of CAMs

A comprehensive list of CAM genes in the mouse was collected by combining data from the Gene Ontology Consortium with manually created lists of CAMs presented in recent publications. The complete list of 1,407 genes associated with the Gene Ontology term cell adhesion (GO:0007155) was downloaded from the Mouse Genome Informatics database (Blake et al. 2021). In addition, 406 genes identified as CAMs by Földy *et al.* were included (Földy et al. 2016). A set of 474 human genes identified as “likely” CAMs (Zhong et al. 2015) were converted to mouse orthologues using the biomaRt R package (version 2.46.2) (Durinck et al. 2009). The lists of genes were merged and duplicates removed, which resulted in a final list of 1,611 CAM genes.

### 2.2 Comparing gene expression in microarray data between cell types

The microarray data processed as described in Chapter 1 section 2.3 was used. To generate the initial set of candidate CAMs, expression of the 1,611 cell adhesion-related genes was compared between corticotrophs and gonadotrophs and between somatotrophs and lactotrophs to reveal cell type-enriched CAMs. The $\log_2$-fold change was calculated for each gene expressed in the microarray dataset according the following formula:

$$\mathrm{Log_2\text{-}fold\ change} = \log_2\left(\frac{\text{expression value in cell type X}}{\text{expression value in cell type Y}}\right)$$

where cell type X is corticotrophs or somatotrophs and cell type Y is gonadotrophs or lactotrophs. If multiple probes mapped to the same gene, the expression value for the gene was taken from the mean expression of those probes. UniProt annotations (The UniProt Consortium 2021) were used to determine if proteins encoded by the genes have a single transmembrane domain.

### *2.2.1 Comparing gene expression in a pituitary cell type to expression in the whole anterior lobe*

To account for the different abundance of cell types in the anterior pituitary, the following equation was used to calculate the fold-change in expression of a gene between a cell type and the entire anterior lobe:

$$\text{Adjusted fold-change} = \frac{\mathrm{x}(1-\mathrm{y})}{\mathrm{z}-\mathrm{yx}}$$

where $\mathrm{x}$ is the expression value for a gene in a cell type, $\mathrm{y}$ is the proportion of cells in the anterior lobe that is of that cell type, and $\mathrm{z}$ is the expression value for the gene in the entire anterior lobe. Corticotrophs and gonadotrophs were assumed to both make up 10% of anterior pituitary cells in male mice, whereas somatotrophs and lactotrophs were considered to make up 25% and 35%, respectively, of the female anterior lobe.

## 2.3 Expression of CAMs in pituitary scRNA-seq data

The integrated pituitary scRNA-seq dataset described in Chapter 1 section 2.1 was used to assess the expression of CAMs in adult mouse pituitaries. Access to aligned scRNA-seq data from postnatal day 7 C57BL/6 male mouse pituitaries was kindly provided by Dr Leonard Cheung (University of Michigan) and a published scRNA-seq dataset from mixed male and female adult Sprague Dawley rats (Fletcher et al. 2019) was downloaded from the Gene Expression Omnibus (aligned files, accession number GSE132224). The data were processed and cell types identified with the same pipeline as the individual adult mouse datasets described in Chapter 1 section 2.1 using the quality control filters indicated in Table 2.

DEGs between corticotrophs and gonadotrophs and between somatotrophs and lactotrophs were determined using the FindMarkers function in Seurat. To calculate the percentage of corticotrophs, gonadotrophs, somatotrophs and lactotrophs expressing the candidate CAMs and mean expression of the genes in each cell type, log-normalised expression values for the candidate genes were extracted from the Seurat objects created for each dataset. Any

cell with an expression value above 0 for a gene was considered to express that gene. Mouse genes were converted to their human and rat orthologues using the HomoloGene tool developed by the National Center for Biotechnology Information (NCBI Resource Coordinators 2014). To determine the correlation between gene expression values, the Pearson correlation coefficient (ρ) was calculated using the following formula:

$$\rho = \frac{\sum(x - \bar{x})(y - \bar{y})}{\sqrt{\sum(x - \bar{x})^2 \sum(y - \bar{y})^2}}$$

where $x$ is the expression value for a gene in a cell type or species and $y$ is the expression value for the gene in another cell type or species. $\bar{x}$ denotes mean expression of all the genes considered in the cell type or species and $\bar{y}$ denotes mean expression of all the genes in the other cell type or species.

# 3 RESULTS

## 3.1 A comprehensive list of CAMs to act as a platform for discovery

As outlined in the introduction, expression patterns of common CAMs are not sufficient to explain the specific homotypic adhesion between cells in the pituitary. Identification of possible molecules mediating homotypic network formation therefore requires an extensive list of CAMs to be used as a reference. As no such list was available, one was gathered by combining all murine genes associated with the Gene Ontology term cell adhesion (GO:0007155) with two complementary curated lists of CAMs from two recent publications (Zhong et al. 2015; Földy et al. 2016). This set of 1,611 cell adhesion-related genes encompasses a broad set of genes among which any candidates involved in homotypic network adhesion are likely to be found.

## 3.2 Comparison of microarray expression data from 4 pituitary cell types identifies an initial set of potential CAMs mediating adhesion in homotypic networks

To explain the selective adhesion between homotypic cells, the different cell types must differ in their expression patterns of CAMs. Some cell types in the pituitary also form frequent heterotypic attachments between them, whereas others rarely associate with each other. Cell types that belong to the latter category are likely to show even further divergence in their expression of CAMs to explain why the cell types remain segregated. These cell types include corticotrophs, which form few connections with gonadotrophs, and somatotrophs, which rarely associate with lactotrophs (Sasaki and Iwama 1988; Cónsole et

al. 1999; Noda et al. 2001). Taking advantage of this expected divergence in CAM expression between the two pairs of cell types, DEGs between corticotrophs and gonadotrophs and between somatotrophs and lactotrophs were identified in mouse pituitary scRNA-seq data. However, no CAMs were differentially expressed above 2 $\log_2$-fold between the cell types. This might be due to the dropout effect in scRNA-seq data (Kharchenko et al. 2014; Jiang et al. 2020).

Since no clear candidate CAMs emerged when analysing scRNA-seq data, candidates were instead generated by comparing expression between the pairs of cell types using microarray data from FAC-sorted pituitary cells. Due to the nature of the dataset, the corticotrophs and gonadotrophs compared were from male mice, whereas the somatotrophs and lactotrophs were from female mice. Of the 1,611 adhesion-related genes, 51 were differentially expressed at a threshold of a $\log_2$-fold change of 2 or more between corticotrophs and gonadotrophs. The 2 $\log_2$-fold change, corresponding to a 4-fold change in raw expression, was used as a threshold to obtain CAMs exhibiting considerable enrichment in a cell type. To filter out most genes that encode proteins involved in regulating cell adhesion without having a role as actual CAMs, only those genes encoding proteins with a single transmembrane domain, a shared property of most CAMs (Földy et al. 2016), were included. This initial screening strategy produced 19 CAMs that showed cell type-enriched expression, of which 12 were upregulated in corticotrophs and 7 in gonadotrophs (Table 8). A similar comparison between somatotrophs and lactotrophs revealed 38 adhesion-related genes differentially expressed between the two cell types above a $\log_2$-fold change threshold of 2. Of the 15 genes encoding single-pass transmembrane proteins, 7 were upregulated in somatotrophs and 8 upregulated in lactotrophs (Table 9). The gene names of all candidate CAMs are given in Table 10.

### *3.2.1 Sex differences in expression of candidate CAMs*

To determine whether there were overt sex differences in expression of these candidate CAMs, mean expression of each gene was compared in whole anterior pituitaries of male and female mice. Even when applying a non-stringent threshold of a 1 $\log_2$-fold difference in expression, only 9 of the 34 candidate genes were expressed in a sex-dependent manner (Table 11). While this does not rule out more detailed sex differences in expression at the level of individual cell types, most of the candidate CAMs were expressed at overall similar levels in male and female pituitaries. Of the 9 genes showing sex differences in expression, 3 were enriched in females (*Epha6*, *Il1rapl2* and *Pcdh10*) and 6 were enriched in males (*Alcam*, *Cdh8*, *Ceacam1*, *Cntnap4*, *Il1r1* and *Ptprj*) (Table 11).

| Gene | Expression in corticotrophs compared to gonadotrophs ($log_2$-fold difference) | Cell type enrichment |
|---|---|---|
| *Cdh8* | 3.96 | Corticotrophs |
| *Jam2* | 3.13 | |
| *Ptprz1* | 3.00 | |
| *Dcc* | 2.94 | |
| *Kit* | 2.91 | |
| *Slitrk6* | 2.80 | |
| *Ceacam1* | 2.67 | |
| *Slitrk1* | 2.65 | |
| *Cntnap5b* | 2.54 | |
| *Pcdh9* | 2.47 | |
| *Cdh10* | 2.27 | |
| *Cntnap5a* | 2.01 | |
| *Cntnap4* | -2.04 | Gonadotrophs |
| *Nrp2* | -2.15 | |
| *Cntnap5c* | -2.28 | |
| *Igdcc4* | -2.33 | |
| *Ncam2* | -2.45 | |
| *Lrrn1* | -2.45 | |
| *St6gal1* | -2.47 | |

**Table 8: Cell adhesion molecule genes identified as enriched in corticotrophs or gonadotrophs based on a 2 $log_2$-fold difference in expression between the two cell types in microarray data.**

| Gene | Expression in somatotrophs compared to lactotrophs ($log_2$-fold difference) | Cell type enrichment |
|---|---|---|
| *Il1r1* | 4.49 | Somatotrophs |
| *Alcam* | 3.24 | |
| *Itga6* | 3.03 | |
| *Tenm3* | 2.49 | |
| *Ncam2* | 2.31 | |
| *Ptprj* | 2.15 | |
| *Tenm4* | 2.14 | |
| *Il1rapl2* | -2.03 | Lactotrophs |
| *Nrp1* | -2.29 | |
| *Pcdhb2* | -2.36 | |
| *Cd44* | -2.43 | |
| *Dscam* | -2.48 | |
| *Pcdh10* | -2.89 | |
| *Epha6* | -3.30 | |
| *Ptpro* | -3.54 | |

**Table 9: Cell adhesion molecule genes identified as enriched in somatotrophs or lactotrophs based on a 2 $log_2$-fold difference in expression between the two cell types in microarray data.**

| Gene symbol | Gene name |
|---|---|
| *Alcam* | Activated leukocyte cell adhesion molecule |
| *Cd44* | CD44 antigen |
| *Cdh10* | Cadherin 10 |
| *Cdh8* | Cadherin 8 |
| *Ceacam1* | Carcinoembryonic antigen-related cell adhesion molecule 1 |
| *Cntnap4* | Contactin associated protein-like 4 |
| *Cntnap5a* | Contactin associated protein-like 5a |
| *Cntnap5b* | Contactin associated protein-like 5b |
| *Cntnap5c* | Contactin associated protein-like 5c |
| *Dcc* | Deleted in colorectal carcinoma |
| *Dscam* | DS cell adhesion molecule |
| *Epha6* | Eph receptor A6 |
| *Igdcc4* | Immunoglobulin superfamily, DCC subclass, member 4 |
| *Il1r1* | Interleukin 1 receptor, type I |
| *Il1rapl2* | Interleukin 1 receptor accessory protein-like 2 |
| *Itga6* | Integrin alpha 6 |
| *Jam2* | Junction adhesion molecule 2 |
| *Kit* | KIT proto-oncogene receptor tyrosine kinase |
| *Lrrn1* | Leucine rich repeat protein 1, neuronal |
| *Ncam2* | Neural cell adhesion molecule 2 |
| *Nrp1* | Neuropilin 1 |
| *Nrp2* | Neuropilin 2 |
| *Pcdh10* | Protocadherin 10 |
| *Pcdh9* | Protocadherin 9 |
| *Pcdhb2* | Protocadherin beta 2 |
| *Ptprj* | Protein tyrosine phosphatase, receptor type, J |
| *Ptpro* | Protein tyrosine phosphatase, receptor type, O |
| *Ptprz1* | Protein tyrosine phosphatase, receptor type Z, polypeptide 1 |
| *Slitrk1* | SLIT and NTRK-like family, member 1 |
| *Slitrk6* | SLIT and NTRK-like family, member 6 |
| *St6gal1* | Beta galactoside alpha 2,6 sialyltransferase 1 |
| *Tenm3* | Teneurin transmembrane protein 3 |
| *Tenm4* | Teneurin transmembrane protein 4 |

**Table 10: Gene symbols and names of all candidate cell adhesion molecules.**

| Gene | Mean expression in the male compared to female anterior lobe ($log_2$-fold difference) |
|---|---|
| *Alcam* | 1.47 |
| *Cd44* | -0.74 |
| *Cdh10* | 0.60 |
| *Cdh8* | 1.37 |
| *Ceacam1* | 1.15 |
| *Cntnap4* | 1.84 |
| *Cntnap5a* | 0.80 |
| *Cntnap5b* | -0.15 |
| *Cntnap5c* | 0.13 |
| *Dcc* | 0.45 |
| *Dscam* | 0.07 |
| *Epha6* | -1.93 |
| *Igdcc4* | -0.02 |
| *Il1r1* | 1.68 |
| *Il1rapl2* | -2.28 |
| *Itga6* | -0.14 |
| *Jam2* | 0.89 |
| *Kit* | 0.08 |
| *Lrrn1* | 0.97 |
| *Ncam2* | 0.55 |
| *Nrp1* | -0.20 |
| *Nrp2* | 0.85 |
| *Pcdh10* | -1.42 |
| *Pcdh9* | -0.45 |
| *Pcdhb2* | 0.14 |
| *Ptprj* | 1.03 |
| *Ptpro* | 0.10 |
| *Ptprz1* | 0.88 |
| *Slitrk1* | 0.89 |
| *Slitrk6* | 0.59 |
| *St6gal1* | 0.59 |
| *Tenm3* | 0.48 |
| *Tenm4* | 0.46 |

**Table 11: Difference in candidate cell adhesion molecule expression between male and female mouse pituitaries.** Mean expression of each gene was compared in microarray data from whole anterior lobes.

### 3.3 Analysis of scRNA-seq data from the mouse pituitary can point to candidates with greater potential to act as CAMs in homotypic networks

Since analyses of scRNA-seq data did not produce candidate CAMs, the datasets were instead used to evaluate the candidates generated using microarray data. This evaluation

was performed as a three-layered process to identify the genes for which there is most evidence in favour of a role as CAMs in homotypic networks. First, the distribution of candidate CAM expression was compared in corticotrophs and gonadotrophs and in somatotrophs and lactotrophs in scRNA-seq data from the adult mouse pituitary. Next, the cell type-specificity of candidate expression was assessed by comparing expression of the candidate CAMs in their respective cell types with expression in the whole pituitary. Finally, expression of the candidates was compared between species and developmental stages to identify any shared expression patterns which might indicate CAMs with important conserved functions in homotypic networks.

Using the integrated adult mouse pituitary scRNA-seq dataset described in Chapter 1, any candidate fitting the following 3 criteria in the dataset was considered a CAM with more potential to act as a homotypic CAM: 1) expression in a large proportion of a cell type, 2) expression in a low proportion of cells of the other cell type that expression was compared against in the previous section and 3) higher expression values in one cell type compared to the other. The first criterion was set because for genes expressed in a low number of cells in scRNA-seq data, it is difficult to know if the gene is truly expressed or if the expression is an artifact of the noisiness of scRNA-seq data for lowly expressed genes (Kharchenko et al. 2014). Furthermore, the more cells of a cell type that express a CAM, the more likely that CAM might be to contribute to the formation of a cohesive homotypic network.

### *3.3.1 Comparison of candidate CAM expression in pairs of cell types in scRNA-seq data*

Enlarged versions of all figures presented in this chapter can be found at the following link (please copy and paste the full address and ensure that the hyphen is copied for it to work): https://uoe-my.sharepoint.com/:b:/g/personal/s1749812_ed_ac_uk/EQeADrnKShVJq96UnNM8Si8BYlu9vGFaR-MinBdtPxtzYQ?e=2s5lQV. Of the genes identified as candidate CAMs in microarray data from corticotrophs and gonadotrophs, the majority were expressed in fewer than 15% of both cell types in the scRNA-seq dataset (Figure 14A). However, *Pcdh9*, *Ptprz1* and *Slitrk1* were both expressed in over 15% of corticotrophs and expressed in few gonadotrophs (Figure 14A). In gonadotrophs, 4 genes were expressed in more than 15% of cells and showed expression in few corticotrophs: *Igdcc4*, *Lrrn1*, *Nrp2* and *St6gal1* (Figure 14A). Similarly, in somatotrophs and lactotrophs most of the genes identified as candidates using microarray data showed expression in fewer than 15% of cells of both types (Figure 14B). However, *Alcam* was both highly expressed and showed enriched expression in somatotrophs compared to lactotrophs and *Dscam* and *Ptpro* showed enriched expression in lactotrophs compared to somatotrophs (Figure 14B).

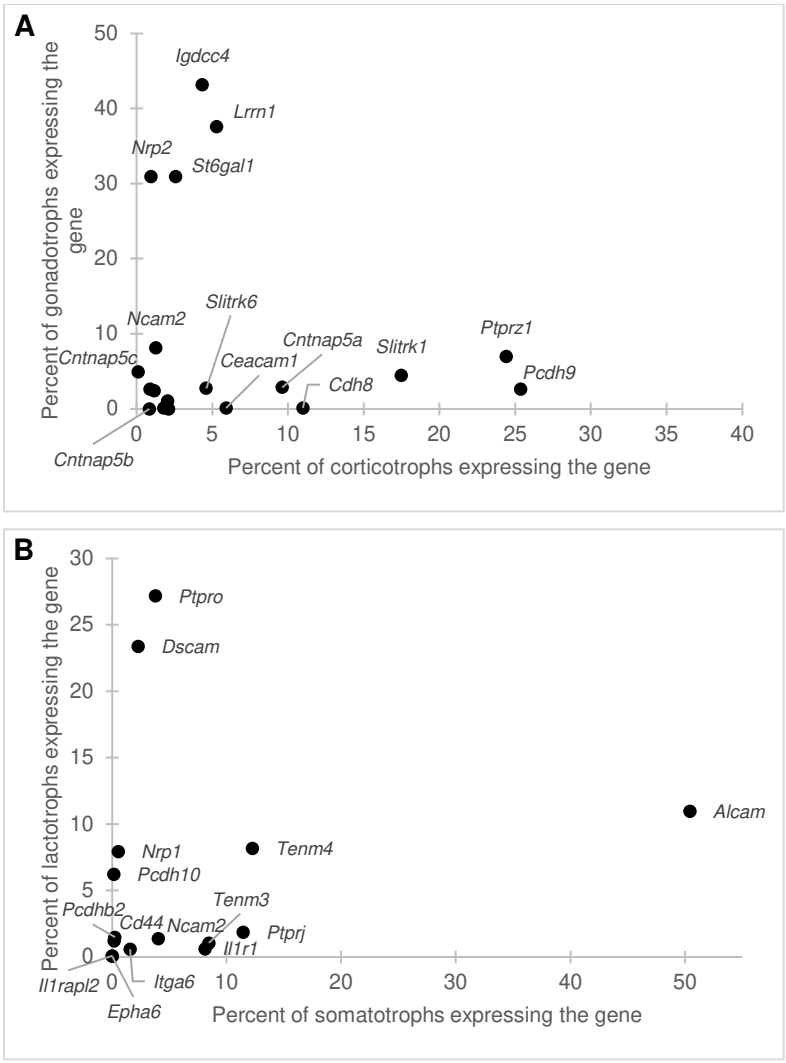


**Figure 14: Percent of corticotrophs and gonadotrophs (A) and somatotrophs and lactotrophs (B) expressing the candidate cell adhesion molecules in single cell RNA-sequencing data from adult mouse pituitaries.**

### *3.3.2 Expression levels of candidate CAMs in scRNA-seq data*

Apart from the cell type distribution of CAM expression, the quantity of CAMs expressed also influences adhesion. However, the mean expression of all candidate CAM in their respective cell types directly followed the proportion of cells expressing the candidates in the scRNA-seq dataset (ρ=0.997 to ρ=1.000, Figure 15), indicating that all candidate CAMs were expressed at a similar level in each cell. For this reason, the following analyses were focused on the patterns of CAM expression rather than the quantities of transcript detected.

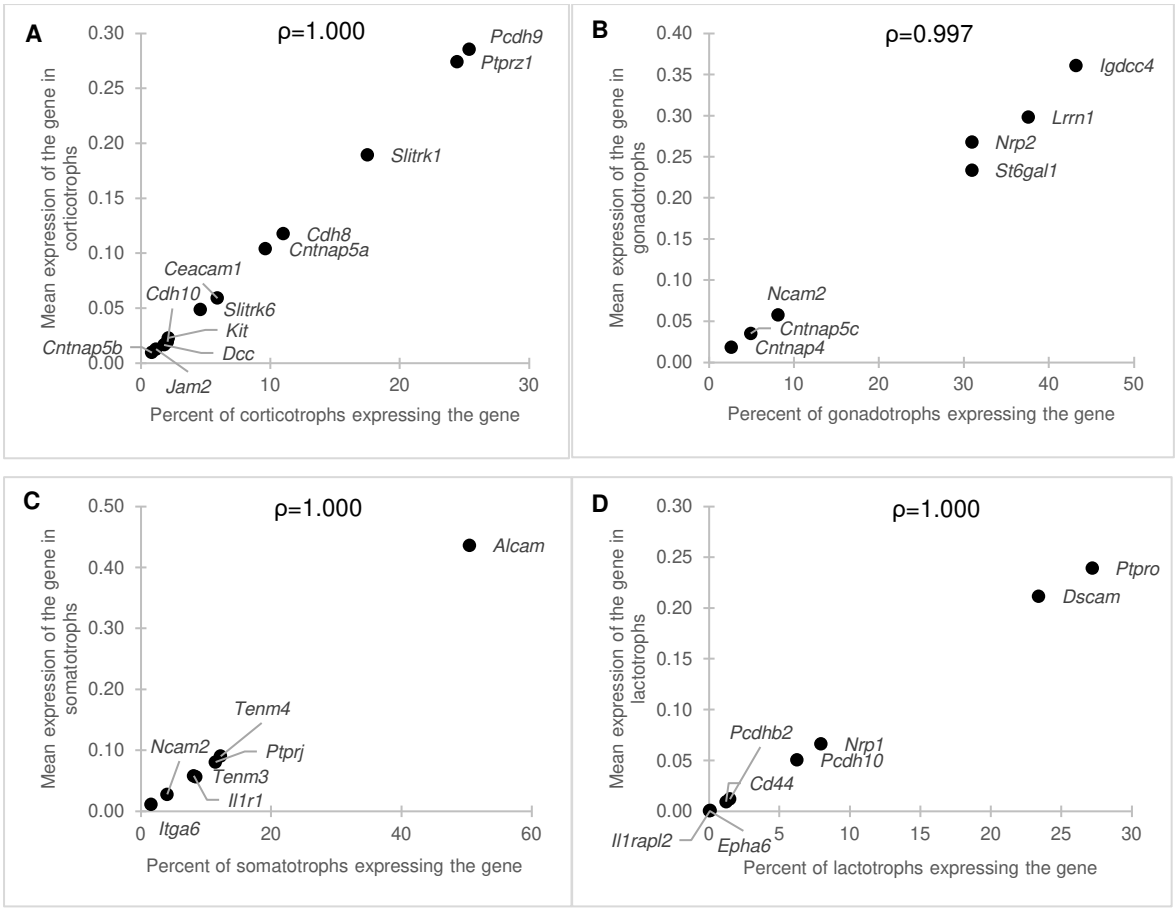


**Figure 15: Correlation between the percent of corticotrophs (A), gonadotrophs (B), somatotrophs (C) and lactotrophs (D) expressing the candidate cell adhesion molecules and the mean expression of those genes in each respective cell type in single cell RNA-sequencing data from adult mouse pituitaries.**

### *3.3.3 Candidate CAM expression is representative in a small subset of each cell type*

Corticotrophs and gonadotrophs make up a similar proportion of roughly 10-20% of pituitary cells *in vivo*, whereas about 25-50% and 15-35% of pituitary cells are somatotrophs and lactotrophs, respectively, depending on sex and physiological state (Ben-Shlomo and Melmed, 2011; Shimon, 2014). However, corticotrophs significantly outnumbered gonadotrophs in 2 of the individual scRNA-seq datasets and somatotrophs were up to 17-fold more abundant than lactotrophs (Table 12), introducing the possibility that cell sampling bias might distort expression of CAMs. While expression in pituitary cells that were not captured for scRNA-seq cannot be known, examining whether candidate CAM expression is similar in a small random subsample of cells compared to all cells of a cell type might indicate whether changes in cell number easily affect candidate expression. A random subsample of 10% of corticotrophs, gonadotrophs, somatotrophs and lactotrophs in the integrated adult mouse dataset were obtained and expression of the candidate CAMs was

compared in the subsample and full number of cells of each cell type. All candidates were expressed in a similar proportion of cells in the subsampled and full population of a cell type (ρ=0.98 to ρ=1.00, Figure 16), suggesting that candidate CAM expression is robust to fluctuations in captured cell number.

| Dataset reference | Number of corticotrophs | Number of gonadotrophs | Number of somatotrophs | Number of lactotrophs |
|---|---|---|---|---|
| (Cheung et al. 2018) | 620 | 562 | 6,585 | 3,580 |
| (Lopez et al. 2021) | 284 | 232 | 3330 | 449 |
| (Mayran et al. 2019) | 878 | 224 | 3099 | 1822 |
| Integrated | 1,893 | 873 | 13,049 | 5,806 |
| (Zhang et al. 2020b) | 180 | 426 | 784 | 109 |
| L. Cheung, unpublished | 322 | 177 | 1,432 | 83 |
| (Fletcher et al. 2019) | 861 | 261 | 1,971 | 482 |

**Table 12: Number of cells identified as corticotrophs, gonadotrophs, somatotrophs and lactotrophs in each of the six single cell RNA-sequencing datasets analysed.**

### 3.4 Exclusive expression of a candidate CAM in one pituitary cell type might indicate involvement in homotypic adhesion

While selective expression of a CAM in a cell type is not necessary for the CAM to be involved in homotypic adhesion, it might indicate a target of interest. To examine cell type specific expression of the candidate CAMs in the microarray dataset, expression of each candidate in its cell type was compared to mean expression of the candidate in the anterior lobe adjusted for cell type abundance. The candidates that showed more than 2 $\log_2$-fold upregulated expression in corticotrophs compared to their mean expression in the anterior lobe were *Cdh8*, *Cntnap5a*, *Cntnap5b*, *Dcc*, *Kit*, *Pcdh9* and *Ptprz1* (Table 13). In gonadotrophs, expression of *Cntnap5c*, *Igdcc4* and *Nrp2* was upregulated above 2 $\log_2$-fold compared to mean expression of the genes in the anterior lobe (Table 13). Using the same cut-off in somatotrophs and lactotrophs, *Il1r1*, *Itga6* and *Tenm4* were upregulated in somatotrophs (Table 13) and *Cd44*, *Dscam* and *Epha6* were upregulated in lactotrophs compared to mean expression in the anterior lobe (Table 13).

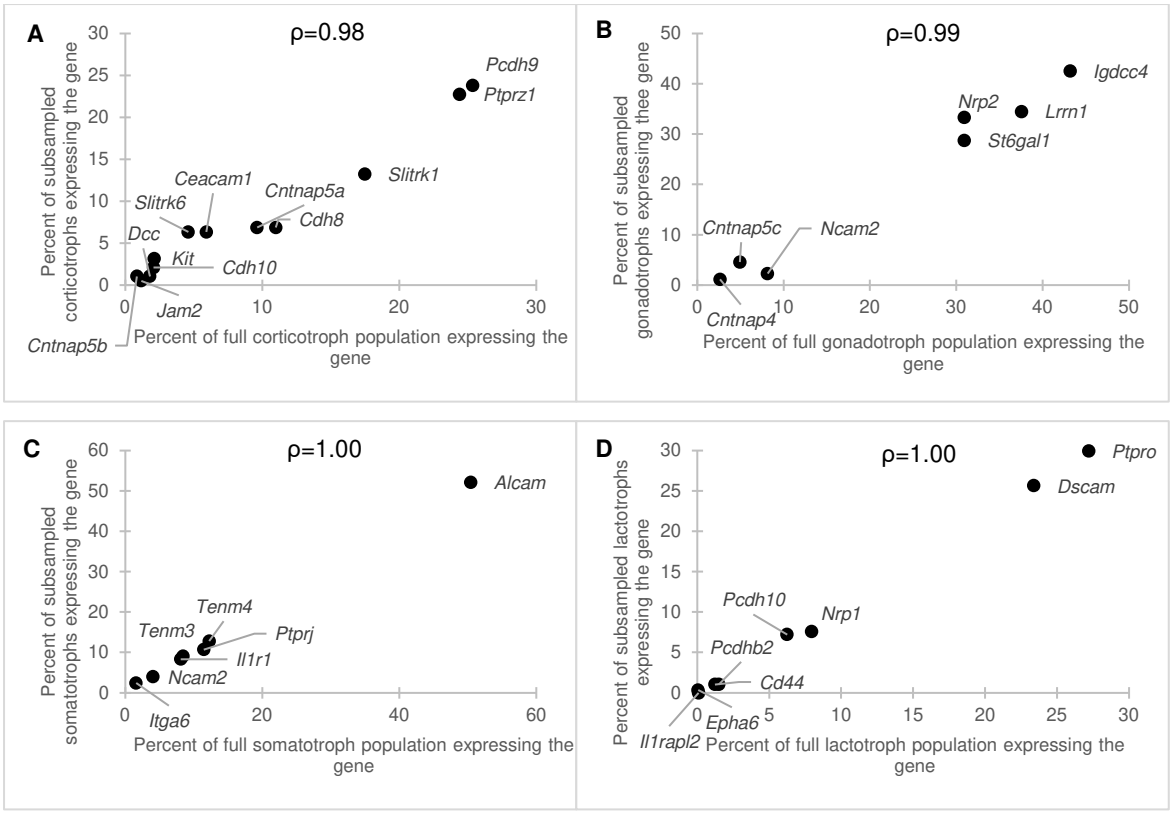


**Figure 16: Expression of the candidate cell adhesion molecules in a random subset of corticotrophs (A), gonadotrophs (B), somatotrophs (C) and lactotrophs (D) correlates well with their expression in the full population of each cell type.** Each cell type in the integrated adult mouse pituitary single cell RNA-sequencing dataset was randomly subsampled to 10% of the original population and the percent of cells expressing the candidate cell adhesion molecules was compared to the percent expressing the candidates in the full cell type.

scRNA-seq data offer another way to assess specificity by showing how expression of the candidate genes is distributed among the different cell types. If a candidate was expressed randomly in the pituitary, the expression distribution between different cell types would be expected to be the same as is the abundance of each cell type in the pituitary. For example, of the cells expressing a gene that is randomly distributed, 10% would be expected to be corticotrophs given that approximately 10% of pituitary cells are corticotrophs. Any candidate CAM expressed in a proportion that is much higher than the abundance of that cell type in the pituitary therefore might represent a CAM that plays a role in homotypic adhesion of that cell type. Corticotrophs and gonadotrophs were taken to make up roughly 10%, somatotrophs 40% and lactotrophs 20% of pituitary cells in male mice.

| Gene | Cell type | Expression in the cell type compared to mean expression in the anterior lobe ($log_2$-fold change) | Percent of pituitary cells expressing the gene identified as the cell type |
|---|---|---|---|
| *Cdh8* | Corticotrophs | 3.02 | 91.2 |
| *Cdh10* | | 1.84 | 18.7 |
| *Ceacam1* | | 1.35 | 22.0 |
| *Cntnap5a* | | 2.96 | 76.8 |
| *Cntnap5b* | | 2.16 | 30.8 |
| *Dcc* | | 2.57 | 13.1 |
| *Jam2* | | 1.71 | 1.6 |
| *Kit* | | 3.04 | 17.9 |
| *Pcdh9* | | 2.36 | 24.7 |
| *Ptprz1* | | 2.71 | 30.8 |
| *Slitrk1* | | 1.86 | 19.6 |
| *Slitrk6* | | 1.53 | 8.2 |
| *Cntnap4* | Gonadotrophs | 0.37 | 2.7 |
| *Cntnap5c* | | 2.09 | 71.7 |
| *Igdcc4* | | 2.86 | 31.7 |
| *Lrrn1* | | 0.75 | 12.9 |
| *Ncam2* | | 0.53 | 9.2 |
| *Nrp2* | | 2.74 | 21.0 |
| *St6gal1* | | 0.65 | 9.4 |
| *Alcam* | Somatotrophs | 1.47 | 65.5 |
| *Il1r1* | | 3.13 | 82.5 |
| *Itga6* | | 5.77 | 17.8 |
| *Ncam2* | | 1.56 | 67.9 |
| *Ptprj* | | 1.79 | 66.8 |
| *Tenm3* | | 0.80 | 48.1 |
| *Tenm4* | | 5.33 | 40.8 |
| *Cd44* | Lactotrophs | 2.79 | 28.0 |
| *Dscam* | | 5.11 | 66.6 |
| *Epha6* | | 2.27 | 60.0 |
| *Il1rapl2* | | 0.80 | 10.0 |
| *Nrp1* | | 0.67 | 24.7 |
| *Pcdh10* | | 1.52 | 82.5 |
| *Pcdhb2* | | 1.86 | 62.3 |
| *Ptpro* | | 1.36 | 63.4 |

**Table 13: Specificity of candidate cell adhesion molecule expression in corticotrophs, gonadotrophs, somatotrophs and lactotrophs of adult mice.** Candidate cell adhesion molecule expression in microarray data from the cell types was compared with mean expression of the genes in the whole anterior lobe. The percent of pituitary cells expressing the candidates that were of the relevant cell type were calculated from single cell RNA-sequencing data. The high enrichment of *Il1r1*, *Itga6* and *Tenm4* in somatotrophs produced a negative fold change value, which was made positive before calculating the $log_2$-fold change.

In corticotrophs, *Cdh8*, *Cntnap5a*, *Cntnap5b* and *Ptprz1* showed cell type-enriched expression with over 30% of all pituitary cells expressing the genes being corticotrophs (Table 13). Gonadotrophs made up more than 30% of cells expressing *Cntnap5c* and *Igdcc4*, indicating cell-type enriched expression (Table 13). More than 60% of cells expressing the somatotroph candidate CAMs *Alcam*, *Il1r1*, *Ncam2* and *Ptprj* were somatotrophs (Table 13) and more than 60% of cells expressing the lactotroph candidate CAMs *Dscam*, *Epha6*, *Pcdh10*, *Pcdhb2* and *Ptpro* were lactotrophs (Table 13).

## 3.5 Candidate CAMs show both shared and divergent expression patterns between species and developmental stages

Pituitary cells are organised into networks early in development with adaptive changes to the networks occurring throughout life as new demands arise (Budry et al. 2011; Hodson et al. 2012). This selective organisation is not unique to the mouse and has been observed in most vertebrate pituitaries studied to date, although some differences in structure and composition exist between species (Santiago-Andres et al. 2021). Together these observations suggest that the CAMs mediating adhesion in homotypic networks might be conserved between species and developmental stages. To address this possibility, expression of the candidate CAMs was analysed in scRNA-seq datasets from foetal human, young mouse and adult rat pituitaries.

### *3.5.1 Candidate CAM expression is more widespread in early human foetal pituitaries*

To first determine whether expression of the human orthologues of the candidate CAMs differed depending on the developmental stage of the human foetal pituitary, candidate expression was compared in pituitary samples obtained during weeks 7-10 and weeks 23-25 post-fertilisation (Zhang et al. 2020b), the earliest and latest time points available, respectively. The total number of cells obtained in both sample groups was similar (weeks 7-10: 1,305 cells, weeks 23-25: 1,360 cells) but the number of cells belonging to each cell type differed between the early and late samples (Table 14). No lactotrophs were identified in the samples from weeks 7-10, consistent with the late emergence of the cell type (Slabaugh et al. 1982). Most candidate CAMs (10/11 candidates in corticotrophs, 5/7 candidates in gonadotrophs and 6/7 candidates in somatotrophs, Figure 17) were expressed in a greater proportion of cells during weeks 7-10 than weeks 23-25. The genes that showed the greatest upregulation at the earlier time point included *DCC*, *KIT* and *SLITRK1* in corticotrophs and *CNTNAP5* and *NCAM2* in gonadotrophs (Figures 17A and 5B). *TENM3* was upregulated at weeks 23-25 in somatotrophs (Figure 17C).

| Sample | Number of cells | | | | |
|---|---|---|---|---|---|
| | Corticotrophs | Gonadotrophs | Somatotrophs | Lactotrophs | Total pituitary |
| Weeks 7-10 | 116 | 94 | 77 | 0 | 1,305 |
| Weeks 23-25 | 30 | 156 | 366 | 37 | 1,360 |

**Table 14: The number of cells of each cell type identified in single cell RNA-sequencing data from human foetal pituitaries obtained 7-10 weeks or 23-25 weeks post-fertilisation.**

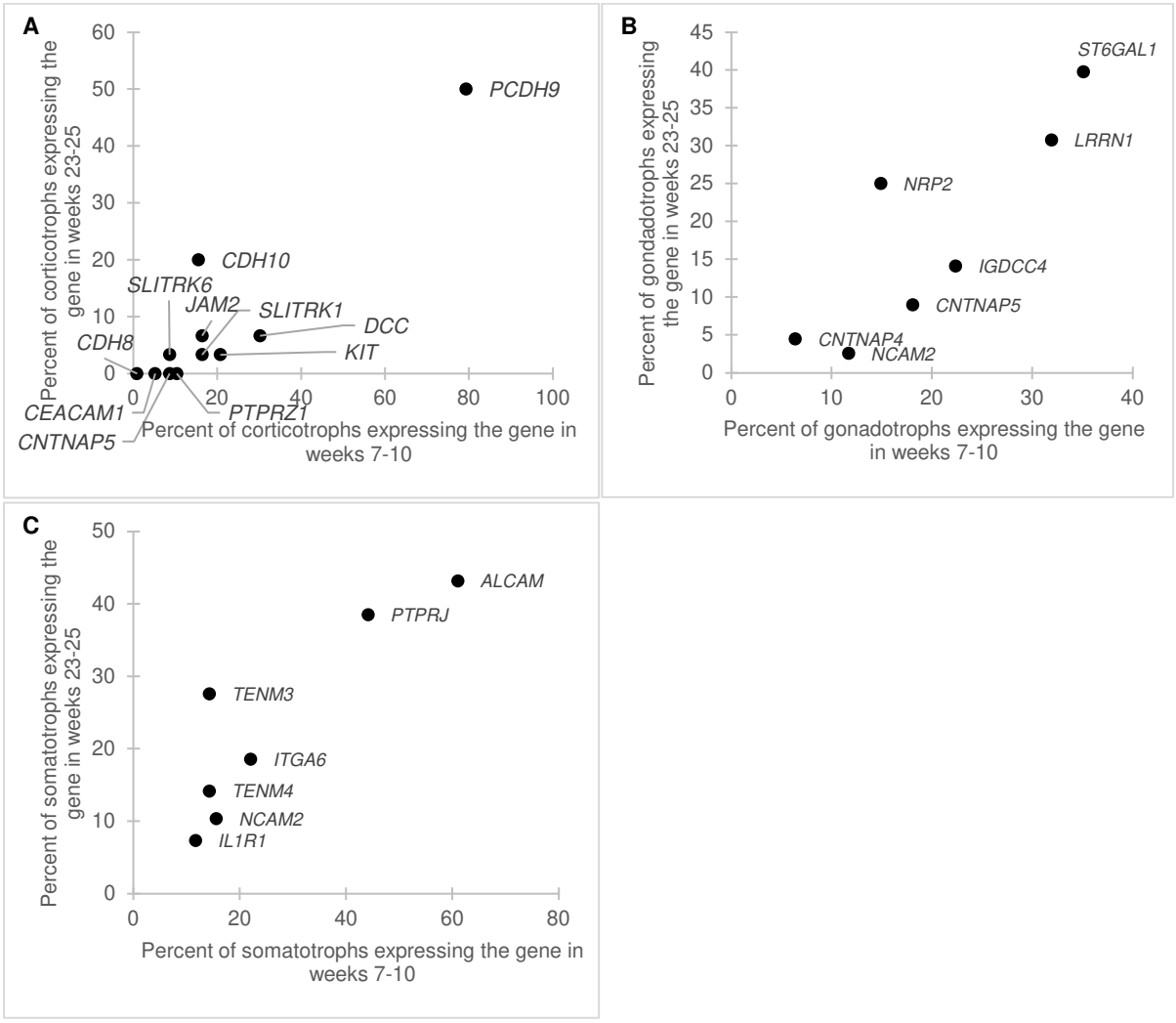


**Figure 17: Percent of corticotrophs (A), gonadotrophs (B) and somatotrophs (C) expressing the human orthologues of the candidate genes in human pituitary samples obtained during weeks 7-10 and 23-25 post-fertilisation.**

### 3.5.2 *Expression patterns of candidate CAMs in the foetal human pituitary indicate targets conserved between species and developmental stages*

To assess whether the expression patterns of candidate CAMs in mice were conserved in human foetal pituitaries, the samples obtained during weeks 23-25 post-fertilisation were used for comparison. Using the latest samples available maximises the probability that homotypic networks are in their established rather than still forming states. In corticotrophs, there was an overall low correlation of candidate CAM expression in the adult mouse and human foetus (ρ=0.41, Figure 18A). All candidates were expressed in fewer than 10% of human foetal corticotrophs except *Cdh10* and *Pcdh9* which were expressed in a greater proportion of human foetal than adult mouse corticotrophs (Figure 18A). None of the candidates showed corticotroph-specific expression in the human pituitary (Table 15). Gonadotroph candidate expression was better conserved (ρ=0.70, Figure 18B). The candidates expressed in a higher proportion of gonadotrophs in the mouse (*Lrrn1*, *Nrp2* and *St6gal1*) were expressed in a similar proportion of gonadotrophs in the adult mouse with the exception of *Igdcc4*, which was more lowly expressed in the human (Figure 18B). *CNTNAP5* and *ST6GAL1* showed biased expression in gonadotrophs (Table 15).

Candidate expression in somatotrophs was moderately correlated overall (ρ=0.69) with *Alcam* showing expression in a large proportion of both adult mouse and foetal human somatotrophs (Figure 18C). The rest of the somatotroph candidates showed expression in a similar or higher proportion of foetal human than adult mouse somatotrophs (Figure 18C). None of the candidates were somatotroph-specific (Table 15). Expression of lactotroph candidates correlated well (ρ=0.82) with the candidates most highly expressed in the adult mouse (*Dscam* and *Ptpro*) expressed in a similar or higher proportion of cells in the human foetus as well (Figure 18D). In addition, *Nrp1* was expressed in a large proportion of cells in human foetal lactotrophs (Figure 18D). None of the lactotroph candidates showed specific expression in the cell type (Table 15).

These results show that candidate expression is better conserved in some cell types than others and that several of the candidates are expressed in a markedly different proportion of cells in adult mouse compared to foetal human pituitaries. While the greater depth of sequencing and lower number of cells sequenced in the human dataset likely account for part of the differences in expression, the fact that this difference was not unidirectional for all candidates suggests that either species or developmental stage also contributed to the differences.

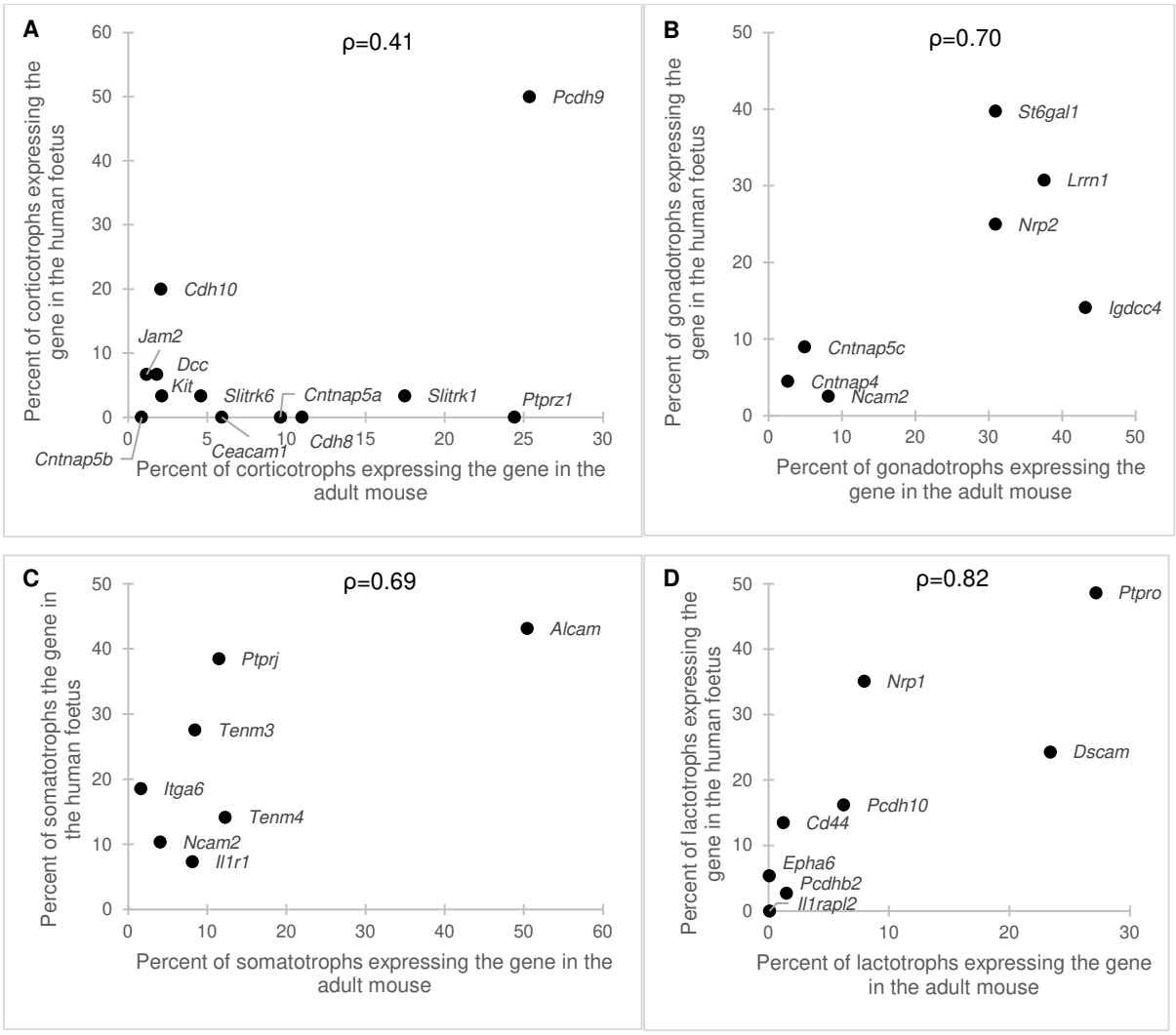


**Figure 18: Percent of corticotrophs (A), gonadotrophs (B), somatotrophs (C) and lactotrophs (D) expressing the candidate cell adhesion molecules in adult mouse and foetal human pituitary single cell RNA-sequencing data.** Human orthologues of the given mouse genes were used to calculate gene expression values in human corticotrophs. The Pearson correlation coefficient (ρ) for all data points is indicated.

### *3.5.3 Contribution of developmental stage and species to expression of CAMs*

To determine the relative contribution of developmental stage and species to the difference in CAM expression, expression of the candidate genes in the foetal human and adult mouse pituitaries was compared with their expression in a scRNA-seq dataset from a young 7-day-old (P7) mouse pituitary (data courtesy of Dr Leonard Cheung, University of Michigan). While the P7 mouse pituitary is not foetal, the pituitary does continue to undergo significant development during the first few weeks after birth (Taniguchi et al. 2001a, b; Yoshida et al. 2011), suggesting that the P7 pituitary might exhibit features more reflective of a developing pituitary than an adult one. Conserved CAM expression between the adult and P7 mouse

pituitaries would therefore indicate that species rather than developmental stage plays a larger role in determining candidate CAM expression.

| Gene | Cell type | Percent of pituitary cells expressing the gene identified as the indicated cell type |
|---|---|---|
| *CDH8* | Corticotrophs | 0.0 |
| *CDH10* | | 13.6 |
| *CEACAM1* | | 0.0 |
| *CNTNAP5* | | 0.0 |
| *DCC* | | 6.1 |
| *JAM2* | | 0.9 |
| *KIT* | | 1.3 |
| *PCDH9* | | 5.5 |
| *PTPRZ1* | | 0.0 |
| *SLITRK1* | | 16.7 |
| *SLITRK6* | | 3.4 |
| *CNTNAP4* | Gonadotrophs | 4.9 |
| *CNTNAP5* | | 38.9 |
| *IGDCC4* | | 13.2 |
| *LRRN1* | | 16.7 |
| *NCAM2* | | 3.6 |
| *NRP2* | | 11.2 |
| *ST6GAL1* | | 26.8 |
| *ALCAM* | Somatotrophs | 23.4 |
| *IL1R1* | | 26.0 |
| *ITGA6* | | 30.6 |
| *NCAM2* | | 33.9 |
| *PTPRJ* | | 37.9 |
| *TENM3* | | 32.4 |
| *TENM4* | | 23.6 |
| *CD44* | Lactotrophs | 1.4 |
| *DSCAM* | | 5.8 |
| *EPHA6* | | 22.2 |
| *IL1RAPL2* | | 0.0 |
| *NRP1* | | 2.6 |
| *PCDH10* | | 2.1 |
| *PCDHB2* | | 1.0 |
| *PTPRO* | | 9.5 |

**Table 15: Percent of all pituitary cells that expressed the candidate cell adhesion molecules that were corticotrophs, gonadotrophs, somatotrophs or lactotrophs in foetal human single cell RNA-sequencing data.**

Expression of the corticotroph and gonadotroph candidate CAMs was better correlated between the adult and P7 mouse (ρ=0.92 for corticotrophs (Figure 19A), ρ=0.89 for gonadotrophs (Figure 19B)) than between the human foetus and P7 mouse (ρ=0.42 for corticotrophs (Figure 19C), ρ=0.53 for gonadotrophs (Figure 19D)). Of the corticotroph and gonadotroph candidates, *Cdh8*, *Pcdh9*, *Ptprz1* and *Slitrk1* were expressed in more than 20% of the cell type in the P7 mouse (Figure 19A), whereas *Igdcc4*, *Lrrn1*, *Ncam2*, *Nrp2* and *St6gal1* were expressed in more than 20% of gonadotrophs in the P7 mouse (Figure 19B).

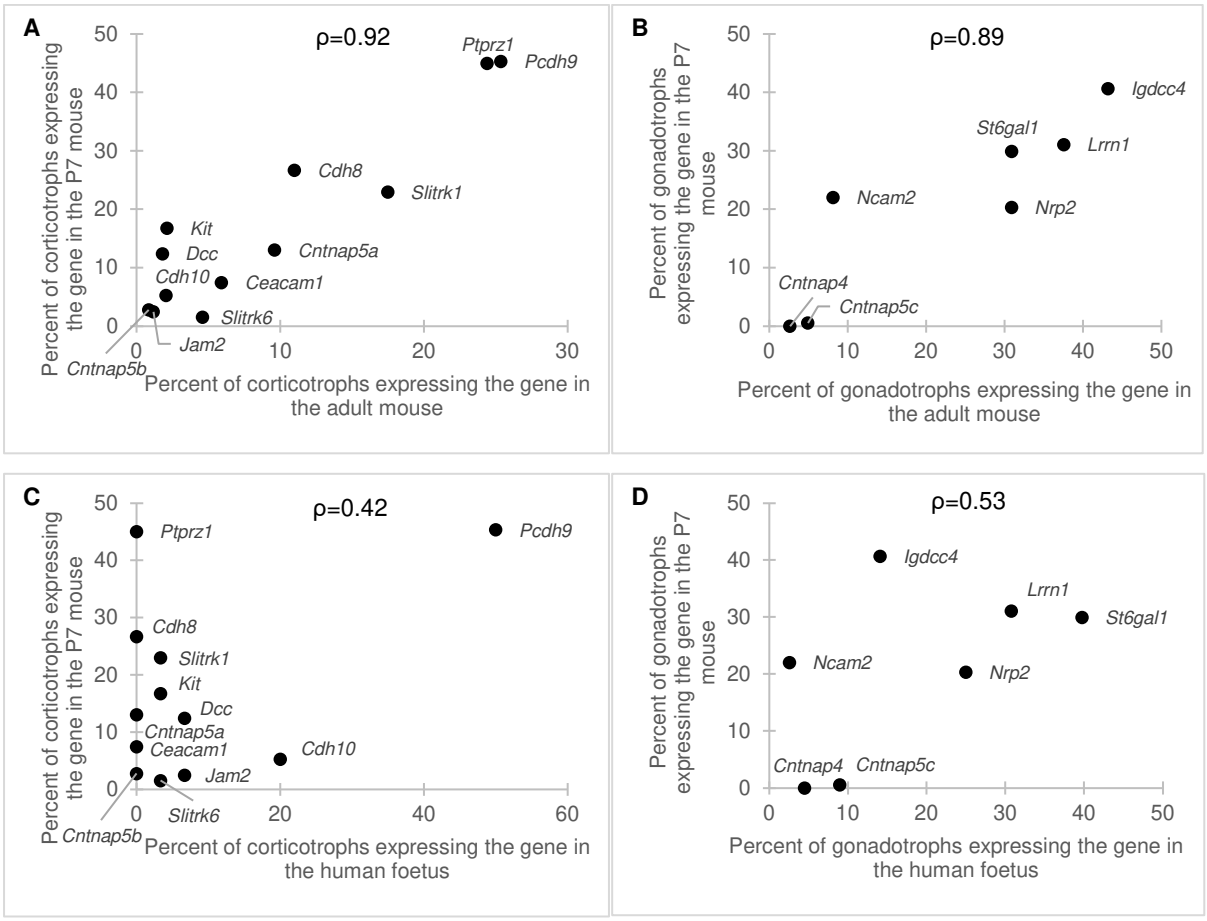


**Figure 19: Percent of corticotrophs (A, C) and gonadotrophs (B, D) expressing the candidate cell adhesion molecules in adult and 7-day-old (P7) mouse (A-B) or foetal human and P7 mouse (C-D) pituitary single cell RNA-sequencing data.** The Pearson correlation coefficient (ρ) for all data points is indicated.

In somatotrophs, there was poor correlation of candidate expression both between the adult and P7 mouse (ρ=-0.06, Figure 20A) and between the human foetus and P7 mouse (ρ=-0.22, Figure 20B). All candidates except *Itga6* were expressed in more than 20% of P7 somatotrophs (Figure 20A). However, *Alcam*, which was highly expressed in the adult mouse was expressed in a lower proportion of somatotrophs in the P7 mouse (Figure 20A).

Expression of the lactotroph candidates was better correlated between the adult and P7 mouse (ρ=0.97, Figure 20C) than between the human foetus and P7 mouse (ρ=0.77, Figure 20D). *Dscam* and *Ptpro* were expressed in an even higher proportion of lactotrophs in the P7 than the adult mouse (Figure 20D).

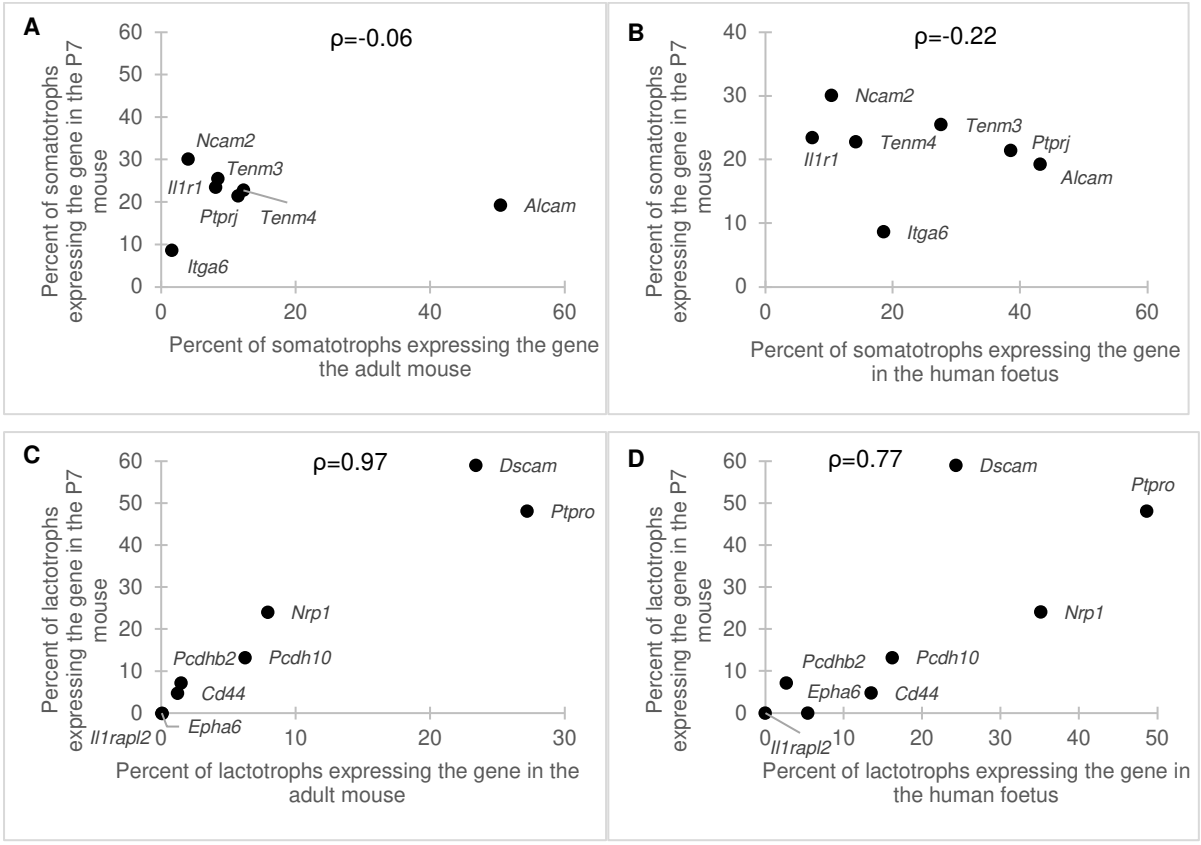


**Figure 20: Percent of somatotrophs (A-B) and lactotrophs (C-D) expressing the candidate cell adhesion molecules in adult and 7-day-old (P7) mouse (A, C) or in foetal human and P7 mouse (B, D) pituitary single cell RNA-sequencing data.** The Pearson correlation coefficient (ρ) for all data points is indicated.

These results indicate that species rather than the stage of development might be the greater contributor to differences in CAM expression between the foetal human and adult mouse corticotrophs, gonadotrophs and lactotrophs. However, expression of somatotroph and candidate CAMs was overall less explained by either factor.

### *3.5.4 Mouse and rat pituitaries exhibit more similarities in candidate CAM expression*

To further examine species differences in candidate CAM expression, a scRNA-seq dataset from adult rat pituitaries (Fletcher et al. 2019) was analysed. As humans and rodents diverged approximately 96 million years ago, whereas this happened approximately 33

million years ago for rats and mice (Nei et al. 2001), patterns of CAM expression might be expected to show more similarities between the rodent pituitaries. Expression of the CAMs in adult mouse compared to rat corticotrophs (ρ=0.84, Figure 21A), gonadotrophs (ρ=0.69, Figure 21B) and somatotrophs (ρ=0.90, Figure 21C) showed a moderately high degree of correlation, but candidate expression in lactotrophs (ρ=-0.18, Figure 21D) was poorly correlated.

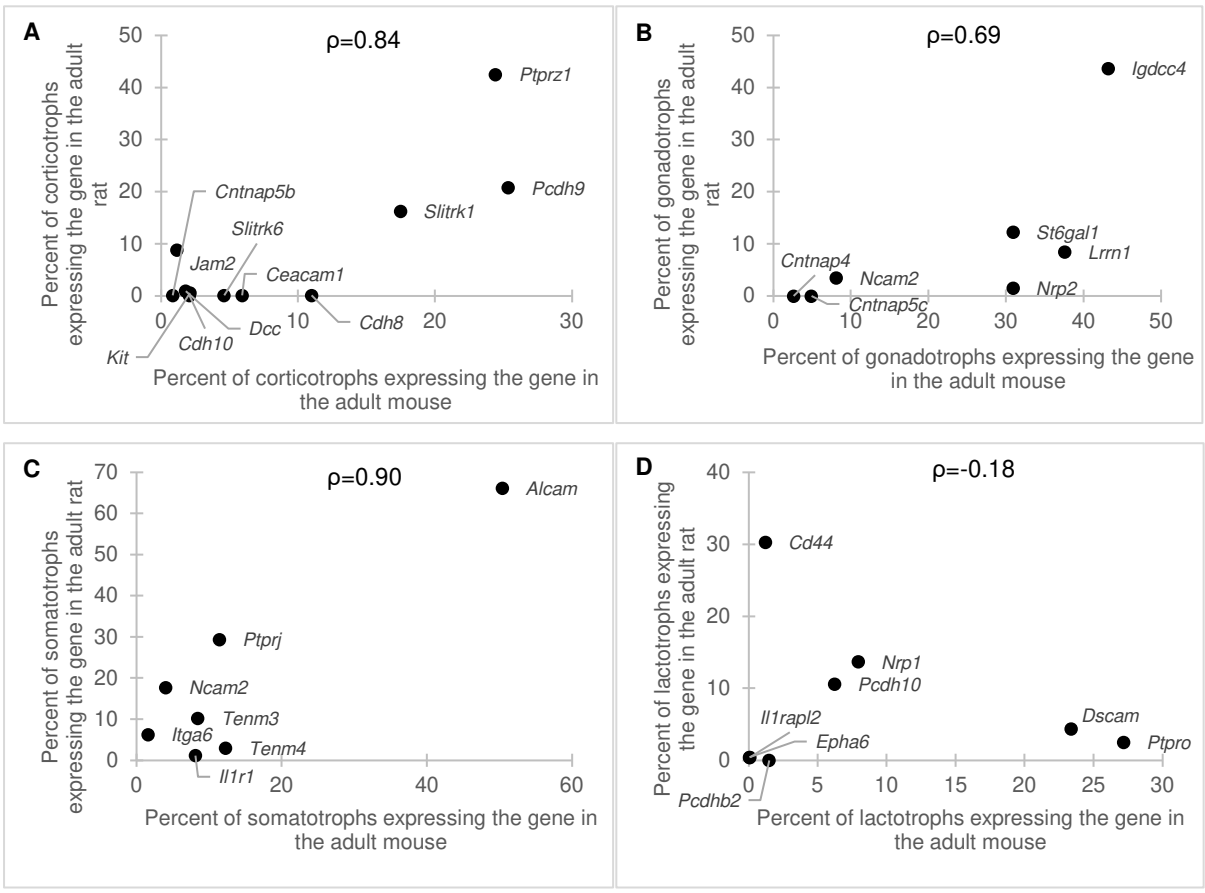


**Figure 21: Percent of corticotrophs (A), gonadotrophs (B), somatotrophs (C) and lactotrophs (D) expressing the candidate cell adhesion molecules in adult mouse and rat pituitary single cell RNA-sequencing data.** The Pearson correlation coefficient (ρ) for all data points is indicated.

Corticotroph candidates *Pcdh9*, *Ptprz1* and *Slitrk1* were expressed in more than 15% of the cell type in the rat (Figure 21A). Of the candidates in gonadotrophs, only *Igdcc4* was highly expressed (Figure 21B). *Alcam* was expressed in a large proportion of rat somatotrophs (Figure 21C) and *Cd44* was the only lactotroph candidate expressed in more than 30% of rat lactotrophs (Figure 21D). *Ptprz1* and *Slitrk1* showed biased expression towards corticotrophs (Table 16) and expression of *Igdcc4* was slightly overrepresented in

gonadotrophs compared to other pituitary cells (Table 16). Expression of *Ncam2* was biased towards somatotrophs and *Pcdh10* towards lactotrophs (Table 16).

| Gene | Cell type | Percent of pituitary cells expressing the gene identified as the indicated cell type |
|---|---|---|
| *Cdh8* | Corticotrophs | 6.3 |
| *Cdh10* | | 9.1 |
| *Ceacam1* | | 2.1 |
| *Cntnap5b* | | 2.0 |
| *Dcc* | | 4.6 |
| *Jam2* | | 5.4 |
| *Kit* | | 4.1 |
| *Pcdh9* | | 12.2 |
| *Ptprz1* | | 40.4 |
| *Slitrk1* | | 39.8 |
| *Slitrk6* | | 2.1 |
| *Cntnap4* | Gonadotrophs | 0.0 |
| *Cntnap5c* | | 0.0 |
| *Igdcc4* | | 24.8 |
| *Lrrn1* | | 5.0 |
| *Ncam2* | | 1.9 |
| *Nrp2* | | 0.3 |
| *St6gal1* | | 4.3 |
| *Alcam* | Somatotrophs | 36.3 |
| *Il1r1* | | 5.8 |
| *Itga6* | | 19.5 |
| *Ncam2* | | 73.7 |
| *Ptprj* | | 46.0 |
| *Tenm3* | | 12.3 |
| *Tenm4* | | 7.9 |
| *Cd44* | Lactotrophs | 11.2 |
| *Dscam* | | 2.8 |
| *Epha6* | | 28.6 |
| *Il1rapl2* | | 4.3 |
| *Nrp1* | | 8.2 |
| *Pcdh10* | | 44.3 |
| *Pcdhb2* | | 0.0 |
| *Ptpro* | | 6.3 |

**Table 16: Percent of all pituitary cells that expressed the candidate cell adhesion molecules that were corticotrophs, gonadotrophs, somatotrophs or lactotrophs in adult rat single cell RNA-sequencing data.**

# 4 DISCUSSION

The approach outlined in this chapter allowed the identification of a set of CAMs that may act to mediate adhesion in homotypic networks of the pituitary. First, an initial list of candidate CAMs was generated by comparing microarray transcriptome data from corticotrophs against gonadotrophs and somatotrophs against lactotrophs. scRNA-seq datasets obtained from different species and at different developmental stages were then used to evaluate the candidates to find the ones with the greatest potential as homotypic CAMs. These candidates represent a useful set of CAMs that can be tested experimentally for involvement in homotypic adhesion.

## 4.1 Limitations of scRNA-seq data for evaluating candidate CAMs

Most of the limitations of the approach used in the present chapter are related to the imperfect nature of scRNA-seq data. First, acquiring single cell suspensions for sequencing requires disruption of cell-cell adhesion, the very phenomenon that is the subject of this study. Unfortunately, the cells that adhere most strongly to each other are also the most difficult to separate and therefore most likely to be discarded as ‘multiplets’ during processing. Loss of these cells with valuable CAM expression profiles might undermine detection of additional important CAMs but does not invalidate the candidates identified in this chapter. The tissue processing steps might also selectively kill or burst cells that depend on adhesion to other cells for survival because these cells are unable to remain alive in single cell suspension or are prone to bursting. Furthermore, tissue dissociation can alter the RNA composition of cells, both due to mechanical damage which allows RNA to leak from cells and due to damage-induced changes in gene expression (Denisenko et al. 2020). CAM genes might be particularly susceptible to this effect as expression of genes associated with the Gene Ontology term ‘membrane’ (including most CAMs) was downregulated in cells damaged by single cell preparation (Ilicic et al. 2016). By removing low quality cells during data processing (see Chapter 1 section 2.1.1), these artifactual changes in expression were minimised but probably not fully eliminated.

An expression value of zero in scRNA-seq data might be due to true absence of expression or only apparent absence of expression (dropout) due to inefficient capture of the low quantities of RNA present in a cell (Jiang et al. 2020). To further complicate the matter, not all transcripts are equally likely to show false negative expression: transcriptional dynamics, the structure of the mRNA molecule and the affinity for proteins might all influence the probability of detecting a transcript (Svensson 2020; Jiang et al. 2020). CAM genes that are more lowly expressed in a cell might be more susceptible to the dropout effect and therefore

appear to be expressed in only a small proportion of cells. However, comparing the expression of examples of candidate CAMs expressed in a high and a low proportion of cells in each cell type shows that the raw counts are within a similar, low range for all genes, except for *Alcam* which is more highly expressed (Figure 22). This would indicate that the dropout effect is unlikely to be the only explanation for why some candidates are expressed in a low proportion of cells. However, as discussed further below, the data are also consistent with the dropout effect leading to the differential probabilities of detecting gene expression. Alternative methods would be required to distinguish between these possibilities. While the present study focused on the more widely expressed candidates, the candidates expressed in a low proportion of cells should not be dismissed as they still might mediate or regulate adhesion in an important subpopulation of a cell type.

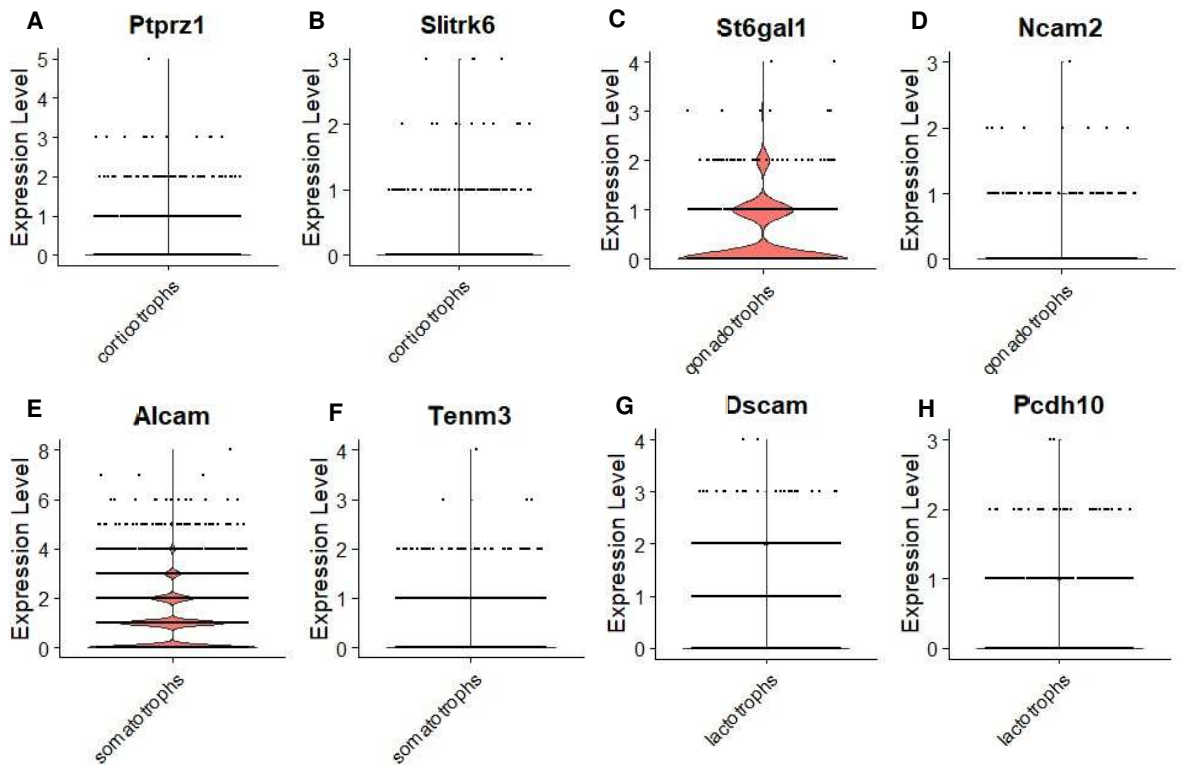


**Figure 22: Raw counts of example candidate cell adhesion molecules expressed in a high and low proportion of each cell type in adult mouse single cell RNA-sequencing data.** *Ptprz1* (A) was expressed in a high and *Slitrk6* (B) in a low proportion of corticotrophs, *St6gal1* (C) was expressed in a high and *Ncam2* (D) in a low proportion of gonadotrophs, *Alcam* (E) was expressed in a high and *Tenm3* (F) in a low proportion of somatotrophs, and *Dscam* (G) was expressed in a high and *Pcdh10* (H) in a low proportion of lactotrophs. Each dot represents the raw expression count for an individual cell.

Low expression levels may also give the appearance that a candidate is highly enriched in a cell type. Selectivity of CAM expression was assessed by determining what proportion of cells expressing a candidate CAM belonged to each cell type (Tables 13, 15 and 16). However, when very few cells in total express a CAM, the candidate may appear to be highly enriched in a cell type when in actuality it is only present in a few cells of that cell type, perhaps by chance, compared to other cell types.

Finally, somatotroph and lactotroph candidate CAMs were generated using microarray data from female pituitaries but the scRNA-seq data used to evaluate these candidates were from male pituitaries. While overt sex differences for candidate CAM expression in the whole anterior lobe were absent (Table 11), it is still possible that differences at the level of individual cell types exist, which may have biased the conclusions drawn regarding the potential of the candidates as homotypic CAMs.

## 4.2 Candidate CAM expression patterns offer clues as to how homotypic adhesion specificity might be achieved

Fundamentally, the cells in the different homotypic networks must show some difference in the properties of the CAMs they express to explain why they selectively adhere to cells of their own type instead of randomly adhering to any other cell. What this difference is and how it generates the specific arrangement of cells in the pituitary is still unclear. However, the expression patterns of the candidate CAMs identified in this chapter might provide some indication as to how adhesive specificity is generated in homotypic networks. The simplest way to achieve specificity is to have each cell type uniformly express a distinct set of CAMs that does not overlap with the set of CAMs expressed by any other cell type (Figure 23A). However, two observations from the expression of the candidate CAMs suggest that this model is unlikely to explain adhesion in the homotypic networks: 1) none of the candidate CAMs were exclusively expressed in one cell type and 2) most of the candidate CAMs were expressed in less than half of a cell type.

The first observation would be consistent with a combinatorial model of CAM expression (Figure 23B). Rather than the cell types possessing their own unique set of homotypic CAMs, they might share some of the molecules and generate specificity by combining expression of the molecules in unique patterns instead. Each cell type then has an “adhesion code” that distinguishes one cell type from another. A variety of tissues including the brain (Bisogni et al. 2018) and olfactory system (Katsunuma et al. 2016) appear to make use of this combinatorial model of CAM expression to achieve specific adhesion between different cell types.

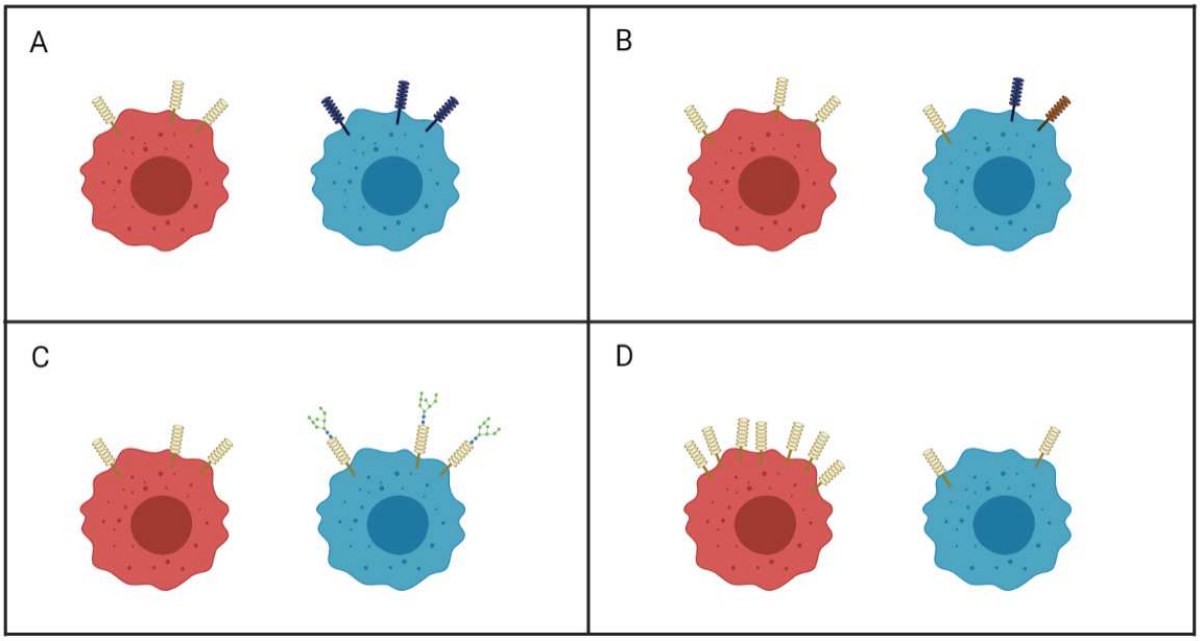


**Figure 23: Different ways to achieve selective adhesion between cells of the same type.** (A) expression of completely different cell adhesion molecules between the cell types, (B) combinatorial expression of a potentially overlapping set of cell adhesion molecules between the cell types so that each cell type is marked by a unique combination of molecules, (C) expression of cell adhesion molecules differentially modified by posttranscriptional or posttranslational mechanisms between the cell types, (D) expression of different quantities of cell adhesion molecules between the cell types.

Further specificity could be added by altering the properties of CAMs through post-transcriptional and post-translational modifications (Figure 23C). Evidence for this type of regulation in the pituitary exists for neural cell adhesion molecule (NCAM), which shows different adhesive ability depending on the splice variant expressed or the type of molecular group added to the CAM (Rutishauser et al. 1988; Doherty et al. 1992). Several of the splice variants have been detected in the pituitary (Lahr et al. 1993) and while NCAM expression is ubiquitous throughout the gland, the presence of polysialylated NCAM is restricted to corticotrophs (Gubkina et al. 2001).

The second observation could be explained by detection limits of scRNA-seq data (i.e., the dropout effect). To address this possibility, the expression distribution of the top candidate CAMs in corticotrophs and gonadotrophs (section 4.3) was examined within the relevant cell type (Figure 24). At least one of the top 3 corticotroph candidates is expressed in 51% of corticotrophs, whereas at least one gonadotroph candidate is expressed in 71% of gonadotrophs (Figure 24). Examining the overlap between the cells that express the different candidate CAMs may help indicate if the candidates are truly expressed in different subpopulations of cells or if all candidates are expressed in the same cells but the dropout effect in scRNA-seq data prevent detection of this.

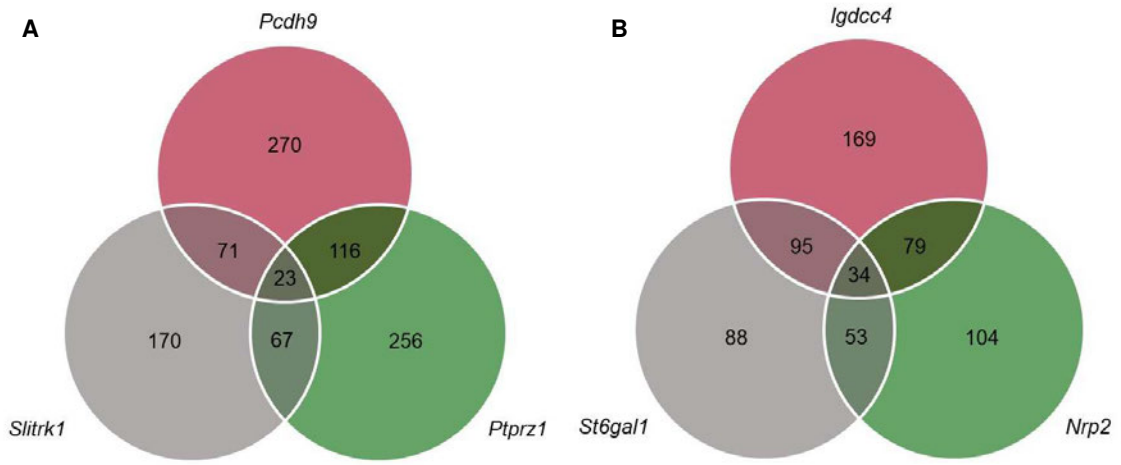


**Figure 24: Overlap of top corticotroph (A) and gonadotroph (B) candidate cell adhesion molecule expression.** The numbers indicate how many cells express the candidate gene in adult mouse pituitary single cell RNA-sequencing data. Of 1,893 corticotrophs, 973 (51%) cells express at least one of the 3 candidate genes, whereas 622 (71%) gonadotrophs out of a total of 873 gonadotrophs express at least of the 3 candidate genes in the cell type.

At the detection limit in the scRNA-seq dataset, the probability of detecting expression of an individual CAM is the number of cells it is detected in divided by the total number of cells that belong to the relevant cell type. For example, for corticotroph candidate *Pcdh9* this probability would be (270 + 71 + 23 + 116)/1,893, which is 0.2536 (Figure 24A). The probability of randomly detecting expression of two candidate CAMs in the same cell would be the product of the individual probabilities of detecting the two CAMs. For example, for *Pcdh9* and *Ptprz1* this would be 0.2536 times 0.2440 which is 0.0619. We would therefore randomly expect to see expression of the two CAMs in 117 cells (0.0619 times 1,893 corticotrophs) (Table 17). This number is very close to the 116 cells truly found to express the two CAMs. Repeating the calculations for the combinations of the other candidate CAMs also gives similar values for the expected and observed numbers of cells that express candidate CAMs (Table 17). While this is not proof that the 3 candidate molecules are expressed in all cells of the relevant cell type, the observed overlap does roughly match the expected dropout rate assuming that all cells express the candidate CAMs.

| CAM combination | Expression in number of cells | |
|---|---|---|
| | Expected | Observed |
| Pcdh9-Ptprz1 | 117 | 116 |
| Pcdh9-Slitrk1 | 84 | 71 |
| Ptprz-Slitrk1 | 81 | 67 |
| Pcdh9-Ptprz1-Slitrk1 | 29 | 23 |
| Igdcc4-Nrp2 | 117 | 79 |
| Igdcc4-St6gal1 | 117 | 95 |
| Nrp2-St6gal1 | 84 | 53 |
| Igdcc4-Nrp2-St6gal1 | 36 | 34 |

**Table 17: Observed and expected numbers of cells that express combinations of candidate CAMs in corticotrophs and gonadotrophs in adult mouse pituitary scRNA-seq data.**

Apart from the identity of the CAMs, the other factor determining adhesion specificity is their level of expression (Figure 23D). Cell populations expressing the same CAMs will segregate if the concentration of molecules is different between the populations (Friedlander et al. 1989), indicating that a quantitative rather than a qualitative difference in CAMs could contribute to generating homotypic networks. While expression levels of the candidate CAMs were similar in scRNA-seq data (Figure 15), this does not exclude that differences might arise at the level of protein expression. Furthermore, the spatial distribution of CAMs on the membrane can alter adhesion. For example, cadherins localised to junctional complexes show different adhesive strengths compared to cadherins distributed throughout the plasma membrane (Esni et al. 1999; Ranscht 2000). Given that a variety of intercellular junctions or the absence of any typical junctional structures have been observed between pituitary cells (Herbert 1979; Saunders et al. 1982; Wilfinger et al. 1984; Krisch and Buchheim 1984), a differential spatial localisation of CAMs to junctional structures or within the plasma membrane might provide another level of specificity.

The expression patterns of candidate CAMs in this study therefore support a model where homotypic cell adhesion is the result of CAMs expressed in more complex patterns than exclusive and uniform expression of a set of CAMs in each cell type. Given that the pituitary networks are actively being remodelled throughout life (Schaeffer et al. 2011; Hodson et al. 2012), adhesions between cells also need to break and re-form to accommodate cells proliferating, dying and moving around. Having diversified expression of CAMs might create a dynamic adhesion system that more easily allows local changes as well as overall fine tuning of the network structure in response to physiological stimuli.

## 4.3 Top candidate CAMs

### *4.3.1 Corticotrophs*

From the evaluation process, 3 CAMs emerged as strong candidates for mediating homotypic adhesion in corticotrophs. *Pcdh9* was expressed in a high proportion of corticotrophs and low proportion of gonadotrophs in the adult mouse (Figure 14A) and microarray data indicated that its expression was upregulated in corticotrophs compared to mean expression of the gene in the anterior lobe (Table 13). It was also highly expressed in foetal human and P7 mouse corticotrophs (Figure 19C). *Pcdh9* belongs to the protocadherin family of CAMs. The size and diversity of the family combined with its predominant expression in the nervous system has led to the hypothesis that combinatorial expression patterns of protocadherins might underly the specificity of neuronal connectivity in the brain (Wu and Maniatis 1999). Given that no other protocadherins emerged as clear targets as homotypic CAMs despite many of them being expressed in the pituitary (Figure 25), combinatorial expression patterns of protocadherins might contribute to the specificity of homotypic adhesion in the pituitary as well.

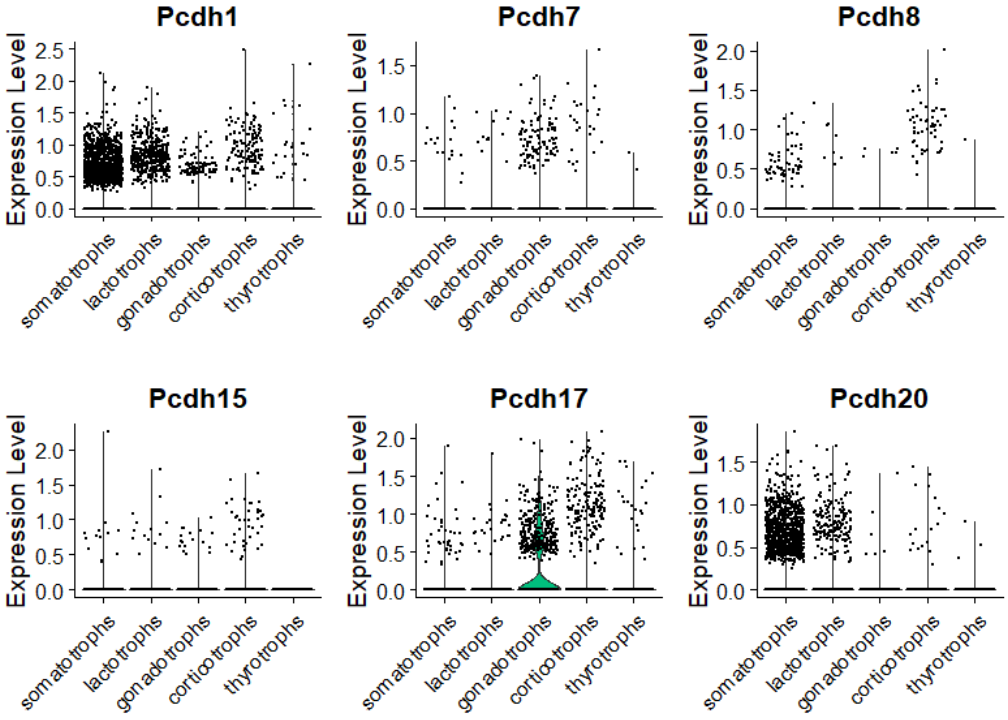


**Figure 25: Expression of protocadherins (Pcdh) in somatotrophs, lactotrophs, gonadotrophs, corticotrophs and thyrotrophs in adult mouse pituitary single cell RNA-sequencing data.** Each dot represents the expression level for an individual cell.

Expression of *Ptprz1* was biased towards adult mouse corticotrophs compared to gonadotrophs (Figure 14A) and both microarray and scRNA-seq data from the adult mouse and scRNA-seq data from the adult rat indicated that expression of the gene was overrepresented in corticotrophs compared to other pituitary cells (Tables 13 and 16). It was also highly expressed in P7 mouse and adult rat corticotrophs (Figures 19A and 21A). *Ptprz1* encodes a receptor with tyrosine phosphatase activity (Ariyama et al. 1995) and is therefore not a traditional CAM. However, in recent years many membrane receptors have been shown to have dual roles in adhesion and signalling (Yona et al. 2008). Indeed, the extracellular part of PTPRZ1 binds a range of CAMs, including NCAM (Milev et al. 1994) and contactin 1 (Lamprianou et al. 2011), which are both broadly expressed in the pituitary (Figure 26A and B). *Ptprz1* mRNA has been detected exclusively in rat corticotrophs and somatotrophs (Fujiwara et al. 2015).

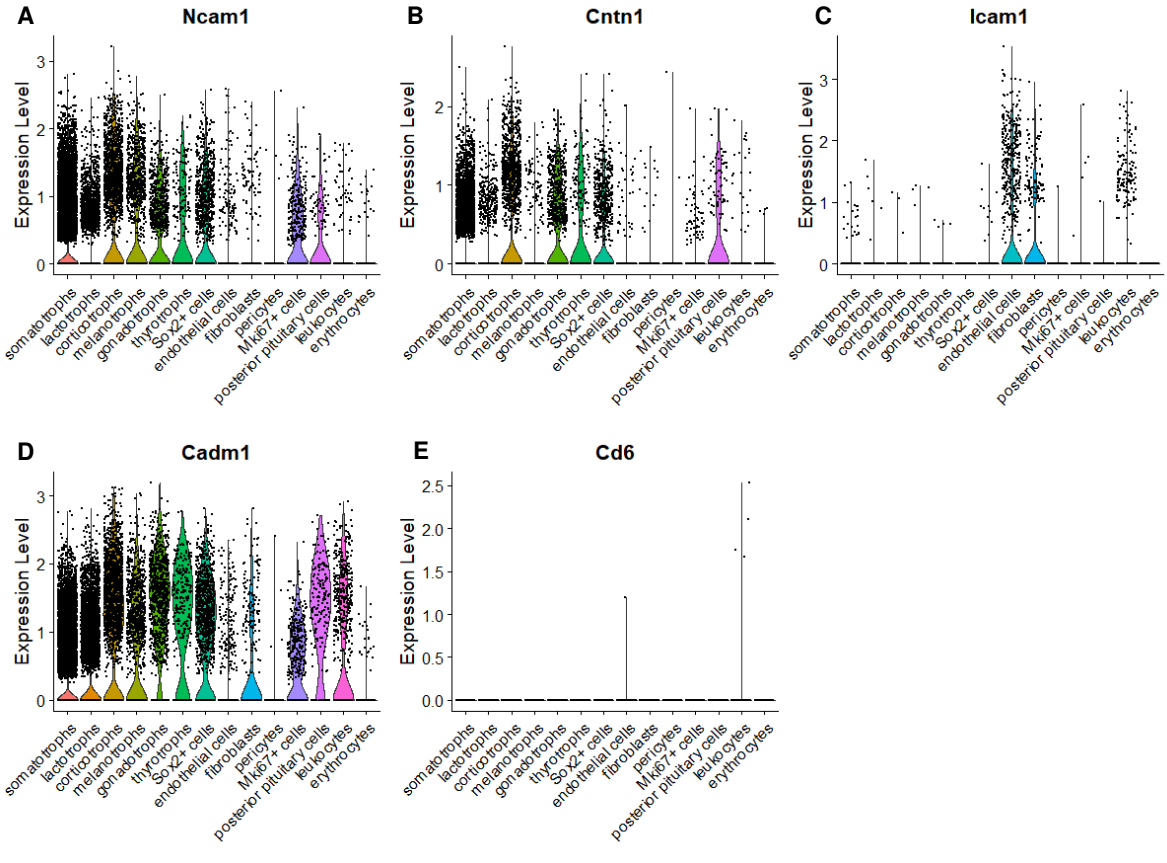


**Figure 26: Expression distribution of *Ncam1* (A), *Cntn1* (B), *Icam1* (C), *Cadm1* (D) and *Cd6* (E) between cell types in pituitary single cell RNA-sequencing data from the adult mouse.** Each dot represents the expression level for an individual cell.

Expression of *Slitrk1* was also biased towards corticotrophs compared to gonadotrophs (Figure 14A). It was expressed in a relatively high proportion of P7 mouse and adult rat corticotrophs (Figures 19A and 21A) and its expression was overrepresented among rat corticotrophs (Table 16). *Slitrk1* is highly expressed in the central nervous system where it encodes a transmembrane protein mediating synapse formation and neurite outgrowth (Aruga and Mikoshiba 2003; Beaubien et al. 2016). Its role in neurite outgrowth is interesting given that corticotrophs extend cytoplasmic projections towards other corticotrophs and blood vessels (Budry et al. 2011). SLITRK1 might therefore have another possible function in controlling the outgrowth of these projections.

### *4.3.2 Gonadotrophs*

In gonadotrophs, 3 CAMs emerged as clear candidates with potential to be involved in homotypic adhesion. *Igdcc4* was the candidate expressed in the highest proportion of gonadotrophs in the adult mouse while also being lowly expressed in corticotrophs (Figure 14A). Its expression was overrepresented among gonadotrophs (Table 13) and it was highly expressed in P7 mouse (Figure 19B) and adult rat (Figure 21B) gonadotrophs. Currently, the only known function of *Igdcc4* is as a potential marker for hepatocellular carcinoma (Zweerink et al. 2021).

*Nrp2* was expressed in a high proportion of gonadotrophs and low proportion of corticotrophs in the adult mouse (Figure 14A) and its expression was upregulated in corticotrophs compared to mean expression of the gene in the anterior lobe (Table 13). It was also expressed in more than 20% of both foetal human and P7 mouse gonadotrophs (Figure 19D). *Nrp2* encodes neuropilin 2, a transmembrane receptor implicated in neural and vascular development (Klagsbrun et al. 2002). It appears to have more of a regulatory role in cell adhesion by promoting the strength of integrin interactions with the extracellular matrix (Goel et al. 2012). Interestingly, *Nrp2* knockout mice are sub- or infertile, which has been attributed to a reduced number of GnRH neurons in the hypothalamus (Cariboni et al. 2007). If neuropilin 2 is involved in gonadotroph cell adhesion, a pituitary-level defect might also contribute to the reproductive phenotype of the *Nrp2* knockouts.

*St6gal1* was expressed in a high proportion of gonadotrophs and a low proportion of corticotrophs in the adult mouse (Figure 14A). It was also expressed in a high proportion of gonadotrophs in the human foetus (Figure 18B) and its expression was biased towards gonadotrophs compared to other cells in the foetal human pituitary (Table 15). It was highly expressed in P7 mouse gonadotrophs (Figure 19D). The protein encoded by *St6gal1* is a sialyltransferase enzyme which does not appear to mediate adhesion on its own but does

alter the properties of CAMs such as intercellular adhesion molecule 1 (ICAM1) (Zhou et al. 2019) and cell adhesion molecule 1 (CADM1) (Minami et al. 2013) via sialylation. In the pituitary, *Icam1* is mostly expressed in non-endocrine cells, whereas *Cadm1* is expressed ubiquitously throughout the gland (Figure 26C and D).

### *4.3.3 Somatotrophs*

*Alcam* emerged as a clear candidate CAM in somatotrophs. It was expressed in 50% of somatotrophs and few lactotrophs in the adult mouse (Figure 14B) and its expression was biased towards somatotrophs compared to other pituitary cells (Table 13). It was also highly expressed in foetal human (Figure 18C) and adult rat (Figure 21C) somatotrophs. ALCAM is generally recognised as the adhesion molecule binding CD6, a surface protein expressed on T-cells (Bowen et al. 1995). However, expression of *Cd6* is almost completely absent in the pituitary (Figure 26E), suggesting that *Alcam* likely also interacts with other partners to mediate cell adhesion. Overexpression of ALCAM in GH3 cells, a growth hormone and prolactin secreting cell line, promoted proliferation and invasion of the cells (He et al. 2017), supporting a role for ALCAM as a CAM in somatotrophs.

### *4.3.4 Lactotrophs*

Of the lactotroph candidates, *Dscam* and *Ptptro* were supported by most evidence from the different stages of evaluation. *Dscam* was expressed in a high proportion of lactotrophs and low proportion of somatotrophs in the adult mouse (Figure 14B). Its expression was overrepresented in lactotrophs compared to the rest of the pituitary (Table 13) and it was also highly expressed in P7 mouse lactotrophs (Figure 20C). *Dscam* encodes a CAM with well-characterised roles in the development of the nervous system (Thiry et al. 2015; Arimura et al. 2020), but any studies on its importance in other tissues are lacking. The multiple isoforms of *Dscam* that exist both in mice and humans (Alves-Sampaio et al. 2010) might provide an additional level of specificity to adhesion mediated by the CAM.

*Ptpro* was the candidate expressed in the largest proportion of lactotrophs in the adult mouse while being lowly expressed in somatotrophs (Figure 14B). Its expression was biased towards lactotrophs compared to other pituitary cells (Table 13) and it was highly expressed in foetal human and P7 mouse lactotrophs (Figure 20D). *Ptpro* encodes a receptor-type tyrosine phosphatase (Avraham et al. 1997). It has recently been found to act as a potent CAM capable of forming new synapses in the brain (Jiang et al. 2017) and also regulates the function of a range of CAMs (Shintani et al. 2006), indicating its importance for cell adhesion.

## 4.4 Sex differences in candidate CAM expression

Apart from differences in the somatotroph network response to GHRH and gonadal steroids in male and female mice (Sanchez-Cardenas et al. 2010; Schaeffer et al. 2011), any sexual dimorphism in pituitary networks is unknown. Comparison of candidate CAM expression in male and female mouse whole anterior lobes showed that most candidates were expressed at a similar level (Table 11). However, 9 candidates showed over 1 $log_2$-fold upregulation in either sex (Table 11). If these CAMs are found to be involved in homotypic adhesion, their sex-dependent expression could indicate that homotypic network structures also differ between the sexes. In particular, all the candidate CAMs that were upregulated in females (*Epha6*, *Il1rapl2* and *Pcdh10*) were lactotroph candidates, suggesting that the lactotroph network might be different in females. The female network undergoes significant changes to support prolactin output during lactation (Hodson et al. 2012), so a different connectivity between the cells at baseline in females might better prepare the network for lactation-induced changes.

## 4.5 Differences in candidate CAM expression between species and developmental stages

Expression of many candidate CAMs differed between species and developmental stages (Figures 17-21). Assuming that the pituitary scRNA-seq data are representative of the pattern of *in vivo* expression in the pituitary, the difference might indicate that 1) the structures of the homotypic networks differ between species and developmental stages and/or 2) the CAMs mediating homotypic adhesion differ between species and developmental stages.

While the human and rat pituitary networks have not been characterised yet, there is some evidence that pituitary networks might differ in structure depending on mouse age. A population of gonadotrophs with very different connectivity to corticotrophs compared with prenatal gonadotrophs emerge postnatally (Budry et al. 2011) and somatotroph cluster density and localisation changes with age (Bonnefont et al. 2005). These changes might be facilitated by differential expression of CAMs. In humans, candidate CAM expression was more widespread in a cell type during weeks 7-10 than weeks 23-25 post-fertilisation (Figure 17). The increased CAM expression could potentially be required to support the initial establishment of homotypic networks.

It is also possible that different CAMs mediate homotypic adhesion in different species or at different developmental stages. For example, one set of CAMs might be involved in initial

establishment of a network and another set might then maintain network integrity and remodelling during later development and postnatal life. This is true for other organs such as the kidney where specific cell types rely on different CAMs to create tissue architecture at different stages of development or adulthood (Perantoni 1999). While the CAMs involved in creating such fundamental structures as the pituitary networks might be expected to be conserved throughout evolution, differences in the CAMs that mediate adhesion in cell types of other tissues have also been reported between species (McKeown et al. 2013). The temporal and interspecies differences in candidate CAM expression could therefore be indicative of changing adhesion patterns within the networks.

## 4.6 Conclusion

Very little is still known about how homotypic networks are constructed in the pituitary. This chapter provides a small dent in the effort to understand the mechanism by generating a set of candidate CAMs that may mediate adhesion between homotypic cells. Examining the expression patterns of these candidates also revealed that potential differences might exist in homotypic networks between sexes, developmental stages and species. Furthermore, expression of the candidates was generally not present throughout a cell type, nor was it cell type selective, indicating that the specific adhesion seen between homotypic cells in the pituitary might be achieved through more complex mechanisms than simply mutually exclusive expression of separate CAMs between cell types. These findings imply that the network organisation of the anterior pituitary might be an intricate system with consequences for how the gland achieves its tightly regulated hormone output.

# CHAPTER 3: A TRANSCRIPTOME-BASED PROFILE OF CORTICOTROPH PARACRINE COMMUNICATION DURING CHRONIC STRESS

## 1 INTRODUCTION

### 1.1 Chronic stress: an HPA axis-mediated response with negative consequences on health

Chronic stress is increasingly recognised as an underlying risk factor for a range of conditions including depression (Hammen 2015), cardiovascular disease (Steptoe and Kivimäki 2012) and metabolic syndrome (Tamashiro et al. 2011). Hyperactivation of the hypothalamic-pituitary-adrenal (HPA) axis and the resulting overproduction of glucocorticoids has been implicated as the main culprit of these deleterious effects (Sharpley 2009). However, elevated glucocorticoids do not fully reproduce the physiological changes elicited by chronic stress (Shors et al. 1989; Rebuffé-Scrive et al. 1992; Makino et al. 1995), suggesting that other mechanisms are also involved.

### 1.2 A pituitary-centric view of stress

Pituitary corticotrophs are instrumental to the stress response given that they secrete ACTH, the major determinant of glucocorticoid output from the adrenal glands. However, any role for the pituitary gland beyond acting as the location for a cell type that facilitates the production of glucocorticoids is rarely considered. This is surprising given the evidence that the pituitary as a whole might contribute to the stress response. FS cells have been suggested to mediate the negative feedback of glucocorticoids (Buckingham et al. 2006), changes in pituitary gland mass may contribute to the pattern of HPA axis activity observed following chronic stress (Karin et al. 2020) and cells other than corticotrophs appear to contain the majority of corticosteroid binding globulin in the pituitary (de Kloet et al. 1984; Möpert et al. 2006). Moreover, ACTH can function as a hormone independently of glucocorticoids, altering metabolism, haemodynamics and immune function by activating melanocortin receptors expressed elsewhere in the body (Zelena and Makara 2012). There is also *in vitro* evidence that other pituitary cells produce paracrine factors (e.g. angiotensin II (Steele et al. 1982), calcitonin gene related peptide (Gon et al. 1990) and C-type natriuretic peptide (McArdle et al. 1994)) which alter ACTH secretion from corticotrophs (Gaillard et al. 1981; Iino et al. 1998a; Guild and Cramb 1999). In turn, HPA axis activation produces local changes to the pituitary which cannot solely be attributed to glucocorticoids or hypothalamic

input (Lakić et al. 2011), suggesting paracrine signalling by corticotrophs. The part played by the pituitary in the stress response may therefore extend beyond controlling glucocorticoid production.

## 1.3 The pituitary gland as a communication hub during chronic stress

Chronic stress alters the activity of all 5 hypothalamic-pituitary-target organ axes (Skuse et al. 1996; Cameron 1997; Helmreich et al. 2005; Levine and Muneyyirci-Delale 2018). Any crosstalk between the axes is generally considered to be mediated by the peripheral effector hormones (Viau 2002). However, the pituitary is a focal point where all the axes converge, making the gland uniquely suited to facilitate local exchange of signals between cells belonging to the different endocrine axes. Intrapituitary communication may therefore contribute to inter-axis crosstalk. So far, a few paracrine factors have been identified as mediators of local communication between corticotrophs and other pituitary endocrine cells (Denef 2008) but whether any of these interactions are altered by chronic stress is unknown. Communication between corticotrophs and non-endocrine cells in the pituitary may also have relevance during chronic stress, for example for regulating the changes to blood flow (Goldman 1963), gland volume (Cooper et al. 2017) and cellular composition (Lopez et al. 2021) that occur during stress. Local interactions at the level of the pituitary might therefore be involved in coordination of the chronic stress response.

## 1.4 A computational approach for predicting communication patterns between different pituitary cell types during chronic stress

scRNA-seq data provide valuable insight into the gene expression profiles of individual cells. As mRNA is a prerequisite for protein synthesis, knowledge of the transcripts present in a cell can help indicate which proteins that cell produces, including any signalling molecules and their receptors. In this chapter, pituitary scRNA-seq data from control and chronically stressed mice were analysed to identify potential chronic stress-induced changes to paracrine communication between corticotrophs and other pituitary cells.

## 2 METHODS

### 2.1 Identification of cell types in scRNA-seq data from control and chronically stressed mouse pituitaries

The dataset used to analyse pituitary cell-cell communication has been previously published (Lopez et al. 2021). In the study, mice were subjected to a chronic social defeat stress paradigm for 3 weeks or kept in control conditions, after which scRNA-seq was performed on the pituitaries. The data were downloaded from the Gene Expression Omnibus (accession number GSE161751) and cell types were identified as described in Chapter 1.

### 2.2 CellPhoneDB: predicting paracrine interactions based on expression of ligands and receptors in scRNA-seq data

Potential cell-cell communication within a tissue can be predicted based on expression patterns of ligands and receptors between cell types. CellPhoneDB (Efremova et al. 2020) integrates a database of 1,396 ligand-receptor pairs with a computational algorithm that scans for expression of these ligands and receptors in different cell types pre-identified from single cell transcriptomics data. To analyse chronic stress-induced changes to pituitary cell-cell communication, CellPhoneDB analysis was applied separately to scRNA-seq data from control and stressed pituitaries and the differences were subsequently quantified.

CellPhoneDB is based on human genes for ligands and receptors. To allow its use on mouse data, mouse-to-human gene orthologue conversion was performed using biomaRt (version 2.46.2) (Durinck et al. 2009) in R (version 4.0.3). The count matrices from control and stressed pituitaries were normalised for library size. Significant interactions between corticotrophs and other pituitary cell types were identified using CellPhoneDB (version 2.1.7) with default parameters ($p<0.05$ and expression of ligands and receptors in at least 10% of a cell type). The differences in significant interactions between control and stressed pituitaries were determined manually from the graphical results produced. The human genes were converted back to mouse orthologues for display of the results.

### 2.3 Pathway enrichment

Pathway enrichment for the ligand-receptor interactions identified by CellPhoneDB as upregulated or downregulated in chronic stress was performed using enrichR (version 3.0), an R package interface to the Enrichr pathway analysis tool (Chen et al. 2013). Databases “KEGG_2021_Human”, “Panther_2016” and “Reactome_2016” were queried for gene

enrichment. The top 10 enriched pathways in each database were extracted separately for upregulated and downregulated interactions using the genes for both ligands and receptors as gene inputs. Shared genes in the top 3 enriched pathways of all 3 databases were extracted from the output tables generated by the enrichr function.

### 2.4 CellChat: predicting altered paracrine interactions based on expression of ligands and receptors in scRNA-seq data using a mouse-specific database

CellChat has inbuilt functions to predict altered cell-cell communication between datasets and is based on a database of ligand-receptor interactions specifically adapted to mouse genes (Jin et al. 2021). Using R package CellChat (version 1.1.3) and its secreted signalling database, interactions were first separately computed for the control and stress pituitary scRNA-seq datasets using the recommended pipeline (sequentially applying functions subsetData, identifyOverExpressedGenes, identifyOverExpressedInteractions, projectData, computeCommunProb with the population.size parameter set to true, subsetCommunication, computeCommunProbPathway, aggregateNet and netAnalysis_computeCentrality to the data). The CellChat objects were merged with the mergeCellChat function which also allows determination of altered cell-cell interactions between the objects. Differences in communication were plotted with the netVisual_bubble function.

### 2.5 NicheNet: predicting functional ligand-receptor interactions based on changes in expression of downstream target genes in scRNA-seq data

To identify potential paracrine interactions with functional consequences in pituitary cells, NicheNet (Browaeys et al. 2020) was used. The tool predicts ligand-receptor interactions responsible for gene expression changes observed during a change of conditions (here exposure to chronic stress) using a ligand-receptor-target gene database compiled from different data sources. A combined Seurat object (Hao et al. 2020) was created from the control and stress datasets and NicheNet analysis (using R package nichenetr, version 1.0.0) was applied to the Seurat object with function nichenet_seuratobj_aggregate and its default parameters. The top 20 ligand-receptor interactions predicted to explain the gene expression signature induced by chronic stress were considered in the results.

### 2.6 Tables and figures

Table 19 was made using flextable (version 0.6.7) in R. Figures were made with BioRender.com or R packages CellChat (function netVisual_bubble) (version 1.1.3), enrichR

(version 3.0), ggplot2 (version 3.3.3) (Wickham 2016) and Seurat (function DotPlot) (version 4.0.0) (Hao et al. 2020).

# 3 RESULTS

## 3.1 Comparison between corticotroph interactions predicted by 3 different methods

To increase the confidence in the ligand-receptor interactions predicted from pituitary scRNA-seq data given the concerns around the differential cell type distribution between paracrine ligand mRNA and protein (Chapter 1), the aim was to use shared predictions made by 3 different computational algorithms: CellPhoneDB (Efremova et al. 2020), NicheNet (Browaeys et al. 2020) and CellChat (Jin et al. 2021). However, among the corticotroph interactions predicted to be altered by chronic stress, none were shared between the algorithms. A closer inspection revealed that CellChat predicted only 9 altered interactions, of which 4 were interactions between pituitary hormones and receptors (Figure 27). Some of the ligands and receptors were also very lowly expressed in the relevant cell types despite being identified as significant interactions, such as the heparin binding EGF-like growth factor in corticotrophs or the atypical chemokine receptor 3 in pericytes (Figure 28). Given these limitations, the predictions made by CellPhoneDB and NicheNet were used for further analyses. CellPhoneDB uses expression values of ligands and receptors to predict cell-cell communication between different cell types (Figure 29A) and NicheNet predicts active ligand-receptor interactions based on downstream gene expression changes produced by receptor activation (Figure 29B), making them complementary tools for predicting active paracrine communication in the pituitary.

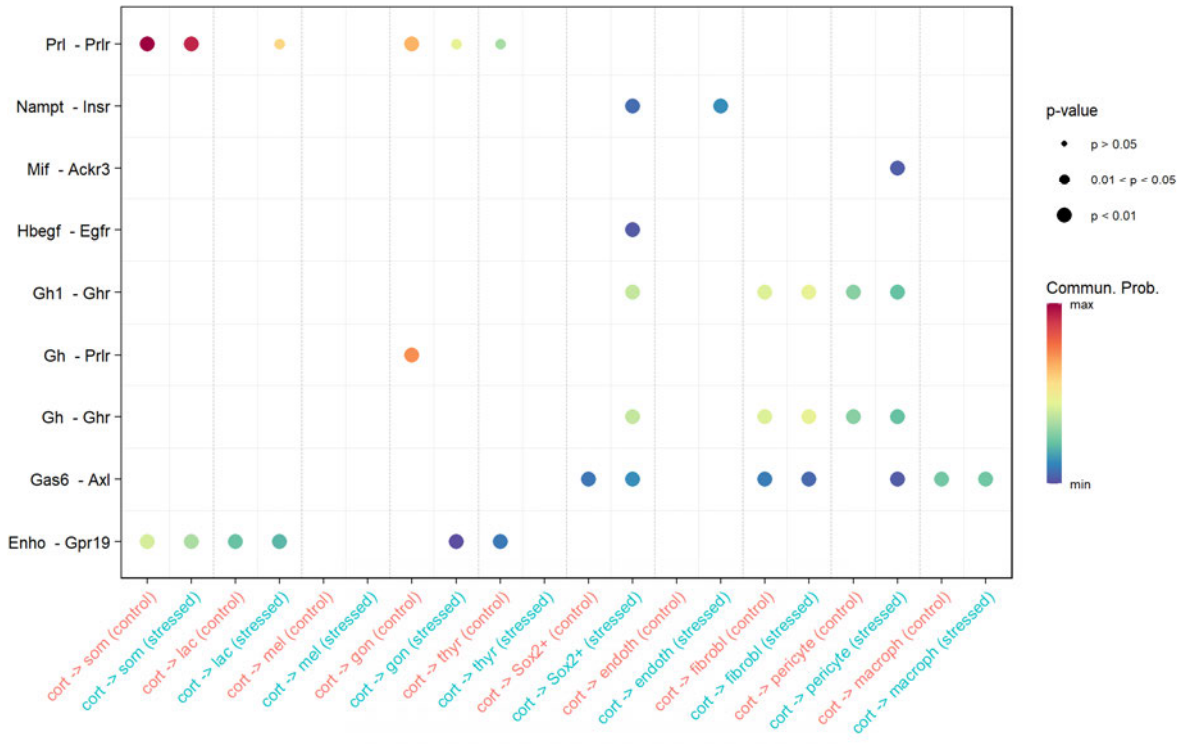


**Figure 27: Chronic stress-induced changes to corticotroph paracrine interactions as predicted by CellChat.** Abbreviations: prolactin (Prl), prolactin receptor (Prlr), nicotinamide phosphoribosyltransferase (Nampt), insulin receptor (Insr), macrophage migration inhibitory factor (glycosylation-inhibiting factor) (Mif), atypical chemokine receptor 3 (Ackr3), heparin binding EGF-like growth factor (Hbegf), growth hormone (Gh), growth hormone receptor (Ghr), growth arrest specific 6 (Gas6), AXL receptor tyrosine kinase (Axl), energy homeostasis associated (Enho), G protein-coupled receptor 19 (Gpr19), corticotrophs (cort), somatotrophs (som), lactotrophs (lac), melanotrophs (mel), gonadotrophs (gon), thyrotrophs (thyr), Sox2+ve cells (Sox2+), endothelial cells (endoth), fibroblasts (fibrobl), macrophages (macroph).

## 3.2 CellPhoneDB reveals chronic stress-induced changes to expression of paracrine ligands and receptors in the pituitary

First, chronic stress-induced changes to corticotroph cell-cell communication based on altered expression of paracrine ligands and receptors was analysed using CellPhoneDB. Quantification of the differences revealed a total of 211 paracrine interactions altered by chronic stress, of which 132 were upregulated and 79 were downregulated. The full list of altered interactions can be accessed online at https://uoe-my.sharepoint.com/:u:/g/personal/s1749812_ed_ac_uk/EWRiDLlgv4dHq968Xyu7BeQBbL9L6ZKmXeLNxTv9QzMLWg?e=xG106H. This transcriptome-based signature of corticotroph paracrine communication was subsequently explored to understand how the relationships between corticotrophs and other pituitary cells may be influenced by chronic stress.

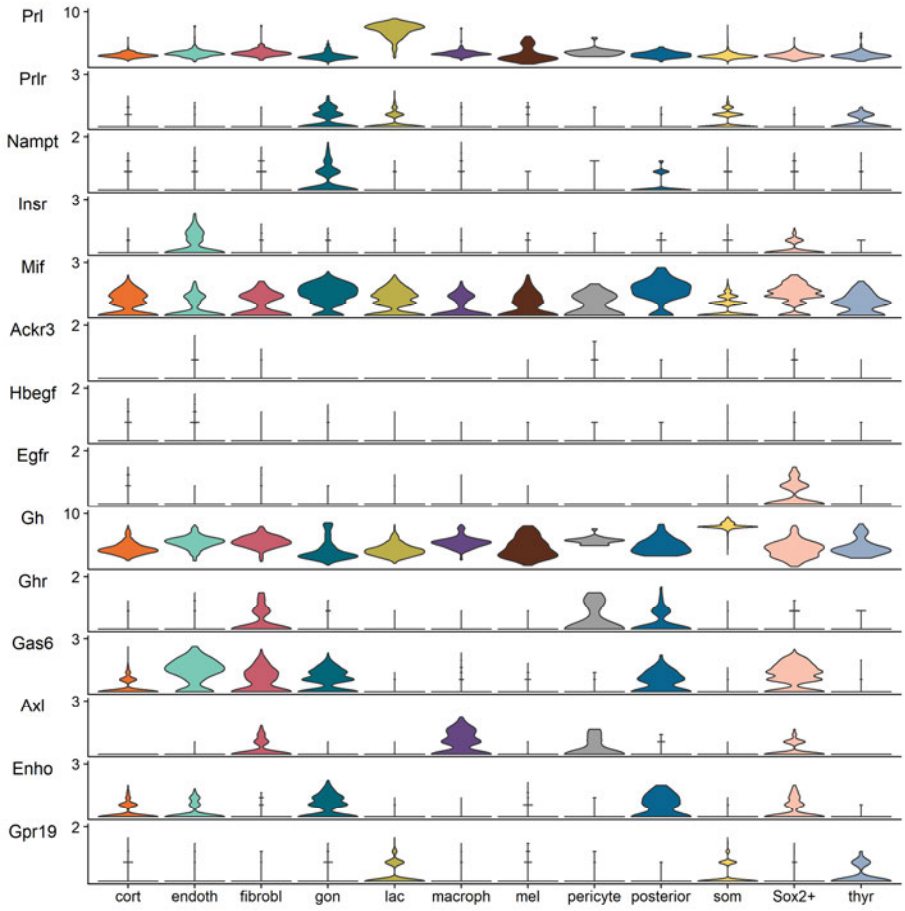


**Figure 28: Expression of paracrine ligands and receptors identified by CellChat as altered by chronic stress in mouse pituitary scRNA-seq data.** The expression values are based on the combined stress and control scRNA-seq datasets. For abbreviations, see legend to Figure 27.

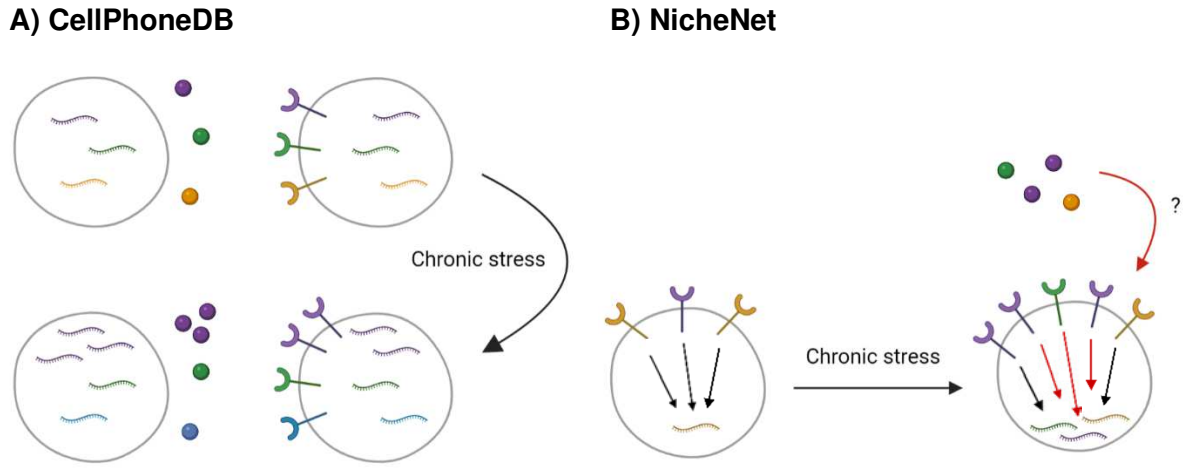


**Figure 29: CellPhoneDB (A) and NicheNet (B) can be used to predict pituitary ligand-receptor interactions altered by chronic stress based on single cell RNA-sequencing data.** CellPhoneDB (Efremova et al. 2020) determines changes in ligand and receptor gene expression to predict shifts in cell-cell communication between different cell types. NicheNet (Browaeys et al. 2020) predicts active ligand-receptor interactions during changing conditions based on their ability to alter the expression of downstream genes that match the differentially expressed genes induced by the change.

### 3.3 Communication between corticotrophs and other pituitary cells may occur increasingly through EGF and FGF and less through ephrin and VEGFA signalling during chronic stress

To identify potential common signalling pathways among the upregulated and the downregulated interactions, pathway enrichment analysis was performed. The top enriched pathways were different depending on the pathway database queried (Figure 30), which is consistent with previous research showing that the choice of database significantly influences pathway enrichment results (Mubeen et al. 2019; Karp et al. 2021). To identify any shared genes in the top enriched pathways in the databases despite different pathway names, genes involved in the top 3 enriched pathways in all 3 databases were identified. Among the upregulated interactions, epidermal growth factor (EGF), fibroblast growth factor (FGF) and their receptors were involved in the top enriched pathways in all 3 databases (Table 18). Ephrins and VEGFA in turn were part of the top 3 enriched pathways among all databases for downregulated interactions (Table 18). This indicates that communication between corticotrophs and other pituitary cell types may overall rely on increased EGF and FGF signalling during chronic stress, while ephrin and VEGF signalling pathways may in turn be downregulated.

### 3.4 Chronic stress predominantly alters corticotroph communication with non-endocrine cell types

To determine the cell types that corticotrophs modified paracrine communication with the most during chronic stress, the total number of altered interactions between different cell types was calculated. Most interactions were changed between corticotrophs and pericytes (36 interactions), Sox2+ve cells (33 interactions), fibroblasts (29 interactions) and posterior pituitary cells (28 interactions), whereas the fewest interactions were altered between corticotrophs and endocrine cell types (Table 19). The number of upregulated compared to downregulated interactions in chronic stress was well balanced for most pairs of cell types (Table 19). However, the number of upregulated compared to downregulated interactions was over 2-fold higher between corticotrophs and pericytes, Sox2+ve cells, somatotrophs and thyrotrophs (Table 19), indicating that the potential for corticotrophs to communicate with these cell types was enhanced during chronic stress. All altered interactions between corticotrophs themselves were upregulated (Table 19), suggesting that self-regulation of the cell type might have increased importance during chronic stress.

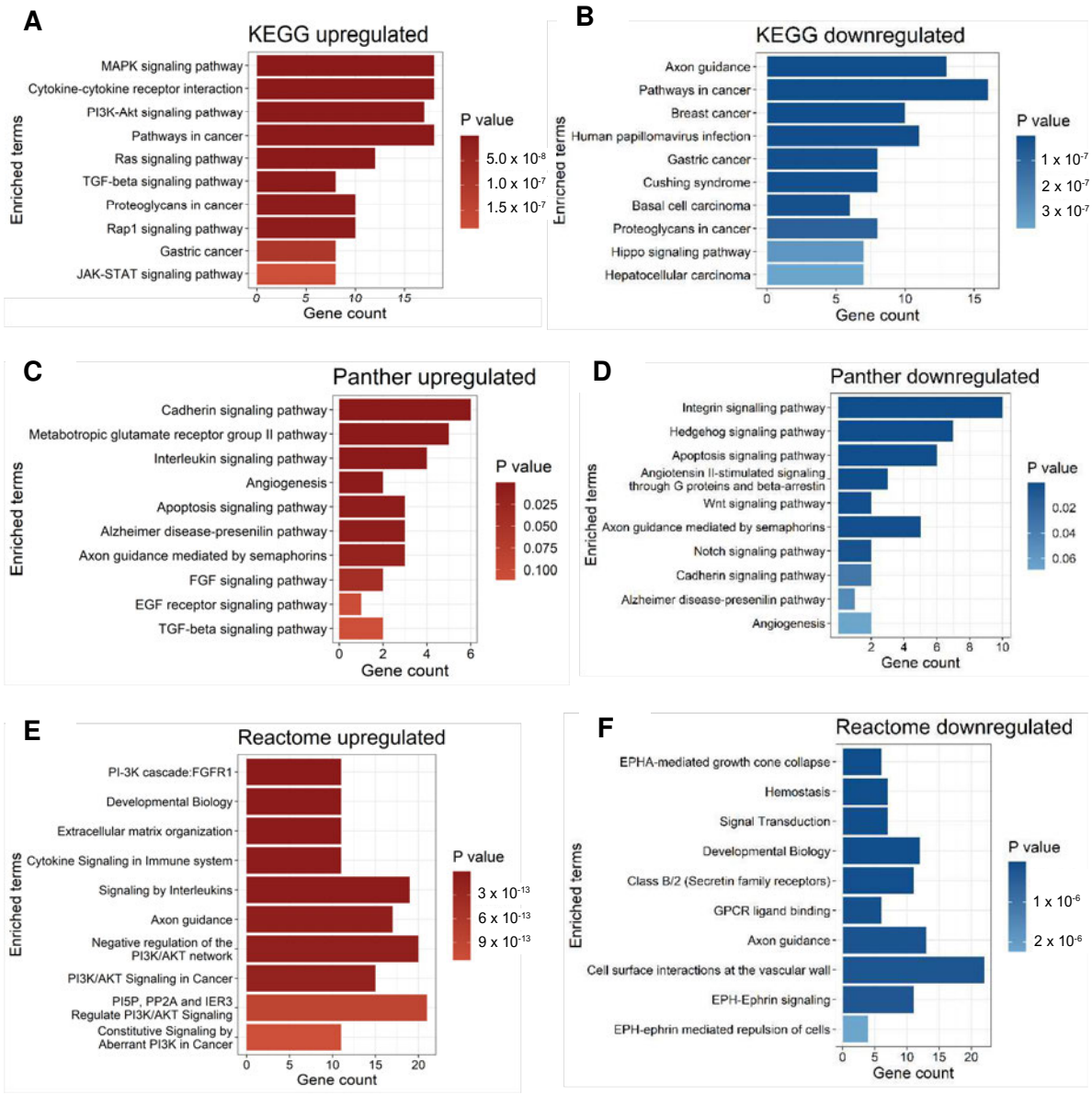


**Figure 30: Enriched pathways among upregulated or downregulated ligand-receptor interactions between corticotrophs and other pituitary cell types in chronic stress.** Pathway enrichment analyses for the ligands and receptors upregulated (A, C, E) and downregulated (B, D, F) by chronic stress were performed using the KEGG (A and B), Panther (C and D) and Reactome (E and F) databases. The top 10 enriched pathways are shown, sorted by p value in ascending order.

| Upregulated | | Downregulated | |
|---|---|---|---|
| **Gene** | **Protein** | **Gene** | **Protein** |
| *Egf* | Epidermal growth factor | *Efnb1* | Ephrin-B1 |
| *Egfr* | Epidermal growth factor receptor | *Efnb2* | Ephrin-B2 |
| *Erbb4* | Receptor tyrosine-protein kinase erbB-4 | *Ephb2* | Ephrin type-B receptor 2 |
| *Fgf1* | Fibroblast growth factor 1 | *Vegfa* | Vascular endothelial growth factor A |
| *Fgf9* | Fibroblast growth factor 9 | | |
| *Fgfr1* | Fibroblast growth factor receptor 1 | | |

**Table 18: Genes shared in the top 3 enriched pathways in KEGG, Panther and Reactome databases for upregulated or downregulated ligand-receptor interactions in chronic stress between corticotrophs and other pituitary cell types.** Gene abbreviations: *Egf* (Epidermal growth factor), *Egfr* (Epidermal growth factor receptor), *Erbb4* (Erb-b2 receptor tyrosine kinase 4), *Fgf1* (Fibroblast growth factor 1), *Fgf9* (Fibroblast growth factor 9), *Fgfr1* (Fibroblast growth factor receptor 1), *Fgfr3* (Fibroblast growth factor receptor 3), *Efnb1* (Ephrin B1), *Efnb2* (Ephrin B2), *Ephb2* (Eph receptor B2), *Vegfa* (Vascular endothelial growth factor A).

## 3.5 Chronic stress reduces the outward flow and increases the inward flow of paracrine signals to corticotrophs

Corticotroph cell-cell communication includes both ligands sent by corticotrophs to other pituitary cells and reciprocally, ligands produced by other cell types acting on receptors on corticotrophs. For the interactions upregulated by chronic stress, corticotrophs were more rarely the sending cell type (Table 19, column 3), suggesting that other cells increase communication with corticotrophs instead of vice versa. In contrast, corticotrophs were the sending cell type for most ligand-receptor interactions downregulated by chronic stress (Table 19, column 4). Corticotrophs may therefore reduce intrapituitary communication with other cell types, receive increased paracrine inputs from other pituitary cells and increase signalling with other corticotrophs during chronic stress.

| | | Number of interactions | | |
|---|---|---|---|---|
| **Cell type communicating with corticotrophs** | **Corticotrophs as senders and/or receivers of ligands** | **Upregulated** | **Downregulated** | **Total** |
| Corticotrophs | | | | |
| | Both | 8 | 0 | 8 |
| | Sender | 8 | 0 | |
| | Receiver | 0 | 0 | |
| Endothelial cells | | | | |
| | Both | 4 | 6 | 10 |
| | Sender | 1 | 5 | |
| | Receiver | 3 | 1 | |
| Fibroblasts | | | | |
| | Both | 18 | 11 | 29 |
| | Sender | 5 | 6 | |
| | Receiver | 13 | 5 | |
| Gonadotrophs | | | | |
| | Both | 7 | 6 | 13 |
| | Sender | 3 | 3 | |
| | Receiver | 4 | 3 | |
| Lactotrophs | | | | |
| | Both | 5 | 6 | 11 |
| | Sender | 1 | 5 | |
| | Receiver | 4 | 1 | |
| Macrophages | | | | |
| | Both | 9 | 5 | 14 |
| | Sender | 1 | 3 | |
| | Receiver | 8 | 2 | |
| Melanotrophs | | | | |
| | Both | 6 | 4 | 10 |
| | Sender | 1 | 2 | |
| | Receiver | 5 | 2 | |
| Pericytes | | | | |
| | Both | 25 | 11 | 36 |
| | Sender | 12 | 6 | |
| | Receiver | 13 | 5 | |
| Posterior pituitary cells | | | | |
| | Both | 15 | 13 | 28 |
| | Sender | 6 | 8 | |
| | Receiver | 9 | 5 | |
| Somatotrophs | | | | |
| | Both | 4 | 2 | 6 |
| | Sender | 0 | 2 | |
| | Receiver | 4 | 0 | |
| Sox2+ cells | | | | |
| | Both | 22 | 11 | 33 |
| | Sender | 10 | 8 | |
| | Receiver | 12 | 3 | |
| Thyrotrophs | | | | |
| | Both | 9 | 4 | 13 |
| | Sender | 1 | 3 | |
| | Receiver | 8 | 1 | |

**Table 19: Number of interactions between corticotrophs and other pituitary cell types predicted to be altered by chronic stress by CellPhoneDB.** The total number of interactions altered by chronic stress is shown for each cell type pair in green. The total number of interactions is further split into upregulated and downregulated interactions as well as into interactions where corticotrophs are the sending and/or receiving cell type for paracrine ligands.

## 3.6 NicheNet predicts active ligand-receptor interactions based on downstream gene expression changes

CellPhoneDB suggested that other pituitary cell types upregulate communication with corticotrophs during chronic stress. To determine which, if any, of these ligand-receptor interactions were active in the pituitary based on their ability to produce downstream changes in gene expression that match the differentially expressed genes in corticotrophs following chronic stress, NicheNet analysis was applied. Of the interactions identified by CellPhoneDB, brain-derived neurotrophic factor (BDNF), ephrin-A5, neuregulin 1 and VEGFA and their receptors expressed on corticotrophs were also among the top 20 interactions predicted as active by NicheNet (Table 20). Neuregulin 1 – lipolysis-stimulated lipoprotein receptor (LSR) and neuregulin 1 – neuropilin and tolloid-like protein 2 interactions were upregulated between corticotrophs. The juxtacrine ephrin-A5 – ephrin type-A receptor 5 interaction was upregulated between pericytes and corticotrophs, whereas VEGFA expressed by gonadotrophs and posterior pituitary cells acting on ephrin type-B receptor 2 on corticotrophs was downregulated. Melanotrophs, pericytes, somatotrophs and thyrotrophs all upregulated BDNF signalling to corticotrophs.

## 3.7 Specific ligand-receptor interactions underlying the changes to paracrine signalling from corticotrophs to fibroblasts and Sox2+ve cells during chronic stress

The greatest number of paracrine interactions were altered by chronic stress between corticotrophs and pericytes (Table 19). However, no genes in pericytes were highly differentially expressed in chronic stress potentially due to the low number of cells, preventing functional NicheNet analysis. Instead, active interactions from corticotrophs to fibroblasts and Sox2+ve cells were determined using NicheNet, given that communication with the two cell types was also extensively altered. Interactions from corticotrophs to fibroblasts that were predicted by both CellPhoneDB and NicheNet as altered by chronic stress included neuregulin 1 – epidermal growth factor receptor (upregulated), vitamin K dependent protein S – tyrosine-protein kinase receptor UFO (downregulated) and protein Wnt4 with various Wnt4 receptors (downregulated) (Table 21). Altered communication from corticotrophs to Sox2+ve cells identified by both CellPhoneDB and NicheNet included upregulation of R-spondin-3 – Leucine-rich repeat-containing G-protein coupled receptor (LGR) 4 and R-spondin-3 – LGR6 as well as downregulation of Wnt4 interactions with 5 different Wnt4 receptors (Table 21).

| Sender | Receiver | Ligand (gene) | Ligand (protein) | Receptor (gene) | Receptor (protein) | Direction |
|---|---|---|---|---|---|---|
| Corticotrophs | Corticotrophs | *Nrg1* | Neuregulin 1 | *Lsr* | Lipolysis-stimulated lipoprotein receptor | Upregulated |
| Corticotrophs | Corticotrophs | *Nrg1* | Neuregulin 1 | *Neto2* | Neuropilin and tolloid-like protein 2 | Upregulated |
| Gonadotrophs | Corticotrophs | *Vegfa* | Vascular endothelial growth factor A | *Ephb2* | Ephrin type-B receptor 2 | Downregulated |
| Melanotrophs | Corticotrophs | *Bdnf* | Brain-derived neurotrophic factor | *Sort1* | Sortilin | Upregulated |
| Pericytes | Corticotrophs | *Bdnf* | Brain-derived neurotrophic factor | *Ntrk2* | BDNF/NT-3 growth factors receptor | Upregulated |
| Pericytes | Corticotrophs | *Efna5* | Ephrin-A5 | *Epha5* | Ephrin type-A receptor 5 | Upregulated |
| Posterior pituitary cells | Corticotrophs | *Vegfa* | Vascular endothelial growth factor A | *Ephb2* | Ephrin type-B receptor 2 | Downregulated |
| Somatotrophs | Corticotrophs | *Bdnf* | Brain-derived neurotrophic factor | *Sort1* | Sortilin | Upregulated |
| Thyrotrophs | Corticotrophs | *Bdnf* | Brain-derived neurotrophic factor | *Ntrk2* | BDNF/NT-3 growth factors receptor | Upregulated |
| Thyrotrophs | Corticotrophs | *Bdnf* | Brain-derived neurotrophic factor | *F11r* | Junctional adhesion molecule A | Upregulated |

**Table 20: Ligand-receptor interactions from other pituitary cell types to corticotrophs identified by both CellphoneDB and NicheNet as altered by chronic stress**. The ligands are expressed by the "sender" cell type and receptors are expressed by the "receiver" cell type. Upregulation or downregulation of the interactions was solely predicted by CellPhoneDB. Gene abbreviations: *Bdnf* (Brain derived neurotrophic factor), *Efna5* (Ephrin A5), *Epha5* (Eph receptor A5), *Ephb2* (Eph receptor B2), *F11r* (F11 receptor), *Lsr* (Lipolysis stimulated lipoprotein receptor), *Neto2* (Neuropilin (NRP) and tolloid (TLL)-like 2), *Nrg1* (Neuregulin 1), *Ntrk2* (Neurotrophic tyrosine kinase, receptor, type 2), *Sort1* (Sortilin 1), *Vegfa* (Vascular endothelial growth factor A).

## 3.8 Wnt4: a potential paracrine factor from corticotrophs to non-endocrine pituitary cells

CellPhoneDB and NicheNet analyses suggested that corticotrophs downregulate Wnt4 signalling to both fibroblasts and Sox2+ve cells during chronic stress. To investigate if downregulated Wnt4 signalling from corticotrophs is a common pattern underlying altered communication with several cell types during chronic stress, expression of *Wnt4* and its

receptor genes were examined across different pituitary cell types. *Wnt4* expression was unique to corticotrophs apart from slight expression in melanotrophs induced by chronic stress (Figure 31). Although the ligand was expressed in only 10% of corticotrophs, expression in those cells was high (Figure 31), indicating that significant amounts of ligand could be secreted. All non-endocrine cell types expressed at least 1 of the 10 receptors for Wnt4 identified in the CellPhoneDB and NicheNet databases, whereas no endocrine cells showed high expression of any of the receptors (Figure 31). Expression of the different receptors was not consistently upregulated or downregulated by chronic stress (Figure 31).

| Sender | Receiver | Ligand (gene) | Ligand (protein) | Receptor (gene) | Receptor (protein) | Direction |
|---|---|---|---|---|---|---|
| Corticotrophs | Fibroblasts | Nrg1 | Neuregulin 1 | Egfr | Epidermal growth factor receptor | Upregulated |
| Corticotrophs | Fibroblasts | Wnt4 | Protein Wnt-4 | Notch1 | Neurogenic locus notch homolog protein 1 | Downregulated |
| Corticotrophs | Fibroblasts | Wnt4 | Protein Wnt-4 | Fzd1 | Frizzled-1 | Downregulated |
| Corticotrophs | Fibroblasts | Wnt4 | Protein Wnt-4 | Smo | Smoothened homolog | Downregulated |
| Corticotrophs | Fibroblasts | Pros1 | Vitamin K-dependent protein S | Axl | Tyrosine-protein kinase receptor UFO | Downregulated |
| Corticotrophs | Sox2+ve cells | Wnt4 | Protein Wnt-4 | Notch1 | Neurogenic locus notch homolog protein 1 | Downregulated |
| Corticotrophs | Sox2+ve cells | Wnt4 | Protein Wnt-4 | Fzd6 | Frizzled-6 | Downregulated |
| Corticotrophs | Sox2+ve cells | Rspo3 | R-spondin-3 | Lgr4 | Leucine-rich repeat-containing G-protein coupled receptor 4 | Upregulated |
| Corticotrophs | Sox2+ve cells | Wnt4 | Protein Wnt-4 | Fzd8 | Frizzled-8 | Downregulated |
| Corticotrophs | Sox2+ve cells | Wnt4 | Protein Wnt-4 | Fzd1 | Frizzled-1 | Downregulated |
| Corticotrophs | Sox2+ve cells | Wnt4 | Protein Wnt-4 | Smo | Smoothened homolog | Downregulated |
| Corticotrophs | Sox2+ve cells | Rspo3 | R-spondin-3 | Lgr6 | Leucine-rich repeat-containing G-protein coupled receptor 6 | Upregulated |
| Corticotrophs | Gonadotrophs | Nrg1 | Neuregulin 1 | Adgrl1 | Adhesion G protein-coupled receptor L1 | Upregulated |
| Corticotrophs | Gonadotrophs | Nrg1 | Neuregulin 1 | Lgr4 | Leucine-rich repeat-containing G-protein coupled receptor 4 | Upregulated |
| Corticotrophs | Gonadotrophs | Nrg1 | Neuregulin 1 | Lsr | Lipolysis-stimulated lipoprotein receptor | Upregulated |

**Table 21: Ligand-receptor interactions from corticotrophs to fibroblasts, Sox2+ cells and gonadotrophs identified by both CellPhoneDB and NicheNet as altered by chronic stress.** The ligands are expressed by the “sender” cell type and receptors are expressed by the “receiver” cell type. Upregulation or downregulation of the interactions was solely predicted by CellPhoneDB. Gene abbreviations: *Adgrl1* (Adhesion G protein-coupled receptor L1), *Axl* (AXL receptor tyrosine kinase), *Egfr* (Epidermal growth factor receptor), *Fzd1* (Frizzled class receptor 1), *Fzd6* (Frizzled class receptor 6), *Fzd8* (Frizzled class receptor 8), *Lgr4* (Leucine-rich repeat-containing G protein-coupled receptor 4), *Lgr6* (Leucine-rich repeat-containing G protein-coupled receptor 6), *Lsr* (lipolysis stimulated lipoprotein receptor), *Notch1* (Notch 1), *Nrg1* (Neuregulin 1), *Pros1* (Protein S (alpha)), *Rspo3* (R-spondin 3), *Smo* (Smoothened, frizzled class receptor), *Wnt4* (Wingless-type MMTV integration site family, member 4).

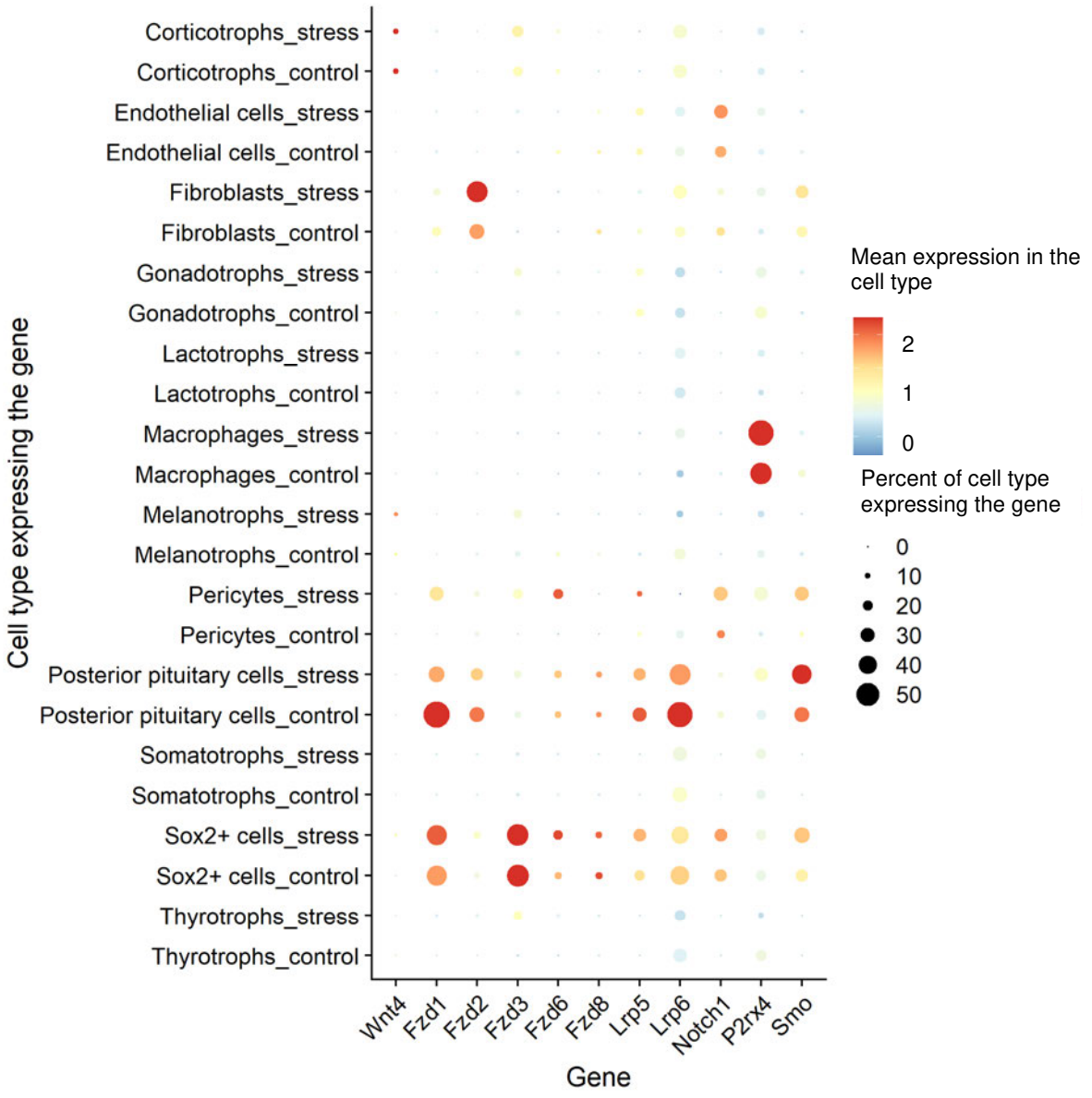


**Figure 31: Expression of Wnt4 and its receptor genes in different pituitary cell types in mouse single cell RNA-sequencing data.** Gene abbreviations: *Wnt4* (Wingless-type MMTV integration site family, member 4), *Fzd1* (Frizzled class receptor 1), *Fzd2* (Frizzled class receptor 2), *Fzd3* (Frizzled class receptor 3), *Fzd6* (Frizzled class receptor 6), *Fzd8* (Frizzled class receptor 8), *Lrp5* (Low density lipoprotein receptor-related protein 5), *Lrp6* (Low density lipoprotein receptor-related protein 6), *Notch1* (Notch 1), *P2rx4* (Purinergic receptor P2X, ligand-gated ion channel 4), *Smo* (Smoothened, frizzled class receptor).

## 3.9 Upregulated neuregulin 1 signalling from corticotrophs to gonadotrophs during chronic stress

Chronic activation of the HPA axis during prolonged stress is associated with inhibition of the hypothalamic-pituitary-gonadal (HPG) axis (Joseph and Whirledge 2017; Acevedo-Rodriguez et al. 2018). The effects have generally been attributed to glucocorticoids (Whirledge and Cidlowski 2010), but local crosstalk between corticotrophs and gonadotrophs at the level of the pituitary could also contribute. To investigate this possibility, the paracrine interactions from corticotrophs to gonadotrophs that were altered by chronic stress were determined. Both CellPhoneDB and NicheNet identified neuregulin 1 as a potential ligand secreted by corticotrophs and acting on adhesion G-protein coupled receptor L1, LGR4 and LSR on gonadotrophs during chronic stress (Table 21).

## 3.10 Chronic stress preferentially targets corticotroph over lactotroph paracrine communication

Given the central role that corticotrophs play in the HPA axis, chronic stress might be expected to alter paracrine communication involving corticotrophs more than that of other pituitary cell types. To examine this hypothesis, the number of paracrine interactions altered by chronic stress to/from corticotrophs was compared with the number altered to/from lactotrophs. Lactotrophs were chosen given their low expression of the glucocorticoid receptor (Ozawa et al. 1999), suggesting that any effects of stress on lactotrophs may be mediated through intrapituitary or hypothalamic communication. The total number of interactions predicted by CellPhoneDB as altered by chronic stress were less than half for lactotrophs than for corticotrophs (102 vs 211, Table 22). The profiles of cell types with which communication was altered also differed for corticotrophs and lactotrophs (Table 22). For example, corticotroph-Sox2+ve cell communication was extensively altered by chronic stress, whereas lactotroph-Sox2+ve cell communication was not a top cell type pair targeted by chronic stress. These results suggest that the corticotroph paracrine interactions predicted by CellPhoneDB are likely not just random interactions given that the same results were not obtained for lactotrophs.

| Cell type | Number of altered interactions | |
|---|---|---|
| | Corticotrophs | Lactotrophs |
| Corticotrophs | 8 | 11 |
| Endothelial cells | 10 | 5 |
| Fibroblasts | 29 | 16 |
| Gonadotrophs | 13 | 7 |
| Lactotrophs | 11 | 1 |
| Macrophages | 14 | 8 |
| Melanotrophs | 10 | 11 |
| Pericytes | 36 | 15 |
| Posterior pituitary cells | 28 | 11 |
| Somatotrophs | 6 | 0 |
| Sox2+ve cells | 33 | 9 |
| Thyrotrophs | 13 | 8 |
| **Total** | **211** | **102** |

**Table 22: Number of interactions between corticotrophs and lactotrophs and other pituitary cell types predicted as altered by chronic stress by CellPhoneDB.** The number of interactions is the sum of both upregulated and downregulated interactions with the cell types as both sources and targets of paracrine ligands.

### 3.11 Absence of established corticotroph paracrine interactions among predicted interactions

At least 31 paracrine factors expressed in the rodent pituitary have been shown to alter corticotroph function *in vitro* (Table 23). However, none of these established interactions were altered by chronic stress according to both CellPhoneDB and NicheNet. When considering all corticotroph interactions predicted by CellPhoneDB whether they were altered by stress or not, 5/31 established literature interactions were present among the predictions (Table 24). To determine why the remaining interactions might have been absent, their coverage in the ligand-receptor database used by CellPhoneDB was first determined. Almost all (37/41) ligands were present and had partners in the database (Table 24), suggesting that it is not the reason for the absence of the interactions. Another possibility is that the ligands and/or receptors are too lowly expressed in corticotrophs and their partner cell type to reach the expression level required by CellPhoneDB (expression in at least 10% of a cell type). If a ligand or its receptor is not expressed in over 10% of any pituitary cell type, then the dropout effect or true absence of expression may explain why the established corticotroph interaction is not detected. This applied to 21/31 ligands (Table 24), suggesting that it is the major reason for the established interactions not being detected. For 5/31 established corticotroph interactions, the interactions were detected between other pituitary cell types but not corticotrophs (Table 24).

| Ligand | Effect on corticotroph function | Support for *in vivo* relevance | Source cell type | Direct or indirect actions proposed |
|---|---|---|---|---|
| Adrenomedullin | Inhibited basal and CRH-stimulated ACTH secretion from dispersed rat pituitary cells (Samson 1998) | i.v. but not i.c.v. infusion reduced plasma ACTH in sheep (Parkes and May 1995) | Widely expressed in the rat pituitary (Montuenga et al. 2000) | |
| Angiotensin II | Increased basal ACTH secretion from dispersed rat pituitary cells in monolayer culture (Sobel and Vagnucci 1982) | | Rat gonadotrophs (Deschepper et al. 1985) | Direct, AT1b receptor mRNA is expressed in corticotrophs (Lenkei et al. 1999) and expression is modulated by restraint stress (Leong et al. 2002) |
| Annexin 1 | Annexin 1 fragments reduced the release of ACTH in response to CRH in rat pituitary culture and antibodies against annexin 1 prevented the inhibitory effects of dexamethasone on ACTH secretion in response to CRH (Taylor et al. 1993) | | Rat FS cells (Traverso et al. 1999) | |
| ANP, BNP, CNP | All inhibited CRH-stimulated ACTH secretion from mouse hemipituitaries (Guild and Cramb 1999), same response in foetal rat pituitaries (Chatelain et al. 2003) | i.v. but not i.c.v. infusion of ANP antiserum in rats increased plasma ACTH (Fink et al. 1991) | | |
| Apelin | Increased basal ACTH secretion from perifused rat pituitary sections (Reaux-Le Goazigo et al. 2007) | - i.c.v administration increased plasma ACTH in rats (Taheri et al. 2002)<br>- KO mice show reduced plasma ACTH response to LPS and reduced plasma corticosterone response to forced swim stressors (Newson et al. 2013) | Rat corticotrophs (Reaux-Le Goazigo et al. 2007) | Direct, apelin receptor mRNA is present in rat corticotrophs (Reaux-Le Goazigo et al. 2007) |
| Bombesin | Increased CRH-stimulated ACTH secretion from dispersed rat pituitary cells (Familari et al. 1988) | s.c. injection increased plasma ACTH in control and cold-stressed rats (Malendowicz and Nussdorfer 1995) | | |
| CCK-8 | Increased basal ACTH secretion from dispersed rat pituitary cells (Reisine and Jensen 1986) | | | |
| CGRP | Increased basal and CRH-stimulated ACTH secretion from rat pituitary monolayer cultures (Iino et al. 1998b) | | | |
| DSIP | Inhibited basal and CRH-stimulated ACTH secretion from dispersed mouse pituitary cells (Bjartell et al. 1989) | | Mouse thyrotrophs (Bjartell et al. 1989) | |
| EGF | - Increased proliferation (Childs et al. 1995)<br>- Increased basal and CRH-stimulated ACTH secretion from dispersed rat pituitary cells (Childs et al. 1991) | | | Direct, effects are still observed in the absence of somatotrophs and lactotrophs, the other pituitary cells capable of binding EGF (Childs et al. 1991) |
| Galanin | Inhibited basal ACTH release (Cimini 1996) | i.c.v. infusion of galanin antiserum increased plasma ACTH in rats (Hooi et al. 1990) | Rat corticotrophs and lactotrophs (Cimini 1996) | |
| GRP(14-27) | Increased basal and CRH-stimulated ACTH release from rat pituitary cells in a cell column system (Hale et al. 1984) | | | |

| Ligand | Effect on corticotroph function | Support for *in vivo* relevance | Source cell type | Direct or indirect actions proposed |
|---|---|---|---|---|
| IFN-γ | Inhibited CRH-stimulated ACTH secretion from rat pituitary monolayer or reaggregate cultures and a neutralising antibody against IFN-γ blocked the effect (Vankelecom et al. 1990) | i.p. injections of IFN- γ in rats caused a phase advance in the circadian peak of plasma ACTH (Cano et al. 2005) | | Indirect, presence of FS cells are required for the inhibitory effects (Vankelecom et al. 1992), possibly via secretion of CXCL10 which acts on CXCR3 on corticotrophs (Horiguchi et al. 2016) |
| IGF-I | Increased proliferation in dispersed mouse pituitary cells (Oomizu et al. 1998) | | | Direct, IGF-I receptor mRNA is expressed in corticotrophs (Honda et al. 1998) |
| IL-1 | Increased basal ACTH secretion from rat pituitary monolayer cultures (Bernton et al. 1987) | Both acute and chronic implants of IL-1 increased plasma ACTH in rats (Sweep et al. 1992) | | |
| IL-2 | Increased basal ACTH release from rat hemipituitaries (Karanth and McCann 1991) | | | |
| IL-6 | Increased basal ACTH secretion from rat hemipituitaries (Lyson and McCann 1991) | i.v. (Naitoh et al. 1988) and i.c.v. (Lyson and McCann 1991) injections increased plasma ACTH in rats | | |
| LIF | - Increased basal ACTH secretion from dispersed rat pituitary cells (Kim and Melmed 1999)<br>- Immunoneutralisation reduced ACTH secretion from mouse and sheep dissociated pituitary cells (Schwartz et al. 1999) | - Intracarotid administration in foetal rhesus monkeys increased plasma ACTH (Akita et al. 1996a)<br>- KO mice have reduced basal plasma ACTH (Akita et al. 1996b) and reduced plasma ACTH in response to i.p. IL-1 injections (Auernhammer et al. 1998) | Rat thyrotrophs and gonadotrophs (Schwartz et al. 1999) | |
| NGF | Increased proliferation in rat pituitary aggregates (Proesmans et al. 1997) | | | |
| NPY | Increased proliferation in rat pituitary aggregates (Tilemans et al. 1992) | | Gonadotroph-conditioned medium could mimic effects of NPY on corticotroph proliferation (Tilemans et al. 1992), suggesting involvement of gonadotrophs | |
| Oncostatin M | Increased basal ACTH secretion in dispersed rat pituitary cells (Kim et al. 2000) | | | |
| PACAP | Increased basal ACTH secretion from dispersed rat pituitary cells (Hart et al. 1992) | | | |
| PAMP | Inhibits basal ACTH secretion from dispersed rat pituitary cells (Samson 1998) | s.c. injections reduced plasma ACTH rise in response to cold stress in rats (Malendowicz et al. 2006) | Rat gonadotrophs (Montuenga et al. 2000) | |
| PreproTRH-(178-199) | Inhibited basal and CRH-stimulated ACTH secretion from dispersed rat pituitary cells (Redei et al. 1995) | | | |
| Substance P | Inhibited basal and AVP-stimulated ACTH secretion from rat pituitaries *in vitro* (Jones et al. 1978) | s.c. injection of substance P antagonist reduced the plasma ACTH rise in response to ether stress (Malendowicz et al. 1996) | | |

| Ligand | Effect on corticotroph function | Support for *in vivo* relevance | Source cell type | Direct or indirect actions proposed |
|---|---|---|---|---|
| TGF-α | Increased proliferation in dispersed mouse pituitary cells (Oomizu et al. 2000) | | Mouse somatotrophs and lactotrophs (Sharma et al. 2003) | Direct, via EGF receptor in corticotrophs (Oomizu et al. 2000) |
| TNF-α | Inhibited CRH-stimulated ACTH secretion from dispersed rat pituitary cells (Gaillard et al. 1990) and increased basal ACTH secretion from dispersed rat pituitary cells or rat hemipituitaries (Milenkovic et al. 1989) | | | |
| Urocortin | Increased basal ACTH secretion in dispersed rat pituitary cells (more strongly than CRH) (Asaba et al. 1998) | i.v. injection increased plasma ACTH in rats (Asaba et al. 1998) | | Direct, mediated by CRHR1 (Asaba et al. 1998) |
| VIP | Increased CRH-stimulated ACTH secretion from rat pituitary fragments (Léonard et al. 1988) | s.c. injection of VIP antagonist in rats inhibited plasma ACTH rise in response to cold stress (Nowak et al. 1994) | | |

**Table 23: Intrapituitary factors with known effects on corticotroph function**. Abbreviations: adrenocorticotrophic hormone (ACTH), atrial natriuretic peptide (ANP), angiotensin II type 1b (AT1b), arginine vasopressin (AVP), brain natriuretic peptide (BNP), cholecystokinin (CCK), calcitonin gene-related peptide (CGRP), C-type natriuretic peptide (CNP), corticotrophin releasing hormone (CRH), corticotrophin releasing hormone receptor 1 (CRHR1), C-X-C motif chemokine ligand (CXCL), C-X-C motif chemokine receptor (CXCR), delta sleep-inducing peptide (DSIP), epidermal growth factor (EGF), folliculostellate (FS), gastrin releasing peptide (GRP), intracerebroventricular (i.c.v.), interferon (IFN), insulin-like growth factor (IGF), interleukin (IL), intraperitoneal (i.p.), intravenous (i.v.), knockout (KO), leukaemia inhibitory factor (LIF), lipopolysaccharide (LPS), nerve growth factor (NGF), neuropeptide Y (NPY), pituitary adenylate cyclase activating polypeptide (PACAP), proadrenomedullin N-terminal 20 peptide (PAMP), subcutaneous (s.c.), transforming growth factor (TGF), tumour necrosis factor (TNF), thyrotrophin releasing hormone (TRH), vasoactive intestinal peptide (VIP).

# 4 DISCUSSION

Prolonged exposure to glucocorticoids has a variety of systemic effects, yet chronic stress is also a local paracrine disorder (Dugaucquier and Segers 2019). Previous research into the contribution of the pituitary to chronic stress has primarily focused on corticotrophs and their role as ACTH factories without paying much attention to the rest of the pituitary. This chapter is an investigation into how the pituitary responds to chronic stress as a community of cells. Using scRNA-seq data from stressed and control pituitaries, paracrine communication between corticotrophs and other pituitary cell types was characterised. Corticotrophs reduced paracrine signalling to other cell types, predominantly non-endocrine cell types, whereas the other cell types in turn enhanced communication with corticotrophs during chronic stress. Individual ligands with potential roles in the pituitary were also identified, such as Wnt4 and neuregulin 1. This computational study of cell-cell communication provides a starting point for future studies aiming to form an integrated understanding of the pituitary under chronic stress.

| Ligand | Interaction predicted by CellPhoneDB | Present in ligand-receptor database | Ligand expressed in at least 10% of one pituitary cell type | Cell type(s) with ligand in over 10% of cells | A receptor expressed in at least 10% of one pituitary cell type | Cell type(s) with receptor in over 10% of cells |
|---|---|---|---|---|---|---|
| Adrenomedullin | No | Yes | Yes | E, F, pos | Yes | E |
| Angiotensin II | Yes | Yes | Yes | G | Yes | C, L, per |
| Annexin 1 | No | Yes | Yes | F, per, Sox | No | |
| ANP | No | Yes | No | | Yes | C, E, G, L, mel, pos, S, T |
| BNP | No | Yes | No | | Yes | C, E, G, L, mel, pos, S, T |
| CNP | Yes | Yes | Yes | G, Sox | Yes | C, E, G, L, mel, pos, S, T |
| Apelin | No | Yes | Yes | pos, Sox | Yes | E |
| Bombesin | No | Yes | Yes | G | No | |
| CCK-8 | No | Yes | No | | No | |
| CGRP | No | Yes | Yes | C | No | |
| DSIP | No | No | No | | No | |
| EGF | Yes | Yes | Yes | C, L, mel, S, T | Yes | C, Sox |
| Galanin | No | Yes | Yes | G, L, T | No | |
| GRP(14-27) | No | Yes | Yes | G | No | |
| IFN-γ | No | Yes | No | | Yes | C, E, F, L, mac, mel, per, pos, Sox, T |
| IGF-I | Yes | Yes | Yes | F, mac, S | Yes | C, E, F, G, L, mel, S, pos, Sox, T |
| IL-1 | No | Yes | Yes | mac | Yes | F, S |
| IL-2 | No | Yes | No | | Yes | E |
| IL-6 | No | Yes | No | | Yes | C, G, mac, mel, S, Sox |
| LIF | No | Yes | No | | Yes | C, E, F, G, mac, mel, pos, S, Sox |
| NGF | No | Yes | Yes | per | Yes | pos |
| NPY | No | No | No | | No | |
| Oncostatin M | No | Yes | No | | Yes | E |
| PACAP | No | No | No | | Yes | F, G, per |
| PAMP | No | Yes | Yes | E, F, pos | Yes | E |
| PreproTRH-(178-199) | No | Yes | No | | Yes | T |
| Substance P | No | No | Yes | Sox | No | |
| TGF-α | Yes | Yes | Yes | Sox | Yes | C, Sox |
| TNF-α | No | Yes | No | | Yes | All |
| Urocortin | No | Yes | No | | Yes | C |
| VIP | No | Yes | No | | No | |

**Table 24: Reasons for absence of established interactions in scRNA-seq data**. Corticotroph interactions predicted by CellPhoneDB based on scRNA-seq data from control mouse pituitaries: angiotensinogen-angiotensin II receptor type 1 from gonadotrophs to corticotrophs, natriuretic peptide C-natriuretic peptide receptor 2 from Sox2+ve cells to corticotrophs, epidermal growth factor-epidermal growth factor receptor from corticotrophs, lactotrophs, melanotrophs, somatotrophs, Sox2+ve cells and thyrotrophs to corticotrophs, insulin-like growth factor 1-insulin-like growth factor 1 receptor from fibroblasts and macrophages to corticotrophs, transforming growth factor α-epidermal growth factor receptor from Sox2+ve cells to corticotrophs. Abbreviations: corticotrophs (C), endothelial cells (E), fibroblasts (F), gonadotrophs (G), lactotrophs (L), macrophages (mac), melanotrophs (mel), somatotrophs (S), pericytes (per), posterior pituitary cells (pos), Sox2+ve cells (Sox), thyrotrophs (T).

## 4.1 Pericytes at the centre of corticotroph-vascular interactions during chronic stress

Hormone secretion and blood flow are coordinated within the pituitary to optimise hormone delivery to the periphery (Schaeffer et al. 2010). A rise in one is paralleled by a rise in the other: GHRH-induced pulses of GH secretion increased local blood flow in the pituitary (Lafont et al. 2010) and changes to the rate of perifusion in culture was followed by changes to GH secretion (Stachura et al. 1990). Pituitary blood flow is responsive to a variety of stimuli, including oestrogen treatment, tamoxifen injections (Stawowy et al. 1989) and acute stress (Goldman 1963). Given that vascular smooth muscle cells appear to be absent in the pituitary (Schaeffer et al. 2010), pericytes are likely the principal cells in control of local blood flow. These contractile star-shaped cells wrap around small blood vessels to regulate vascular tone and permeability (Kutcher and Herman 2009). Their position at the crossroads between the parenchyma and the vasculature makes them ideal mediators of communication between the two compartments (Gaceb and Paul 2018).

Corticotrophs have a close relationship with the vasculature, frequently extending cytoplasmic processes towards nearby capillaries (Nakane 1970; Yoshitomi et al. 2016). Following adrenalectomy, corticotrophs increase their contact surface area with blood vessels (Kubba and McNicol 1987; Itoh et al. 2003), suggesting that glucocorticoids may alter the relationship between corticotrophs and the vasculature. In chronic stress, the greatest changes to paracrine communication potential were seen between corticotrophs and pericytes (Table 19). More interactions were upregulated than downregulated and both cell types were senders and receivers of paracrine signals to similar extents, suggesting increased bidirectional crosstalk between corticotrophs and pericytes in response to chronic stress. The upregulated juxtacrine ephrin interactions between corticotrophs and pericytes during chronic stress (Table 20) could suggest an altered physical association between the two cell types given that ephrins play important roles in facilitating pericyte adhesion to other cell types (Foo et al. 2006). Ephrin-mediated adhesion between corticotrophs and pericytes may also help explain the lack of typical junctional structures observed between corticotrophs and blood vessels (Yoshitomi et al. 2016).

It is tempting to speculate that the overall purpose of the enhanced coordination between corticotrophs and pericytes is to modify blood flow to support the altered pattern of ACTH secretion in chronic stress. Given their ability to regulate vascular permeability, pericytes may also allow increased passage of ACTH into the bloodstream or regulate the entry of substances from the circulation into the pituitary in response to signals from the nearby corticotrophs. Finally, it is interesting to note that practically all paracrine factors known to be

produced by corticotrophs also have the capacity to alter blood flow (including adrenomedullin 2 (Bell and McDermott 2008), neuromedin U (Gardiner et al. 1990), apelin (Mughal and O'Rourke 2018), urocortins (Davidson and Yellon 2009) and acetylcholine (Yamada et al. 2001)), suggesting that corticotrophs may have a particularly strong influence on the vasculature through paracrine signalling. Expression of all these known corticotroph paracrine factors was very low or undetectable in scRNA-seq data.

Pericytes and several other cell types upregulated BDNF signalling to corticotrophs during chronic stress (Table 20). BDNF is an important regulator of HPA axis activity and its expression is altered by chronic stress at multiple levels of the HPA axis (Smith et al. 1995; Naert et al. 2006, 2011, 2015). Consistent with the present results, elevated pituitary BDNF content and mRNA expression has previously been observed in chronic stress (Smith et al. 1995; Naert et al. 2011). Yet, the functional significance of increased BDNF in the pituitary during chronic stress remains unknown. The lack of corticotroph proliferation or hypertrophy in mild chronic stress models suggests that the effects of any enhanced BDNF signalling in corticotrophs may not be related to the typical trophic effects of the peptide. However, BDNF does induce axonal growth, including in proopiomelanocortin (POMC)-expressing neurons (Liao et al. 2015), which may be relevant given that corticotrophs have many axon-like cytoplasmic projections. Alternatively, BDNF may alter ACTH secretion since it also regulates hormone secretion in other tissues (Fulgenzi et al. 2020).

## 4.2 Chronic stress may bring corticotrophs into closer communication with each other

ACTH secretion assays performed in the 1980s and 1990s provided evidence for stimulatory and inhibitory paracrine communication among corticotrophs (Schwartz et al. 1989; Jia et al. 1992). A number of paracrine factors with the ability to alter ACTH secretion *in vitro* have subsequently been identified in corticotrophs (Table 23) but their role *in vivo* remains unexplored. The present results identified neuregulin 1 as a potential paracrine factor upregulated by chronic stress in corticotrophs (Table 20). Rats with a disrupted neuregulin 1 gene show altered HPA axis responses to acute stressors (Taylor et al. 2011b) and mice heterozygous for a neuregulin 1 deletion mutation display lower plasma corticosterone responses following chronic stress (Chohan et al. 2014). Whether the pituitary contributes to the stress phenotype has not been studied. It should, however, be noted that one study found no expression of neuregulin 1 protein in corticotrophs in female rats (Table 6, (Zhao and Ren 2011b)), but the presence of neuregulin 1 mRNA in the cell type in both mice and

rats (Table 7) indicates that the peptide has the potential of being produced, perhaps in conditions such as stress.

CellPhoneDB predicted exclusively upregulated paracrine interactions among corticotrophs (Table 19). The sustained responsiveness of corticotrophs during chronic stress has been attributed to enhanced vasopressinergic activity (Aguilera and Rabadan-Diehl 2000), but later studies relying on vasopressin receptor ablation or antagonism have challenged the contribution of the vasopressin system (Aguilera et al. 2008; Chen et al. 2008). Although the signals responsible for the altered corticotroph activity are therefore still unclear, increased inter-corticotroph communication may be a way of synchronising or coordinating the cells to support their sustained responsiveness during chronic stress. For example, paracrine signals passed between corticotrophs may help coordinate the proportion of cells responsive to hypothalamic stimulation or the number of cells actively secreting ACTH. Interestingly, this "antisocial" behaviour of corticotrophs during chronic stress is similar to the antisocial behaviour exhibited by both humans and rodents exposed to chronic stress early in life (Haller et al. 2014).

### 4.3 Corticotrophs retreat into splendid isolation during chronic stress but remain under heavy paracrine bombardment from other pituitary cells

In contrast to the increased paracrine communication within the corticotroph population, chronic stress tended to overall reduce corticotroph communication with other pituitary cell types (Table 19). Perhaps there is a paracrine signalling programme tonically activated by corticotrophs during basal conditions that allows open communication with other pituitary cells. In response to chronic stress, this communication may be suppressed to allow corticotrophs to act more like an autonomous network and focus on maintaining the sustained HPA axis response. In contrast, other pituitary cell types in general upregulated communication with corticotrophs (Table 19). This may reflect a collective attempt to either inhibit the hyperactive corticotrophs or support the corticotrophs to ensure their continued function in the face of a chronic challenge. Alternatively, the altered paracrine signalling may be entirely maladaptive, simply a sign of growing disharmony within the pituitary that only contributes to the deleterious effects of chronic stress.

### 4.4 Wnt4: a multi-target paracrine signal produced by corticotrophs?

Selective expression of Wnt4 in corticotrophs and its receptors in non-endocrine cells (Figure 31) indicated that the ligand may mediate signalling from corticotrophs to supporting

cells in the pituitary. This was supported by transcriptional effects of Wnt4 being observed in Sox2+ve cells and fibroblasts (Table 21). In the adult pituitary, Wnt signalling has been linked to cell proliferation and tissue remodelling (Willems et al. 2016; Russell et al. 2021a). Several Wnts are expressed in the pituitary (Colli et al. 2013; Russell et al. 2021a), but studies attempting to discern their distribution among the different cell types have obtained ambiguous results. For example, Wnt4 protein was found either exclusively in somatotrophs and thyrotrophs (Miyakoshi et al. 2009) or in all endocrine cell types (Giles et al. 2011) in female rats. In humans, expression was restricted to gonadotrophs (Miyakoshi et al. 2008). Expression in the mouse pituitary is unknown. The presence of several Wnts throughout the adult pituitary may indicate that they contribute to maintaining homeostasis in the adult gland by balancing expansion of different cell populations (Cox et al. 2017). Specific members of the Wnt family may act as messengers between different cell types, such as Wnt4 between corticotrophs and non-endocrine cells.

### 4.5 Neuregulin 1 as a paracrine agent between the HPA and HPG axes

The HPA and HPG axes must be tightly co-regulated to ensure a balance between the survival of the organism and propagation of the species. During chronic stress, resources are poured into survival at the expense of reproduction through a mechanism involving suppression of the HPG axis (Joseph and Whirledge 2017; Acevedo-Rodriguez et al. 2018). Inhibitory signals from corticotrophs to gonadotrophs may contribute to this suppression and here neuregulin 1 was identified as a possible inhibitory ligand secreted by corticotrophs (Table 21). Interestingly, the only other pituitary cell type also expressing neuregulin 1 was the gonadotroph (Figure 32) and the expression was reduced by chronic stress (mean expression: 0.35 (control), 0.23 (stress)). Potential receptors for neuregulin 1 identified in the CellPhoneDB and NicheNet databases were also expressed in corticotrophs (Figure 32). The reproductive phenotype of *Nrg1* mutants has not been characterised but there are clear sex differences in behaviours between male and female rodents with disrupted *Nrg1* function (O'Tuathaigh et al. 2006; Deakin et al. 2009; Taylor et al. 2011a). Expression of *Nrg1* in the female rat pituitary also exhibits changes across the reproductive cycle (Zhao and Ren 2011a). It is therefore possible that neuregulin 1 regulates gonadotroph function in the pituitary. Given that neuregulin 1 was also identified as a paracrine factor among corticotrophs, it may be a multi-purpose ligand activated by chronic stress, both coordinating corticotrophs and suppressing gonadotroph function.

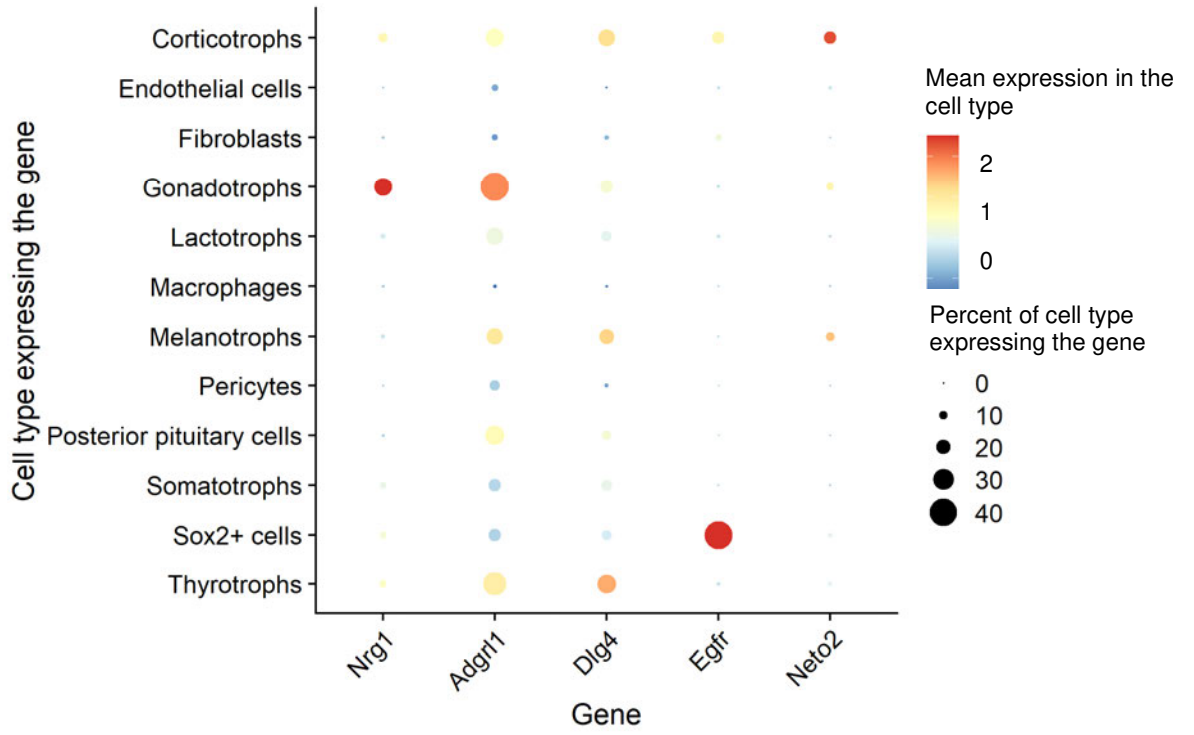


**Figure 32: Neuregulin 1 (*Nrg1*) is expressed in corticotrophs and gonadotrophs with potential receptors for the ligand also found in corticotrophs in mouse single cell RNA-sequencing data.** The gene expression values are combined from control and chronically stressed pituitaries. Gene abbreviations: *Nrg1* (Neuregulin 1), *Adgrl1* (Adhesion G protein-coupled receptor L1), *Dlg4* (Discs large MAGUK scaffold protein 4), *Egfr* (Epidermal growth factor receptor), *Neto2* (Neuropilin (NRP) and tolloid (TLL)-like 2).

## 4.6 No established corticotroph interactions among the predicted interactions altered by chronic stress

None of the paracrine interactions known to alter corticotroph function *in vitro* (Table 23) were among the interactions predicted to be altered by chronic stress. This was unexpected since several of them have been implicated in stress. Perhaps the most established is annexin 1 which is selectively expressed in rat pituitary FS cells (Traverso et al. 1999). It has been shown to be quickly externalised from FS cells in response to glucocorticoids and consequently inhibit ACTH secretion from rat pituitaries *in vitro*, leading to the proposal that it mediates the early negative feedback of glucocorticoids on ACTH secretion (Buckingham et al. 2006).

Other established paracrine factors with potential relevance in stress are the proadrenomedullin-derived peptides adrenomedullin and proadrenomedullin N-terminal 20 peptide (PAMP). Adrenomedullin is widely synthesised in the rat pituitary, whereas PAMP

expression is restricted to gonadotrophs (Montuenga et al. 2000). Adrenomedullin inhibited both basal and CRH-stimulated ACTH release and PAMP inhibited basal ACTH release from dispersed rat pituitary cells (Samson 1998). In sheep, intravenous infusion of adrenomedullin reduced plasma ACTH concentrations whereas no effect was seen with intracerebroventricular infusion (Parkes and May 1995), suggesting action at the level of the pituitary. Subcutaneous injections of PAMP in rats reduced the plasma ACTH rise in response to cold stress (Malendowicz et al. 2006).

Apelin has been proposed to be an autocrine factor acting on corticotrophs. It is highly expressed in the anterior pituitary with 97% of immunoreactive cells identified as corticotrophs in the rat (Reaux-Le Goazigo et al. 2007). Apelin receptor mRNA is also expressed in corticotrophs in rats (Reaux-Le Goazigo et al. 2007). In perifused rat anterior pituitary sections, apelin increased basal ACTH secretion (Reaux-Le Goazigo et al. 2007). Centrally administered apelin increased plasma ACTH in rats (Taheri et al. 2002). While this likely indicates a hypothalamic effect, additional action at the pituitary cannot be excluded. The ACTH response to lipopolysaccharide administration was reduced in apelin receptor knockout mice, as was the corticosterone response to acute forced swim stressors (Newson et al. 2013).

There are a variety of explanations for the absence of these established interactions among the predictions. Firstly, evidence for their relevance has mostly been obtained using rat pituitaries (Table 23) so it is unclear whether they also influence corticotroph function in mice pituitaries which were the source of the scRNA-seq datasets. A species difference may be supported by the different cell type distributions that were found among paracrine factors in rat and mouse pituitary scRNA-seq datasets (Table 7). Another possible reason is that effects of the established ligands on corticotroph function are mediated indirectly by other pituitary cell types. For example, the inhibitory effects of interferon γ (IFN-γ) on ACTH secretion *in vitro* requires the presence of FS cells (Vankelecom et al. 1992), possibly because the cell type is a source of CXC chemokine ligand 10 that inhibits ACTH secretion (Horiguchi et al. 2016). Alternatively, the established paracrine ligands may act via atypical receptors not included in the ligand-receptor databases used by the prediction algorithms.

The major reason, however, is likely to be the inability of scRNA-seq to detect lowly expressed genes. Of the 31 established corticotroph interactions, 21 did not have the ligand or a receptor expressed in more than 10% of any pituitary cell type (Table 24). Notably, 5 established interactions were predicted to mediate communication between other pituitary cell types but not corticotrophs. This does not prove the absence of the interaction in

corticotrophs but suggests that many of the intrapituitary interactions may be active between several cell types instead of acting exclusively between specific cell types.

### 4.7 Limitations of predicting paracrine interactions from scRNA-seq data

The corticotroph ligand-receptor interactions predicted to be altered by chronic stress were highly dependent on the prediction algorithm used: 3 common methods (CellChat, CellPhoneDB and NicheNet) provided predictions that showed no overlap. A recent comprehensive comparison of the available tools also obtained very different interactions from the same dataset (Dimitrov et al. 2021). While these tools can deliver useful predictions as evidenced by the number of recent publications that have successfully employed them to identify physiologically relevant ligand-receptor interactions (Armingol et al. 2020), the divergent predictions also indicate a need to evaluate the suitability of the tools for individual datasets and the importance of verifying predictions by experimental data.

It is very possible that the lack of correlation between mRNA and protein found for the 13 ligands examined (Tables 6 and 7) also extends broadly to all paracrine ligands in the pituitary, including the ones predicted in this chapter. For this reason, NicheNet, a tool incorporating functional consequences of ligand-receptor interactions on downstream gene expression was included in the analyses. While this is more likely to predict active paracrine interactions, it is also based on the assumption that ligands induce the same transcriptional responses across tissues and biological conditions. This was recently shown not to be true – transcriptional responses are highly cell type dependent (Innes and Bader 2021).

Finally, ligands encoded by genes are not the only paracrine signals capable of initiating responses in nearby cells. Purines, fatty acids, nitric oxide and various neurotransmitters are also paracrine agents, all of which have been implicated in local control of pituitary function (Denef 2008). These may play important roles in the pituitary response to chronic stress but were not examined in the present analyses. It is also important to point out that there is no such thing as *a* response to chronic stress. Even the same chronic stress paradigm, yet alone different ones, produce heterogenous stress responses in the same group of animals (Marin et al. 2007; Schmidt et al. 2010). The altered pituitary paracrine transcriptome observed here in response to a chronic social defeat stress paradigm is therefore not necessarily a universally generalisable response.

## 4.8 Conclusion

This chapter provided a glance into the pituitary gland under chronic stress, revealing changes to the paracrine communication network that links corticotrophs and the rest of the pituitary. The central role that corticotrophs play in the stress response makes it essential to characterise how they interact with their cellular environment, the rest of the pituitary, during chronic stress. The present results contribute to this endeavour by providing a bird's eye view of shifts to communication patterns between corticotrophs and other cell types and by predicting individual paracrine interactions susceptible to alteration by chronic stress. The pituitary is therefore both an endocrine and a paracrine gland and exploring the latter definition is important to attain a comprehensive understanding of pituitary function during the major physiological challenge posed by chronic stress.

# A GENERAL DISCUSSION OF THE FINDINGS AND THEIR IMPLICATIONS

## 1 PAST AND PRESENT PERSPECTIVES ON THE PITUITARY GLAND

The image of the anterior pituitary as a passive, externally regulated gland that relays information from the hypothalamus to the periphery is slowly changing. Increasing evidence supports that the pituitary actively participates in regulating activity of the neuroendocrine axes that the gland is part of through intragland coordination. The pituitary can uncouple its hormone secretion from hypothalamic influence as shown by the absence of secretory response despite the arrival of hypothalamic releasing hormone into the gland (Glanowska et al. 2014) or the production of hormone pulses despite a lack of hypothalamic input (Engler et al. 1990). Dispersed somatotrophs *in vitro* secrete GH in response to GHRH in quantities that are several-fold lower compared to *in vivo* (Painson and Tannenbaum 1991; Sugimoto et al. 1991) and the plating density of pituitary cells in culture have a major influence on basal hormone secretion or the response of the cells to a variety of factors (Perez et al. 1995). Pituitary cells also rely on each other for development (Seuntjens et al. 1999; Pulichino et al. 2003; Waite et al. 2010) and for the response to extra- and intrapituitary signals *in vitro* (Denef and Andries 1983; Vankelecom et al. 1992; De Paul et al. 2000). Cellular interactions within the gland therefore appear to have an important role to play in modulating pituitary output.

In some ways this emerging active, whole-gland view of the pituitary is a renaissance of how the gland was viewed in the 19$^{th}$ and early 20$^{th}$ centuries (Severinghaus 1937). Early research on the pituitary was largely conducted on whole glands or hemipituitaries without the ability to separate out or identify the individual cell types currently known to inhabit the gland (although the presence of different cell types was recognised). Many of the studies were observational, revealing changes to the entire pituitary upon distinct physiological states (e.g., pregnancy or stress), target organ ablation or peripheral hormone implantation. There was overall an interest in the relationships between cells in the gland and how those influenced cellular activity. A thorough review on this early perspective can be found in (Severinghaus 1937).

There were perhaps 3 major factors that contributed to the transition into a view of the pituitary where each cell type was considered separately away from the local context provided by the rest of the gland. Firstly, Nakane provided immunohistochemical proof that different pituitary hormones were stored in separate cell types (except for FSH and LH which could be found within the same cells) (Nakane 1970) and a few years later, it became

possible to separate the cell types based on their differential sedimentation through protein gradients (Hymer et al. 1973), which catalysed efforts to characterise the purified cell types *in vitro* without the constraints of whole-tissue research. Second, removing each cell type from its pituitary microenvironment was not deemed problematic as pituitary cells did not appear to be organised into separate regions but formed random intermingled cords that filled the gland together with a dense network of blood vessels. Third, the anterior pituitary was an endocrine gland and at the time endocrine glands were thought to be regulated by humoral factors and hence separating pituitary cells from their neighbours was not thought to influence their function (Perez et al. 1995).

There is no question that studies on individual pituitary cells and cell types have provided immense value in terms of increasing our understanding of the molecular processes contributing to pituitary cell function. The whole-gland perspective adopted in this dissertation is not meant to displace these studies on isolated cells or cell types but provide a complementary angle from which to study the pituitary.

## 2 OUTCOMES OF THE DISSERTATION

### 2.1 Identification of candidate intrapituitary factors

The overarching aim of this dissertation was to identify candidate molecules that may mediate cell-cell interactions in the pituitary. In chapter 1, I first created an integrated mouse pituitary scRNA-seq dataset to act as a platform for the identification of candidate molecules. I showed that shared cell types could be identified from different mouse pituitary scRNA-seq datasets and that the integrated dataset contained expression information for a majority of genes relevant to cell-cell communication and adhesion. Combining the integrated scRNA-seq dataset with pituitary datasets generated by other transcriptomics techniques further increased the depth of information regarding expression of cell-cell communication-relevant genes. In chapter 2, I then took advantage of the gene expression data to identify a set of CAMs that may allow corticotrophs, gonadotrophs, somatotrophs and lactotrophs to adhere to cells of the same type based on selective expression in the cell type. By comparing the expression of these CAMs in different species and at different developmental stages, I further narrowed the candidates down to 1-3 per cell type. In chapter 3, I analysed chronic stress-induced changes to cell-cell communication between corticotrophs and the rest of the pituitary based on altered expression of paracrine ligands, receptors and downstream targets of the ligand-receptor interactions. These analyses revealed both changes to the extent of signalling between corticotrophs and other cell types during stress and indicated the individual candidate paracrine interactions that underlie these changes. Together, these

CAMs and ligand-receptor interactions represent a robust set of candidates that can now be subjected to experimental testing to establish whether they have a role in homotypic cell adhesion and corticotroph paracrine communication in stress, respectively.

### 2.2 Uncovering inconsistencies with and within the literature

Perhaps the most surprising results of this dissertation were the considerable discrepancies that emerged when the paracrine factors detected in pituitary scRNA-seq data were compared to the literature. Established corticotroph paracrine interactions in the literature were largely absent among the interactions predicted from scRNA-seq data (Tables 23 and 24) and the distribution of the paracrine ligands in the literature did not always match the distribution of ligand mRNA in scRNA-seq data (Tables 6 and 7). As discussed in the relevant chapters, these discrepancies may be due to limitations of scRNA-seq data, including the detection bias against low-abundance transcripts, and the variable predictions produced by different bioinformatics tools. However, results within the literature were also inconsistent: the pituitary cell type distribution of paracrine ligands was highly variable between studies (Tables 6). Examining the expression of established corticotroph ligand-receptor interactions in pituitary scRNA-seq data also suggested that individual interactions may be active between a variety of different cell types (Table 24). To understand the potential reasons behind all these heterogeneous results, it may be helpful to examine the substantial literature on intrapituitary interactions.

## 3 WHAT DOES THE LITERATURE SUGGEST ABOUT PARACRINE COMMUNICATION IN THE PITUITARY?

Since the 1980s, over 100 ligands with paracrine activity have been detected in the anterior pituitary gland (Denef 2008). Their cell type distribution, regulation of secretion and functional effects in the pituitary have been investigated in a large number of *in vitro* studies, comprehensively reviewed over the years (Schwartz and Cherny 1992; Houben and Denef 1994; Schwartz 2000; Denef 2008). While the studies highlight the capacity of the ligands to alter pituitary function, what emerges from the literature perhaps more than anything is an abundance of paradoxical results, particularly regarding the distribution of the paracrine ligands and their effects on pituitary hormone secretion. The following section is an attempt to reconcile the different results and consider what they might indicate about paracrine regulation of the pituitary.

## 3.1 Inconsistent localisation of pituitary paracrine ligands in the literature

### *3.1.1 Protein distribution*

In Chapter 1, the protein localisation of 13 paracrine ligands also detected in scRNA-seq data was found to be inconsistent between studies (Table 6). As an example, substance P was expressed in lactotrophs and gonadotrophs but not somatotrophs or thyrotrophs in one study (Morel et al. 1982b), whereas another study found the exact opposite localisation using pituitaries from the same sex and species of animal (Brown et al. 1991). A wider examination of the literature suggests that the inconsistent expression distribution between studies is not limited to these 13 ligands but is true for a large proportion of ligands in the pituitary (prominent examples in Table 25). The differences cannot be solely attributed to methodological or obvious biological factors since experiments performed using antiserum prepared with the same method and with pituitaries from animals of the same age, sex and strain still obtain inconsistent results (Carrillo and Phelps 1992). Instead, it may be that intrinsic biological factors such as the endocrine status of the animal affect the expression of paracrine ligands. Expression of vasoactive intestinal peptide (Steel et al. 1989), substance P (Coslovsky et al. 1985) and galanin (O'Halloran et al. 1990) is hormonally regulated but it remains to be determined whether the endocrine environment also regulates the cell type distributions of the ligands. In humans, high inter-individual variation in pituitary staining for endothelin 3 has also been observed (Naruse et al. 1992). The inconsistent distributions of ligands in the pituitary could therefore be a consequence of differences in the physiological states of the animals.

### *3.1.2 mRNA vs protein distribution*

Even when considering the ligands with the most consistent protein distribution in literature studies, their distribution still differed from the mRNA distribution in scRNA-seq data (e.g., neuromedin U and VEGFA, Tables 6 and 7). While the discrepancies may be explained by technical factors such as the proteins being secreted and taken up by different cells compared to mRNA which stays within a cell, two lines of evidence would support the true presence of an mRNA-protein discordance. First, a lack of overlap between distributions of mRNA and protein has been observed for pituitary hormones (Shivers et al. 1986; Childs et al. 1994; Böckers et al. 1996) as well as for pituitary hypothalamic releasing hormone receptors (Nikodemova et al. 2002). Secondly, there are several examples in the literature of independent regulation of mRNA and protein for pituitary paracrine factors (Table 26). A particularly well-studied example is galanin, for which mRNA and protein appear particularly

uncoupled in response to experimental manipulation. Castration and adrenalectomy in rats altered pituitary galanin content without any change in mRNA expression, thyroid hormone treatment reduced mRNA without changing protein content and treatment with dexamethasone and somatostatin analogue SMS 201-995 produced a contradictory response where galanin mRNA expression decreased while protein content increased (O'Halloran et al. 1990; Hyde and Howard 1992) (Table 26). In these examples, total pituitary mRNA and protein for the ligands were measured, precluding deduction of whether changes occurred in specific cell types. A comparison between the cells that express ligand mRNA and protein in the same pituitary section, for example by combined immunocytochemistry and in situ hybridization (Shivers et al. 1986), would help determine whether there truly is a lack of overlap between the two.

| Ligand | Cell type | Species, sex and age if known | Reference |
|---|---|---|---|
| Neuropeptide Y | Only in thyrotrophs | Male rat | (Jones et al. 1989) |
| Neuropeptide Y | All endocrine cell types except thyrotrophs | Male rat | (Chabot et al. 1988) |
| Vasoactive intestinal peptide | Only in lactotrophs | Male rat | (Morel et al. 1982a) |
| Vasoactive intestinal peptide | Cell types that are not lactotrophs | Male rat | (Carrillo and Phelps 1992) |
| Cocaine and amphetamine regulated transcript | Gonadotrophs only | Adult male rat | (Kuriyama et al. 2004) |
| Cocaine and amphetamine regulated transcript | No gonadotrophs, mostly lactotrophs | Adult male rat | (Smith et al. 2006) |
| Endothelin 3 | Only gonadotrophs | Adult human | (Naruse et al. 1992) |
| Endothelin 3 | All endocrine cell types | Adult human | (Lange et al. 1994) |
| Thyrotropin-releasing hormone | Somatotrophs only | Adult male rat | (Bruhn et al. 1994) |
| Thyrotropin-releasing hormone | Gonadotrophs, thyrotrophs and corticotrophs but no somatotrophs | Adult male rat | (May et al. 1987) |
| Leptin | Thyrotrophs and gonadotrophs, negligible staining in other endocrine cell types | Adult female rat | (Jin et al. 2000) |
| Leptin | Largely somatotrophs | Adult female rat | (McDuffie et al. 2004) |

**Table 25: Inconsistent cell type distributions of pituitary paracrine ligands in the literature.**

| Ligand | Experimental manipulation | Change | Reference |
|---|---|---|---|
| Galanin | Castration | Decreased protein, no change in mRNA | (O'Halloran et al. 1990) |
| | Dexamethasone treatment s.c. | Decreased protein, increased mRNA | |
| | Adrenalectomy | Increased protein, no change in mRNA | |
| | Thyroid hormone ($T_4$) treatment s.c. | Decreased mRNA, no change in protein | |
| | Somatostatin analogue SMS 201-995 treatment s.c. in OVX rats treated with oestradiol s.c. | Decreased mRNA, increased protein | (Hyde and Howard 1992) |
| Chromogranin A | Adrenalectomy | Decreased mRNA, no change in protein | (Grino et al. 1989) |
| Neuromedin B | Dexamethasone treatment s.c. | Increased protein, no change in mRNA | (Jones et al. 1992) |
| Secretogranin II | Oestradiol added to culture | Decreased mRNA, no change in protein | (Anouar and Duval 1992) |
| Substance P | Dopamine agonist bromocriptine treatment s.c. | Increased mRNA, no change in protein | (O'Halloran et al. 1991) |

**Table 26: A variety of experimental manipulations differentially alter mRNA and protein expression of pituitary paracrine ligands.** Abbreviations: subcutaneous (s.c.) and ovariectomy (OVX).

## 3.2 Inconsistent effects of pituitary paracrine ligands on hormone secretion in the literature

The results from studies examining functional effects of pituitary paracrine factors *in vitro* appear to disagree more times than they agree. Instead of simply listing the numerous inconsistencies, I have attempted to identify underlying factors that may explain the different functional responses and give examples that illustrate their influence.

### *3.2.1 Species*

Studies directly comparing species differences in the effects of paracrine factors are rare but indirect support can be found from studies that are similar except for the species of cells used. Pituitary cells from rabbits responded to neuropeptide Y (NPY) with sustained release of LH (Khorram et al. 1988), whereas NPY had no effect on LH release in cultured rat pituitary cells (Kalra et al. 1988) or bovine pituitary cells (Chao et al. 1987). Further conflicting evidence for NPY comes from studies indicating a stimulatory effect of the peptide on basal prolactin release in rat pituitary culture (Chabot et al. 1988), but not in cultures of bovine pituitary cells (Chao et al. 1987). GnRH-induced LH release was not affected by

activin A in cultures of rat pituitary cells (Kitaoka et al. 1988), whereas activin A had an inhibitory effect on LH release in cultures of ovine pituitary cells (Muttukrishna and Knight 1991). Several studies omit information regarding the species from which the ligand was derived (Chabot et al. 1988; Kalra et al. 1988; Kitaoka et al. 1988), potentially further affecting the results.

### *3.2.2 Sex*

Two studies have investigated sex differences in the response to paracrine factors. Substance P stimulated LH secretion in pituitary cells from adult female rats but not from adult male rats (Shamgochian and Leeman 1992) and carbachol increased prolactin release in pituitary aggregates from adult female rats but inhibited prolactin release in aggregates from adult male rats (Carmeliet et al. 1989).

### *3.2.3 Hormonal milieu*

Some of the sex differences in the effects of paracrine factors may be explained by different gonadal hormone environments. Other hormones, including glucocorticoids and thyroid hormones, also have an influence: enabling, disabling or altering the extent of pituitary hormone release in response to the factors. NPY stimulated LH secretion from intact, but not from ovariectomised macaque pituitary fragments (Pau et al. 1991), indicating that gonadal steroids were necessary for NPY to have its effects. Also measuring LH release, Shamgochian and Leeman found that the ability of substance P to stimulate LH secretion was reduced in adult female rats exposed to androgens for 6 weeks compared to control animals (Shamgochian and Leeman 1992). Carbachol promoted prolactin release in serum-free culture of pituitary cells from 14-day-old rats but the presence of dexamethasone or triiodothyronine in culture turned the influence of carbochol to inhibitory (Carmeliet et al. 1989). Neuromedin C-stimulated GH secretion in rat pituitary culture is also highly dependent on the hormonal environment, with oestradiol increasing the response over 6-fold above basal, whereas dexamethasone and dihydrotestosterone barely affected the response (Houben and Denef 1990). The enhancing effect of bombesin on CRH-stimulated ACTH release in perifused rat pituitary cells is dependent on glucocorticoids (Familari et al. 1988) and preincubation of rat pituitary cells with dexamethasone disabled the inhibitory effects of IGF-1 on GH release (Lamberts et al. 1989). Oestrogen increased fibroblast growth factor-induced prolactin secretion by a factor of 9 (Baird et al. 1986). Thus, the endocrine environment has a significant influence on the effects of a paracrine factor on pituitary hormone secretion.

### *3.2.4 Age*

The responses to pituitary paracrine ligands also appear to be highly age dependent. Substance P induced LH release (4-fold over basal) in culture when pituitary cells were obtained from male and female rats 30-35 days of age but no effect of substance P was observed in cells from animals that were 20-30 days old (Shamgochian and Leeman 1992). Epidermal growth factor promoted male pituitary cell migration in 90-day-old rats but not in 13 to 14-day-old rats (González et al. 2004). Angiotensin II inhibited GH release in response to GHRH in pituitary aggregates from adult female animals but promoted the GH response in aggregates from 14-day-old animals (Robberecht and Denef 1988). Galanin increased basal GH secretion in pituitaries obtained from 5 and 10-day-old male rats but had no effect or decreased basal secretion from 40-day-old pituitary cells depending on the dose used (Torsello et al. 1990).

### *3.2.5 Ligand concentration*

Finally, the responses are also determined by the ligand concentration used. In cultured pituitary cells from adult female rats, substance P only increased LH release at high ligand concentrations (100-1000 nM) (Shamgochian and Leeman 1992), whereas increasing galanin concentrations in turn decreased GH release in pituitary cells from 40-day-old rats (Torsello et al. 1990). Examples of peculiar dose-response relationships also exist: prolactin release in response to endothelins in cultures of adult male rat pituitary cells was biphasic and interacted with the time in culture (Dymshitz et al. 1992).

## 3.3 Interpretation of the inconsistent results

Typically, inconsistent results between studies are attributed to methodological differences that affect the system under study. While technical factors have been invoked to explain contrasting results in the pituitary paracrine field, the evidence discussed above indicates that the differences may also arise from biological variability. Whatever the cause, it appears that paracrine signalling in the pituitary is highly context dependent: where in the pituitary a ligand is expressed and what its functional effects are is determined by the circumstances the pituitary is subjected to. This is the definition of plasticity – the capacity to alter physiological behaviour in response to changes in the environment.

### *3.3.1 Paracrine factors as mediators of pituitary plasticity*

The anterior pituitary is tiny, yet it is responsible for secreting several hormones in specific patterns that vary across the lifespan. It has been suggested that this challenging task is facilitated by significant plasticity present at different levels within the gland (Childs et al. 2020). A paracrine system that responds to a change in demand, communicated via hormonal or age-related signals, may be an important mechanism mediating plasticity in the pituitary. How the plasticity is achieved on a molecular level is less clear but may involve changes to receptor expression and/or affinity, altered intracellular signalling, calcium handling and ligand modifications (Fontaine et al. 2020). Overall, paracrine receptors in the pituitary have received much less attention than the ligands. However, a study on GPCRs, one of the most common classes of paracrine receptors, indicated that the pituitary expresses the greatest diversity of GPCRs out of 30 different human tissues (Marti-Solano et al. 2020), indicating the potential for a rich paracrine receptorome in the gland.

### *3.3.2 Paracrine factors, noise and redundancy*

An alternative interpretation of the evidence would be that the complex results reflect a very noisy system. Instead of a neatly engineered system where each individual paracrine factor is employed under highly specific circumstances to fulfil a particular role, many of the factors may simply exist in the pituitary to provide noise into the system. Biological noise is increasingly recognised to serve important functional roles in a variety of contexts, including paracrine signalling (Lee et al. 2009; Handly et al. 2015). In support of such a function, several of the paracrine factors expressed in the pituitary seemingly have no effects on hormone secretion (Heisler et al. 1986; Simard et al. 1986; Morel et al. 1989; Houben and Denef 1991) or produce the same response under the same conditions (Auernhammer and Melmed 1999; Auernhammer et al. 2004). While these ligands with overlapping functions may have distinct roles under specific conditions and the ligands that produced no effect may affect other functions not studied in these publications, they may also be part of the considerable redundancy that appears to have been built into the pituitary. Ablating large proportions of different pituitary cell types does not appear to produce major deleterious functional effects (Waite et al. 2010; Roose et al. 2017), a variety of genes important for pituitary cell differentiation can still be conditionally knocked out without effects on the phenotype (Charles et al. 2008; Stallings et al. 2022) and deleting individual paracrine ligands with established functions in the pituitary frequently does not lead to any significant changes in function (Klein et al. 1998; Roh et al. 2001; Kariagina et al. 2004). The function of

such redundancy may be to provide robustness to the paracrine system in the pituitary, allowing the gland to maintain its function in the case that individual factors are disrupted.

### *3.3.3 The purpose(s) of a pituitary paracrine system*

All in all, the pituitary appears to contain a paracrine system that is highly sensitive to even minor perturbations, be those of biological or technical nature. What would be the purpose of such a system? Paracrine communication has the advantage of being rapidly initiated and terminated compared to endocrine signalling and it can reach a broader target of cells compared to gap junction mediated signalling. It might be that the dynamic rapid-on, rapid-off paracrine system provides the pituitary with its own line of defence against systemic changes, allowing the gland to react quickly and autoregulate, maintaining function and preventing overburdening of the cells as well as ensuring harmony between the cell types. Such a self-regulatory function was proposed as the ultimate purpose of paracrine interactions in the kidney (Leipziger and Praetorius 2020). The multitudes of paracrine factors activated by injury to the gland may also support a self-protective role for the pituitary paracrine network (Willems et al. 2016; Vennekens et al. 2021).

Another potential important function of paracrine communication may be to regulate the extent of heterogeneity in the pituitary. Pituitary cell heterogeneity is an important mechanism allowing plasticity in hormone secretion (Gracia-Navarro et al. 2002; Clay et al. 2021) but population heterogeneity must also be constrained to maintain response fidelity. Research in other systems has demonstrated that paracrine signalling fulfils both of these functions, allowing for variability within a cell type but also promoting robust collective responses (Shalek et al. 2014; Handly et al. 2015).

## 3.4 Paracrine communication and physical networks: two sides of the same coin

Paracrine communication is, however, not the only way pituitary cells interact. Homotypic and heterotypic contacts between cells and the gap junction-mediated communication they allow forms another layer of the pituitary interactome. In fact, it is becoming increasingly clear that cell adhesion and paracrine interactions are part of a cell-cell communication spectrum that cannot easily be divided into separate compartments. Far from passive structural components, many typical CAMs such as cadherins also transduce intracellular responses (Doherty et al. 1991). Some of them also moonlight as paracrine receptors, or as paracrine ligands if cleaved off from the cell membrane (Bosenberg and Massagué 1993). Similarly, some typical paracrine ligands and receptors can also mediate cell-cell adhesion. Of the candidate homotypic CAMs identified in Chapter 2, PTPRZ1 and PTPRO also have

receptor functions. This dual nature of the molecules might provide additional plasticity to the cell-cell interactions.

Paracrine communication and cell adhesion regulate each other. Both expression (Voort et al. 1997; Lamouille et al. 2014) and affinity (Chigaev et al. 2008) of different CAMs is regulated by a variety of paracrine factors, suggesting that paracrine signalling in the pituitary may regulate the formation and/or maintenance of physical connections between cells. Cell adhesion in turn may influence paracrine communication in the pituitary. The specific network arrangement of pituitary cells acts as a “blueprint” that first of all determines which cells neighbour each other, constraining the physical range of action of a paracrine ligand secreted from a particular cell. The adhesion complexes that form between pituitary cells may regulate the diffusion of paracrine factors (Krisch and Buchheim 1984) and tight junctions may partition different proteins, including paracrine receptors, to specific membrane compartments (Schwartz and Perez 1994), which is relevant given the distinct cAMP microdomains shown to exist in pituitary cells (Wachten et al. 2010). Pituitary cell-cell contact also appears to be a requirement for some paracrine interactions. Cholinergic agonist carbachol was only able to influence prolactin release when pituitary cells were in close contact in culture (Carmeliet et al. 1989), angiotensin II altered GH release in pituitary cell aggregates but not in weakly dispersed cells (Robberecht and Denef 1988), β-adrenergic stimulation of prolactin release was abolished in dispersed cells (Perkins et al. 1985) compared to aggregates (Denef and Baes 1982) and E-cadherin mediated adhesion is necessary for Notch signalling in the pituitary (Batchuluun et al. 2017). The observation that every gonadotroph positive for calcitonin was attached to cup-shaped lactotrophs also suggests an association between paracrine interactions and cell-cell relationships (Chronwall et al. 1996). Finally, adhesion and paracrine signalling may together have synergistic influence over pituitary cells. For example, cadherins and Wnts, both shown to play a role in the pituitary (Himes et al. 2011; Russell et al. 2021b), converge to regulate another important component of pituitary cells: β-catenin (Youngblood et al. 2018).

# 4 FUTURE DIRECTIONS: FROM REDUCTIONISM TO A SYSTEMS UNDERSTANDING OF INTRAPITUITARY COMMUNICATION

The past four decades of research into the pituitary paracrine system has relied on a reductionist approach, identifying individual paracrine factors that are produced in the pituitary and influence gland function *in vitro*. While this body of evidence provides a valuable resource of the potential individual factors involved, it has not got us much closer to understanding how the paracrine interactions work together *in vivo* to regulate pituitary function. I want to finish this dissertation by discussing why moving away from the *in vitro*

study of individual interactions to focusing on whole-animal approaches and applying a systems perspective may allow a better understanding of intrapituitary regulation.

### 4.1 The pituitary *in vitro* is not the pituitary *in vivo*

The evidence presented in section 3 suggests that the pituitary culture environment significantly alters the effects of paracrine interactions. However, results vary not just depending on the environment, but the type of culture itself. Pituitary cell lines are often used to study cell-cell interactions; however, in terms of paracrine responses they differ considerably from the cell types they were derived from. The TtT/GF FS cell line does not mount a calcium response to acetylcholine nor does it have a functional nitric oxide system as native FS cells do (Morris and Christian 2011). Bombesin stimulated GH and prolactin secretion in $GH_4C_1$ cells (Westendorf and Schonbrunn 1982), but no such effect was observed in native rat pituitary cells (Rivier et al. 1978). Activin A suppressed basal ACTH release by 50% in AtT20 cells (Bilezikjian et al. 1991) but had no effect in intact rat pituitary cells (Kitaoka et al. 1988).

Cell lines are perhaps expected to diverge from native pituitary cells but there are also considerable differences in the effects of paracrine factors between native pituitary culture systems. A comparison of the effects of angiotensin II, atrial natriuretic peptide, oxytocin and noradrenaline on dispersed pituitary cells immediately after dispersion, after 4 days of static culture in a microperifusion system, after 4 days of culture in a static suspension system and after 4 days of monolayer culture showed that the type of culture system had a significant influence over the response to the paracrine factors (Watanabe and Orth 1988). A variety of paracrine factors, including interleukin 6 (Gloddek et al. 2001), ciliary neurotrophic factor and interleukin 11 (Castro et al. 2001), altered hormone secretion in reaggregates but not in monolayer culture, perhaps indicating that the arrangement of pituitary cells is important for paracrine responses. That the effect of angiotensin II on prolactin release was markedly lower in purified gonadotroph and lactotroph co-cultures compared to cultures containing all pituitary cell types (De Paul et al. 2000) may in turn indicate that the context provided by the rest of the pituitary is important for proper functioning of the interlinked paracrine system. Dissociation of pituitary cells also alters their morphology and gene expression, which may influence paracrine responses. Gonadotrophs and corticotrophs exhibit particularly large morphological changes after dissociation, including altered size, shape and distribution of cytoplasmic granules (Gaisán et al. 1997). Dispersion of pituitary cells upregulates expression of paracrine ligand osteopontin (Bjelobaba et al. 2019) and the ability of TRH to increase *Tshb* expression in rat pituitary fragments is lost when the cells are dispersed

(Bargi-Souza et al. 2015). Rat pituitary cells in monolayer culture respond to $\alpha_1$-adrenergic stimulation, whereas perfused reaggregates of rat pituitary cells instead respond to $\beta_2$-adrenergic ligands (Giguere et al. 1981; Perkins et al. 1985), suggesting that the type of culture alters the expression of factors involved in paracrine communication.

Comparing the direct effects of paracrine factors on the pituitary *in vivo* and *in vitro* is complicated by the effects that the factors have on hypothalamic hormone release *in vivo*. However, one study using hypothalamus-lesioned rats to remove the influence of hypothalamic factors found that pituitary adenylate cyclase activating polypeptide (PACAP) stimulated prolactin and GH secretion *in vivo* in rats, whereas it inhibited prolactin secretion and had no effect on GH secretion in primary rat pituitary cell culture (Jarry et al. 1992). Together, all the evidence suggests that taking the pituitary out of its native environment alters the paracrine system to an extent where it no longer recapitulates what happens *in vivo*.

### 4.2 Pituitary paracrine factors are interlinked into a complex network

Paracrine interactions in the pituitary have mostly been studied one at a time (although exceptions exist (Evans et al. 2001)) but in reality they do not act in isolation. Instead, all the factors in the pituitary are interlinked into an intricate paracrine network that collectively determines the response in pituitary cells. To illustrate the complexity of this system, Figure 33 shows known interactions among 36 paracrine ligands that influence LH secretion from the rat pituitary *in vitro*. For example, substance P induces the release of β-endorphin from the pituitary (Matsumura et al. 1982) and it inhibits the release of pituitary γ-aminobutyric acid (Afione et al. 1990) while itself being regulated by endothelins (Calvo et al. 1990). The total effect of a paracrine factor on hormone secretion is not always consistent: adenosine itself inhibits both basal and GnRH-stimulated LH secretion (Picanço-Diniz et al. 1989) but it also induces the release of pituitary interleukin 6 (Ritchie et al. 1997) which increases basal LH secretion (Yamaguchi et al. 1990). To add further complexity, many of these interactions are influenced by the gonadal steroid environment, as discussed previously. Somatostatin induces pituitary galanin release but this effect is oestradiol dependent (Hyde and Howard 1992) and the influence of galanin on GnRH-induced LH secretion can be either stimulatory (López et al. 1991) or inhibitory (Todd et al. 1998) depending on the presence of oestrogens (Peters et al. 2009). Studying paracrine interactions separately ignores these complex relationships that exist between the factors.

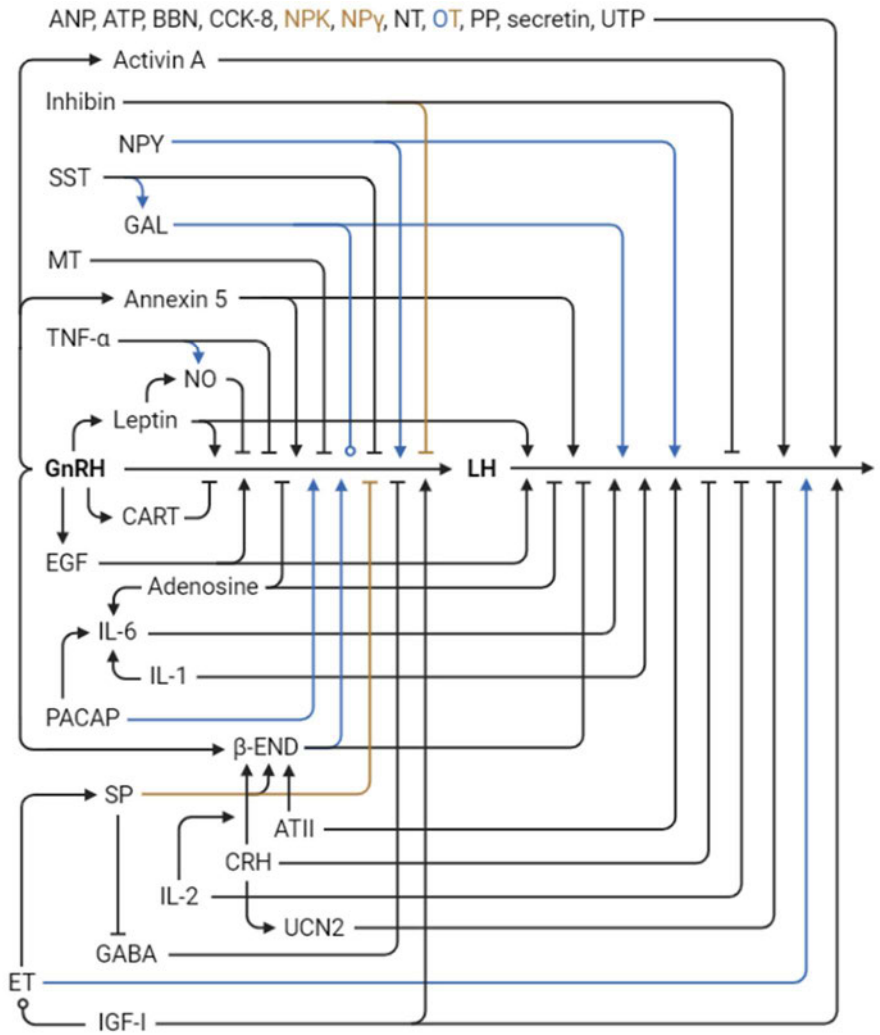


**Figure 33: Complex interactions among paracrine factors influencing LH secretion from the rat anterior pituitary *in vitro*.** The paracrine factors influence gonadotrophin releasing hormone (GnRH)-induced LH secretion (the arrow leading from GnRH to LH) and/or basal LH secretion (the arrow leading from LH). Arrowheads indicate that the paracrine factor has a stimulatory influence, a perpendicular line end indicates an inhibitory influence and a round line end indicates that evidence exists for both stimulatory and inhibitory influence. Blue lines indicate that oestrogens or progesterone significantly modulate the influence of the paracrine factor on LH secretion and brown lines indicates a significant influence of testosterone. Where one ligand influences the secretion of another ligand in the rat pituitary, arrows point to the direction of the influence. All factors in the diagram apart from GnRH are made locally in the rat anterior pituitary. Names of each ligand and evidence for their influence is presented in Table 27.

| Abbreviation | Name | Influence and reference |
|---|---|---|
| | Activin A | ⬆ basal LH (Attardi and Miklos 1990) |
| | Adenosine | ⬆ IL-6 (Ritchie et al. 1997), ⬇ basal and stimulated LH (Picanço-Diniz et al. 1989) |
| ATP | Adenosine triphosphate | ⬆ basal LH (Chen et al. 1995) |
| ATII | Angiotensin II | ⬆ β-END (Kraft et al. 1984) and ⬆ basal LH (Steele et al. 1981) |
| | Annexin 5 | ⬆ basal and stimulated LH (Kawaminami et al. 2002) |
| ANP | Atrial natriuretic peptide | ⬆ basal LH (Horvath et al. 1986) |
| BBN | Bombesin | ⬆ basal LH (Morley et al. 1979) |
| CCK-8 | Cholecystokinin-8 | ⬆ basal LH (Morley et al. 1979) |
| CART | Cocaine and amphetamine regulated transcript | ⬇ stimulated LH (Baranowska et al. 2003) |
| CRH | Corticotrophin releasing hormone | ⬆ β-END (Sweep and Wiegant 1989) and UCN2 (Nemoto et al. 2007), ⬇ basal LH (Blank et al. 1986) |
| ET | Endothelin | ⬆ SP (Calvo et al. 1990), ⬆ basal LH (Stojilković et al. 1990) depending on time of exposure to oestradiol and progesterone (Ortmann et al. 1993) |
| EGF | Epidermal growth factor | ⬆ basal (Przylipiak et al. 1988) and stimulated LH (Miyake et al. 1985) |
| GAL | Galanin | ⬆ (López et al. 1991) or ⬇ (Todd et al. 1998) stimulated LH depending on the oestrogen environment (Peters et al. 2009) and ⬆ basal LH(López et al. 1991) to an extent that is determined by the oestrus stage of the animal (Coen et al. 1990) |
| GnRH | Gonadotrophin releasing hormone | ⬆ activin A (Liu et al. 1996), annexin 5 (Kawaminami et al. 2002), leptin (Akhter et al. 2007), CART (Kappeler et al. 2006), EGF (Mouihate et al. 1995), β-END |
| | Inhibin | ⬇ basal LH (Jakubowiak et al. 1991), ⬇ stimulated LH (Campen and Vale 1988), effect on stimulated dependent on testosterone (Campen and Vale 1988) |
| IGF-1 | Insulin-like growth factor 1 | ⬆ or ⬇ ET (Matsumoto et al. 1990), ⬆ basal and stimulated LH (Kanematsu et al. 1991) |
| IL-1 | Interleukin-1 | ⬆ IL-6, ⬆ basal LH (Yamaguchi et al. 1990) |
| IL-2 | Interleukin-2 | ⬆ CRH-induced β-END, ⬇ basal LH (Karanth and McCann 1991) |
| IL-6 | Interleukin-6 | ⬆ basal LH (Yamaguchi et al. 1990) |
| | Leptin | ⬆ NO (Yu et al. 1997b), ⬆ basal and stimulated LH (Ogura et al. 2001) |
| MT | Melatonin | ⬇ stimulated LH (Martin and Sattler 1982) |
| NPK | Neuropeptide K | ⬆ basal LH (Kalra et al. 1992), depending on androgens (Kalra et al. 1992) |
| NPY | Neuropeptide Y | ⬆ basal (Chabot et al. 1988) and stimulated LH (Bauer-Dantoin et al. 1993), effects on both are oestrogen-dependent (Crowley et al. 1990; Bauer-Dantoin et al. 1993) |
| NPγ | Neuropeptide γ | ⬆ basal LH (Kalra et al. 1992), depending on androgens (Kalra et al. 1992) |
| NT | Neurotensin | ⬆ basal LH (Billiard 1996) |
| NO | Nitric oxide | ⬇ stimulated LH (Ceccatelli et al. 1993) |
| OT | Oxytocin | ⬆ basal LH (Evans et al. 1992), enhanced by oestradiol and inhibited by progesterone or testosterone (Evans et al. 1992) |
| PP | Pancreatic polypeptide | ⬆ basal LH (Morley et al. 1979) |
| PACAP | Pituitary adenylate cyclase-activating polypeptide | ⬆ IL-6 (Tatsuno et al. 1991), ⬆ stimulated LH (Culler and Paschall 1991) depending on the stage of the oestrus cycle (Szabó et al. 2004) |
| | Secretin | ⬆ basal LH (Morley et al. 1979) |
| SST | Somatostatin | ⬆ GAL depending on presence of oestrogens (Hyde and Howard 1992), ⬇ stimulated LH (Yu et al. 1997a) |
| SP | Substance P | ⬆ β-END (Matsumura et al. 1982), ⬇ GABA (Afione et al. 1990), ⬇ stimulated LH (Duval et al. 1996) but is reversed into ⬆ in the absence of dihydrotestosterone (Shamgochian and Leeman 1992) |
| TNF-α | Tumour necrosis factor α | ⬆ NO synthase activity in an oestradiol-dependent manner (Candolfi et al. 2004), ⬇ stimulated LH (Gaillard et al. 1990). |
| UTP | Uridine triphosphate | ⬆ basal LH (Chen et al. 1995) |
| UCN2 | Urocortin 2 | ⬇ basal LH (Nemoto et al. 2009) |
| β-END | β-endorphin | ⬆ stimulated LH depending on oestradiol and progesterone in culture (Kandeel and Swerdloff 1997), ⬇ basal LH (Blank et al. 1986) |
| GABA | γ-aminobutyric acid | ⬇ stimulated LH (Lux-Lantos et al. 2001) |

**Table 27: Evidence that different pituitary paracrine ligands regulate each other and secretion of luteinising hormone (LH).** Arrow up signifies an increase and an arrow down signifies inhibition.

### 4.3 Practical steps forward

To acquire an integrated understanding of intrapituitary regulation, it may therefore be necessary to study the system *in vivo* where the pituitary both retains its organisation and where it is exposed to its complex physiological environment. Given the expense of *in vivo* work, performing initial screening using bioinformatics, as was done in this dissertation, may be a valuable way to narrow down candidates for *in vivo* testing.

To study the involvement of candidate paracrine factors or homotypic CAMs, one approach could be to knock out or knock down the candidates in the pituitary cell type of interest using Cre-controlled CRISPR-Cas9 mutagenesis (Hans et al. 2021). However, deletion of individual CAMs (Hiraga et al. 2020) or paracrine factors (Ingman and Jones 2008; Lin et al. 2022) frequently produce no change in phenotype due to genetic compensation. Compensation is more frequently observed with knockouts compared to knockdowns (El-Brolosy and Stainier 2017), suggesting that the latter may work better.

Alternatively, overexpression of the candidates may be a more suitable approach that would also to an extent circumvent the effects of compensation. The involvement of homotypic CAMs could be studied by overexpressing them in a different cell type, for example by CRISPR-Cas9-mediated activation of a corticotroph candidate homotypic CAM in gonadotrophs. If the CAM is responsible for homotypic adhesion in corticotrophs, the overexpression would be expected to cause increased mixing between corticotrophs and gonadotrophs. Overexpression of paracrine factors in an entire pituitary cell type, however, can have rather drastic effects on the pituitary. When nerve growth factor was overexpressed in mouse prolactin cells, the pituitary grew to 10-100 times its normal size (Borrelli et al. 1992). A more nuanced approach might involve focal overexpression of a paracrine factor in a small set of pituitary cells via adeno-associated viral vectors and observing functional changes in nearby cells. Ultimately, any modifications to pituitary network arrangement or hormone output upon changes to the expression of a candidate would indicate that it has a role in intrapituitary regulation.

## 4.4 Outstanding questions

As a final look into the future, the following questions may be important to consider in the endeavour to understand intrapituitary regulation.

### *4.4.1 What is the role of the extracellular matrix (ECM) in pituitary cell networks and paracrine communication?*

Apart from an influence on pituitary hormone secretion (Horacek et al. 1992; Denduchis et al. 1994; Diaz et al. 2002), the ECM may also facilitate connections between pituitary cells and regulate paracrine interactions. Expression patterns of different ECM components are developmentally regulated in the pituitary (Murray et al. 1997; Horiguchi et al. 2013; Ramadhani et al. 2014), infantile pituitary cell aggregation is increased in the presence of collagen, and migration rates of infantile pituitary cells differ depending on the collagen composition of the ECM (González et al. 2004), suggesting that the ECM may contribute to the arrangement of pituitary networks during development. The presence of ECM in culture is also an important requirement for the formation of cytoplasmic extensions between different adult pituitary cells *in vitro* (de Carvalho et al. 2000; Horiguchi et al. 2010; Azorín et al. 2014), supporting a role for the ECM in maintenance of heterotypic connections between cells.

The ECM binds a variety of different paracrine ligands and it has been suggested that the release of a ligand from a cell is less important for its function than is its liberation from the ECM (Roy and Kornberg 2015). The responses of pituitary cells to paracrine factors such as epidermal growth factor are dependent on the presence of ECM in culture (Toral et al. 2007) and secretion of vascular endothelial growth factor from lactotrophs is regulated by the ECM *in vitro* (Alfer et al. 2015). The ECM may also facilitate gap junction-mediated communication since gap junctions only form between FS cells in primary culture in the presence of laminin (Horiguchi et al. 2011).

### *4.4.2 Do cellular extensions contribute to paracrine communication and the formation of pituitary cell networks?*

All pituitary cell types extend cellular processes but the extensions of corticotrophs, gonadotrophs and FS cells appear to be particularly prominent. In cultures of medaka pituitary cells, gonadotrophs extended cytoplasmic projections that brought somas closer together, apparently facilitating clustering of the gonadotrophs (Grønlien et al. 2021). In mammalian gonadotrophs, cellular migration was associated with the formation of

cytoplasmic extensions in response to GnRH (Navratil et al. 2007), perhaps indicating that the projections could have a sensory function in network formation and/or maintenance. The cellular projections are often proposed to mediate hormone secretion since they project to blood vessels, however, they also connect pituitary cell types to each other (Fletcher et al. 1975; Liu and Ju 1998; Paden et al. 2006), suggesting that they may be involved in cell-cell communication. The projections may allow directional communication given that rat corticotrophs, for example, always send projections towards lactotrophs rather than vice versa (Liu and Ju 1998).

### *4.4.3 Do networks and paracrine interactions have importance beyond primarily regulating pituitary hormone secretion?*

A diversity of intriguing possibilities regarding the functions of pituitary networks and paracrine communication have been proposed, a few of which are mentioned here. Radiolabelled ANP is taken up into mitochondria in gonadotrophs after intravenous injection (Morel et al. 1989), suggesting that it may play a role in mitochondrial metabolism (Domondon et al. 2019). The corticotroph homotypic network that forms first during development may act as a scaffold directing the formation of the other pituitary cell networks (Budry et al. 2011). Pituitary cell adhesion appears to regulate cell proliferation as overexpression of E-cadherin and/or N-cadherin inhibits GH3 cell proliferation (Heinrich 1999). Future study of pituitary networks and paracrine regulation should therefore not be limited to influence on hormone secretion.

### *4.4.4 Does disruption of network architecture or paracrine communication in the pituitary contribute to disease?*

Disrupting pituitary homotypic cell networks or paracrine communication has been suggested to alter hormone secretion (Golan et al. 2016) or lead to tumour formation (Arzt et al. 1999) but concrete evidence is still scarce. Given the prevalence of pituitary disorders (Regal et al. 2001; Ezzat et al. 2004), determining how intrapituitary mechanisms contribute to such pathology should be a priority.

## 4.5 Conclusion

This dissertation has merged an old field with new technological approaches. Pituitary cell-cell interactions have long been known to exist, but they have thus far been analysed with more traditional laboratory techniques. The studies presented in this dissertation were aimed to identify candidate molecules involved in intrapituitary interactions using bioinformatics analyses of pituitary transcriptomics data. In a first study, candidate cell adhesion molecules that may mediate homotypic adhesion between pituitary cells were generated. Their expression patterns were explored within and between cell types as well as across development and species to evaluate their potential as candidate molecules. A second study was designed to identify candidate paracrine interactions that may play a role in the pituitary response to chronic stress. By applying different cell-cell communication prediction algorithms to pituitary scRNA-seq data, altered interactions between corticotrophs and other pituitary cell types were inferred. Together these candidate intrapituitary factors represent a set of novel molecules that can now be subjected to experimental validation.

Despite the successful generation of candidate molecules, this body of work has also highlighted limitations regarding what can be learned about pituitary biology from scRNA-seq data. The expression of some paracrine factors in scRNA-seq data conflicted with their expression in the literature, suggesting that different technical or biological factors have significant influence over the expression of intrapituitary factors. Moreover, the dropout effect in scRNA-seq technology prevents detection of potentially large numbers of relevant transcripts, which means that the absence of a factor in scRNA-seq data is not a useful signal i.e., the absence of evidence is not evidence of absence. These bioinformatics analyses extend our current knowledge on the interactome that connects pituitary cells into complex physical and paracrine networks.

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