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30 Years of Neuroendocrinology: technological advances pave the way for molecular discovery

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Since the 1950's (1) the systems level interactions between the hypothalamus, pituitary and end organs such as the adrenal, thyroid and gonads have been well known, however it is only over the last three decades that advances in molecular biology and information technology have provided a tremendous expansion of knowledge at the molecular level. Neuroendocrinology has benefitted from developments in molecular genetics, epigenetics and epigenomics, and most recently optogenetics and pharmacogenetics. This has enabled a new understanding of gene regulation, transcription, translation and post-translational regulation, which should help direct the development of drugs to treat neuroendocrine related diseases.

Understanding the molecular mechanisms of steroid receptor signalling is a very important aspect of gaining information about the underlying causes of diseases associated with aberrant signalling, such as in hormone sensitive cancers or the side-effects of steroid treatment. One of the most important neuroendocrine systems is the hypothalamic-pituitary-adrenal axis, which regulates the release of glucocorticoid hormones from the adrenal glands and is critical for the maintenance of homeostasis. Glucocorticoids are clinically widely used due to their potent anti-inflammatory effects. Prolonged use of high doses of glucocorticoids is associated with many unwanted side-effects and often therapy is complicated by the development of glucocorticoid resistance. A better understanding of the molecular mechanisms of glucocorticoid receptor (GR) signalling will provide the rationale for better therapy, as well as limiting adverse side-effects and the development of resistance. Similarly, understanding the molecular mechanisms of intracellular receptors for estrogen (ER), progesterone (PR) and androgen (AR) may aid in the development of more specific therapies for diseases such as breast cancer or prostate cancer respectively, where dysregulation of signalling can promote disease development and progression. The importance of this was recognised 30 years ago when the journal of Neuroendocrinology was established!

The human genome project has enabled scientists to examine the entire complement of human genes, as well as their RNA and protein expression, instead of the conventional time-consuming methods focusing on individual components. The development of more advanced ways to assess chromatin structure and interactions such as receptor binding is enhancing our knowledge of these cellular processes as well as providing a new understanding of the dynamics of how hormone receptors interact with the chromatin landscape. In addition to this, new ways to edit the genome such as CRISPR, have become powerful tools in assessing the function of proteins. Optogenetics and Pharmacogenetics are also helping determine the specific roles of subsets of neurons as well as identifying neuron circuitry. Together these techniques have begun to enable molecular biology to advance the practice of medicine by determining the molecular alterations which promote disease, thereby leading to the future design of specific, more targeted treatments.

High throughput sequencing and steroid receptor action

High throughput sequencing techniques have become a major tool for the identification of molecular mechanisms across many scientific fields. Previously RNA analysis was restricted to analysis of known transcripts while modern sequencing enables analysis on a genome-wide scale requiring no prior knowledge of which genes are expressed. Many now believe RNA-Seq to be a more sensitive technique allowing more accurate measurements of transcript levels (2, 3). Despite some controversy, it is clear this technique can form a basis for most studies and unveil potential directions which can be further explored using the conventional techniques including microarrays and real-time quantitative polymerase chain reaction (qPCR). RNA-Seq is proving useful in characterizing differences in gene expression between specific cell types and in determining the changes which exist promoting glucocorticoid resistance (4-6). It has for instance recently been used to show how cyclosporine A, a drug frequently used to rescue glucocorticoid resistant diseases, can promote a genome-wide shift attenuating the responsiveness of a cell line prone to promoting resistance (4). This is important as patients which become glucocorticoid resistant often require high doses leading to several side effects. Understanding the biological mechanisms that cause resistance may help identify other therapies to aid glucocorticoid treatment of these patients in the future. Similarly, RNA-Seq is helping identify gene expression changes in response to estrogen, progesterone and androgens across different types of cancers (7-10) and has been used as an unbiased tool for transcriptome analysis in MCF7 breast cancer cells to determine ER regulatory pathways (7). Advanced analysis techniques are also enabling hierarchical clustering of genes according to hormone-dependent expression patterns, such as SupraHex analysis (5, 11), and functional pathway analyses providing detailed information about the genome-wide expression profiles of individual cells. Another advantage of RNA-Seq is that it can be used to identify splice variants and mutations such as short nucleotide polymorphisms (SNPs). In a study using palmitate treated human islets of Langerhans, more than 3000 splice variants that had not been detected in previous microarray studies were identified (12). Advances in information technology combined with accurate mapping of RNA-Seq reads (13, 14) has aided the identification of SNPs in diseases such as cancer (15, 16). In addition to RNA-Seq, a technique referred to as 'Drop-seq' analyses mRNA transcripts from individual cells and determines the transcripts' cell of origin, which is incredibly useful for expression profiling of heterogenous cell populations *in-vivo*. In mouse retinal tissue, 39 transcriptionally unique cell populations were found from over 40000 cells (17). This shows how large-scale single cell analysis can help improve our understanding of individual cell populations in complex neural tissue.

Advances in protein sequencing techniques using mass spectrometry have aided the examination of global protein expression. This is improving our understanding of the processes occurring from hormone-mediated transcriptional expression to translation. It is also enabling scientists to identify the proteins present in individual cell types and determine interactions between proteins using coimmunoprecipitation techniques. By comparing protein expression in disease state to a control, this enables determination of which proteins gain or lose expression or interactions with other proteins, for example in cancer due to specific mutations. Using liquid chromatography mass spectrometry (LC-MS) proteomics has identified differences in proteins present in androgen-dependent and independent cancer cells compared to normal prostate epithelium (18). Proteins interacting with ERalpha have

been determined using LC-MS in human breast cancer (19). This technique can thereby hasten the identification of novel drug targets.

Steroid receptor binding and chromatin accessibility

Understanding the chromatin landscape can help inform us of the genomic response of transcription factors by determining transcription factor binding as well as predicting regulatory regions by assessing chromatin accessibility, nucleosome mapping and dynamic long-distance chromatin conformations, including looping. The first studies to suggest nuclear hormone receptor interactions with chromatin appeared as early as the 1960s (20-22). However direct evidence for this came later and was first reported using binding assays. Since then, chromatin immunoprecipitation (ChIP) developed in the 1990s, has become a widely used technique for assessing protein-DNA interactions. This technique has greatly advanced our understanding of the binding of nuclear receptors, co-factors, and other components of the transcriptional machinery such as Polymerase II, to specific DNA regulatory sites of a few well-known candidate genes. ChIP has been used to assess time-dependent transcription factor binding in live cells and *in-vivo* due to its ability to fix proteins at any point in time. Early studies by Chen and colleagues (23), demonstrated recruitment of ER as well as co-factors to target genes following treatment with estradiol in MCF7 cells. Here, they showed that although ER binding remained, time dependent changes in recruitment of co-factors such as acetyltransferases CBP and p300 unexpectedly correlated to a decrease in transcription of c-Myc and cathepsin D (CTD). This study and similar more recent studies (24-27), have demonstrated the sequential recruitment of co-factors including histone acetyltransferases, histone methyltransferases and subunits of the chromatin remodelling BRG1 associated factor (BAF) complex to promote transcription. The advance of ChIP sequencing (ChIP-Seq) has allowed us to identify genome-wide binding sites for transcription factors such as GR. Figure 1 shows ChIP-Seq data from rat hippocampal tissue, highlighting a selection of GR binding events induced by acute stress and exogenous corticosterone treatment (28).

Additionally, ChIP-Seq has enabled the genome-wide assessment of how binding profiles for GR, ER, PR and AR are highly cell-type specific and subject to alteration with changing environmental conditions (29-36) as well as being dependent upon other regulatory proteins such as AP-1 and Foxa1 (30, 37). ChIP-Seq has revealed insights into loss of protein-DNA associations occurring in disease states, such as cancer, increasing our understanding of disease development and potentially leading to the development of more specific therapies. Recently advances in ChIP protocols has seen the development of a robust ChIP-exo technique referred to as ChIP-nexus which uses an exonuclease for high nucleotide resolution and an additional DNA self-circularization step during library preparation (38). This produces high quality data with high resolution at the single nucleotide level (38). Future studies are already focusing on improving the ChIP technique further using UV Laser ChIP-Seq (39) to improve the identification of proteins that are in immediate contact with nucleic acids rather than being tethered to other proteins.

Other studies have focused on the interactions of steroid receptors and alterations in the chromatin architecture. For GR, early studies have involved the extensive use of the MMTV promoter as a model system (40-43). The advance of DNase I techniques to assess chromatin accessibility also showed GR to induce '*de novo*' chromatin remodelling of the MMTV array

by interactions with the SWI/SNF complex (44-46). DNase I cuts regions of more open chromatin which result from changes in nucleosome structure (47, 48). The combination of ChIP and DNase I studies with sequencing has recently become a powerful tool. DNase I sequencing again is a genome-wide approach and requires no previous knowledge of the sequence or the protein of interest (49). Alongside this has been the development of Formaldehyde-Assisted Isolation of Regulatory Elements sequencing (FAIRE-Seq) to assess open chromatin and nucleosome occupancy (50), micrococcal nuclease sequencing (MNase-Seq) (51, 52) to determine nucleosome positions, and most recently ATAC-Seq (Assay for Transposase-Accessible Chromatin using sequencing) (Figure 2). ATAC-Seq uses hyperactive Tn5 transposase to catalyse the movement of transposans to assess chromatin accessibility (53, 54). Tn5 cuts and inserts sequencing adaptors to open chromatin sites which can then be directly sequenced (55). This method is now seen as a quicker and more sensitive alternative to DNase-Seq for assessing chromatin accessibility and an alternative to MNase-Seq for assessing nucleosome positions (37, 56). These techniques have been important in determining the fundamentals of steroid receptor binding in combination with chromatin organisation, unveiling the complex dynamics of steroid receptor mediated transcription. For example, before recent studies revealing nuclear receptors can bind at a great distance from target genes (57-59) it was believed that regulatory regions were confined to the proximal promoter region. In mouse 3134 cells it has been observed that 93% of GR binding sites occur more than 2.5kb distal to the Transcription start site (TSS) (49). Using transcriptome profiling with GR ChIP-Seq, Uhlenhaut et al. (32) showed that less than 6% of GR binding sites occurred at proximal promoters in lipopolysaccharide activated macrophages. In HeLa cells it has also been demonstrated that 24% of GR binding sites are > 25kb from the TSS with only 7% of binding sites located in the promoter region (60). Similarly, in rat hippocampal tissue few GR binding sites have been found in promoter regions (28, 61). In MCF7 cells, the majority of ER binding has also been shown to occur at great distances from TSS (34, 57). This has promoted suggestions of complex chromatin looping to enable interactions between receptors and other transcriptional co-factors to promote transcription (34, 62).

These techniques have been key in elucidating the roles of steroid receptor-mediated transcription. In addition to this they have also revealed the complexity of chromatin organisation around regulatory sites, including identification of epigenetic marks and the characteristics of inducible and repressible chromatin configurations. This aids our understanding of not only how transcription factors, regulatory complexes and proteins interact at the chromatin level but also how the dynamic chromatin structure is important for aiding interactions between proteins, for example by looping, to regulate a transcriptional response. Future applications of sequencing data combined with predictive modelling techniques may even form a basis for further investigations into how this chromatin structure aids regulation.

Advances in Fluorescent In-situ Hybridisation (FISH) techniques

FISH uses probes consisting of nucleotide sequences incorporated with fluorophores to recognise the presence of complementary sequences in RNA or DNA. This technique which started being used in the 1980s has now been widely used to determine the genetic content and RNA expression of individual cells. RNA ISH (in situ hybridisation) enabled analysis of RNA using conventional brightfield or standard fluorescence microscopes but lacked sensitivity

and specificity to accurately visualise single molecules. Recently RNAscope®, a novel multiplex RNA ISH technology, which uses unique probes to promote simultaneous signal amplification with low background, represents a major advance allowing more specific visualisation of gene expression in complex tissues (64). Another major advantage of the system is detection of low abundance mRNA expression. Therefore, for neuroendocrinology, this technique could enable us to co-localise hormone-dependent transcriptional responses with neurotransmitter and/or receptor mRNA distribution in a wide range of tissues. Figure 3 shows the heterogenous distribution of mRNA expression of GR and MR, along with co-localization to the glucocorticoid-target transcript Per1 in the dentate gyrus of the rat hippocampus. This increased resolution to the cellular level overcomes previous limitations associated with analysing heterogenous transcription in different cell types within the complex architecture of brain tissue.

A further example of this includes the Angiotensin receptor mRNA, which has recently been visualised in the paraventricular nucleus of the hypothalamus on corticotrophin releasing hormone (CRH) neurons enabling studies assessing correlations between loss of these receptors and levels of CRH mRNA (65-67).

Gene transcription has also been shown to occur in bursts with refractory periods (68-72) and single molecule RNA ISH (73, 74) can be used to study transcriptional bursts. By determining the frequency and size of these bursts (quantity of RNA produced) this technology can provide information into gene regulation in response to hormones in different brain regions. Visualisation of RNA in living cells is a further challenge crucial for obtaining important spatial and temporal data about RNA expression, localisation and storage. New techniques are now focused on tracking RNA in living cells and we may see a major advance in this over the next decade (75, 76).

Another advance in FISH techniques is the use of 3D DNA FISH to assess chromatin reorganisation in response to hormone receptor action. Jubb and colleagues (77) used 3D DNA FISH to assess glucocorticoid mediated chromatin decompaction at several loci. This provides extra information about speed and duration of chromatin decompaction which is difficult to determine using DNase I or ATAC-Seq techniques. This technique may similarly be used to investigate other hormone inducible changes in chromatin organisation during acute-phase transcriptional regulation.

Editing the genome to determine a functional role

The introduction of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9 system has greatly advanced genome editing/engineering. CRISPR genome editing uses a system found in the prokaryotic immune system which introduces double strand breaks on viral DNA as a defence mechanism (78). A single guide RNA, complementary to the viral DNA sequence, directs Cas9 nuclease towards the viral DNA where it promotes a double strand break. This technology has been adapted and can be used to introduce mutations at specific sites in the genome. In 2013 the system was first used for multiplex engineering in eukaryotic cells (79) and has rapidly become a widely used genome editing tool. CRISPR has already been used to introduce mutations into ERs (80, 81) to determine the importance of these mutations in endocrine resistance. Combined with RNA-Seq and ChIP-

Seq a single mutation Y537S in ER has been shown to promote constitutive ER activity promoting estrogen independent growth (81). This use of CRISPR demonstrates how it can be used to help identify what outcomes certain mutations can cause. CRISPR editing to disrupt AR has been shown to inhibit the growth of androgen sensitive prostate cancer cells (82). This could suggest another important use of CRISPR as a therapeutic target for prostate cancer. CRISPR editing of the PR has recently questioned the importance of PR in the role of female reproductive cyclicity in rodents (83) which remained despite other expected outcomes of PR deletion. This shows how CRISPR can be used to further decipher the roles of hormone receptors. Overall CRISPR is a powerful tool for introducing or editing mutations to further understand the roles of genes and known mutations in neuroendocrine disease.

Optogenetic and Chemogenetic tools

The recent advances in optogenetics and chemogenetics is now enabling neuroscientists to modulate the activity of specific subsets of neurons using light or designer drugs. These are useful tools for helping functionally dissect which cells are important for specific roles, for example which subset of cells specify certain behaviours, emotions or are specific to certain motor functions. Optogenetics use Opsin-based receptors which are selectively expressed in cells of interest. The most frequent receptor used is channelrhodopsin which can be expressed in specific cell phenotypes. *In-vivo*, viruses can be used to deliver Cre dependent receptors to transgenic Cre-driver rodent lines (84). In neuroendocrinology, optogenetics has been valuable in determining control of pituitary hormone secretion (84). Gonadotropin releasing hormone (GnRH) neurons expressing channelrhodopsin have been used to show pulsatile release of luteinising hormone (LH) (85). Optogenetics has contributed to further understanding of important neural networks including those involved in homeostasis as well as for higher cognitive functions and emotional behaviours (86).

Pharmacogenetics, similarly to optogenetics, looks at variation in gene responses to stimulation of receptors, but by using pharmacological agents. This often begins with the discovery of an unexpected response to a drug and then looks at the genetic cause often by introducing selected genes to specific brain targets to alter cell function. An example of this is the treatment of Parkinson's symptoms, usually treated with dopamine agonists, in rats by increasing GABA concentrations in the subthalamic nucleus by transferring the gene for gamma-amino butryic acid (GAD) which is the rate limiting enzyme in GABA production (87). Clinical trials on patients have since been trialled (88, 89).

More recently a new chemogenetic approach to studying specific cell populations utilizes Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) and is termed 'pharmacosynthetics'. DREADDs involve the use of receptor proteins designed through mutagenesis of endogenous G protein coupled receptor DNA. These 'Designer Receptors' can be targeted to specific neuronal subtypes by engineering to cell-specific endogenous promoter sequences, ensuring that only the specific neuronal population will be stimulated by the 'Designer Drug' clozapine-*n*-oxide (CNO). The specificity of DREADDs can be seen in the elegant studies of Yoshimura et al. (90) using the AVP-hM3Dq-mCherry transgenic rat. In Figure 4, acute systemic CNO treatment results in highly specific c-fos induction, indicating neuronal activation (91), within vasopressin neurons (hM3Dq-mCherry positive, AVP positive) in the supraoptic (SON) and the paraventricular nuclei (PVN). DREADDs can also be inhibitory

when hM4Di (92-94), a variant of the endogenous M4 muscarinic acetylcholine receptor, is utilized. DREADDs technology has been suggested to have several therapeutic applications, these include metabolic disorders (95), diabetes (96), depression and stress disorders (94, 97) and inflammatory disorders (98). For Parkinson's disease, pharmacogenetics could re-engineer neurons to produce neuroprotective neurotrophins to protect dopaminergic cells while optogenetics could promote cell type specific control for repair of dysfunction (99).

Insight to the future

Recent technological advances in the field of quantitative fluorescence microscopy are allowing new insight into the biological activities taking place inside living cells. It is now possible to track individual proteins enabling scientists to obtain information such as transcription factor binding times and dissociation rates. GR has already been viewed in this way, labelled with a new generation of fluorescent tags with increased brightness and better photostability to aid visualisation at the single molecule level (100). This technology is only getting started but may very soon increase our understanding of the dynamics of how transcription factors such as GR interact with the chromatin landscape to promote transcription. This could promote a further expansion of our knowledge of molecular processes in living cells, potentially confirming previous findings and revealing a new understanding of dynamic cellular processes. In addition to this a surge in molecular data has also enabled an advance in mathematical modelling in the field of neuroendocrinology, to help us predict dynamic interactions of different proteins. Mathematical modelling can provide clues to what may be occurring in live cells based on previously acquired knowledge and may help direct future experiments and technology to further improve our molecular understanding. Combining these advances in single molecule tracking and mathematical modelling with further improvements to ChIP, chromatin accessibility, CRISPR and pharmacogenetics, the next 30 years will likely again see another explosion in molecular data and further advance our understanding of hormone regulation.

Summary

The recent advance in sequencing techniques and the application of next generation sequencing to RNA, ChIP, DNase I, ATAC, MNase and FAIRE studies has revealed new insights into gene regulation in neuroendocrine systems. This enhances our understanding of biological pathways and importantly can identify events that have a role in causing various diseases, such as the development and progression of certain cancers. The introduction of CRISPR as well as similar genome editing techniques now enables scientists to study known mutations to model diseases, promoting a better understanding of the causes of certain diseases and the pathways involved in their progression. Optogenetic and chemogenetic techniques also provide huge potential for the research of neurological diseases, enabling the visualisation of neural networks and the design of receptors to regulate neuronal activity, respectively. The future of molecular neuroendocrinology will see a further expansion in knowledge with the aid of new technologies such as single molecule tracking to visualise the

movement of proteins and the subsequent biological processes in living cells. With this more detailed understanding of the molecular basis of normal and abnormal function at the neuro-hormonal interface, we expect to identify novel pathways as well as new therapeutic targets. This will ultimately lead to the development of new therapies to benefit patients with cancer or neuroendocrine dysfunction.

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Figure 1: Genome Browser Shots showing GR binding sites (peaks) detected in regulatory elements of glucocorticoid-target genes in the rat hippocampus after acute stress (30min restraint) or exogenous corticosterone treatment (30min intravenous infusion). Genome-wide GR binding events were found to be highly similar between stress (red trace) and corticosterone treatment (blue trace) conditions. Peaks were detected at sites upstream, intronic and intergenic for genes encoding Period1 (a), Metallothionien1 / 2 (b), DNA damage inducible Transcript 4 (c), Camk2a (d), Bdnf (e), and Thra (f).

Figure 2: Comparisons of sequencing techniques. a) ChIP-Seq reveals genome-wide interactions between proteins, such as hormone receptors, with specific DNA sequences. b) RNA-Seq is highly sensitive and enables analysis of gene expression across the genome. c) Protein sequencing can be performed using Liquid chromatography mass spectrometry (LC-MS) to show protein expression, protein-protein interactions and post-translational modifications. d) DNase-Seq and ATAC-Seq map the more accessible, exposed, chromatin regions across the genome whereas FAIRE-Seq assesses proteins of the nucleosome depleted DNA. DNA regulatory areas where transcription factors bind and protect against enzyme digestion leave a footprint which can also be detected from DNase-Seq and ATAC-Seq data (63). e) MNase-Seq can be used to map where nucleosomes are present and therefore also where nucleosome free areas are found.

Figure 3: Cellular distribution of mRNA for GR, MR and Per1 in the dentate gyrus of the rat hippocampus. RNA Fluorescent in situ hybridisation (RNAscope® Multiplex Fluorescent Reagent Kit v2 2.0; Advanced Cell Diagnostics Inc., Hayward, CA, US) was used to detect GR,

MR and Per1 mRNA according to the manufacturer's protocol. Probes used were: GR Rn-NR3C1, (catalog no. 466991), MR Rn-NR3C2 (custom designed probe targeting 1175-2062 of NM_013131.1; catalog no. 320269-C2) and Rn-Per1/466161(catalog no. 300031-C3), all from Advanced Cell Diagnostics Inc. The NR3C1 probe set was used in channel 1 with TSA Plus Fluorescein (NEL741001KT PerkinElmer). The NR3C2 probe set was used in channel 2 with TSA Plus Cyanine 3 (NEL744001KT PerkinElmer). The Per1 probe set was used in channel 3 with TSA Plus Cyanine 5 (NEL745001KT PerkinElmer). Fluorescent microscopy was performed on Leica SPE single channel confocal laser scanning microscope system at 40x magnification. Solid state lasers: 405nm (25mW), 488nm (10 mW), 532nm (10mW) and 635nm (18mW) were used to detect Dapi, Fluorescein, Cyanine 3 and Cyanine 5 respectively. Images were captured on a Leica DFC365FX monochrome digital camera (1392x1040 6.45µm pixels, 8 or 12 bit, 21 fps full frame).

Figure 4: Fos immunoreactivity (Fos-ir) in SON and PVN mCherry positive (hM3Dq-mCherry) vasopressin (AVP) neurons a) This illustrates the DREADD technique used in this study. b) Images showing Fos induction following 90min intraperitoneal administration of CNO targeting hM3Dq-mCherry expressing AVP neurons of the SON and PVN (90). Scale bars indicate 200µm; OT, optic tract; 3rd V, third ventricle.