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How technical progress reshaped behavioral neuroendocrinology during the last 50 years... and some methodological remarks

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Abstract

The first issue of *Hormones and Behavior* was published 50 years ago in 1969, a time when most of the techniques we currently use in Behavioral Endocrinology were not available. Researchers have during the last 5 decades developed techniques that allow measuring hormones in small volumes of biological samples, identify the sites where steroids act in the brain to activate sexual behavior, characterize and quantify gene expression correlated with behavior expression, modify this expression in a specific manner, and manipulate the activity of selected neuronal populations by chemogenetic and optogenetic techniques. This technical progress has considerably transformed the field and has been very beneficial for our understanding of the endocrine controls of behavior in general, but it did also come with some caveats. The facilitation of scientific investigations came with some relaxation of methodological exigency. Some critical controls are no longer performed on a regular basis and complex techniques supplied as ready to use kits are implemented without precise knowledge of their limitations. We present here a selective review of the most important of these new techniques, their potential problems and how they changed our view of the hormonal control of behavior. Fortunately, the scientific endeavor is a self-correcting process. The problems have been identified and corrections have been proposed. The next decades will obviously be filled with exciting discoveries in behavioral neuroendocrinology.

Keywords

Radioimmunoassay; In situ autoradiography; Stereotaxy; Gene knock-out; Optogenetic

1. Introduction

The first issue of *Hormones and Behavior* was published 50 years ago in 1969 and I started my PhD research investigating the control by hormones of male sexual behavior in ducks in 1972. My scientific career has therefore overlapped extensively with the history of this journal and I have directly witnessed the technical and conceptual changes that took place

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during this period. I recently reviewed the conceptual shifts in the field that crystalized since 2000 and include for example the “direct” effects of genes and the implication of immune responses in sexual differentiation, the discovery that the brain is also a site of steroid synthesis, the role of steroid receptor-co-regulators and the implication of rapid changes in production and action of steroids in the control of sexual behaviors. I indicated on this occasion that these conceptual changes had not been mainly driven by the appearance of new techniques during that period but rather by the ingeniousness of investigators (Balthazart, 2019).

The situation is however quite different if we look back to the late 1960s when *Hormones and Behavior* published its first issue. One must remember indeed what was the technical situation at that time. Biochemical hormone assays were just beginning, there was no personal computer and calculations had to be made either with a slide ruler or on a main frame with punched cards, and... there was no Internet.

The years that followed saw the emergence and progressive generalization of key techniques that allowed investigating questions that were in everybody’s mind but could not be really studied due to the lack of suitable techniques. They are in fact at the origin of the conceptual changes that I discussed in the other recently published review (Balthazart, 2019). I will briefly review here the most important of these techniques focusing largely on techniques that provided insights in the control of reproductive behavior by sex steroids, one of the main topics that was investigated in the studies historically published in *Hormones and Behavior*.

2. Measuring concentrations of steroids and other hormones

Before 1969, there were only few ways in which hormones could be measured. The specific but insensitive bio-assays (injection of hormone extract into a live animal and assessment of a physiological response: e.g., the prolactin assay via the measure of crop sac growth in pigeons, (Nicoll, 1967)) had been followed by a number of chemical methods (fluorimetric assays, paper chromatography, spectrophotometry, gas liquid chromatography; e.g., (Jallageas and Attal, 1968)). These methods were expensive and time consuming and they were only slightly more sensitive so that they could often be used only for hormones circulating in relatively large quantities in big animals producing samples of very large volume.

As a result, the 4 issues of *Hormones and Behavior* volume 1 (1969) only contain a couple of articles in which hormones were measured. One paper reports on 17-hydroxycorticosteroids concentrations in the urine of rhesus monkeys as measured by a colorimetric assay (Sassenrath, 1970). A single paper describes concentrations of several sex steroids including 17-ketosteroids, androsterone and etiocholanolone but the assays were performed in urine samples collected over 24 hours in humans (Margolese, 1970).

In 1959, Solomon Aaron Berson and Rosalyn Yalow developed the first radioimmunoassay (RIA) that allowed measuring insulin in small volumes of blood (Yalow and Berson, 1959). This initiated a real revolution in biological and medical research even if this discovery was

first received with great resistance. Journals initially refused to publish papers describing the discovery of insulin antibodies because scientists generally believed that antibodies could not recognize such a small molecule. These researchers went on to set up assays for other molecules present in low concentrations such as vitamins and viruses and eventually Rosalyn Yalow's contribution was awarded the Nobel Prize in 1977. Berson had died a few years before in 1972. She shared that Nobel Prize with Roger Guillemin and Andrzej Schally, two other major figures in endocrinology.

It took however another 10 years before the first RIAs were established for steroids. These small molecules are indeed not immunogenic, so researchers had to develop a method for coupling them in a stable manner with a larger molecule (initially bovine serum albumin, later replaced by other options such as keyhole limpet haemocyanin to reduce possibilities of cross-reactions with vertebrate proteins) so that this complex could be injected in a rabbit or a mice in order to raise antibodies. The first assays were published at the end of the 1960s (for estradiol: (Abraham, 1969; Mikhail et al., 1970); for testosterone: (Furuyama et al., 1970); see for review: (Nieschlag and Wickings, 1975)). The exquisite sensitivity of this approach (down to a few picograms i.e., 10^{-12} g) opened the door to repeated measurements of plasma hormones in small animals such as rats, guinea pigs and a variety of avian species. Most steroids could indeed be quantified by RIA in sample as small as 20 to 100 μ l of plasma.

Most notably, in 1975 John Wingfield and Donald Farner developed a method allowing to separate by celite column chromatography and then separately assay 5 steroids in a small volume of avian plasma (Wingfield and Farner, 1975). Based in part on this technique Wingfield launched a vast program investigating changes in plasma concentrations of sex steroids in free-living birds exposed to the full complexity of environmental control factors. This was the start of a new field of investigation known as “field endocrinology” that has in the past 45–50 years unraveled how reproductive cycles are integrated with the social and non social environment in birds and also in other vertebrates. Hundreds of papers have been published in this topic (for review see (Romero and Wingfield, 2016; Wingfield, 2006; Wingfield, 2017)).

RIAs thus provided an easy way to measure steroids in a large number of very small samples thanks to its exquisite sensitivity. The technique is however associated with a couple of potential problems. First the use of radioactivity in a laboratory comes with a number of practical constraints that were potentially limiting the use of the technique. The more recent introduction of enzyme-immunoassays (EIA) in which the radioactive hormone is replaced by hormone labeled by an enzyme has essentially solved this problem and EIAs have now largely replaced RIAs around the world. The second problem relates to the specificity of the technique and is more difficult to solve. RIAs and EIAs are indeed based on the displacement of the binding of labeled hormone to a specific antibody by the unlabeled hormone to be assayed or present in known amount in a standard curve used for calibration. The specificity of the antigen–antibody reaction ensures a relatively high degree of specificity, but the weakness is that any decrease in bound labeled hormone will be interpreted as unlabeled hormone. A decrease in binding can actually be induced in a non-specific manner by chemical contaminating the assay media (e.g., low concentration of

detergent left in the vials) and by a variety of compounds present in the assayed sample such as fat (see Figure 1). A partial purification of the sample before assay often provides an adequate solution but it is not always implemented. As an example, the plasma of an avian female in the process of egg laying contains incredibly large concentrations of fat and this fat will decrease the binding of any steroid to its antibody. Many studies have reported increases in a variety of steroid at the time of ovulation in female birds but without adequate validation these data are highly suspicious. The same problem applies to assays of steroids in brain tissue that also contains high amounts of fat.

There is also an extensive metabolism of steroids, in particular testosterone, that produces metabolites differing by only a few atoms and they can potentially cross-react with the antibody that is designed to recognize the parent hormone. The companies producing antibodies for use in RIAs/EIAs usually quantify and report cross-reactivity with common steroids that are similar to the target molecule but no such test can be carried out against a steroid that has not been identified. This problem can become critical when establishing an assay in a new species. For example, most antibodies against testosterone report a minimal cross-reactivity (a few percent) with its metabolite 5 α -dihydrotestosterone (5 α -DHT). A testosterone assay without previous separation from 5 α -DHT presumably provides reasonably accurate values because 5 α -DHT is present in low concentrations in most mammals but in birds another closely related metabolite, 5 β -DHT is present in very large concentrations and if an antibody against testosterone cross-reacts just by a few percent, this might provide a completely false evaluation of its concentration. We recently showed that 5 β -DHT is approximately 10 times more concentrated than testosterone in the brain of male Japanese quail (Liere et al., 2019). If there is just a small cross reactivity with 5 β -DHT of an antibody against testosterone, this might significantly distort the results but suppliers never report cross-reactivity with this steroid.

All these problems of specificity can be solved by purifying samples before assay and specificity can be evaluated by running validation tests. These validations include the assay of known amounts of the target steroid when placed in the biological medium considered ("spiking") or running a test for parallelism in which a sample is assayed at various dilutions that should in theory provide the same concentration per milliliter. This kind of validation used to be a pre-requisite for publication when RIAs first appeared but, unfortunately, these demands have more recently been relaxed when assay kits became broadly available. These validations should still be requested when an assay is established in a new species or for a new type of samples (e.g., brain vs. blood vs. feces).

The recent years have seen the emergence a completely new approach to steroid assay based on a sample purification by high performance liquid chromatograph (HPLC) or gas chromatography (GC) followed by steroid identification and quantification by mass spectrometry (MS) (Koren et al., 2012; Liere et al., 2000; Lopez-Rodriguez et al., 2016). On the positive side, these techniques identify steroids with a high degree of confidence, allow measuring dozens of steroids in the same sample and they are as sensitive if not more than RIAs. They require however an expensive equipment that is technically challenging to operate and they only allow measuring one sample at a time versus several hundred samples

in one single RIA. The combined use of RIA/EIA and mass spectrometry provides however unprecedented opportunities for investigation hormone-behavior relationships.

3. Identifying brain sites of action

3.1. The localization of receptors

In 1969, the brain sites of steroid action for the control of behavior were largely unknown. The availability of radioactive steroids led around that time to the development of procedures that provided within a decade a fairly precise view of where sex steroids bind in the brain. Almost simultaneously, Don Pfaff at the Rockefeller University in New York and Walther Stumpf at the University of North Carolina in Chapel Hill developed the *in vivo* autoradiography technique by which a live animal is injected with a bolus of tritiated steroid and its brain is collected about one hour later when most of the steroid has been cleared except if fixed to a specific high affinity receptor. The brain is then sectioned and an autoradiogram of the sections is prepared to identify sites that have accumulated radioactivity. Microscopic examination of these autoradiograms together with the underlying counterstained tissue then provides a cellular localization of binding sites that are presumed to be high affinity receptors.

This technique provided for the first time a precise localization of the brain binding sites for testosterone (Pfaff, 1968a; Sar and Stumpf, 1972) and estradiol (Pfaff, 1968b; Stumpf, 1970) in the rat brain and soon thereafter in a number of other vertebrate species including birds, reptiles, amphibians and fishes (Morrell et al., 1975). This eventually resulted in the mapping of sex steroid binding sites in the vertebrate brain and the realization that these sites are very well conserved among all classes of tetrapods ((Kelley and Pfaff, 1978; Morrell and Pfaff, 1978)). The densest populations of cells accumulating radioactive sex steroids are located mainly in the medial preoptic area, the bed nucleus of the stria terminalis, the amygdala, various nuclei of the medio-basal hypothalamus and parts of the optic tectum in the midbrain.

The technique was however not without problems. It was first necessary to inject large amounts of radioactivity into live animals, which created problems of waste disposal and was expensive, thus limiting the procedure to relatively small species. The procedure was also technically complex and tedious. It involved sectioning frozen brains on a cryostat in a dark room and placing brain sections directly on microscopic slides coated with photographic emulsion. The film then had to be exposed to the radioactive sections for several months if not years to produce an accurate representation of the binding sites. And last and not least, the chemical specificity of the receptors could not be determined by this approach. In particular, testosterone is readily aromatized in the brain into estrogens and their binding to estrogen receptors is not distinguished in this technique from the binding of testosterone to androgen receptors.

However, during the following years (late 1970s and early 1980s), sex steroid receptors were isolated and purified first from peripheral tissue and then identified in the brain (for review see: (Gorski and Gannon, 1976)). Purified receptors were then used to develop specific antibodies that could be used to localize these receptors at the cellular or even sub-cellular

level of anatomical resolution by immunocytochemistry. In general, immunocytochemical studies using mono- or polyclonal antibodies against the steroid receptors molecules confirmed the initial results obtained by *in vivo* autoradiography. Interestingly, the overall anatomical distribution of androgen-concentrating cells is similar to that of estrogen-concentrating cells. However, limited differences were observed in the intensity and the number of cells labeled as well as in their precise distribution within a given nucleus. A colocalization between both types of receptors was often identified but specific populations expressing only one of these receptor types have also been reported. These immunocytochemical techniques also solved the question of the subcellular localization of the receptors by showing that the vast majority of these proteins were present in the cell nucleus (Yamashita, 2001) even if a fraction of the receptors is located in the neuronal perikarya and in cell processes including dendrites, axons and presynaptic boutons (Blaustein, 1992; Blaustein et al., 1992; Blaustein and Turcotte, 1989).

When techniques for cDNA library screening/ cloning and DNA sequencing became routine in the 1980s, a first receptor cDNA encoding the glucocorticoid receptor was cloned by Evans and collaborators (Hollenberg et al., 1985). The cloning of cDNAs encoding receptors for estrogens (Green et al., 1986), progestins (Conneely et al., 1986), and androgens (Chang et al., 1988; Lubahn et al., 1988) followed soon thereafter. This information could then be used to establish *in situ* hybridization procedures allowing the visualization of the corresponding mRNA with a cellular level of anatomical resolution. The maps describing the localization of the mRNAs coding for androgens, estrogens and progestins receptors produced in this way were extremely similar and often identical to the maps derived from studies performed by immunohistochemistry or *in vivo* autoradiography. This similarity is intellectually rewarding given that the three technical approaches are based on completely different aspects of the steroid receptors: their binding affinity, the immunoreactivity of the protein and the corresponding mRNA. This example convinces me that the scientific approaches actually reveals aspects of the real world in a reliable manner.

One must finally notice that, since the initial studies described the evolutionarily conserved dense populations of sex steroid receptors, substantial increases in the sensitivity of the visualization methods have taken place. Better antibodies became available and more sensitive *in situ* hybridization procedures were developed. These new approaches demonstrated that sex steroid receptors expression is in fact no limited to the diencephalon and limbic system but a low level of expression is present in many other brain areas including large parts of the telencephalon and the hippocampus. These receptors, despite their low local concentration, are functionally relevant and mediate for example effects of estrogens on memory processes (Ervin et al., 2013; Frick, 2020; Luine, 2014).

3.2. Where are steroids acting to activate behaviors?

When *Hormones and Behavior* published its first issue, stereotaxic surgery was still in its infancy. The discovery of multiple binding sites of steroids in the brain begged the question of the specific role played by steroids at each of these locations. It seemed reasonable to assume that only a subset of the total number of binding sites would be implicated in the control of reproductive behaviors. These critical sites were over the years identified by a

combination of lesion experiments and of stereotaxic implantation of steroids directly in the brain of gonadectomized subjects.

The first stereotaxic implantations of steroids in the brain of live animals were performed by Robert D. Lisk (Lisk, 1960; Lisk, 1962) and Richard P. Michael (Michael, 1962) who placed estrogens in the diencephalon of rats and cats respectively and observed as a result increases in female sexual receptivity. This approach remained relatively uncommon during that decade and the very first issue of *Hormones and Behavior* actually published the first paper describing stereotaxic implantation of testosterone in an avian species, the domestic fowl (Barfield, 1969) followed soon afterwards by a similar study in ring doves (Barfield, 1971). At that time, stereotaxic devices were not readily available for studies in birds. Ron Barfield thus had to go see David Kopf who had recently started the Kopf instrument company that was among the first to produce high quality stereotaxic frames. Kopf asked for a few rooster heads and personally modified an existing stereotaxic device to be compatible with work in chickens (Ball and Balthazart, 2018).

In conjunction with stereotaxic lesion experiments and with tract-tracing studies analyzing the connections between brain areas, these approaches have over the years build a reasonably complete model of the relevant brain circuits that mediate steroid action on reproductive behaviors. In general, the medial part of the preoptic area and the ventromedial nucleus of the hypothalamus appear as critical and sufficient sites for the activation by steroids of respectively male and female sexual behaviors. Additional sites, however, are also implicated. For example, progesterone acts both in the ventromedial hypothalamus and in the ventral tegmental area to promote lordosis (Pleim et al., 1990). Androgen actions in parts of the limbic system such as the nucleus of the stria terminalis and the amygdala also plays a significant role in the activation of male sexual behavior (Newman, 1999). Extensive overviews of this circuitry mediating male and female sexual behavior have been published (Hull and Dominguez, 2015, 2019; Pfaus et al., 2015).

4. Relating genetic information to behavior

The last quarter of the 20th century also saw an unprecedented development of techniques that greatly facilitated manipulations of nucleic acids (DNA and RNA) and analysis of their implication in the control of behaviors. Key landmarks only will be mentioned here.

The structure of DNA was identified in 1953 by James Watson and Francis Crick (Watson and Crick, 1953) but it is only in 1977 that Frederick Sanger (Nobel Prize in 1980) developed the “dideoxynucleotide” chain-termination method that provided a reasonably easy way to determine the order of occurrence of the 4 nucleic acids that constitute a molecule of DNA (Sanger et al., 1977). However, this approach allowed sequencing only short stretches of DNA in a single gel chromatography (less than one kilobase in length) so that sequencing a long chain of DNA required sequencing multiple overlapping DNA fragments, which was time consuming. Since that time, second- and third-generation sequencing methods have been developed and these methods are by far much easier, faster and cheaper (Heather and Chain, 2016; Shendure et al., 2017). This technical progression continues to this date: it is namely attested by the contrast in the long-lasting effort (about 13

years) that was required to produce the first human genome in 2001 (Lander et al., 2001; Venter et al., 2001) with the current sequencing facilities that can read a similar genome in less than one day for a much reduced price (about 300 millions to one billion spent over 13 years versus approximately one thousand US dollars; Figure 2 for a graph illustrating the rapid decrease in the cost of sequencing).

The increasing availability of these techniques has permitted research programs that were absolutely unthinkable in 1969. As an example, the sequencing of 48 avian species representing all major extant clades has provided an enormous amount of information about the evolution and adaptation to different life styles (Zhang et al., 2014), including for example the history of the beak development (Meredith et al., 2014) or of the acquisition of song learning (Pfenning et al., 2014)... and more ambitious programs are ongoing!

Multiple techniques were also developed during the last five decades to analyze and quantify the expression of genes in individual subjects and correlate this expression with behavior. Following the identification of a DNA polymerase isolated from the bacteria *Thermus aquaticus* that retains its enzymatic activity at temperatures above 75°C (Chien et al., 1976), Kary Mullis after multiple attempts identified a procedure called polymerase chain reaction (PCR) that can almost indefinitely amplify a specific nuclei acid segment (either DNA or RNA) to produce quantities that can then easily be sequenced and characterized (Mullis et al., 1986). Mullis was awarded the Nobel Prize in 1993 for this discovery that changed the world of molecular biology.

By monitoring “on line” the amplification of the target transcript, this method can also be made quantitative. The technique is then called real-time polymerase chain reaction or quantitative polymerase chain reaction (qPCR)(Logan et al., 2009). The PCR products are here quantified in real time by following either fluorescent dyes that intercalate in any double-stranded DNA or fluorescent reporter probes detecting only the DNA containing the sequence complementary to the probe. The method is highly specific and accurate provided a number of basic controls are performed including namely a verification of the amplification efficiency and an analysis of the melting curve and eventually sequencing of amplified products to make sure they correspond to the target transcript (Bustin et al., 2009). These validations are unfortunately sometimes lacking in the published papers.

Microarray techniques then appeared by which purified RNA is first reverse-transcribed, eventually amplified by PCR, and then hybridized to microscopic spots of DNA (probes) attached to a plate. The hybridized products are detected and quantified by detection of a fluorophore or by chemiluminescence. This approach has been used broadly to measure the expression level of a large number of genes at the same time. Its weakness is of course that it can only measure genes for which probes have been spotted on the plate. Other problems of this approach include cross-hybridization artifacts and poor quantification of genes expressed at low levels. Arrays were initially developed for mice and rats but later also produced for less common model species such as the zebra finch (Replogle et al., 2008).

The progress in next-generation sequencing, however, led to the progressive replacement of microarrays by the RNA-Seq technology (Weber, 2015) that is based on the complete

sequencing and quantification of the whole transcriptome. In this approach, the entire set of RNA is reverse-transcribed, amplified and then sequenced. The great advantage is that this technique makes no a priori selection of the transcripts that will be quantified (represented by probes on the microarray plate) and does not require the pre-existence of a sequenced genome. In addition the dynamic range of RNA-Seq is twice as large as in microarrays and this allows accurate quantification of transcripts present in low abundance among populations of very abundant transcripts. This technique also identifies sequence variants (both splice variants and single nucleotide polymorphism), small and micro RNAs and transfer RNA (Wang et al., 2009).

Together these tools stimulated significant progress in behavioral neuroendocrinology. For example, it was realized in 1996 that there are in fact two separate estrogen receptors (ER) resulting from the transcription of two different genes. A second type of ER cDNA was cloned in rats (Kuiper et al., 1997) and soon after shown to be present in humans (Mosselman et al., 1996) and many other species including birds (Foidart et al., 1999). The discovery of this new receptor labeled ER beta (ER β) generated a lot of excitement (Kuiper et al., 1998) since it revealed new ways in which estrogens can affect behavior and it could explain some of the paradoxical effects of estrogens (Paech et al., 1997) and their presence in tissues that were apparently devoid of the previously known ER now renamed ER α . *In situ* hybridization indeed demonstrated that ER β was expressed in some brain regions also expressing ER α but also in other areas and tissues (Bernard et al., 1999; Shughrue et al., 1997).

Molecular biology also revealed the existence of multiple isoforms of essentially all steroid receptors (Keightley, 1998) and functional studies often revealed that even though they are derived from the same gene, these proteins have different neuroanatomical distributions, different affinities for their ligand and therefore they play different physiological roles. In the context of behavior control, it was for example demonstrated that the two isoforms of the progesterone receptor, PR-A and PR-B display differential responses to progesterone and to the dopamine agonists SKF81297 and consequently play a differential role in the activation of hormone-dependent and hormone-independent sexual receptivity (Mani et al., 2006)

5. Manipulating gene expression and action: the ascent of pharmacology and its partial replacement by molecular biology techniques

With the exception of the stereotaxic procedures described in 3.2, the techniques discussed so far only provided a detailed description of the endocrine system but they were not directly addressing its causal significance. By combining them with the castration-replacement procedure that predates the period we are discussing here, it was possible to obtain a first degree of causal analysis by analyzing which hormone is implicated in the control of a given behavior but this analysis remained relatively crude. Tools were therefore needed and were progressively developed to investigate in finer grain the causal implications of hormones in behavior activation.

In a first step these tools were of pharmacological nature. Due to the implication of sex steroids in a number of clinical conditions (e.g., the trophic role of estrogens and androgens

in the growth of some tumors), many pharmaceutical companies initiated extensive research programs to identify compounds that could selectively block the production or action of these steroids in humans. Behavioral endocrinology indirectly benefited from this research.

As an example, I was for about a decade in contact with researchers in a Belgian Pharmaceutical company, Janssen Pharmaceutica, which was, like several other companies around the world, trying to design a potent aromatase inhibitor that could be used to block or at least slow-down the progression of estrogen-dependent tumors. They eventually identified in the late 1980s a very effective compound that had little or no side effect, known at that time by its internal name R76713 (Wouters et al., 1989). They quickly realized that R76713 contained two isomers of which one only was active, the R83842, now nicknamed Vorozole (Wouters et al., 1990; Wouters et al., 1994). While they were still in clinical trial, they made these compounds available to my laboratory and to others so that it became possible to better characterize the organizational and activational roles of estrogens in our favorite model species, the Japanese quail (Balthazart et al., 1990; Foidart et al., 1992) and in others (e.g., (Rissman et al., 1996)). For the anecdote, some of the other companies that were also developing similar compounds (anastrozole or Arimidex™, exemestane or Aromasine™, formestane or Lentaron™, letrozole) successfully completed clinical trials before Janssen Pharmaceutica and started selling their products on the market. As a consequence, Janssen stopped its research program that had been ongoing for several decades. It should be noted that the internal name of the compounds at Janssen (R76713) reflected the number of different molecules they developed and tested before identifying those that were effective. This gives an idea of the cost of pharmaceutical research !

In a similar vein, the discovery of two ERs discussed in section 4 stimulated research to develop agonists and antagonists that would selectively interact with these receptors (selective estrogen receptors modulators or SERM). The DNA binding domains of ER α and ER β are highly homologous but there is only about 60% similarity in the ligand-binding domains, suggesting that specific ligands could be identified that would preferentially bind with one or the other ER (Enmark et al., 1997). Two compounds were indeed identified, propyl-pyrazole-triol (PPT) and diarylpropionitrile (DPN) that bind and activate in a relatively selective manner the ER α and ER β respectively (Malamas et al., 2004; Manas et al., 2004). PPT is more than 400 times more selective for ER α than for ER β but conversely DPN only shows a 70-fold higher selectivity for ER β than for ER α . These differences allow meaningful comparisons *in vitro* but when tested *in vivo*, it is sometimes difficult to make sure that one receptor type only has been activated. Since the two agonists might have different pharmacokinetics, the relative concentrations at the receptor might be substantially different from the relative doses that have been injected (see discussion in (Seredynski et al., 2011; Seredynski et al., 2015)).

A tendency therefore developed to switch to alternative approaches based on molecular biology. A technique allowing to specifically inactivate expression of a specific gene in mice was initially established in 1989 (Capecchi, 1989) and soon knock-out (KO) mice were developed for various genes of interest in the field of reproduction and behavioral endocrinology such as ER α (Lubahn et al., 1993) and ER β (Krege et al., 1998) or aromatase (Fisher et al., 1998; Honda et al., 1998). This strategy could not be used for the

androgen receptor since it is located on the X chromosome and it is critical for male fertility. These new mouse models rapidly caught the attention of behavioral neuroendocrinologists and their behavioral phenotype was characterized in detail (e.g., (Bakker et al., 2002; Ogawa et al., 1999; Ogawa et al., 1997; Rissman et al., 1997)).

However, this approach also had a number of practical problems. By observing differences between “normal” (wild-type) and KO mice it is possible to infer the function of the inactivated gene but the reverse manipulation (function recovery) is often impossible except in cases such as the enzyme aromatase where the lack of endogenous estrogens can be compensated by the delivery of exogenous hormone (Bakker et al., 2004). Importantly, for a long time and for practical reasons KO mice were developed using embryonic stem (ES) cells from the 129 inbred strain of mice and this specific strain is peculiar. For example they often lack a corpus callosum (Wahlsten, 1982), which makes them unsuitable for some types of experiments including behavior. It is therefore usual to backcross KO mice into another strain but the subsistence of 129 genes around the knockout segment can represent a confounding factor, the flanking-gene effect. Methods have been presented to control for this problem (Crusio et al., 2009; Wolfer et al., 2002) but... they are not systematically (rarely?) implemented! Note however that ES cells derived from the C57BL/6 inbred strains are now available that allow easy generation of KO in C57BL/6 background (Pettitt et al., 2009).

More importantly KO mice produced in this original way lack expression of the target gene throughout development and adulthood. As far as steroid action is concerned, it is therefore impossible to distinguish between lasting developmental (organizational) and acute (activational) effects. Furthermore the absence of a protein during development can activate compensatory mechanisms. If was for example shown that mice in which the steroid receptor coactivator 1 has been inactivated (SRC1-KO) only have a limited phenotype despite the well-established role of this protein (Apostolakis et al., 2002; Auger et al., 2000; Molenda et al., 2002), because they compensate during ontogeny by over-expressing the related SRC2 and SRC3 (Apostolakis et al., 2002; Nishihara et al., 2003; Xu et al., 1998).

The solution to this last problem came with the advent of conditional knock-outs in which the DNA modification that will block expression of the target gene is present from the start of embryonic development but is only activated after the subject has been exposed to a specific chemical stimulus. The most common technique used to achieve this goal is based on the Cre-Lox recombination system in which an enzyme, the Cre recombinase (Cre for “causes recombination”), specifically recognizes two *loxP* loci flanking a given gene and causes recombination (i.e., deletion or inversion) of the corresponding DNA segment (Sauer and Henderson, 1988). This can be achieved by crossing a mouse carrying a floxed gene with a mouse bearing a copy of the Cre recombinase. Expression of the target gene will be suppressed in the offspring. This suppression by recombination of the target gene can be temporally controlled by either regulating the expression of the Cre recombinase with a transcriptional activator (via injections of the antibiotic tetracyclin) or by restraining Cre transport to the nucleus, where it is required for the recombination event, thanks to a ligand-binding domain fused to the Cre (via injections of the estrogen receptor antagonist tamoxifen, a compound that is not produced by the organism and will bind to the estrogen receptor associated with Cre) (Zhang et al., 2012). Note that this last method of induction

could be problematic in behavioral endocrinology studies if the dependent variable of interest is sensitive to estrogens and needs to be measured relatively soon after the tamoxifen injection. A reporter gene (e.g., Green Fluorescent protein, GFP) can additionally be added downstream from the stop codon of the target gene. It will be transcribed only when the stop codon has been removed and will in this way specifically mark all cells bearing the gene deletion.

It has also appeared that deleting expression of one gene in the entire organism could lead to problems of specificity and interpretation. It thus became desirable to suppress gene expression in a specific organ, tissue or cell type. This spatial control (i.e., targeted deletion in specific tissue or cell type) can be obtained by using a tissue/cell specific promoter driving Cre expression (Figure 3). For example, placing Cre expression under the control of nestin or synapsin will specifically limit expression of the Cre enzyme and thus the gene deletion to the brain parts. Cre expression under pro-opiomelanocortin (POMC) control will limit deletion of a floxed gene to the POMC neurons. There are several pitfalls in these powerful and sophisticated techniques. Numerous publications have for example explained the need for assessment of artifactual effects of the Cre-lox system but “*a wealth of literature continues to be generated in which these controls are omitted*” (Harno et al., 2013).

A host of variations can be introduced in these procedures including the general deletion of a gene and its replacement in a tissue-specific manner by the original gene or by a modified version of this gene (the Knock-In technique)(Gibson and Muse, 2009) but these go beyond this short historical overview. We now have a variety of mice models that allow addressing very specific questions in behavioral neuroendocrinology such as inducible knock-outs for the androgen receptor (Yeh et al., 2002) including a conditional brain specific knock-out (Raskin et al., 2009), mice specifically lacking ER β in the central nervous system (Naule et al., 2016) or ER α -KO mice in which the native receptor has been replaced by a mutated receptor that cannot bind to the DNA but retains activity in ERE-independent pathways (McDevitt et al., 2008) or by an ER α that cannot be translocated to the cell membrane due to the mutation of the palmitoylation site so that signaling at the membrane is abolished (Adlanmerini et al., 2014).

It should also be pointed out that the establishment of the CRISPR-Cas9 technique has in the last few years greatly facilitated modifications of the genome in a simple, targeted and efficient manner (Doudna and Charpentier, 2014). This has opened the door to the development of knock-out models in any species while this technology was so far restricted almost exclusively to mice. Although the main pitfalls of this technology are the “off-site effects”, i.e. introduction of mutations at non-specific loci that are difficult to predict and identify, it will obviously lead to major advances in all fields of biological and biomedical research including behavioral endocrinology.

6. Manipulating neuronal activity

Most of the manipulations described in section 5 have long-term if not permanent effects. To study the brain mechanisms controlling behavior it was additionally desirable to design techniques to transiently increase or inhibit the activity of neurons, and if possible specific

sub-types of neurons. This goal that looked nearly impossible at the end of last century has now been achieved by at least three completely different approaches.

A variety of RNA-based transient gene knock-down techniques have been developed in which complementary or modified RNA sequences including for example anti-sense RNA (asRNA), small/short interfering RNA (siRNA), morpholinos,.... are injected directly into brain targets where, to the surprise of the first researchers who tested the technique, they are taken up by neurons and then temporarily decrease expression of a target gene (McCarthy et al., 1993a). The technique has for example been used to block brain masculinization by injection of an antisense to the estrogen receptor (McCarthy et al., 1993b) or demonstrate the role of SRC-1 in the sexual differentiation (Auger et al., 2000) and activation of female sexual behavior in rats (Molenda et al., 2002) and male sexual behavior in quail (Charlier et al., 2005).

Sophisticated tools combining chemical engineering and genetic (chemogenetic) were also developed including the Designer Receptor Exclusively Activated by Designer Drugs (DREADD) that permit temporally and anatomically specific control of neuronal activity via activation of an artificial G-protein-coupled receptor (GPCR) (Armbruster et al., 2007; Dobrzanski and Kossut, 2017; Roth, 2016). The technique is based on an artificial GPCR engineered to respond exclusively to a synthetic ligand that is not physiologically present in an organism. Clozapine N-oxide (CNO) was the prototypical DREADD activator but many other modified GPCR and ligands have since been developed that can stimulate or inhibit neuronal activity (Roth, 2016).

Expression of the DREADD can be induced in the whole brain or in specific cell types by the knock-in technique described in the previous section but these receptors can also be added to adult brains using viral vectors (transgenic vs. viral approach). Sophisticated virus vectors have been designed in which expression of the DREADD either can be allowed in the entire brain area where the injection took place or can be conditioned by the activation of a specific promoter (only in neurons, only in excitatory neurons, ...) or can be dependent on the presence of Cre recombinase itself possibly under the control of a specific promoter, or can be targeted to a specific location such as synapses (Campbell and Marchant, 2018; Dobrzanski and Kossut, 2017). The expression of the DREADD can also be associated with expression of a fluorescent (Green Fluorescent Protein, mCherry, Tomato, ...) or enzymatic (β -galactosidase) marker so that post mortem histology can detect which cells were expressing the receptor during the experiment.

This technique creates incredible experimental opportunities but is not without caveats. One of them is that a small fraction of the administered CNO can potentially be metabolized to clozapine, an antipsychotic drug that affects the activity of multiple endogenous receptors including 5-HT, dopamine and histamine receptors (Mahler and Aston-Jones, 2018). If clozapine ends up being present in sufficiently high concentrations, behavioral effects can thus be induced by injection of CNO even in subjects devoid of the DREADD. Proper controls are therefore essential such as comparing effects of the DREADD agonist in DREADD-expressing versus non-DREADD-expressing subjects and using the lowest possible doses of DREADD agonist (CNO).

Similarly, the development of optogenetics has opened the door to a real-time control of neuronal activity even in freely-moving animals (Boyden et al., 2005; Deisseroth et al., 2006). The approach is similar to chemogenetics but in this case, a light-sensitive channel is incorporated into neurons, again either by genetic manipulations or by viral transfection. The different forms of viral transfection that are described in the previous paragraphs for DREADD can of course also be implemented to transfect a light-sensitive channel into specific cells or synapses and provide great experimental versatility.

The prototypical protein used these studies in channelrhodopsin-2 that will depolarize (activate) a neuron when exposed to a light of specific wavelength but channels that hyperpolarize neurons have also been designed (e.g., halorhodopsin) so that it is currently possible to increase or decrease electrical activity of specific neurons with a millisecond scale temporal precision. To achieve this goal, a discrete illumination with a selected wavelength must be applied with an adequate periodicity to the transfected neurons ideally located with a specific brain nucleus. This is usually achieved by implanting an optic fiber deep into the brain just dorsal to the target area and delivering at high frequency (2–20 Hz) pulses of light produced by light-emitting diodes (LED) or diode-pumped solid state (DPSS) light sources connected to a computer. This will produce synchronized periodic discharges in neurons expressing channelrhodopsin or conversely inhibit spontaneous firing activity in neurons expressing halorhodopsin (Aravanis et al., 2007). Wireless systems of light stimulation have even been developed (Wentz et al., 2011).

More recently, optogenetic tools have even been created that allow manipulation in behaving animals of the concentration of specific intracellular messengers such as cyclic AMP or IP3 via illumination of a transfected opsin fused to a specific GPCR (Airan et al., 2009). There is thus a vast repertoire of emerging optogenetic techniques that now allow temporally precise and cell-type-specific control of many aspects of neuronal activity and correlatively behavior.

This brief presentation only concerns the most commonly used chemogenetic and optogenetic techniques and obviously ignores in the interest of space all the variations and refinements that have been introduced into these approaches during the last decade. This has already allowed to this date the development of truly amazing research programs. For example, a combination of neurons silencing by DREADD and optogenetic neuronal activation has clearly demonstrated the key and specific role of aromatase-expressing neurons in the bed nucleus of the stria terminalis in the control of sex recognition in male mice (Bayless et al., 2019). The association of genetic knock-out, viral tracing, viral ablation with the use of an adeno-associated virus expressing caspase 3 and optogenetic stimulation demonstrated that separate neuronal populations of kisspeptin neurons control the olfactory-driven mate preference and the copulatory behavior in female mice (Hellier et al., 2018).

Very recently researchers even succeeded in implanting “artificial memories” in the brain by repeated optogenetic stimulation. In young male zebra finches that had not yet been exposed to the song of a tutor, they injected a virus expressing channelrhodopsin-2 into the song control nucleus Nif (nucleus interfascialis of the nidopallium) that is at the interface between the sensory and motor processes mediating vocal learning and production. The young birds

were then implanted with an optic fiber directly on the Nif to HVC synapses and they were exposed during a few days to pulses of light that had durations either shorter or longer than a typical song syllable. When adults, the song of these birds reflected the duration of the light pulses they had been exposed to: birds exposed to short duration pulses produced songs with shorter than usual song elements and vice-versa (Zhao et al., 2019).

All these techniques alone or in combination thus allow specific manipulations of defined neuronal populations at various time scales ranging from the millisecond to the entire life of the subject. The sky appears to be the limit at this point!

7. Computers and big data

One should also mention in finishing that many of these technical developments were made possible by the parallel development of computers and sophisticated electronic equipment. In 1969, calculations had to be made by hand or with a slide ruler! The only alternative was the use of unwieldy mainframe computers using punched cards for data acquisition. The appearance in the 1970s of hand-held calculators and soon thereafter of personal computers and the internet created new facilities to rapidly analyze data, prepare manuscripts for publication, communicate with colleagues and access bibliographic databases. This computing power not only facilitated basically all aspects of scientific research but it also made feasible investigations that would have otherwise been impossible. Sequencing facilities and RNA-Seq programs now generate billions of data points in just a few days and their analysis by hand would of course not be possible. Computers are also associated with more in depth recording and analyses of behavior by allowing the recording at the same time of multiple aspects of the behavior of a subject via automated devices eventually using the rapidly developing tools of artificial intelligence. Many studies also now include complex experimental plans and the statistical analysis of their results requires sophisticated statistical techniques that can only be performed with computers.

8. Conclusions

The last 50 years have seen the development of incredibly powerful techniques that have permitted addressing questions in behavioral (neuro)endocrinology at a level that clearly sounded like science fiction when *Hormones and Behavior* published its first issue. Some of these approaches are still relatively recent and have obviously not yet been fully exploited. Our understanding of the endocrine controls of behavior has nevertheless been markedly expanded during this period and new knowledge has been accumulating at a constantly increasing speed.

This technical transformation of the field has obviously been very beneficial for Science in general but it did also come with some negative consequences. The facilitation of scientific investigations, data analysis and manuscript preparation has indirectly generated an obsession for speed that came with a relaxation of methodological exigency. RIAs and EIAs are performed with kits that have not been validated for the type of samples concerned, immunohistochemistry uses antibodies with poorly characterized specificity (Saper, 2005; Saper and Sawchenko, 2003) and complex gene knock-out experiments, chemogenetic or

optogenetic analyses are used in the absence of key control procedures. Multiple statistical approaches can quickly be used to analyze a same set of data, which generates the temptation of p-hacking, i.e., manipulation of data in a way that produces a desired (low) p value (Raj et al., 2018; Ulrich and Miller, 2015). The easy access to the entire literature has also generated a fascination for rankings and for publication in high impact factor journals of perfect, often underpowered, stories (Burt et al., 2017; Button et al., 2013) that sometimes represent selections of positive data ignoring the many failed attempts, which creates a false impression of the current state of knowledge (Brembs et al., 2013).

Taken together, these problems have generated what has been called a reproducibility crisis, i.e. the realization that a substantial part of the published results cannot be independently reproduced (Ioannidis, 2005; Munafo et al., 2017). Multiple solutions have been proposed including changes in the way data are reported in publications and *Hormones and Behavior* has enforced some of these recommendations. They include the publication of the effect size of differences in addition to the associated probabilities, presentation of individual data points in graphs and more detailed description of experimental methods (Ioannidis et al., 2014). But quite fortunately, the scientific endeavor is a self-correcting process; the problems have been identified and corrections have been proposed. The next decades will obviously be filled with exciting discoveries in behavioral neuroendocrinology.

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Highlights

- Most of the current techniques in behavioral endocrinology did not exist in 1969
- Hormones and gene expression can now be quantified in small biological samples
- The activity of specific neurons can also be manipulated at various time scales
- These new techniques are briefly presented together with their potential limitations
- These techniques should still lead to exciting discoveries in neuroendocrinology

Figure 1. Schematic illustration of the tests for specificity in radioimmunoassays.

The calibration (standard) curve for the assay illustrated with back dots shows the decrease in binding (B) of the labeled hormone as the concentration of unlabeled hormone (X axis) increases from 1 pg to 10 ng/tube. Binding in the absence of unlabeled hormone (B_0) is set at 100%. The parallel curve with open diamonds refers to another hormone that specifically cross-reacts with the target hormone (parallel inhibition of binding) while the curve with open triangles shows a non-specific non-parallel inhibition of binding by a contaminating compound (fat, detergent). The open circles illustrate the test for specificity performed by assaying the target hormone at increasing dilutions of a sample of the biological material. This dilution curve should be parallel to the standard curve or in other terms the concentration per unit volume should be independent of the volume used for the assay. This type of control test was a standard procedure when RIAs were first introduced (e.g., Brian Follett used to assay each sample for luteinizing hormone in duplicate at 2 or 3 dilutions (Follett et al., 1972; Gibson et al., 1975), which might be considered as excessively cautious) but this control tended to disappear over time while it would clearly be needed at least each time a new assay is applied to a new kind of sample.

Figure 2. Illustration of the decrease in the cost of sequencing during the last 20 years. The cost of the first sequencing of the human genome was over 100 millions US dollars while the same work can now be performed for about one thousand \$. Redrawn from data in <https://www.genome.gov/about-genomics/fact-sheets/Sequencing-Human-Genome-cost>

Figure 3. Diagram illustrating how to generate a conditional knock-out mouse.

In this example, a mouse containing the Cre gene under the control of a tissue-specific promoter is bred with a mouse containing the target gene inserted between two loxP sites. In the F1 generation, Cre will be expressed in the target tissue only and will induce recombination of the floxed target gene. This will at the same time remove a stop codon leading to the expression of a reporter gene, here the green fluorescent protein, that will allow identification of specific cells where the target gene is no longer expressed. In other tissues, Cre will not be expressed and expression of the target gene will therefore be normal.