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New concepts in the study of the sexual differentiation and activation of reproductive behavior, a personal view

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ABSTRACT

Since the beginning of this century, research methods in neuroendocrinology enjoyed extensive refinements and innovation. These advances allowed collection of huge amounts of new data and the development of new ideas but have not led to this point, with a few exceptions, to the development of new conceptual advances. Conceptual advances that took place largely resulted from the ingenious insights of several investigators. I summarize here some of these new ideas as they relate to the sexual differentiation and activation by sex steroids of reproductive behaviors and I discuss how our research contributed to the general picture. This selective review clearly demonstrates the importance of conceptual changes that have taken place in this field since beginning of the 21st century. The recent technological advances suggest that our understanding of hormones, brain and behavior relationships will continue to improve in a very fundamental manner over the coming years.

1. Introduction

Purified, and shortly after synthetic, sex steroids became available for experimentation during the first half of the 20th century (Gallagher and Koch, 1929; David et al., 1935; Ruzicka and Wettstein, 1935; Butenandt and Hanisch, 1935). This initiated an intense research effort to understand the endocrine controls of various physiological and behavioral phenomena. At the behavioral level, a first synthesis of available evidence was produced by Frank Beach in 1948 already (Beach, 1948). This research endeavor quickly led to major progress in our understanding of how sex steroids control the expression of reproductive behavior (Balthazart et al., 2018). One decade later, the seminal paper of Phoenix and collaborators identified the basic notion of the early organizational effects of testosterone on copulatory behavior (Phoenix et al., 1959) and this again generated an entire field of research aimed at deciphering the mechanisms of sexual differentiation of brain and behavior. Thousands of papers were published during the second half of the 20th century dealing with endocrine controls of behavior (more than 40,000 papers retrieved in PubMed with the search terms "Hormones" AND "Behavior") that were nicely synthesized in the first edition of Hormones, Brain and Behavior, a 5 volume book gathering reviews on all topics in this field (Pfaff et al., 2002). One could therefore have imagined that by the end of the century all major concepts in behavioral neuroendocrinology had been formulated and extensively documented. Nothing is however more remote from the reality.

The "Torino meeting" on Steroids and the Nervous system was launched in 2001 and celebrated in February 2019 its 10th issue. We experienced during these 10 meetings major conceptual shifts and witnessed the identification of completely novel mechanisms of behavior control by steroids. This review has therefore two goals. First, I will briefly introduce these new concepts that have appeared since the beginning of the 21st century coinciding with the first Torino meeting and were extensively documented during these scientific presentations. In parallel, I will summarize how the work in my laboratory at the University of Liege has contributed to these new developments. I will focus exclusively in this presentation on changes that concerned the mechanisms underlying the sexual differentiation and the activation by sex steroids of reproductive behaviors ignoring similar ideas concerning purely neuroendocrine studies that will be covered by other contributions.

2. Sexual differentiation of behaviors

During the first half of the 20th century it was broadly believed that males and females were displaying different types of sexual behaviors largely because they were exposed as adults to different steroid hormones, essentially testosterone (T) in males and estradiol (E2) associated or not with progesterone (P) in females. A few studies had however demonstrated that estrogens are able to activate male-typical

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copulatory behaviors in males, a finding that was inconsistent with this general theory (Södersten, 2012; Sodersten, 2015). In 1959, Phoenix, Goy, Gerall and Young provided an alternative explanation for the behavioral sex differences by showing that early actions of testosterone modify in an enduring manner the responsiveness of the brain to the activating effects of sex steroids. This paper and the numerous studies that followed rapidly gave rise to the concept of organizational effects of sex steroids according to which the early exposure to T masculinizes and defeminizes in a permanent manner the developing brain in mammals. Brains of genetic males that are exposed in early life to T, or its metabolite E2, acquire a high degree of responsiveness to the activation effects of adult T on male-typical copulatory behavior and lose the ability to display the lordosis behavior in response to E2 ± P. In this model, females would spontaneously (in the absence of any hormonal stimulation) develop the ability to express lordosis in response to E2 ± P and would never acquire the capacity to display male copulatory behavior (for synthesis see (Gerall et al., 1992)). The females could therefore be considered as the neutral or default sex developing without hormonal stimulation. According to this model the male and female-typical behavior would thus develop under the influence of mechanisms similar to those that had been postulated for the development of sexually differentiated morphological structures such as the reproductive tract, penis or syrinx of ducks (Jost et al., 1973; Jost, 1978).

Research in a few species of birds focusing mainly on Japanese quail (*Coturnix japonica*) later confirmed the existence of similar organizing effects of sex steroids on the brain and behavior but indicated that the differentiation of copulatory behavior in birds is controlled by mechanisms that are a mirror image of mechanisms described in mammals (Adkins, 1975, 1978; Balthazart and Adkins-Regan, 2002). Specifically in quail it is the male-typical behavior that develops in the absence of any hormonal influence (the neutral sex) and the ability to display this behavior is lost in females under the early influence of ovarian estrogens (Adkins-Regan, 1985b, 1985a; Balthazart and Ball, 1995). These notions essentially remain true but they have been expanded and refined at several levels. Three main groups of concepts have emerged during the last 20 years.

2.1. Neuro-endo-immunology

Research in the second half of the 20th century had convincingly demonstrated that T can be metabolized into E2 in the brain (Naftolin et al., 1975; Balthazart and Ball, 2013) and that a large part of the organizing effects of T on brain and behavior is due to E2 at the cellular level (McEwen et al., 1977; McCarthy, 2008). The sexual differentiation of sex behavior is taking place in parallel with changes in the neural circuits mediating this behavior, prominently including modifications of the organization of the preoptic area (POA) by selective apoptosis, reorganization of connectivity and modifications of synaptic and dendritic organization (McCarthy et al., 2010). The downstream events leading from an exposure to E2 to the brain and behavioral changes were however completely unknown until recently.

In 2002–2004, research in the laboratory of M.M. McCarthy demonstrated that the masculinization by E2 of the behavioral and preoptic anatomical sex differences in perinatal females is mimicked by a single administration of prostaglandin-E2 (PGE2). Conversely pharmacological inhibition of cyclo-oxygenase-2 (COX-2), the PGE2 synthesizing enzyme, by injection of indomethacin inhibits the masculinization of male-typical behavior and of the POA physiologically induced in males by the testicular androgen and its estrogenic metabolite (Amateau and McCarthy, 2002, 2004). Since inflammatory episodes resulting in increased PGE2 brain concentrations or absorption of aspirin and similar drugs that are known inhibitors of the COX enzymes are common events in humans, this discovery rightly raised serious concerns related to human sexual differentiation, concerns that have not been properly addressed to this date.

This set of studies involving the immune system in the process of steroid-induced sexual differentiation was only the start of an extensive quest that has over the last 15 years identified neuroanatomically-specific mechanisms underlying the sexual differentiation of different brain areas. These studies have initially implicated the microglia in these processes (Lenz et al., 2013; Lenz and McCarthy, 2015; VanRyzin et al., 2018) and more recently the brain mast cells (Lenz et al., 2018). This research program firmly establishing the interactions between the immune and the (neuro)endocrine systems in the control of sexual differentiation in rats and mice will be described in another article of this issue (see (McCarthy et al., 2017), McCarthy et al., 2019 this issue). It would now be important to determine whether similar mechanisms are present in other mammalian species (including humans) and in non-mammalian vertebrates. Ongoing research in Liege suggests that microglia could also be a player in the control of sexual differentiation in quail (see Section 2.4.2).

2.2. "Direct" genetic effects

The chain of events leading to the adult male or female phenotype in mammals was broadly accepted at the end of the 20th century. This model initially developed based on studies of sexually differentiated morphological traits (Jost, 1978) was extended to the controls of sexual behavior by a host of studies that followed the seminal paper of Phoenix and collaborators (Phoenix et al., 1959). According to this model, the chromosomal sex (XX or XY) determines the gonadal sex via the sry gene present on the Y chromosome leading, with other contributing genes, to testicular formation. The embryonic or neonatal testes are then secreting T that is directly, or via its transformation into E2, responsible of the development of sexually differentiated morphological, physiological or behavioral traits. There is thus a chain leading from the genetic to the gonadal to the hormonal to the phenotypic sex.

A similar scenario was also shown to take place in birds even if the genes of the sex chromosomes were until recently not identified. Recent research now indicates that a dosage effect of the DMRT1 gene located on the Z chromosome is responsible in chickens for the development of the undifferentiated gonadal anlage into a testis or an ovary (one copy of the gene in females, two in males) (Smith et al., 2009; Ayers et al., 2013).

In Japanese quail (*Coturnix japonica*), the most studied avian species in this context, after the gonadal differentiation the differential exposure to steroids during the early phases of life determines, like in mammals, the phenotypic morphological and behavioral sex. The underlying mechanisms are however a mirror image of what happens in rodents since the male appears to be the behavioral sex developing in absence of endocrine influence while ovarian estrogens demasculinize male-typical copulatory behavior in females (Balthazart and Adkins-Regan, 2002; Balthazart et al., 2017). By the beginning of the 21st century, it thus seemed that the story was complete and the process of sexual differentiation fully understood in birds and mammals at least at the endocrine level. Additional work "just" had to identify the intracellular mechanisms mediating the so-called organizing effects of steroids (see previous section).

The study of a very unusual songbird was going to shake these convictions. In the early 2000, the laboratory of Art Arnold at UCLA published a study investigating a zebra finch (*Taeniopygia gutata*) that was half male and half female, a condition called gynandromorphism. This bird showed the typical male plumage including the red cheek and the black and white stripes on the right side of the breast but an absence of these features as normally observed in females on the left side (Agate et al., 2003). This bird was singing and courting females although he never succeeded in fertilizing the eggs produced by these females. He showed at autopsy a dysfunctional testis on the right side but ovarian tissue on the left side. *In situ* hybridization revealed that the male/female difference also concerned the entire brain tissue. The gene ASW that is located on the W sex chromosome of females (females have ZW

while males have ZZ sex chromosomes in birds) was expressed exclusively on the left side of the brain. Correlatively, the song control nucleus HVC was 80% larger on the male right side of the brain than on the female left side, an asymmetry that had never been observed in control zebra finches. Expression of the androgen receptor was also more abundant on the male side (Agate et al., 2003).

Because both sides of the brain had obviously been exposed to similar concentrations of gonadal sex steroids during the entire life of the bird (blood passing through the left and right gonads is mixed in the heart), the existence of this asymmetry clearly indicated that genetic factors exert substantial effects on the brain phenotype that are not mediated by a differential exposure to gonadal steroids. A differential expression of genes in the male and female brain sides was thus presumably responsible in a more of less direct manner for the asymmetric phenotype. This did not exclude, however, an action of steroids. Genes could indeed differentially control the synthesis of steroids by the two brain sides or induce a differential expression of sex steroid receptors that would modulate the action of peripheral hormones in a specific manner in each brain side.

A similar asymmetry has been observed on a few rare occasions in other avian species such as chicken (Zhao et al., 2010; Morris et al., 2018) or Northern cardinals, *Cardinalis cardinalis* (Peer and Motz, 2014) (See also references in these articles for other examples), but gynandromorphism has not, to my knowledge, been observed in other vertebrate classes. The general significance of this singularity was therefore difficult to establish even if a partial gynandromorphism can be experimentally induced by grafting parts of a chicken embryo into a chicken of the opposite sex (Clinton et al., 2012).

The analysis of this phenomenon could however not be further explored based on the rare birds that are spontaneously affected. Art Arnold and several colleagues then decided to make use of a mouse model that had been developed in England by Robin Lovel-Badge and Paul Burgoyne to investigate these “direct” genetic effects on brain and behavior differentiation. In this model, 4 separate genotypes allow separating the differentiating effects of gonadal steroids from the effects of the sex chromosome complements (XX vs. XY). The so-called four core genotype model indeed includes mice with the two usual phenotypes, i.e. XX females developing ovaries and XY males developing testes following expression of the sry gene, in addition to XY “females” in which the sry gene is mutated so that the undifferentiated gonad develops as ovary (XY^{SRY-} “females”) and “males” in which the sry gene has been experimentally inserted in an autosome so that XX individuals develop ovaries (XX^{sry} males). It is therefore possible in this model to differentiate between sex differences that are due to the organizational effects of gonadal hormones (mice with testes, i.e. XY and XX^{sry} will be different from mice with ovaries, i.e., XX and XY^{sry-}) from sex differences due to the sex chromosome complements independently of the type of gonads (XX and XX^{SRY} mice different from XY and XY^{sry-}).

A host of comparative studies of these four phenotypes have now revealed that in general, behavioral and neuroanatomical traits directly related to reproduction become sexually differentiated mostly under the organizing influence of gonadal steroids as explained before (XY and XX^{sry} males different from the XX and XY^{sry-} females). However the sex difference in a number of other neurobehavioral responses such as the density of catecholaminergic cells of the mesencephalon (Carruth et al., 2002), the susceptibility to specific neural diseases or some aspect of pain sensitivity seem to depend on the complement of sex chromosomes rather than on the sex of the gonads (XY and XY^{sry-} subjects different from the XX and XX^{sry} subjects) (see (Arnold and Chen, 2009) for review). In a few cases, an interaction between these different genotypes and the activating effects of sex steroids in adulthood has also been identified. For example, in adult sexually mature mice, the testes bearing subjects (XY and XX^{sry}) have a larger body weight that the subjects with ovaries (XX and XY^{sry-}). However, after gonadectomy, the XY subjects become heavier than all XX subjects independent of whether they had testes or ovaries during their development before gonadectomy (Chen et al., 2013). Future research will have to establish to what extent this mode of genetic control of sexual differentiation applies to different sexually differentiated traits. This research also raises the question of whether sex differences related to the genotype rather than to the gonadal type are caused by the expression of genes from the Y chromosome or by a dosage effects due to the presence of two X chromosomes in females versus one single X chromosome in males. Different genetic models have been developed to answer this question (e.g., X0 vs. XXY vs. XY vs. XX). A detailed presentation of these studies is beyond the scope of the present overview but reviews are currently available (e.g., Arnold and Chen, 2009; Chen et al., 2013; Arnold, 2017).

2.3. Epigenetic mechanisms

We cannot conclude this section without mentioning the prodigious development during the last 20 years of studies investigating the epigenetic controls of behavior. Work carried out in many laboratories has now unequivocally demonstrated that early life events, including the exposure to sexually differentiated concentrations of sex steroids are able to modify in a enduring manner the expression of specific genes, which will in turn have lasting effects on behavior (e.g., McCarthy et al., 2009; Bale, 2015; Forger, 2016). Emerging evidence even indicates that some of these effects can be trans-generational, i.e., affect offspring that were not exposed to the modifying events up to the 3rd generation (Rissman and Adli, 2014; Forger, 2016). The expression of genes is indeed deeply affected by specific biochemical modifications targeting either the DNA itself (e.g. methylation of cytosine residues) or the surrounding histone proteins (acetylations, methylations, phosphorylations…). These modifications of the DNA or of the associated histones can be induced by life events such as chronic stress or changes in feeding regime but also by sex steroids and a variety of compounds that mimic or inhibit their action, globally grouped under the name of endocrine disruptors.

The process of sexual differentiation of brain and behavior actually seems to be mediated, at least in part, by steroid-induced long-lasting changes in gene expression that could be identified through new techniques such as RNA-Seq (McCarthy et al., 2010). Quite surprisingly, it was recently discovered that the female brain in rodents is not simply the result of a passive process that would take place in the absence of any hormonal influence – females would be the so-called neutral or anhormonal or default sex (McCarthy and Arnold, 2011) – but actually results from the active repression of male-typical genes via DNA methylation (Nugent et al., 2015). The role of testosterone, or its metabolite estradiol, during sexual differentiation would not be to stimulate the expression of male-specific genes but rather to remove an inhibition that was preventing this expression. Neonatal female brains have a higher activity of DNA methyltransferase enzymes (Dnmts) than males and early exposure of males to testosterone reduces the activity of the Dnmts thus decreasing DNA methylation and releasing male genes from epigenetic repression. Accordingly the effects of testosterone on sexual differentiation can be mimicked by a pharmacological inhibition of Dnmts or by a conditional knock-out of one isoform of these enzymes.

The study of these epigenetic mechanisms is however in its early days even if it is already impossible to cover this work in an exhaustive manner in the present broad review. It is difficult at this stage to determine their full importance in the control of sexual differentiation of brain and behavior. Studies in other species would also be needed. It would in particular be fascinating to test whether the discrepancy in the effects of estrogen on sexual differentiation in rats (masculinization of females) and quail (demasculinization of males) is related to a differential methylation of the genome in these two species.

It is important to mention that the process of sexual differentiation also results in the organization of a differential connectivity between brain regions. The projection from the bed nucleus of the stria

terminalis to the anteroventral periventricular nucleus is, for example, more developed in male than in female rats (Hutton et al., 1998). This difference is organized during embryonic development under the action of sex steroids (Simerly, 2002). The basis of this differential connectivity is understood in only a limited number of model systems such as the projections of the motoneurons to the penile muscles in rats which rely on the availability of specific trophic factors related to neuronal activity and hormonal stimulation (Sengelaub and Forger, 2008). In the brain, complex interactions are obviously implicated (Morris et al., 2004; McCarthy et al., 2010). How these different processes (hormonal, genetic and epigenetic controls, differential connectivity) interact to generate adult subjects of two sexes with specific functional characteristics is certainly not fully understood at present.

2.4. Contribution of the neuroendocrine group in Liège

2.4.1. Studies in genetically modified mice

When she joined the laboratory in 2000, Julie Bakker initiated studies on the sexual differentiation of sexual behavior in aromatase knock-out mice (ArKO). These mice represent an interesting knock-out because, contrary to the knock-out of steroid receptors, the deficit in enzymatic activity can be bypassed via an injection of exogenous estradiol. By injecting the ArKO mice with estradiol it is therefore possible to test whether their behavior suffers from organizational deficits that cannot be corrected by adult hormone replacement. As anticipated, male ArKO mice displayed pronounced deficits in male-typical copulatory behaviors that could not be corrected by treatment with exogenous testosterone (Bakker et al., 2002b) but were largely though not completely corrected by adult replacement with exogenous estradiol benzoate plus dihydrotestosterone propionate (Bakker et al., 2004).

Surprisingly however, females also showed deficits in sexual receptivity: treatments with estradiol plus progesterone that fully activate lordosis in wild type mice largely failed to restore this behavior in ArKO females even after multiple testing (Fig. 1A). This finding therefore suggested that estrogens are required during development to support a normal development of the female brain (Bakker et al., 2002a). This was directly confirmed in experiments comparing the behavior of female ArKO mice that had been treated during ontogeny with estrogens or with their solvent. Control females treated with sesame oil replicated the previously identified adult deficit in lordosis behavior in response to ovarian hormones. However administering estradiol during post-natal days 15–25 (i.e., just before puberty) significantly enhanced the ability of ArKO mice to display lordosis behavior in response to ovarian hormones in adulthood (Fig. 1B). A similar treatment administered earlier during post-natal days 5–15 was however ineffective in improving lordosis behavior but in contrast enhanced male-typical mounting after testosterone treatment in adulthood (Brock et al., 2011). Together these data are consistent with the classical notion that estrogens derived from testosterone aromatization masculinize sexual behavior during the perinatal period but the same hormones are required during the prepubertal period for the full feminization of females. The female phenotype therefore does not develop in the absence of hormonal stimulation contrary to what had been broadly accepted previously (Baum, 1979; MacLusky and Naftolin, 1981; De Vries and Simerly, 2002).

Julie Bakker also addressed using a different strain of knock-out mice another basic question that had never been properly solved in the context of sexual differentiation. Estrogens derived from central aromatization of testosterone during the perinatal period were known to masculinize and defeminize the behavior of males but why females were not similarly affected by maternal estrogens remained a bit unclear. The accepted theory was that alpha-fetoprotein (AFP), a plasma glycoprotein produced in large quantities during fetal life, was binding circulating estrogens thereby preventing their action in the brain (McEwen et al., 1975) but other studies suggested that AFP was rather actively transporting estrogens in the brain since it was identified in the central nervous system but apparently not synthesized there as suggested by the failure to detect the corresponding messenger RNA (Benno and Williams, 1978; Schachter and Toran-Allerand, 1982). Analysis of the behavior of AFP-KO mice showed that females in this strain are masculinized and defeminized as expected if AFP was protecting them from estrogen action (Fig. 1C) (Bakker et al., 2006). Furthermore, when estrogen production was blocked during embryonic life by treatment of the dams with an aromatase inhibitor, the female phenotype of these mice was restored thus confirming that the masculinization/feminization of the AFP-KO female mice was indeed due to the lack of protection from estrogens (Fig. 1D). The presence in the brain of the AFP protein in absence of the corresponding mRNA therefore presumably reflected a lack of sensitivity of the detection methods and AFP does not transport estrogens from the periphery into the brain.

2.4.2. Quail sexual differentiation

It had been clearly established during the second half of the 20th century that sexual differentiation in birds is also based on early organizational actions of sex steroids. The specific processes involved are however a mirror image of what happens in mammals: instead of a masculinization and defeminization of male mammals under the influence of testosterone and estradiol, in birds the male phenotype develops spontaneously in the absence of hormonal influences and the female phenotype is induced by ovarian estrogens which irreversibly block male-typical copulatory behavior. This process of demasculinization takes place in quail, the best studied model, between days 6 and 12 of incubation (out of a 17 days incubation period). It can be mimicked in males by injection of an estrogen before incubation day 12 or blocked in females by injection of an aromatase inhibitor at the beginning of the sensitive period (see for review (Balthazart and Adkins-Regan, 2002)).

The estrogen receptor responsible for this demasculinization process had however not been identified. We tackled this question by injecting into quail eggs on day 7 of incubation either 25 μg of estradiol benzoate (EB) or 300 μg of the estrogen receptor alpha (ERα) agonist propyl-pyrazole-trisphenol (PPT) or 300 μg of the ERβ agonist diarylpropionitrile (DPN) or their solvent propylene glycol. Birds were then gonadectomized at three weeks post-hatch and they received subcutaneous Silastic™ implants filled with testosterone two weeks later. When adult, they were then tested for male typical copulatory behavior. As anticipated based on previous studies, control males displayed an intense copulatory behavior characterized by large numbers of mounts and cloacal contact movements observed after very short latencies following the introduction of a female. These behaviors were essentially absent in control females and in males treated with EB. They were also largely suppressed in males injected *in ovo* with DPN while males injected with PPT essentially showed a normal copulatory activity (Court et al., 2018). These data quite surprisingly suggest that the demasculinizing effects of estrogens in quail are largely if not exclusively mediated by an interaction with ERβ and not with ERα as most commonly reported for the sexual differentiation of mice (Ogawa et al., 1997; Wersinger et al., 1997; Ogawa et al., 1998) even if a some observations also point for a role of ERβ in the defeminization of mice sexual behavior (Kudwa et al., 2005; Bodo and Rissman, 2006).

Charlotte Cornil and Charlotte Delage have also initiated a series of studies to test whether the estrogen-induced demasculinization in quail is mediated, like the sexual masculinization of rodents, by changes in microglial activity and in prostaglandin secretion (Amateau and McCarthy, 2004; Lenz et al., 2013; Lenz and McCarthy, 2015). To this date, it has been established that the density of microglia in the medial preoptic area varies significantly during the critical period of sexual differentiation and does so in a sexually differentiated manner; this density is larger in female than in male embryos between incubation days 9 and 12. In addition, the injection in quail eggs on incubation day 7 or 9 of indomethacin, a blocker of the cyclooxygenases that catalyze the production of prostaglandin E, inhibits the development of adult

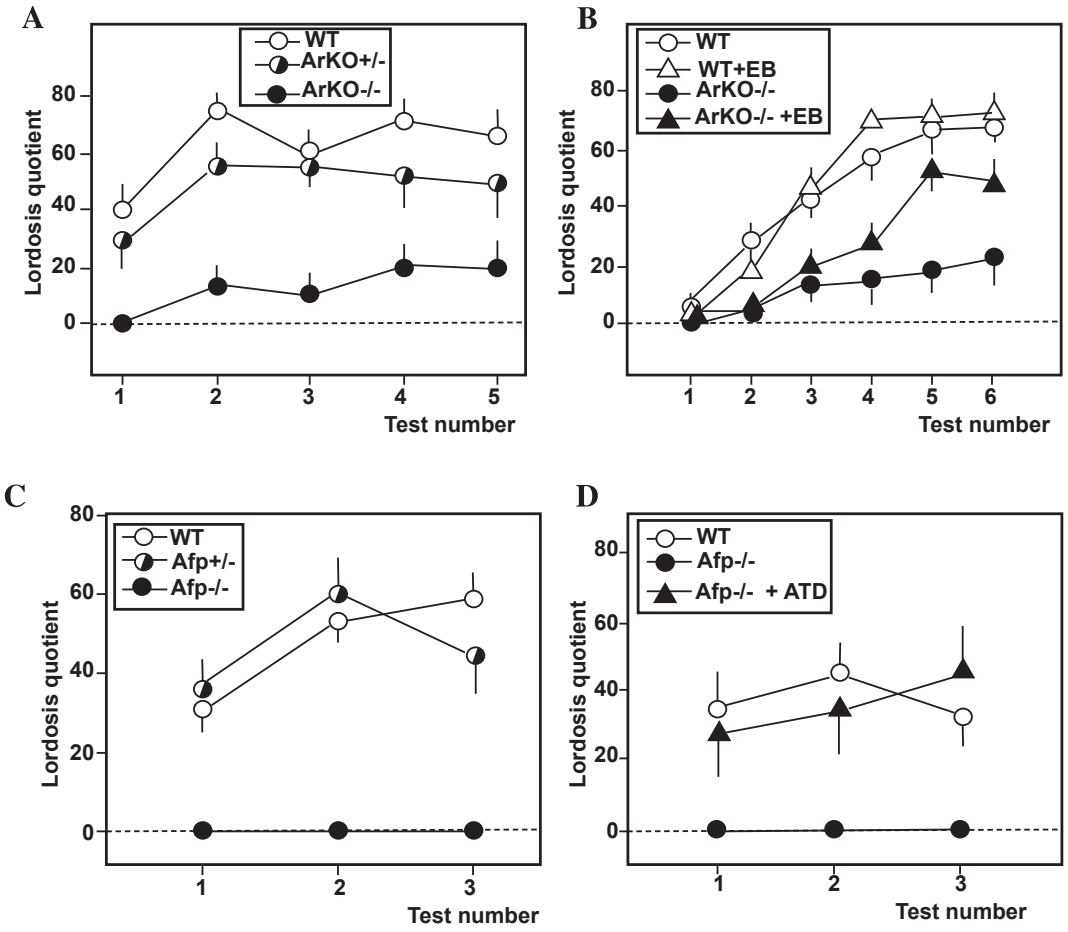

Fig. 1. Summary of experiments demonstrating a role for postnatal estrogens in female feminization and a role of prenatal estrogens in defeminization in mice. (A) Aromatase knock-out (ArKo−/−) female mice show deficits in lordosis behavior even if treated in adulthood with estradiol plus progesterone by comparison with wild type (WT) and heterozygous (ArKo+/−) subjects. (B) Treatment of ArKo−/− mice with estradiol benzoate (EB) before puberty (post-natal days 15 to 25) largely corrects these deficits. (C) Female mice genetically modified to block expression of alpha-fetoprotein (Afp−/−) are unable to show female sexual behavior (lordosis) in adulthood. (D) Treating these females with the aromatase inhibitor ATD restores the behavior which clearly demonstrates that Afp−/− are defeminized by the estrogens produced by the embryos and most importantly their mother. Redrawn from data in Bakker et al. (2002a), Brock et al. (2011) and Bakker et al. (2006).

male copulatory behavior (Delage et al., 2017). Even if these data suggest a role for microglia and prostaglandins in the sexual development of quail, the physiological significance of these effects remains at this time a bit unclear. The available data do not yet provide a clear understanding of how immune reactions could at the same time mediate the masculinization of male behavior by estrogens in rodents and its demasculinization by the same steroids in quail. Additional research will be needed to establish a functional model of these effects.

2.4.3. Studies of another gynandromorphic zebra finch

We recently discovered the existence of another gynandomorphic zebra finch in the animal vivarium of Dr. Barbara Caspers (formerly laboratory of Hans-Joachim Bischoff) at the University of Bielefeld, Germany. The bird, which was 11 years old, was generously given to us and we recorded its vocalizations during several two hours sessions in sound attenuated chambers while the bird was either alone or in the presence of a female. The bird and several male and female conspecifics of similar age were then injected with bromodeoxyruridine and their brains were collected 3 weeks later after being perfused with 4% paraformaldehyde. Brain sections and the recorded vocalizations are currently under study.

This new gynandromorph was interestingly male of the left and female on the right side while the bird studied in the Arnold laboratory had an opposite lateralization, right male and left female (Agate et al., 2003). Our bird from Germany had successfully inseminated females who laid fertile clutches and successfully raised their young while the bird from Agate and collaborators never sired any chicks. Our bird produced superficially normal songs but also emitted songs that were made of more than 10 repetitions of a same syllable.

Brain sections were stained so far only for perineuronal nets (PNN) that are aggregations of extracellular material mostly around inhibitory neurons expressing the calcium-binding protein parvalbumin (see also Section 3.5). These PNN were previously shown to be expressed in high densities in the song control nuclei HVC and RA (the robust nucleus of the arcopallium) (Balmer et al., 2009; Cornez et al., 2015) and to be present in much higher densities in males than in females (Cornez et al., 2015, 2018b). Our preliminary quantifications indicate that (a) the total volume of the telencephalon is larger on the male compared to the female side, (b) the volume of HVC and to a lesser extent RA is larger on the male side of the brain than on the female side and (c) the total number of PNN and of parvalbumin-immunoreactive neurons in HVC is also larger on the male side.

These initial data are therefore consistent with the previous study of Agate and collaborators suggesting that despite exposure to the same concentrations of gonadal steroids, the two brain sides in a gynandromorph display different developmental trajectories, presumably due to the local expression of male or female-specific genes. Additional studies are currently ongoing attempting to further refine this

conclusion.

3. The activation of sexual behavior by steroids

The last 20 years have also seen substantial changes in the way we consider the activating effects of sex steroids on reproductive behavior. I would like to highlight here 5 major conceptual shifts that developed during this period. In this section I will fully integrate work performed in the Balthazart/Cornil laboratory of Liège with the global changes in concepts because our research often played a significant role in the global evolution of thinking.

3.1. The "Balkanization" of neuroendocrinology

It was traditionally assumed that steroids produced by the gonads act on their targets in the brain to activate sexual behavior. It was also established that steroids, in particular testosterone and progesterone, could be transformed within the brain into behaviorally active (e.g., by aromatization or 5α-reduction of testosterone) or inactive (e.g., by 5β-reduction of testosterone) metabolites (Balthazart, 1989; Melcangi et al., 1999; Balthazart, 2017). The end of the 20th century coincided with the emergence of the notion that steroids can also be synthesized directly in the brain from cholesterol (Baulieu and Robel, 1990; Robel and Baulieu, 1994; Baulieu, 1998). It was indeed demonstrated in multiple species including mammals and birds that the mRNA of all enzymes needed to produce sex steroids from cholesterol are expressed in the brain (for review: (Robel and Baulieu, 1994; Schumacher and Baulieu, 1995)). In selected cases, it was also shown that these enzymes are active in brain homogenates and efficiently catalyze the transformation of their substrate into the corresponding products (e.g., Baulieu et al., 1999; Tsutsui et al., 1999; Tsutsui and Schlinger, 2002; London et al., 2009). Additionally several studies showed that some steroids, such as pregnenolone, are present in higher concentration in the brain than in the periphery and their concentration does not decrease following castration (Baulieu et al., 1987; Tsutsui and Yamazaki, 1995). This led to the concept of neurosteroids, i.e. steroids locally produced in the brain and acting locally to modulate neuronal activity (Baulieu and Robel, 1990; Tsutsui et al., 1999).

The broader availability of assay techniques such as mass spectrometry coupled with either gas chromatography (GC–MS) or high-pressure gas chromatography (HPLC-MS) more recently allowed quantifying a large variety of steroids in various brain structures (Liere et al., 2000; Caruso et al., 2008; Melcangi et al., 2017) (see also chapter by Melcangi, this issue). Multiple studies also suggested that many of these steroids presumably produced in the brain affect a variety of physiological processes in health and disease (Caruso et al., 2010; Melcangi et al., 2012; Caruso et al., 2014; Mitro et al., 2014). In my opinion, the full demonstration that steroids completely produced in the brain from cholesterol, independently from any contribution of steroids produced in the periphery by the gonads or adrenals, have a physiological impact on brain function is however only available in a very small number of selected cases (e.g., Koenig et al., 1995). For example, allopregnanolone (or 3α,5α-tetrahydroprogesterone), one of the most studied "neurosteroids", is clearly able to modulate brain function associated with pain, stress or several psychiatric conditions via its interaction with type A aminobutyric acid (GABAa) receptors (see chapters by Guy Mensah et al. and by Monique Vallée et al. in this issue) but it is unclear to what extent this metabolite of progesterone is derived from progesterone secreted in the brain or in the periphery. In other terms, we often do not know whether allopregnanolone is a true neurosteroid or simply a neuroactive steroid derived for gonadal progesterone. A role for true neurosteroids is particularly unclear in my opinion when considering the control of reproductive behaviors since these behaviors disappear or are severely reduced in most animal models following castration.

One very interesting case of behavioral impact of steroids produced in the brain from a steroid substrate synthesized in the periphery was however discovered during the study of autumnal aggression in one songbird species, the song sparrow (*Melospiza melodia*). In this species males are aggressive and defend a territory all year long except during the summer when they are molting and photorefractory. In the spring this aggression is associated with high concentrations of testosterone in the blood but this steroid becomes undetectable in the fall while the behavior remains present (Wingfield and Hahn, 1994). Quite surprisingly it was shown that the fall aggression is inhibited when birds were treated with an androgen receptor antagonist, flutamide combined with the aromatase inhibitor, androstatrienedione (Soma et al., 1999, 2000). This suggested that the behavior was still steroid-dependent while circulating concentrations were extremely low. In an elegant series of experiments, Kiran Soma and his collaborators demonstrated that, during the fall, aggressive behavior is actually activated by androgens and estrogens derived from the brain metabolism of dehydroepiandrosterone (DHEA). This steroid is produced by the adrenals and remains present in high concentration in the blood throughout the year. In the fall, the activity of the 3β-hydroxysteroid dehydrogenase (3β-HSD) in the brain markedly increases and the enzyme catalyzes the transformation of DHEA into behaviorally active local concentrations of testosterone and estradiol (Soma and Wingfield, 2001; Soma et al., 2002, 2008).

This demonstration that testosterone and estradiol can be produced in the brain by local metabolism of DHEA of adrenal origin greatly added to the complexity of the mechanisms of neuroendocrine control. This example clearly showed that behavior could be controlled by steroids coming from the periphery, but also by steroids synthesized in the brain from circulating active or inactive precursors or even from cholesterol. Soma and colleagues proposed that this increased complexity represents a "Balkanization" of the endocrine system whereby individual tissues or organs are able to autonomously synthesize active steroids, in some case independently of the systemic concentrations (Schmidt et al., 2008; Soma et al., 2008).

We recently investigated by GC–MS the concentrations of 25 steroids in 4 brain regions (the preoptic area-hypothalamus [POA], entire telencephalon, cerebellum and optic lobes) and in the serum of male and female quail, and the impact of castration associated or not with a replacement therapy by T or E2 on these concentrations (Liere et al., 2019). Indeed if the presence of mRNA for most steroid-synthesizing enzymes and the in vitro activity of some of these enzymes in this widely used animal model had been well documented (Tsutsui et al., 2006; Tsutsui, 2011), the profile of steroid concentrations in their brain remained unknown. Such a profile was available for a few mammalian species, mostly rodents (e.g., Labombarda et al., 2006; Schumacher et al., 2015), but had never been established in any avian species to our knowledge. Results of these studies were recently published (Liere et al., 2019) and they contain a number of major surprises.

First, although sex steroids are lipophilic and classically supposed to freely enter in lipophilic tissues such as the brain, a number of steroids were found in larger concentrations in all brain regions of both sexes than in the blood. This was the case for the steroid that results from the first enzymatic transformation of cholesterol, namely pregnenolone and its metabolite 20α-dihydropregnenolone (Fig. 2). In addition pregnenolone sulfate was specifically present in the preoptic area but undetectable in the other brain regions and in the serum thus suggesting a specific local metabolism of pregenenolone by a sulfotransferase in this brain region. These concentrations that are much larger in the brain than in the serum suggested that these steroids were produced in the brain independently from peripheral production, a fact that was also supported by the observation that the brain concentrations of pregnenolone were not decreased following castration in males, nor after treatment of the castrates with exogenous testosterone or estradiol (Fig. 3).

Quite surprisingly, three estrogens, estradiol, estrone and estriol were also detected in the POA (0.2–0.6 $ng.ml^{-1}$) and telencephalon (0.03–0.3 $ng.ml^{-1}$) of both sexes while they were present in extremely

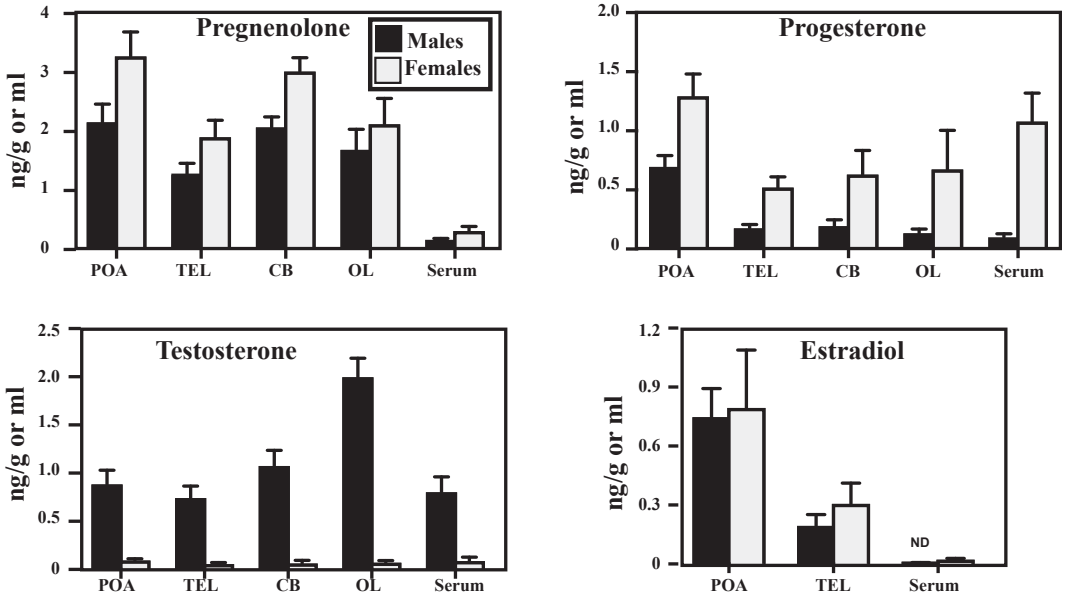

Fig. 2. Concentrations of pregnenolone, progesterone, testosterone and estradiol in four brain areas and in the serum of adult sexually mature male and female Japanese quail as measured by gas chromatography coupled with mass spectrometry. POA = Preoptic area; TEL = Telencephalon; CB = Cerebellum; OL = Optic Lobes; ND = not detectable. Redrawn from data in Liere et al. (2019).

low or undetectable concentrations ($< 0.02\,ng.ml^{-1}$) in the serum, even in females. These low systemic concentrations are clearly discordant with the results of previous studies based on radioimmunoassays (RIA) that had identified systemic concentrations of approximately 0.1 and 0.2–0.4 $ng.ml^{-1}$ estradiol in the blood of sexually mature males and females respectively (Delville et al., 1986; Balthazart et al., 1987). Whether this difference represents a problem with the GC–MS assay of estrogens or a non-identified cross-reactivity in the RIA is unclear at present.

Many other steroids were present in relatively similar concentrations in the serum and in all brain areas considered. This was namely the case for corticosterone and testosterone but also multiple other androgens including DHEA, androstenedione, 3α,5α-tetrahydrotestosterone and 3α,5β-tetrahydrotestosterone. These steroids are presumably secreted in periphery and highly diffusible. This is clearly the case for corticosterone secreted by the adrenal glands and testosterone by the testes. In addition, androstenedione and 5α- or 5β-reduced androgens could be formed in the brain. Previous in vitro work with radioactive T has indeed identified an active 17β-HSD and an extremely high 5β-reductase activity in the brain (Balthazart et al., 1983; Schumacher and Balthazart, 1986). A fraction of these steroids could thus be produced in the brain by transformation of testosterone and secondarily released in the periphery as shown previously for estrogens in zebra finches (Schlinger and Arnold, 1992, 1993).

Progesterone, which is secreted by the ovaries and adrenals, was also roughly in equilibrium between the periphery and the brain. It was however present in higher concentration in the POA than in other brain areas indicating that either progesterone did not reach these areas at the same rate or it was rapidly metabolized in a region-specific manner.

Interestingly also, many of these steroids displayed reliable sex differences both in the periphery and in the brain. Progesterone and its metabolites were significantly more concentrated in females than in males in all samples while the reverse was true for testosterone, its metabolites and DHEA. It is notable that the sex difference in testosterone serum concentrations (0.803 ± 0.159 in males vs. $0.089 \pm 0.038\,ng.ml^{-1}$ in females, means ± SEM) was by far more pronounced here than in previous work based on RIA (females showing about 50% of the male concentrations with some overlap between values in the two sexes) (Balthazart et al., 1983, 1987; Dickens et al., 2011). The RIA used in previous studies was thus possibly affected in females by a non-identified cross-reaction.

In general, the GC–MS and HPLC-MS techniques have demonstrated that brain tissue contains a very large number of steroids that are only separated after optimization of the chromatographic procedure (e.g., Liere et al., 2000). Hundreds of peaks are often present, which is easily understandable when considering the complexity of the steroid-metabolizing pathways (Akawa et al., 1990) and only a few of them have been so far identified and quantified. It is therefore not surprising that undetected cross-reactivities are affecting quantifications by RIA and explain the discrepancies that have been recently observed between different experimental approaches.

These experiments also analyzed whether the activation of male copulatory behavior by testosterone and its metabolite estradiol locally produced in the POA were paralleled by the expected changes in brain concentrations of these steroids (Fig. 3). Castration decreased testosterone and estradiol concentrations in the serum and POA to low or undetectable values and the subcutaneous testosterone Silastic™ implants restored the preoptic testosterone concentrations to the values observed in sexually mature males. In birds implanted with Silastic™ capsules filled with estradiol there was however no increase of estradiol in the POA nor in the TEL with the lowest dose (40 mm implant) that raised serum concentrations to about $1.5\,ng.ml^{-1}$. It is only the higher dose (80 mm implants) raising serum levels above $2\,ng.ml^{-1}$ that succeeded in increasing brain estrogens concentrations.

The discrepancy between systemic and central estradiol concentrations could result from an extensive metabolism of the steroid in the brain (Balthazart et al., 1994; Balthazart and Ball, 2006; Cornil et al., 2006b) or reflect a failure to penetrate the brain. This might explain why a systemic treatment with exogenous estradiol has little effect on copulatory behavior unless huge doses with systemic toxic effects are used (Adkins and Nock, 1976; Balthazart et al., 1985; Watson et al., 1990) while the behavior is clearly estrogen-dependent, as indicated namely by the fact that aromatase inhibitors implanted in the POA efficiently block the behavior (Adkins et al., 1980; Watson and Adkins-Regan, 1989; Balthazart et al., 1990a; Balthazart and Surlemont, 1990). Preoptic aromatization might produce high local concentrations of estrogens that are nearly impossible to reach by systemic administration of estradiol (see (Liere et al., 2019) for additional discussion).

The picture emerging from all these results clearly highlights the complexity of the endocrine brain in its interaction with behavior.

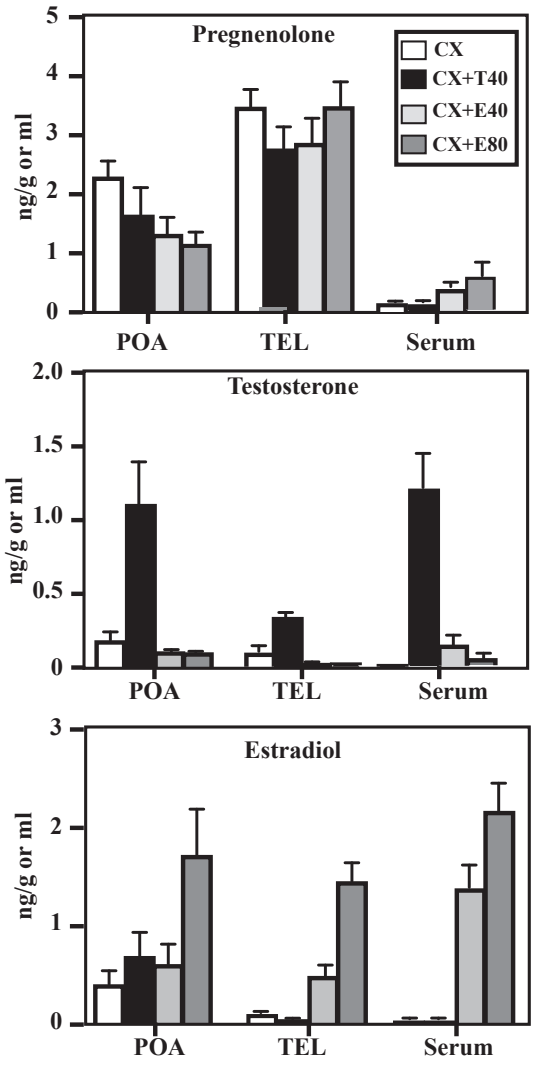

Fig. 3. Concentrations of pregnenolone, testosterone and estradiol in the preoptic area (POA), telencephalon (TEL) and serum in male Japanese quail that were castrated and treated with empty Silastic implants (CX), 40 mm long implants filled with testosterone (CX + T40), or 40/80 mm long implants filled with estradiol (CX + E40/80). Redrawn from data in Liere et al. (2019).

These data also support the idea that true neurosteroids play little or no role in the control of sexual behavior: castration did not decrease the brain concentration of pregnenolone but almost completely depleted the brain from testosterone and estradiol and this condition is known to be associated with a suppression of male copulatory behavior. Why testosterone but also progesterone and androstenedione concentrations decrease in these conditions when their precursor pregnenolone is present and we know that the 3β-HSD and CYP17 (or 17α-hydroxylase/17,20-lyase) are expressed and active in the quail brain (Tsutsui et al., 2006; Tsutsui, 2011) remains unclear at present. The answer may rest in an anatomical separation of the different enzymes making the synthesis chain from cholesterol to androgens inefficient or inactive. More research is obviously needed on this topic.

3.2. Steroid receptor co-regulators

During the second half of the 20th century, intracellular (nuclear) receptors for all sex steroids were identified and it was demonstrated with the use of receptor antagonists and transcription or translation inhibitors that steroids activate behaviors at least in part via the binding to these receptors, association of the occupied receptors with specific DNA binding sites and the subsequent activation of the transcription and translation of genes that in turn modulate neuronal activity (Blaustein and Olster, 1989; Auger, 2004; Mani and Blaustein, 2012). Over the years, the neuroanatomical distribution of these receptors was mapped with increasing precision and detailed correlations could be established between changes in receptor densities and behavior. A number of situations were however progressively identified in which behavior intensity failed to correlate with steroids concentrations or with the density of their receptors. It was proposed to explain these discrepancies by the fact that, in some conditions, nuclear receptors require specific co-activators and that different receptors compete for these co-factors which then become limiting, a phenomenon named squelching. The first confirmation of the existence of such coactivators was obtained in the O'Malley laboratory in 1995 (Onate et al., 1995). These researchers cloned and sequenced a protein they called steroid receptor coactivator-1 (SRC-1) that was shown to be closely associated with the progesterone receptor and is essential for the full transcriptional activity of this receptor, …but also of the androgen receptor. SRC-1 was the first identified protein of a family that has grown to more than 300 members and includes both co-activators and co-repressors (see: https://nursa.org/nursa/molecules/index.jsf). After being recruited, these coactivators enhance target gene activation and transcription by remodeling the DNA (via histone acetylations and methylations) but also by recruiting and stabilizing the general transcription machinery, and ultimately the RNA polymerase.

These mechanisms had been largely elucidated by the turn of the century (McKenna et al., 1999; McKenna and O'Malley, 2000, 2002; Charlier and Balthazart, 2005) but most of this work had been performed in vitro in cell cultures with the exception of one study showing that targeted disruption of the SRC-1 gene induced a partial steroid hormone resistance in mice (Xu et al., 1998). The potential role of steroid receptor coactivators in the control of behaviors was as a consequence unexplored and it is only during the last 20 years that this topic has been a focus of study.

A first series of experiments demonstrated that reducing SRC-1 expression via the central infusion of specific antisense oligodeoxynucleotides in the hypothalamus on the day of birth and on the two following days interfered with the defeminizing actions of estrogens in rats. Male and androgenized female rats displayed higher levels of lordosis when infused with the SRC-1 oligodeoxynucleotides. This treatment also significantly decreased the volume of the sexually dimorphic nucleus of the preoptic area in androgenized females (Auger et al., 2000). A number of research programs also developed knock-out mice models for the first three steroid receptor coactivators (SRC-1, -2 and -3) and identified various deficits in steroid action but little attention was paid to steroid-sensitive behaviors (Xu et al., 1998, 2000; Gehin et al., 2002). Because these studies disrupted the coactivators expression during ontogeny, they were also faced with the problem of developmental compensation. For example, the SRC-1 knock-out male mice were surprisingly fertile, females showed proceptive as well as receptive sexual behaviors, they were fertile and they produced and fed normal litters of pups (Xu et al., 1998; Monks et al., 2003). It was later shown that this resistance is probably explained by an overexpression of the other coactivator with similar function, SRC-2 (Xu et al., 1998).

Because genetic inactivation blocks the expression of the target gene during both development and in adulthood, it was also impossible to differentiate between effects of these coactivators on the sexual differentiation and on the activation of behavior. To avoid these problems, the role of coactivators was thus analyzed by antisense technology to decrease their expression in a specific brain area in a specific time frame. Reducing SRC-1 or SRC-1 and CBP (CREB Binding protein) or SRC-2 expression in the ventromedial nucleus of the hypothalamus (VMN) of female rats significantly decreased the expression of the progesterone receptor normally induced by the estrogenic activation of estrogen receptors (Apostolakis et al., 2002; Molenda et al., 2002). Given that this induction conditions the expression of female behaviors,

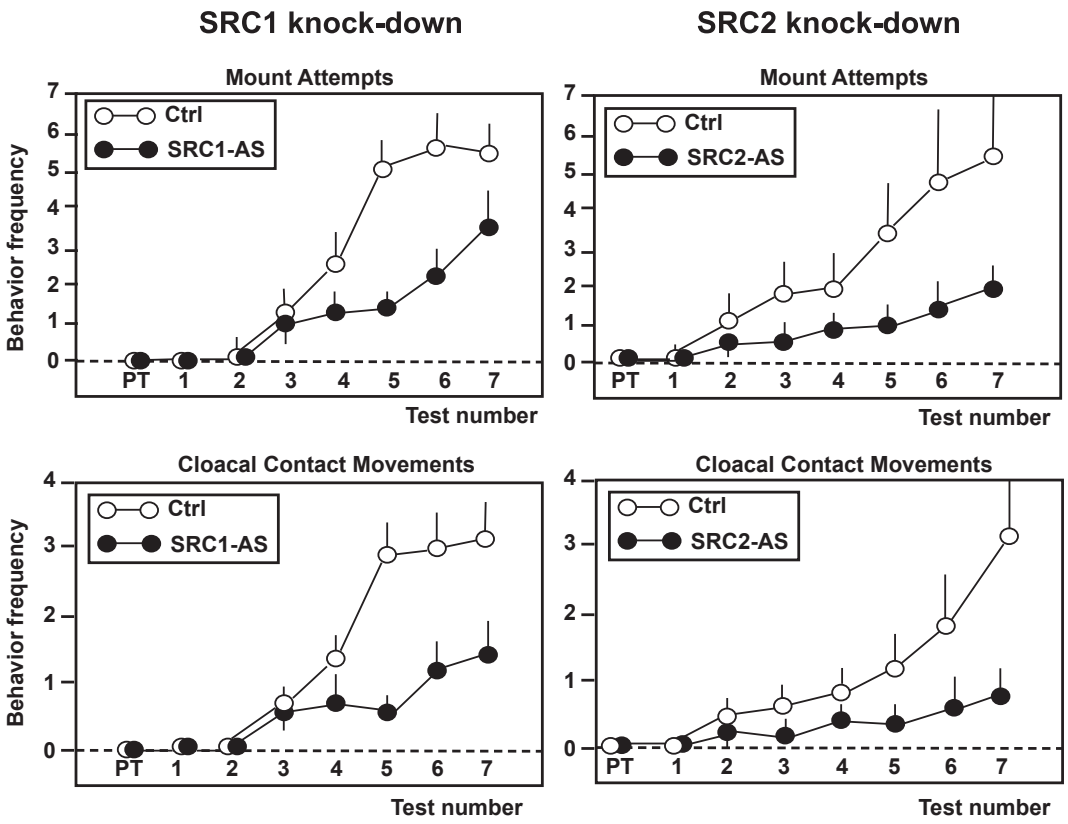

Fig. 4. Behavioral effects of the inhibition of SRC1 or SRC2 expression by repeated daily injections in the third ventricle of locked nuclei acid-derived antisense oligodeoxynucleotides. Castrated males were implanted with a Silastic capsule filled with testosterone and repeatedly tested for copulatory behavior, as assessed by the frequency of mount attempts and cloacal contact movements. PT = Pretest; Ctrl = Control injection of scrambled oligodeoxynucleotides; AS = Antisense.redrawn from data in Charlier et al. (2005) and Niessen et al. (2011).

there was also in parallel an inhibition of estrogen- and progesterone–dependent female sexual behaviors (Apostolakis et al., 2002; Molenda et al., 2002). Additional studies showed that neurons expressing the estradiol-induced progesterone receptors in the VMN also co-express SRC-1 (Molenda-Figueira et al., 2008). This type of colocalization, which is required for the coactivators to exert their actions on receptors, was later extended to other coactivators, other brain nuclei and to both estrogen and progesterone receptors (Tognoni et al., 2011). SCR-1 was actually shown to interact directly with steroid receptors in brain issue (Molenda-Figueira et al., 2008). All this work has been reviewed on multiple occasions (Tetel et al., 2009; Tetel and Acharya, 2013).

In Liege, we studied the role of SRC-1 and SRC-2 in the control by testosterone of male sexual behavior in our favorite avian model species, the Japanese quail (Fig. 4). Locked nuclei acid-derived antisense oligodeoxynucleotides were injected daily in the third ventricle of castrated males treated with exogenous testosterone and were shown to down-regulate the expression of SCR-1 in the medial preoptic nucleus (POM), a key center in the activation of male copulatory behavior by testosterone. This treatment also markedly inhibited the activation by testosterone of both the copulatory sequence and the frequency of struts, a pre- and post-copulatory display in this species (Charlier et al., 2005). Because the copulatory sequence is activated by a combined action of androgens and estrogens derived from testosterone aromatization while struts are strictly androgen-dependent, the observed behavioral changes suggested that SRC-1 interacts with both androgen and estrogen receptors. SRC-1 injections also decreased the volume of the POM and the expression of aromatase in this nucleus. The density of vasotocin-immunoreactive fibers, another marker of testosterone action, was also reduced in the brain of these males. When the SRC-1 injections were interrupted a rebound of the SRC-1 concentration and of copulatory behavior was observed within two days. Another study later showed that the down-regulation of SCR-2 by a similar anti-sense treatment also inhibits copulatory behavior and decreases the POM volume in testosterone-treated castrated male quail (Niessen et al., 2011).

Together, all these data demonstrate that receptor coactivators of the SRC- and CBP- family regulate the expression of reproductive behaviors activated by androgens, estrogens and progestins in males and females. Effects were observed in one avian and one rodent species suggesting existence of a broadly distributed phenomenon that was potentially shared by common ancestors to these two vertebrate classes (see (Katz, 2019) for a recent and stimulating discussion of the concept of conservation during evolution). However, the role of only a few coactivators of steroid receptors has barely been examined. Many other behavioral responses should be considered and the potential influence of many coactivators and corepresors in this large family of proteins should be investigated.

3.3. Rapid changes in aromatase activity

While running preliminary studies to optimize the radio-enzymatic assay of aromatase in quail brain homogenates, we serendipitously discovered that this enzymatic activity is inhibited within minutes by exposure to increased but physiological concentrations of divalent cations (Ca^{2+} and Mg^{2+}) as previously described for other enzymes (Albert et al., 1984) and even suggested for aromatase (Hochberg et al., 1986; Onagbesan and Podie, 1989; Steimer and Hutchison, 1991). This inhibition was markedly enhanced in the presence of adenosine triphosphate (ATP) but blocked by addition of agents that chelate divalent ions such as EGTA or EDTA and by inhibitors of protein A or C kinase (Fig. 5A) (Balthazart et al., 2001, 2003). These data strongly suggested

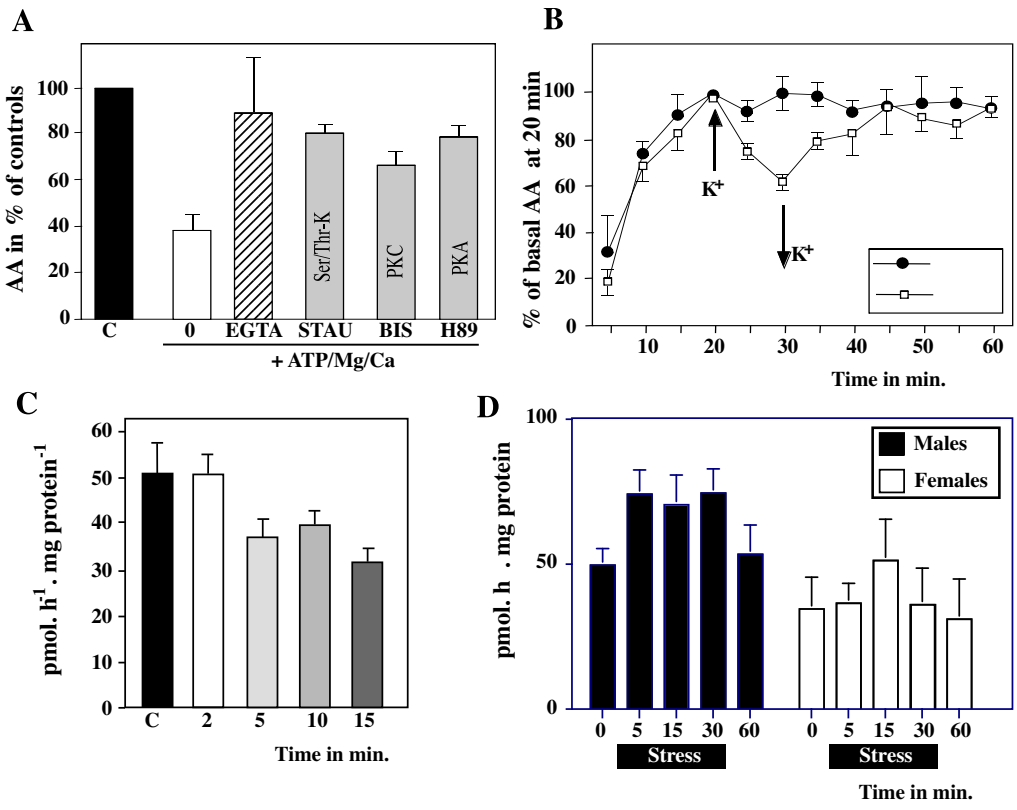

Fig. 5. Rapid changes in aromatase activity (AA) observed *in vitro* and *ex vivo* in the preoptic area-hypothalamus (HPOA) of male Japanese quail after various experimental manipulations. (A) Phosphorylating conditions (addition of ATP/Mg/Ca) to HPOA homogenates inhibits AA within 15 min and this effect is blocked by the addition of EGTA, which chelates calcium or of various kinase inhibitors such as staurosporin, bisindolylmaleimide or H89 that respectively inhibit the serine/threonine kinases, phosphokinase C or phosphokinase A. (B) Rapid and reversible inhibition of AA in paired HPOA explants maintained in vitro and exposed from minute 20 to 30 to an increased concentration of potassium (K^+). (C) Inhibition of AA in the medial preoptic nucleus dissected by the Palkovits punch technique from the brain of male quail that were exposed to and had the opportunity to copulate with a female for 2, 5, 10 or 15 min. (D) Rapid increase in AA in the medial preoptic nucleus dissected by the Palkovits punch technique from the brain of male or female quail that had been exposed to an acute restraint stress from minute 0 to 30. The increase was more rapid in males than in females but AA returned to baseline with 15 min after birds were replaced in their home cage. Redrawn from data in Balthazart et al. (2001), Balthazart et al. (2003), de Bournonville et al. (2013), Dickens et al. (2011).

that phosphorylation processes rapidly modulate quail brain aromatase activity.

The sequencing of the quail aromatase gene indeed identified 15 phosphorylation consensus sites including two sites corresponding to the specificity of protein kinases A and C (Balthazart et al., 2003) indicating that the phosphorylation processes modulating the enzyme activity potentially concern the enzymatic protein itself. This was confirmed by in vitro studies of the HEK296 cell line transfected with aromatase demonstrating that the enzymatic inhibition induced by phosphorylating conditions (increased ATP, Mg^{2+} and Ca^{2+} concentrations) is associated with an increase of phospho-serine and an increased incorporation of ^{32}P in the presence of $\gamma^{32}P$-ATP in the Western blot band corresponding to immunoreactive aromatase (Charlier et al., 2011a).

We then wondered whether similar inhibitions would be detected in intact brain cells. We established an *in vitro* culture of preoptic-hypothalamic explants in which we could repeatedly (every 5 or 30 min) quantify the total aromatase activity by measuring the release of tritiated water generated by the aromatization of [1β-^{3}H]-androstenedione into estrone (Balthazart et al., 2001, 2003). After approximately 20 min needed to reach a steady state level, the release of tritated water then remained relatively stable for a few hours, which allowed us to evaluate the impact of diverse experimental manipulations on the enzymatic activity.

Changes in intracellular Ca^{2+} concentrations stimulated by a K^+-induced depolarization or by addition of thapsigargin, a drug that mobilizes Ca^{2+} intracellular pools, inhibited within 5 min aromatase activity in the explants and this effect was rapidly reversed after washout (Fig. 5B) (Balthazart et al., 2001). Similar transient decreases in enzymatic activity were also observed after addition to the incubation medium of glutamate agonists, in particular α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid [AMPA], kainate or, to a lower extent, N-methyl-D-aspartic acid [NMDA] (Balthazart et al., 2006). The effect of kainate could be blocked by a pre-incubation with antagonists to this receptor, which was previously shown to be expressed on aromatase-immunoreactive cells of the quail preoptic area (Cornil et al., 2004). Together these data suggested that the glutamatergic neurotransmission directly modulates aromatase activity in the quail brain with latencies of a few min, if not less, given that technical limitations prevented us from analyzing shorter latencies. Subsequent work tested whether such rapid variations of aromatase activity can be detected in live subjects exposed to a variety of experiences.

Tissue blocks containing the preoptic area and hypothalamus collected immediately after adult male quail had been allowed to copulate freely with a female for 1, 5 or 15 min exhibited a decrease in enzymatic activity that was noticeable after 1 min, maximum after 5 min and had returned to control values at 15 min (Cornil et al., 2005). To identify the specific population of aromatase cells affected by the behavioral performance and the time course of the phenomenon, this experiment was repeated but birds were now allowed to interact with a female for 2, 5, 10 or 15 min and aromatase activity was specifically quantified in six nuclei dissected by the Palkovits punch technique (Palkovits, 1973) adapted for the quail brain (Schumacher and Balthazart, 1987; Cornil et al., 2011). A significant decrease in enzymatic activity was detected after only 2 min of interaction in the tuberal hypothalamus (Fig. 5C). A similar decrease was present in the POM after 5 min and the maximal inhibition was observed at 15 min in both regions. The simple visual interaction with a female also transiently decreased aromatase activity in the bed nucleus of the stria terminalis (de Bournonville et al., 2013). These changes were potentially mediated by an endogenous release of glutamate given that copulation was shown to increase the extracellular concentration of glutamate in the preoptic area as measured by *in vivo* dialysis and injection of glutamate in the preoptic area of anesthetized quail locally decreased this enzymatic activity (de Bournonville et al.,

2017a).

In contrast, exposing male quail to an acute restraint stress induced a rapid increase of aromatase activity in the POM (within 5 min) and in the VMN (after 15 min) and the enzymatic activity returned to baseline 30 min after the stress cessation (Fig. 5D) (Dickens et al., 2011). Females exposed to the same treatment exhibited a moderate increase of aromatase activity in the POM but a very profound enzymatic down-regulation in the tuberal hypothalamus that did not recover even after 30 min of recovery in their home cage.

Together all these data demonstrate that the activity of the testosterone metabolizing enzyme aromatase can be modulated in a matter of minutes if not faster in the brain. Experiments in another avian species, the song sparrow also indicated that this type of rapid variations is not limited to aromatase but also concern the androgen-synthesizing enzyme 3β-HSD. We described in a previous section (see 3.1) how this enzyme produces behaviorally active concentrations of androgens and estrogens from DHEA and how this maintains aggressive and territorial behavior in the fall when plasma concentrations are basal. Additional experiments demonstrated that the brain 3β-HSD activity is up-regulated within 30 min by social interactions in males exposed to a simulated territorial intrusion triggering extensive aggressive behavior (Pradhan et al., 2010). This increase was positively correlated with the amount of time that birds spent within one meter of the intruder decoy and thus with the intensity of the aggressive behavior of the territory owner. Interestingly this up-regulation of 3β-HSD activity by aggressive interactions was observed when the enzymatic activity was quantified without addition of the enzyme cofactor NAD^+ (nicotinamide adenine dinucleotide). Addition in the assay of saturating concentrations of this cofactor obliterated this regulation indicating that it is possibly mediated by a change in cofactor availability (Balthazart, 2010; Pradhan et al., 2010).

This study provides a second example of rapid modulation of a steroid metabolizing enzyme activity by social interactions. This new phenomenon might actually be widespread given the multiple biochemical mechanisms that could mediate it such as the phosphorylation of the enzymatic protein, any other type of allosteric modulation, changes in co-factor availability or in pH.

These studies suggested, though they did not prove, that local concentrations of steroids might change in a dynamic fashion in the brain. It was until fairly recently thought that this conclusion would be impossible to test since brain areas cannot be repeatedly collected from a same subject and inter-individual variations would probably obscure the limited changes taking place locally. This notion was challenged by a study demonstrating that changes in local concentrations of estradiol can be repeatedly assessed by the technique of *in vivo* microdialysis associated with ultra-sensitive immuno-enzymo assays (Remage-Healey et al., 2008). These experiments showed that the local concentration of estradiol increases within 30 min in the secondary auditory area NCM (medio-caudal nidopallium) of male zebra finches during social interactions with females or after exposure to conspecific song. Retrodialysis of glutamate also caused a rapid decrease in local estradiol concentration in the dialysates (Remage-Healey et al., 2008).

NCM expresses unusually high levels of aromatase enzyme (Balthazart et al., 1990b, 1996; Saldanha et al., 2000) and of aromatase activity (Vockel et al., 1990; Schlinger and Arnold, 1991, 1992) and it could be feared that this approach by *in vivo* microdialysis could not be extrapolated to other steroids or animal models. Encouraged by this study, we decided however to test whether estradiol could similarly be measured by *in vivo* dialysis in the quail POA, another model system where aromatase activity is relatively high. With the help of a super sensitive RIA based on radioactive iodine we were able to demonstrate that there is within the medial POA a transient rise in concentration of immunoreactive estradiol when males are allowed to copulate with a female. A similar rise was also observed in the bed nucleus of the stria terminalis in males that were only allowed to interact visually with a female and this increase was actually of a longer duration (de Bournonville et al., 2017b). With this ultrasensitive RIA we were even able to limit the collection periods to 10 instead of 30 min thus providing an unprecedented time resolution in the analysis.

How such increases in extracellular concentrations of estradiol can be reconciled with the decrease in aromatase activity observed by *ex vivo* assays in POA dissected immediately after the sexual interaction remains unclear at present. This was recently discussed in full detail (Balthazart, 2017; de Bournonville et al., 2017a; Cornil et al., 2018) and the possible explanations will not be reiterated here. Suffice to say that if aromatase predicts changes in estrogen concentrations over long periods of time (e.g. across seasons), in the short term, aromatase activity does not seem to be a reliable proxy for the local estrogen concentrations present in the brain (Charlier et al., 2011b; Dickens et al., 2014). Phenomena such as diffusion from the synthesis site or catabolism presumably obscure this correlation.

In the microdialysis experiments designed to measure estradiol, we always perfuse the subjects overnight at the rate of 1 μl/min before initiating behavioral experiments in the morning in order to make sure that steroids would be in steady state concentrations. We decided recently to investigate by GC–MS whether these pooled overnight samples collected over 500 min would contain other steroids in detectable concentrations. Quite interestingly 16 steroids could be detected in these samples including pregnenolone, DHEA, estradiol, testosterone and eight of its metabolites. Their concentration was one or two orders of magnitude smaller than the concentrations measured in the entire tissue but they were nevertheless reliably detected (Liere et al., 2019). These results show that this approach could be used to analyze changes over time of multiple steroids in specific brain areas. The currently available sensitivity of the assays would not permit measuring these steroids more than once every 10–12 h which means that dynamic changes in response to brief events such as social interactions could not be studied unless new more sensitive assays (e.g., GC–MS/MS) can be implemented. However this approach would already allow within individual studies of day-to-day changes in the brain hormonal milieu in response to experimental manipulations such as castration, changes in photoperiod or various drug treatments. This could lead to significant increases in our understanding of steroid action in the brain.

In summary, it has become clear during the last 20 years, that two independent mechanisms are controlling brain aromatase activity and probably the activity of other steroid metabolizing enzymes: the well-established relatively slow increase of transcription by sex steroids of the corresponding gene resulting in an increased concentration of the enzymatic protein and the faster modulation by phosphorylations of the activity of a stable concentration of enzymatic molecules. As a consequence, brain concentrations of neuroactive steroids probably change in a much more dynamic fashion than previously believed. This finding also led to another conceptual change concerning the way steroids act on behavior that will be discussed in the next section.

3.4. Rapid membrane-initiated actions of steroids on behavior

The history of the rapid, presumably membrane-initiated, effects of steroids in the brain dates back to the 1970ies when Martin Kelly working in the laboratory of Bob Moss in Texas discovered that estradiol is able to modify the firing of preoptic neurons in vitro within minutes if not seconds (Kelly et al., 1976). During a quarter of century, evidence accumulated demonstrating that steroids have a variety of effects on brain function that are way too rapid to be mediated by changes in gene transcription triggered via the binding to intracellular receptors (for reviews, see namely (Schumacher, 1990; McEwen, 1994; Ramirez et al., 1996; McEwen and Alves, 1999; Ronnekleiv and Kelly, 2002; Rudolph et al., 2016)). To my knowledge, this type of rapid action of sex steroids had barely been investigated in the context of the control of behavior, in particular reproductive behavior, before the beginning of this century, although work in the laboratory of Frank Moore had clearly established that corticosterone inhibits within

minutes the copulatory behavior (clasping reflex) of one species of newt, *Taricha granulosa* (Moore and Miller, 1984; Moore and Orchinik, 1991). In 1999, one first study on male rats demonstrated that a single injection of estradiol increases genital olfactory investigations and mounts with latencies of 20–30 min (Cross and Roselli, 1999). This prompted us to research in quail whether the rapid changes brain aromatase activity that we had identified had any behavioral consequences.

Our initial work based on systemic treatments indicated that a single injection of a high dose (500 μg/kg) of estradiol increases with 15 min male-typical sexual behavior in castrated males provided they have been pre-treated with a sub-threshold, behaviorally inactive, dose of testosterone (Cornil et al., 2006a). Conversely a single injection of the aromatase inhibitor Vorozole™ decreased the expression of copulatory behavior and of one measure of sexual motivation, the rhythmic contractions of the cloacal sphincter muscles, RCSM (Seiwert and Adkins-Regan, 1998; Thompson et al., 1998; Ball and Balthazart, 2010) after latencies of 30–45 min (Cornil et al., 2006b). The magnitude and reproducibility of these effects was however limited and it still did not seem that these rapid effects of estrogens on copulatory behavior of quail were very significant compared to the slower effects mediated by nuclear receptors.

In the meantime, research coming from a number of laboratories investigating multiple animal species and different behavioral variables had accumulated supporting a widespread existence of rapid behavioral effects of (neuro)estrogens (see (Cornil et al., 2012a) for review). Research on the rapid modulation of the central production of estrogens had also progressed quite substantially as described in the previous section. Therefore we initiated a new series of experiments in which estrogens as well as aromatase inhibitors and estrogen receptor antagonists were administered by intra-cerebro-ventricular (ICV) injection in the third ventricle in order to affect more directly the brain centers controlling behavior (Seredynski et al., 2013). These experiments demonstrated that:

(a) in birds deprived of estrogens by a chronic treatment with Vorozole™, a single ICV injection of estradiol increases within 15 min the expression of two responses indicative of the sexual motivation, the RCSM and the learned social proximity response (Domjan and Hall, 1986a,b; Ball and Balthazart, 2010),
(b) these effects are mimicked by an injection of estradiol coupled to bovine serum albumin suggesting that they are induced at the membrane level,
(c) a single injection of an anti-estrogen (Tamoxifen or ICI-182,720 = Faslodex™) (Fig. 6A) or of an aromatase inhibition (Vorozole™ or ATD, androstatrienedione) (Fig. 6B) significantly decreases the rate of RCSM within 15–30 min,
(d) a single ICV injection of Vorozole™ inhibits RCSM but also inhibits the expression of the learned social proximity response; these effects of Vorozole™ are blocked if this injection is preceded by an injection of estradiol or of estradiol coupled to biotin, another membrane-impermeable form of estrogen (Fig. 6C).

All these effects on measures of sexual motivation were obtained while the consummatory part of the behavior (copulation *sensu stricto*) was not affected at least when birds were tested in a small arena where copulation was achieved while the male did not have to search for and pursue the female (Seredynski et al., 2013).

We then wondered which type of membrane estrogen receptor(s) was/were mediating these rapid changes in RCSM frequencies. The acute inhibition induced by Vorozole™ was rescued by the ERβ agonist DPN, but not by the ER α agonist PPT nor by the GPR30 agonist G1 nor by the Gq-mER agonist STX (Fig. 6D) (Seredynski et al., 2015). These effects of DPN were also blocked by the concurrent administration of the specific antagonist of the metabotropic glutamate receptor of type 1a (mGluR1a) LY367,385 (Fig. 6E) (Seredynski et al., 2015) indicating that the occupied membrane estrogen receptors presumably activate sexual motivation via a transactivation of mGluR1a, as suggested for the rapid controls of lordosis in female rodents (Meitzen et al., 2013).

Similar rapid effects of estrogens have now been reported in a large number of experimental models and now concern aggressive behaviors (Trainor et al., 2008; Heimovics et al., 2015), male and female sexual behaviors with a special emphasis of male sexual motivation (Dewing et al., 2007; Cornil et al., 2018; Micevych and Sinchak, 2018), vocal communication in species as diverse as midshipman fishes and canaries (Remage-Healey and Bass, 2004, 2006; Alward et al., 2016a), processing of acoustic signals in songbirds (Remage-Healey, 2013; Krentzel and Remage-Healey, 2015; De Groof et al., 2017), nociception (Evrard and Balthazart, 2004; Amandusson and Blomqvist, 2013; Liu et al., 2017; Storman et al., 2018) and memory formation in rodents (Luine and Frankfurt, 2013; Phan et al., 2015; Sheppard et al., 2017; Frick et al., 2018). The membrane receptors and the intracellular signaling cascades implicated in these behavioral responses have been identified in a fraction of these cases. This field of investigation is now quite extensive and actually justified the recent publication of an entire issue of Hormones and Behavior containing no less than 20 full review papers (see (Balthazart et al., 2018) for the Introduction to this special issue, Vol 104, 2018).

At the conceptual level, rapid actions of estrogens and other steroids are now well established and it is this clear that estrogens in particular have a dual mode of action including rapid effects presumably initiated at the neuronal membrane level and slower effects resulting from changes in the transcription of specific genes regulated by the occupied nuclear receptors (Cornil et al., 2015). Neurosteroids might thus act both as regular hormones but also in a manner more akin to neurotransmitters or at least neuropeptides (Balthazart and Ball, 2006). In the specific case of estrogens, a review of the literature suggests that the rapid actions might concern more specifically motivational aspects of behaviors while the slower presumably nuclear actions would rather modulate the consummatory aspects of these behaviors (Cornil et al., 2015). This distinction seems fairly well established for the control of sexual behaviors in male quail (Seredynski et al., 2013; Seredynski et al., 2015) but may also apply to sexual behavior in rodents (Pfaus, 1996; Sachs, 2007; Taziaux et al., 2007; Vasudevan and Pfaff, 2007; Ball and Balthazart, 2008) and to other forms of motivated behaviors such as drug addiction and possibly auditory processing in songbirds or learning and memory (see (Cornil et al., 2015) for discussion).

A major remaining question would then concern the neuroanatomical organization both at the cellular and subcellular level of this dual mode of action of estrogens. At the macroscopic level, it is indeed not well established whether the slow and fast actions of estrogens are controlled in the same brain nuclei. The distribution of the membrane ER has been poorly investigated: a few papers describe the distribution of GPR30 (in rats: (Brailoiu et al., 2007; Hazell et al., 2009), in zebra finches: (Acharya and Veney, 2012)) but where and in which density the ERα and ERβ are translocated to and signal from the membrane is not well understood. Aromatase is known to be expressed in the neuronal perikarya but also in cells processes including the full length of dendrites and axons (Naftolin et al., 1996; Peterson et al., 2005; Rohmann et al., 2007). Some of these projections extend far away from the perikarya as observed for example in quail where aromatase-immunoreactive neurons massively project to the periaqueductal gray in the mesencephalon (Absil et al., 2001; Carere et al., 2007). It has also been suggested that the hypothalamic aromatase cells could produce estrogens that would be secreted in the third ventricle (Storman et al., 2018). The brain nuclei where estrogens exert their fast membrane-initiated effects therefore do not need to be the same as for the nuclear effects.

At the subcellular level, it is interesting to note that aromatase and ERα are not always colocalized in the same cells: in the quail brain, about 70% of the aromatase positive cells co-express ERα in the VMN but this percentage drops to 16% in the POM and only 4% in the bed

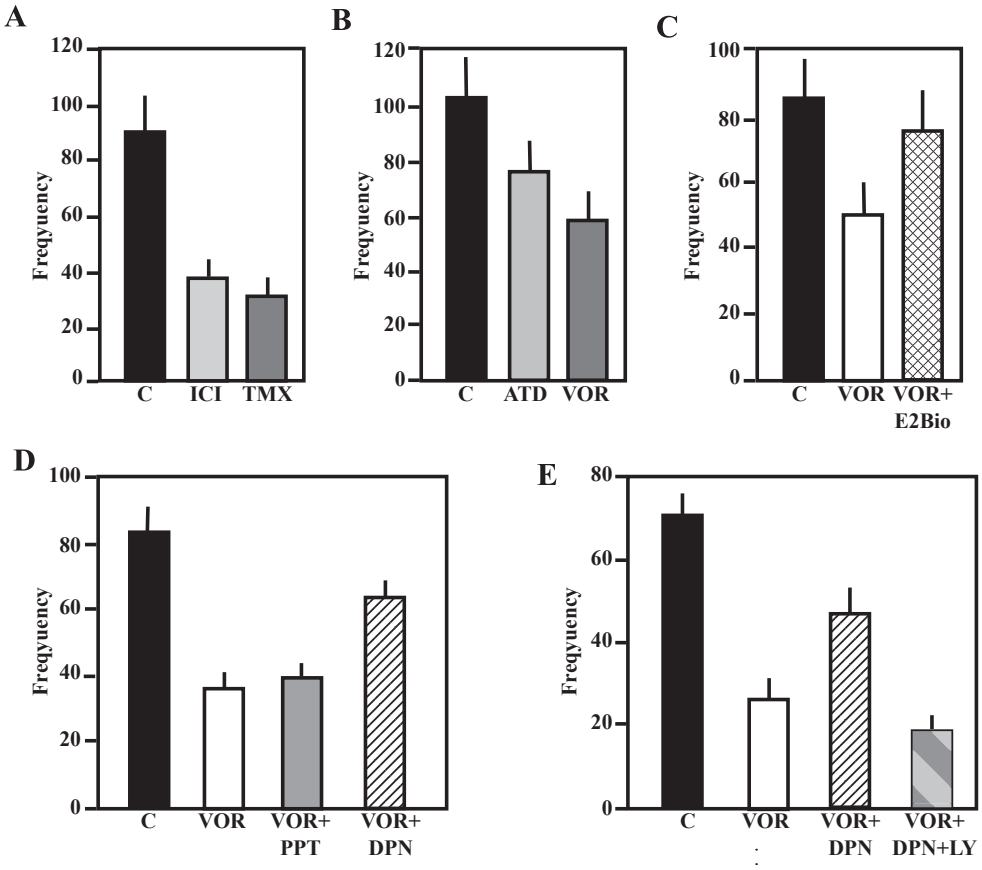

Fig. 6. Rapid effects of estrogens on the appetitive component of male sexual behavior in quail, as measured by the frequency of rhythmic contractions of the cloacal sphincter muscles (RCSM). (A) The frequency of RCSM is inhibited after 15 min by a single injection of an antiestrogen, ICI-182,780 (Fulvestran, ICI) or tamoxifen (TMX). (B) The frequency of RCSM is similarly inhibited 30 min after an injection of a steroidal (ATD) or a non-steroidal (VOR) aromatase inhibitor. (C) The effects of an acute deprivation of estrogens produced by a single injection of VOR 30 min before the test are counteracted by a single injection of the membrane-impermeable E2-biotin performed 15 min before testing. (D) The acute inhibition of RCSM induced by a single injection of VOR is partially reversed by a single injection of the ERβ agonist DPN but not the ERα agonist PPT. (E) Restoration of the RCSM frequency by DPN following its acute inhibition by VOR is blocked by a single injection of the mGluR1 antagonist LY-367,385. Redrawn from data in Seredynski et al. (2013) and Seredynski et al. (2015).

nucleus of the stria terminalis (Balthazart et al., 1991). Furthermore, electron microscopy combined with immunocytochemistry has revealed the presence of both ERα (Blaustein, 1992) and of aromatase (Naftolin et al., 1996; Rohmann et al., 2007) in presynaptic terminals and aromatase is enzymatically active at this level (Steimer, 1988; Schlinger and Callard, 1989; Cornil et al., 2012b). Whether they interact at this level and mediate rapid effects of estrogens is unknown at present. Furthermore a recent study demonstrated by co-immunoprecipitation that the membrane fractions of ERα (mERα) and of aromatase (mARO) associate (oligomerize) to various degrees in different parts of the central nervous system (15% in the hypothalamus but over 90% in the spinal chord) and that this degree of association correlates with the enzymatic activity of aromatase (Storman et al., 2018). Non-genomic actions of estrogens identified so far are compatible with both pre- and post-synaptic modulation of synaptic transmission (Cornil, 2009). The specific subcellular localization of where estrogens are produced and act in a rapid fashion thus remains essentially unknown.

3.5. Steroids and brain plasticity

The idea that the brain is plastic and this plasticity is modulated by steroids obviously predates this century. A broad synthesis of the field was actually published in 2009 (Garcia-Segura, 2009) and this research was extensively covered during the Torino Steroid meetings (Panzica et al., 2012). This namely included talks on effects of neuroestrogens produced by brain aromatase (Azcoitia et al., 2001, 2011), on the neuroprotective effects during brain anoxia of estrogens (Wise, 2002; Suzuki et al., 2006) and progesterone (Stein, 2011).

Adult neurogenesis was (re)discovered in the early eighties in songbirds during studies of seasonal changes of singing behavior performed by Fernando Nottebohm and his colleagues at the Rockefeller University (Goldman and Nottebohm, 1983; Alvarez-Buylla et al., 1988; Goldman, 1998). Birds have since been very useful research subjects in this field namely because they display much larger changes in gonadal activity than mammals between the breeding and non-breeding season. The testes size for example varies 10 to a 100 fold between these two extremes in many species of birds while only one or two fold changes, if any, are generally observed in mammals. Large changes in sex steroids concentrations are similarly observed across seasons in birds and it is therefore not surprising to see in parallel large changes in brain structure and function. For example, the volume of the POM in quail increases by 50–80 percent when castrated males are treated with exogenous testosterone (Panzica et al., 1987, 1996) and the number of aromatase-immunoreactive cells in the nucleus increases 4 to 6 fold following this endocrine treatment (Panzica et al., 1996). Significant changes in these steroid-dependent variables can already be observed after one or two days of exposure to testosterone (Charlier et al., 2008).

Most of our work on adult brain plasticity has however focused on canaries (*Serinus canaria*), a songbird species which shows extensive brain plasticity in response to changes in photoperiod or to treatments with exogenous sex steroids (Balthazart and Ball, 2016). Several studies analyzed in particular the incorporation of new neurons in the adult song control nucleus HVC (formerly acronym for High Vocal Center, now used as a proper name, see (Reiner et al., 2004)). Neurogenesis was assessed by quantification of the exogenous DNA marker bromodeoxyuridine (BrdU) and the endogenous marker of new neurons doublecortin (DCX) (Balthazart et al., 2008; Balthazart and Ball, 2014).

DCX is a microtubule-associated protein that forms a part of the cellular machinery supporting the migration of neurons (Bai et al., 2003; Jin et al., 2004; Moores et al., 2004) and is broadly used in mammals as a marker of young post-mitotic neurons (Francis et al., 1999; Brown et al., 2003; Rao and Shetty, 2004). We identified large populations of DCX-immunoreactive (ir) neurons in areas of the canary brain known to incorporate large numbers of new neurons in adulthood and validated that large fractions of these neurons were double-labeled

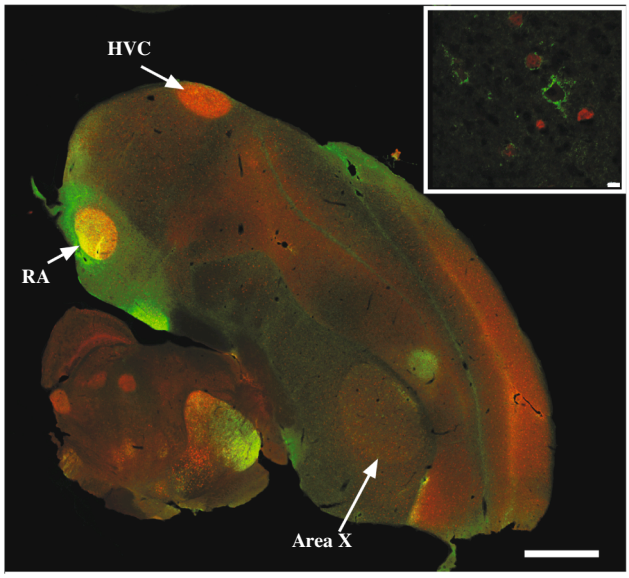

Fig. 7. Photomicrograph of a sagittal section through the male zebra finch brain illustrating the distribution of perineuronal nets (PNN), stained in green by an antibody directed against chondroitin sulfate, and of parvalbumin-immunoreactive neurons (red). Both markers are co-expressed at high density in the three song control nuclei, HVC, RA and Area X. The insert shows a few PNN surrounding or not parvalbumin neurons. Magnification bar = 1 mm in the large panel and 10 μm in the insert. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with BrdU if the DNA analog was injected 10 days before brain collection (Boseret et al., 2007). Like in mammals two types of DCX-ir neurons were present in the canary brain: some displayed a characteristic fusiform shape and represent migrating cells while others had a multipolar shape and represent slightly older neurons during their early stages of differentiation. Both types of neurons were present in particularly high density in HVC and allowed the limits of the nucleus to be outlined.

In multiple experiments, the number of new DCX-ir neurons in HVC was demonstrated to increase in physiological situations associated with an increase in HVC volume including treatment with exogenous testosterone (Balthazart et al., 2008) or its androgenic or estrogenic metabolites (Yamamura et al., 2011) and in photostimulated compared to photorefractory birds (Balthazart et al., 2008). In addition, castrated males in which plasma testosterone concentrations were clamped to a stable level by a subcutaneous implant of testosterone, had more fusiform and round DCX-ir cells in HVC if living in a cage with a female (M − F) than if living with another male (M − M) (Balthazart et al., 2008). These data thus indicate that steroids but also social stimuli, independently of steroid action, modulate neurogenesis in HVC and they do this is an anatomically specific maner since DCX-ir cell densities were usually not affected in the adjacent parts of the nidopallium.

This notion of socially- or activity-driven brain plasticity was further supported by the PhD research of Beau Alward in the laboratory of Gregory F. Ball at the University of Maryland. Beau demonstrated that the singing motivation of male canaries is largely controled by testosterone acting in the medial preoptic area. Stereotaxic implantation of testosterone in the POM indeed activates an intense singing activity in castrated male canaries (Alward et al., 2013), even if the quality of the song produced is low unless testosterone is simultaneously implanted in HVC (Alward et al., 2013, 2016b). Quite interestingly, the singing activity triggered by testosterone action in the POM was positively correlated with a bilateral increase of HVC volume and of the density of DCX-ir cells in this nucleus. Since there is no direct connection between the POM and HVC and since unilateral implantation of testosterone in POM drives a bilateral increase in HVC volume and a moderate increase in DCX-ir cells (Alward et al., 2016b), these data provide a strong support for the notion of activity-driven brain plasticity. Earlier work has suggested that HVC growth is activated by both testosterone and by the singing activity itself with both effects being mediated by an increased secretion of the brain derived neurotrophic factor, BDNF (Rasika et al., 1994, 1999; Alvarez-Borda and Nottebohm, 2002). The present experiments confirm thus dual mechanisms of control.

Together these studies illustrate the usefulness of using an endogenous marker of neurogenesis, eventually combined to the injection and detection of a DNA analog. DCX provides an integrated view of all new neurons present at a given location at a given time while numbers of BrdU-positive cells provide a snap-shot of a selection of these cells that were born at a specific time (within a few hours after the injection, since BrdU is rapidly metabolized and thus labels cells during only a short period) (Packard et al., 1973; Miller and Nowakowski, 1988; Boswald et al., 1990); see (Barker et al., 2013) for similar data in canaries). Although both approaches have their specific advantages and drawbacks (Balthazart and Ball, 2014), taken together they provide a more complete view of the dyamics of neurogenesis and should allow progress in the understanding of how this complex process is modulated by hormones and social stimuli.

During the last few years we also paid attention to another form of plasticity that had been hypothesized to control the connectivity in the song control nuclei and in this way to regulate song crystallization during ontogeny, the perineuronal nets (PNN). PNN are aggregations of extracellular matrix components, including chondroitin sulfate proteoglycans, tenascin R, hyaluronic acid and binding proteins, that form a scaffold mainly around fast spiking GABAergic interneurons expressing parvalbumin (Deepa et al., 2006; Wang and Fawcett, 2012).

In mammals, PNN are supposed to play an important role in the closing of sensitive periods for sensory learning by limiting the associated synaptic plasticity (Hensch, 2004, 2005; Wang and Fawcett, 2012; Werker and Hensch, 2015) in areas such as the somatosensory cortex (Nakamura et al., 2009) and the visual cortex where they develop in an experience-dependent manner following visual stimulation (Liu et al., 2013; Ye and Miao, 2013). In several experimental models, the experimental degradation of PNN has been shown to restore functional plasticity (e.g., (Bradbury et al., 2002; Pizzorusso et al., 2002; Galtrey and Fawcett, 2007; Karetko and Skangiel-Kramska, 2009; Wang and Fawcett, 2012).

PNN were shown to be expressed in very high densities in the song control nuclei of zebra finches in particular in HVC, RA and Area X (Fig. 7) (Balmer et al., 2009; Cornez et al., 2015). One initial study showed that expression of PNN, and in particular PNN located around parvalbumin-positive neurons in HVC, correlates with song development. They are present in much higher densities in adult than in 33 day-old males and delaying song crystallization by tutor deprivation additionally decreased PNN expression (Balmer et al., 2009).

In a series of experiments, we recently collected evidence indicating the PNN development correlates in multiple ways with song crystallization. PNN density is higher in adult males who sing a crystalized song than in females who do not sing in the main song nuclei HVC, RA and Area X (Meyer et al., 2014; Cornez et al., 2015). In adult males in full reproductive condition, this density varies widely between species with different learning abilities (Fig. 8). European starlings who are open-ended learners and can learn new songs at any phase of their reproductive cycle (Bohner et al., 1990; Chaiken et al., 1994) have the lowest PNN expression; canaries another open-ended learning species showing lower singing plasticity are intermediate and zebra finches, a closed-ended learning species have the highest densities of PNN in their song control nuclei (Cornez et al., 2017b).

A detailed study analyzing every 10 days the PNN appearance in the developing zebra finch brain showed that it correlates with the timing of sensitive periods for song learning (Cornez et al., 2018b) and the same conclusion was recently reached in the study of song development

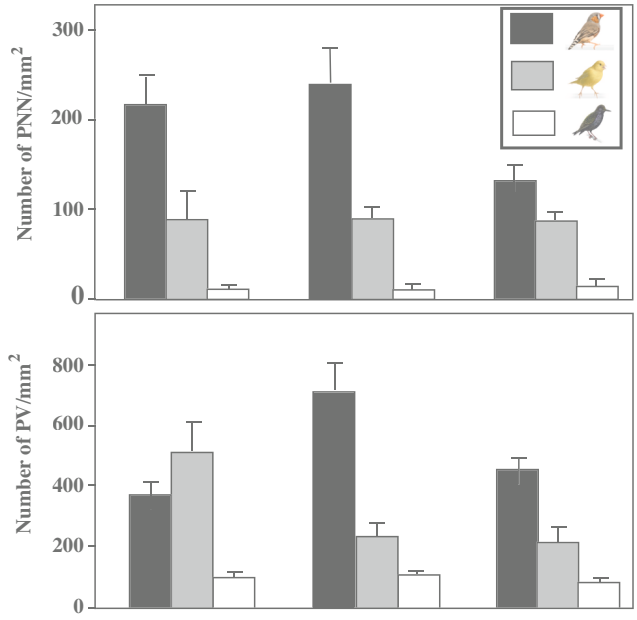

Fig. 8. Comparison of the density (numbers/ mm^2) of perineuronal nets (PNN) and of parvalbumin-immunoreactive (PV) neurons in three song control nuclei, HVC, RA and Area X, of male starlings, canaries and zebra finches that were all in full reproductive condition. Redrawn from data in Cornez et al. (2017b).

in canaries (Cornez et al., 2018a). PNN expression was quantified during the first year of life in male canaries at time points corresponding to specific developmental stages of singing: first spring (subsong), summer (early plastic song), fall (plastic song), winter (ongoing song crystallization), and second spring (fully crystallized song). PNN reached their maximum density in the fall in HVC but only in the winter in RA and Area X. In an additional group treated with testosterone in winter there was no further enhancement of PNN expression over what was observed in untreated birds at the same age. Total song duration and song developmental score only reached their maximum in the spring and were enhanced by testosterone in the winter but energy or band width were already at the adult levels in the winter. Thus PNN seem to contribute to song crystallization that starts in the winter and is completed at the onset of spring.

Although PNN expression did not seem to vary seasonally in European starlings (Cornez et al., 2017b), the open-ended learner showing the largest level of singing plasticity (Chaiken et al., 1994), the canary, a songbird that is also able to modify its song across seasons each year but to a lesser extent, seems to display such seasonal changes in PNN expression (Gilles Cornez and Jacques Balthazart, unpublished data). Canaries thus seem to provide a better model to explore the role of PNN in supporting seasonal variations in singing behavior. We therefore tested in this species whether testosterone, the most prominent endocrine stimulus controlling seasonal cycles, would affect the expression of PNN in male and female canaries.

Treatment of castrated males with a subcutaneous implant of testosterone increased within a few days the song rate of the subjects and increased the density of PNN in HVC, RA and Area X in brains collected after three weeks of treatment (Cornez et al., 2017a). The treatment of females with testosterone similarly increased PNN density in these three song control nuclei. In this case, brains were however collected and studied after 1, 2, 9 and 21 days of exposure to testosterone and the changes in PNN were only significant in the samples collected after 21 days (Cornez et al., 2017a). This effect thus develops only relatively slowly.

All these experiments are consistent with the notion that increases in PNN expression in the song control nuclei, in part under the influence of testosterone, occur in parallel with and presumably induce the development and crystallization of song. We are currently testing this conclusion in a more directly causal manner with a technique initially developed in the laboratory of Teresa Nick based on the dissolution of the PNN by local application of chondroitinase ABC. This approach was shown to deplete PNN in the HVC of zebra finches (Best et al., 2011) and canaries (our unpublished data) for periods up to a few weeks. We are now evaluating whether these manipulations modify the form of established song and/or eventually allows adult male to acquire more easily new song types.

4. Conclusions and perspectives

This brief and selective review of recent research in behavioral neuroendocrinology focusing in part on research performed in the Balthazart/Cornil laboratory in Liege clearly demonstrates the importance of conceptual changes that have taken place in this field since the beginning of the 21st century. These changes were extensively covered in the 10 meetings on Steroids in the Brain that took place every other year in Torino. One could be tempted, as one was in 2000, to think that the conceptual framework of this field is now mature and will not further evolve in the coming years. This is however unlikely to be the case.

We have acquired during the last few years a number of extremely powerful investigation tools such as improvements in molecular biology (RNA seq, CHIP Seq, CRISPR/Cas9, …), optogenetics, designer receptor exclusively activated by designer drugs (DREADD), viral tract-tracing and viral cell type-specific manipulations, *in vivo* imaging for small animals (magnetic resonance imaging, MRI and positron emission tomography, PET). When used alone or in combination, these techniques allow us to address questions that were essentially pure science fiction twenty years ago and these techniques have clearly not yet produced their full effects at the conceptual level.

Multiple new findings based on these techniques have already focused our attention on new ideas and processes. For example, in the domain of the organization and sexual differentiation of behavior, epigenetic studies based on RNA-Seq associated with pharmacological and endocrine manipulations have changed our view of sexual differentiation in rodents by showing that the development of the female brain is not a passive process but involves the chronic repression of a set of male-typical genes and that brain masculinization involves the suppression of this inhibition after exposure to testosterone (Nugent et al., 2015). On another front, molecular biology and biochemical techniques have identified mTOR (the mechanistic Target Of Rapamycin) as a key player in the process of socially-mediated song learning in zebra finches (Ahmadiantehrani and London, 2017), which has opened a brand new avenue of research.

In the study of the activation of adult behavior, viral tract-tracing combined with optogenetics have provided a much more detailed description of the neural circuits mediating lordosis behavior and demonstrated the causal implication of kisspeptin in the control of this behavior (Hellier et al., 2018). Similarly, the combination of genetically modified mice with viral transfections and optogenetics recently led to the clear demonstration that neurons expressing aromatase in a subdivision of the bed nucleus of the stria terminalis mediate sex recognition and social behaviors in naïve male mice (Bayless et al., 2019).

It can thus be expected that our understanding of hormones, brain and behavior will continue to improve in a very fundamental manner over the coming years. Recently progress was largely made in mice models due to technical limitations. In fact, during the 20th century, research has progressively focused on a decreasing number of animal models (Logan, 2002) and this tendency increased with the availability of molecular techniques that could be applied to mice only. These few animal models (mice and rats in mammals, zebra finches and chicken in birds, anolis in reptiles, zebra fish in fishes) have a specialized biology and it is therefore difficult and adventurous to generalize findings made in these species (see for example (Smale et al., 2005)). Fortunately,

genetic manipulations became more recently possible in other organisms and it can be anticipated that new findings will now be expanded to include other species including humans. Let's hope that the Torino meeting on Steroids and the Nervous System will still be there to report on these findings.

Acknowledgments

The research from my laboratory, now the laboratory of Charlotte Cornil since I became Emeritus, described in this review was performed over the years with the collaboration of a large number of undergraduates, PhD students, post-docs and external collaborators who are too numerous to be listed here. I sincerely thank them for their contribution and for all the discussions we had about behavioral neuroendocrinology that have deeply influenced my thinking and the present review. This research was financially supported by multiple grants from the Belgian FNRS (Fonds National de la Recherche Scientifique) and from the University of Liège and by grants from the National Institutes of Health to Gregory F. Ball, Charlotte Cornil and myself (MH50388, NS035467 and NS104008).

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