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Rapid changes in auditory processing in songbirds following acute aromatase inhibition as assessed by fMRI

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Abstract

This review introduces functional MRI (fMRI) as an outstanding tool to assess rapid effects of sex steroids on auditory processing in seasonal songbirds. We emphasize specific advantages of this method as compared to other more conventional and invasive methods used for this purpose and summarize an exemplary auditory fMRI study performed on male starlings exposed to different types of starling song before and immediately after the inhibition of aromatase activity by an i.p. injection of Vorozole™. We describe how most challenges that relate to the necessity to anesthetize subjects and minimize image- and sound-artifacts can be overcome in order to obtain a voxel-based 3D-representation of changes in auditory brain activity to various sound stimuli before and immediately after a pharmacologically-induced depletion of endogenous estrogens. Analysis of the fMRI data by assumption-free statistical methods identified fast specific changes in activity in the auditory brain regions that were stimulus-specific, varying over different seasons, and in several instances lateralized to the left side of the brain. This set of results illustrates the unique features of fMRI that provides opportunities to localize and quantify the brain responses to rapid changes in hormonal status. fMRI offers a new image-guided research strategy in which the spatio-temporal profile of fast neuromodulations can be identified and linked to specific behavioral inputs or outputs. This approach can also be combined with more localized invasive methods to investigate the mechanisms underlying the observed neural changes.

Highlights

-fMRI detects rapid changes in brain activity induced by acute aromatase inhibition

-Effects of aromatase inhibition on starling brain auditory activity vary across seasons

-Some effects of aromatase inhibition are lateralized to the left auditory cortex

-Effects of aromatase inhibition are stimulus-specific

-fMRI is an outstanding tool to localize rapid effects of steroids in the brain

1. Introduction

Estrogens exert pleiotropic effects on a variety of morphological, physiological and behavioral responses in all vertebrate classes ranging from fishes to mammals. Effects of estrogens in the brain concern reproductive behaviors (aggression, copulation, sexual differentiation, control of gonadotropins secretion) but also pain perception, language acquisition, various aspects of cognition (verbal and spatial memory, mood), as well as more broadly brain plasticity (neurogenesis, synaptogenesis, spinogenesis, angiogenesis, neuroprotection), cancer cell growth or immune function (Amandusson and Blomqvist, 2013; Au et al., 2016; Ervin et al., 2015; Fortress and Frick, 2014; Luine and Frankfurt, 2013; Marrocco and McEwen, 2016; Srivastava et al., 2013) (for more information see two full books on this topic: (Balthazart and Ball, 2013; Etgen and Pfaff, 2009)).

It has long been thought that effects of estrogens on behavior rely on the same genomic mechanisms as morphological effects: estrogens regulate the expression of a variety of transmitters synthesizing or metabolizing enzymes and of neurotransmitters receptors, which in turn affect behavior expression. Estrogens like other steroids are indeed known to bind to intracellular receptors that then act as transcription factors to regulate gene expression (Jensen et al., 1968; McEwen and Alves, 1999). As such, these effects are relatively slow since they involve the transcription of specific genes into mRNA, their translation and then often posttranslational modifications of the resulting proteins and their incorporation into functional units (e.g. at the neuronal membrane). As a whole these processes take hours and sometimes days and as a result these genomic effects of steroid on behavior usually have latencies that range between a few hours and a few days.

Faster actions of estrogens were however detected first at the cellular level (Kelly et al., 1976) (for reviews see:(McEwen, 1994; Schumacher, 1990)) and then more recently in terms of behavior control ((Cross and Roselli, 1999); reviews in (Balthazart and Ball, 2013; Cornil et al., 2012)). There was consequently a major paradigm shift in how we consider estrogens and more generally steroid action (Balthazart et al., 2018). Multiple reviews have dealt with this new way of thinking about steroid action (e.g. (Balthazart, 2017; Cornil et al., 2015; Foradori et al., 2008; Ronnekleiv and Kelly, 2017; Rudolph et al., 2016; Saldanha et al., 2011; Vasudevan and Pfaff, 2008). The current

Special Issue of Hormones and Behavior focuses on these fast actions of estrogens, and more broadly of steroids, on brain and behavior. In this context, we review here recent data demonstrating rapid actions of estrogens on stimulus processing in animals considering in particular our recent work analyzing rapid estrogen action on auditory processing in songbirds as assessed by functional magnetic resonance imaging (fMRI)(De Groof et al., 2017).

2. Sex steroids and social communication

Estradiol and testosterone have long been known to modulate both the production and the processing of external stimuli, in particular stimuli relevant to reproductive behaviors (Ball and Balthazart, 2009). It is for example clear that the production of rodent pheromones that control sexual interactions (Baum and Bakker, 2013; Petrulis, 2013) and the vocal activity of some fishes, amphibians and songbirds are markedly influenced by androgens and estrogens (Bass, 2008; Brenowitz, 2004, 2008; Zornik and Kelley, 2011). Similarly, androgens drastically modify the electric organ discharges that are used by gymnotiform electric fishes for sex recognition (Bass and Zakon, 2005).

Additionally, a host of studies demonstrate that steroids modify the perception and processing of sensory signals. This topic was recently covered in a special issue of Frontiers in Neuroendocrinology considering olfaction, vision, nociception and most importantly audition, which is the focus of this review (Balthazart, 2013). These effects of steroids are largely mediated by changes in the processing and interpretation of sensory signals by the brain, more than by changes in the sensory receptors themselves. There are only few examples where steroids were shown to modify the sensitivity or specificity of sensory detectors. One exception concerns neotropical electric fishes in which tuning of electrosensory receptors to changes in electrical organ discharges is affected by sex steroids. Similarly, estrogens modulate the inner ear capacity for encoding frequencies in female midshipman fishes, *Porichthys notatus* (Bass and Zakon, 2005). Accordingly, estrogen receptors and sometimes the estrogen producing enzyme, aromatase, are expressed in the inner ear of a wide variety of species ranging from fishes to birds, rodents and probably humans (Bass and Zakon, 2005; Chariditi and Canlon, 2010; Munaut et al., 2001; Noirot et al., 2009). Sex steroids could thus act directly on the inner ear but there is to date limited evidence that this is the case, possibly because this possibility has not been extensively investigated.

There are in contrast many studies demonstrating that sex steroids modulate brain processing of stimuli related to reproduction and this correlates with the observation that steroid receptors and aromatase are expressed in many brain nuclei that are part of the olfactory, visual and auditory pathways. Nearly 50 years ago, Pfaff and Pfaffmann already discovered that sexually relevant olfactory inputs only reach the preoptic area of male rats if they are exposed to elevated concentrations of testosterone (Pfaff and Pfaffmann, 1969). Castration does not modify the detection of these signals by the olfactory receptors as shown by the fact that they elicit neural activity in the olfactory bulbs, but the surgery completely blocks the transfer of this information to the preoptic region, and this effect is reversed by a treatment with exogenous testosterone. Importantly, this effect of testosterone is specific to olfactory signals controlling sexual behavior (pheromones): other olfactory signals never reach the preoptic area independently of the endocrine condition of the subjects. This concept (central rather than peripheral controls) is supported by multiple studies based namely on the detection of brain activity via the demonstration of increased immediate early gene expression (fos or egr-1) that have subsequently confirmed the role of sex steroids in the selective processing of sexually relevant olfactory stimuli (Baum and Bakker, 2013; Petrulis, 2013; Wood and Newman, 1995).

Other sensory modalities are similarly affected by sex steroids. This is not extensively documented for the visual system (Little, 2013) and in this specific case effects of estrogen seem to be mostly impacting the eye (Gupta et al., 2005; Hutchinson et al., 2014). The work of Richmond Thompson and collaborators in goldfishes (*Carassius auratus*) similarly demonstrates rapid actions of estrogens on the processing of visual stimuli and effects are mediated at least in part in the eye although this work does not exclude so far additional effects in the central nervous system (Lord et al., 2009; Mangiamele et al., 2017; Yue et al., 2018). In contrast, there is an extensive literature demonstrating effects of sex steroids on the perception and processing of somatosensory inputs, in particular pain stimuli. This topic is however complex and the modulatory effect of sex steroids on nociception seems to vary as a function of the sex of the subjects, the type of painful stimuli and other specific aspects of the experimental protocols. This topic has been reviewed multiple times recently (see for recent reviews: (Amandusson and Blomqvist, 2013; Gintzler and Liu, 2012; Traub and Ji, 2013)) and is beyond the scope of the present paper.

The modulation of audition by steroids has also received a substantial attention in a variety of animal models including humans (Caras, 2013) and there is in particular a very substantial literature of the effect of estrogens on the processing of auditory stimuli, which is the focus of this review.

3. Estrogens and audition

There is multidimensional correlative evidence suggesting that estrogens modulate auditory function in humans. Women demonstrate a better perceptual sensitivity for high frequency sounds compared to males, their auditory function fluctuates in parallel with circulating estrogen concentrations during the menstrual cycle, and auditory function is decreased during pregnancy and after menopause but can be restored by a treatment with exogenous estrogens (reviewed in (Caras, 2013)). The causal value of these correlations has not been tested in humans for obvious ethical reasons but there is a variety of animal studies in fishes, frogs, birds and mammals demonstrating in a causal way the impact of estrogens on the processing of auditory stimuli (Caras, 2013; Maney and Pinaud, 2011; Miranda and Liu, 2009; Sisneros, 2009; Wilczynski et al., 1993; Zornik and Kelley, 2011). We briefly review here the available information related to songbirds that are the focus of this review and were used in our functional Magnetic Resonance Imaging (fMRI) studies.

Songbirds are outstanding models in the study of vocal communication (Maney and Pinaud, 2011). Following the pioneering studies of William H. Thorpe and Peter Marler on the development of song in chaffinches (*Fringilla coelebs*) and white-crowned sparrows (*Zonotrichia leucophrys*) (Marler, 2004; Thorpe, 1954), Nottebohm and colleagues identified the neural network mediating song production in oscines (Nottebohm, 1980; Nottebohm et al., 1976). The auditory inputs to this circuitry were later described (Mello et al., 2004; Theunissen et al., 2008) and formed the basis of the functional studies of estrogens on audition. As already mentioned, estrogen receptors alpha and aromatase are expressed in the zebra finch (*Taeniopygia guttata*) inner ear (Noirot et al., 2009). They are also expressed together with estrogen receptor beta at high densities in a major telencephalic second-order auditory area of songbirds, the caudomedial nidopallium, NCM (Jeong et al., 2011; Metzdorf et al., 1999; Peterson et al., 2005; Saldanha and Coomaralingam, 2005; Saldanha et al., 2000). Additionally, the membrane G protein-coupled estrogen receptor GPER1, also known as GPR30, is

expressed in broad areas of the zebra finch telencephalon (Acharya and Veney, 2012; Krentzel et al., 2018).

Three types of evidence indicate that systemic estradiol presumably acting through these estrogen receptors regulate auditory sensitivity and selectivity. First, the auditory brainstem response (ABR) recorded immediately after the onset of auditory stimuli have an increased amplitude during the spring when circulating estradiol concentrations are high in a number of songbird species (Henry and Lucas, 2009; Lucas et al., 2007). Opposite effects have however been observed in white-breasted nuthatches (*Sitta carolinensis*) and downy woodpeckers (*Picoides pubescens*) (Lucas et al., 2002; Lucas et al., 2007). Seasonal plasticity thus seems to affect the peripheral auditory processing, possibly with estrogenic mediation of inner ear function (Caras, 2013). Accordingly, in another seasonal songbird, the Gambel's white-crowned sparrow (*Zonotrichia leucophrys gambelii*), females in breeding condition (i.e. maintained under long day length and implanted with a subcutaneous estradiol pellet), the ABRs displayed elevated threshold and longer peak latencies compared to ABRs from females in non-breeding condition (short day length and no estradiol implant) (Caras et al., 2010).

Secondly, this role of estrogens is confirmed by electrophysiological recording of neurons in telencephalic auditory areas. The comparison of extracellular single unit responses to pure tones or to conspecific songs in white-crowned sparrows (*Zonotrichia leucophrys)* brought into breeding or non-breeding condition as described above identified highly specific effects of estradiol (Caras et al., 2012). Estradiol increased the spontaneous firing rates, the maximally evoked firing rates and the auditory response strength across a wide range of stimuli specifically in one class of cells showing a monotonic response to stimuli. The response properties of these cells were additionally correlated with circulating estradiol concentrations. These effects were not present in the non-monotonic cells that display suppressed firing rates at higher sound intensities. Other studies based on single- and multi-units recordings in canaries (*Serinus canaria*) have additionally reported enhanced spontaneous activity and neural selectivity for the bird own song (BOS) as a function of season in the song control nucleus HVC (used as a proper name) that receives indirect auditory inputs from the telencephalic auditory areas (Del Negro and Edeline, 2002; Del Negro et al., 2000; Del Negro et al., 2005). Similarly in zebra finches, increases in neuroestrogens in the auditory area NCM enhance song selectivity in HVC whereas opposite effects are observed following

inhibition of neuroestrogens production in NCM (Remage-Healey and Joshi, 2012).

Thirdly and finally, multiple studies have analyzed the activation of neurons in the auditory telencephalic areas of songbirds in response to various auditory stimuli. Neuronal activation was identified by the increased expression of immediate early genes such as fos or ZENK (also know as zif-268, egr-1, NIFI-A or krox-24)(Maney and Pinaud, 2011; Mello et al., 2004). These studies clearly demonstrate that the neuronal activation in these auditory area is specifically related to the nature of the sound stimuli ((Gentner et al., 2001; Leitner et al., 2005; Mello and Clayton, 1994; Mello and Ribeiro, 1998; Monbureau et al., 2015), reviewed in (Maney and Pinaud, 2011)).

In addition, this approach has shown that estrogens modulate the immediate early gene response in telencephalic auditory area mainly in NCM and in the caudomedial mesopallium, CMM. In females of a seasonally-breeding species, the white-throated sparrow (*Zonotrichia albicollis*), the systemic administration of estradiol resulted in a higher density of ZENK-immunopositive cells in NCM and CMM in response to song exposure as compared to pure tones but this stimulus specificity was absent in untreated birds (Maney et al., 2006; Sanford et al., 2010). Similarly, species-specific songs and calls induced a higher density of ZENK–positive cells than heterospecific vocalizations in the NCM of male black-capped chickadees (*Poecile atricapilus*) during the breeding season but not in birds that were not in reproductive condition (Phillmore et al., 2011)

Taken together, these studies indicate that estrogens modulate the auditory inputs reaching HVC. How much of this regulation mirrors changes in the inner ear that are reflected in the ABR versus central changes taking place in the telencephalic auditory areas remains somewhat unclear but recent work focusing on rapid effects of estrogens brings direct support to the latter of these options. This work has been reviewed multiple times (Krentzel and Remage-Healey, 2015; Remage-Healey, 2012, 2013; Remage-Healey et al., 2012) and is also considered in detail in this special issue (see Remage-Healey and Vahaba, this volume). It will thus be mentioned here only briefly.

The development of *in vivo* dialysis and of ultrasensitive radioimmunoassays for estradiol recently allowed investigations of endogenous fluctuations of estradiol concentrations in the NCM of awake zebra finches. These studies revealed that local estradiol concentrations significantly increase within 30 min in NCM but not in adjacent

areas when males engage in social interactions with females (Remage-Healey et al., 2008). Additional experiments showed that acoustic playback of male songs is sufficient to produce rapid increase in NCM estradiol concentrations (Remage-Healey et al., 2008). Because these changes were not reflected in the periphery, they presumably reflect a local production of neuroestrogens.

Retrodialysis of estradiol or of aromatase inhibitors was then combined with electrophysiology to analyze the functional significance of these rapid changes in estradiol concentration in NCM. Retrodialysis of estradiol in anesthetized males rapidly (within minutes) increased the auditory-evoked activity of NCM neurons and this response returned to baseline in the subsequent wash-out condition (Remage-Healey et al., 2010). This global increase was caused by the switch of some neurons from a tonic, isolated firing pattern to a burst firing pattern under the influence of estradiol. Conversely when the aromatase inhibitor Fadrozole™ was retrodialyzed, the rate of burst firing in neurons was significantly inhibited and again the effect rapidly disappeared at wash-out. Similar rapid electrophysiological effects of estradiol were observed in females although in this sex, elevated neuroestradiol concentrations were observed only in response to auditory stimuli whereas they were detected in response to visual and auditory stimuli in males (Remage-Healey et al., 2012).

Another heroic set of studies combining retrodialysis with electrophysiological recording at two distinct brain sites further indicated that the estradiol-induced increase in auditory-evoked firing in NCM also enhanced the selectivity of auditory-evoked responses in the downstream sensorimotor nucleus HVC (Remage-Healey and Joshi, 2012). Because NCM is not mono-synaptically connected to HVC, additional research was designed to investigate the pathway mediating this effect. This research demonstrated that the rapid effects of estrogens in NCM influence HVC via the nucleus interfacialis of the nidopallium (Nif) that has a direct input to HVC. Acute administration of estradiol to NCM increased the baseline firing rate and auditory-evoked firing rates in Nif while blockade of estradiol synthesis in NCM decreased selectivity of Nif and HVC neurons (Pawlisch and Remage-Healey, 2015).

It was also shown that these changes in electrophysiological activity in NCM and HVC have functional consequences at the behavioral level. Retrodialysis of Fadrozole™ into the left NCM of awake male zebra finches caused within 30 min an acute suppression of their preference for their own song versus a conspecific song and after

30 min of wash-out the preference for the familiar versus unfamiliar song was restored. Surprisingly, no such effect was observed after retrodialysis of Fadrozole™ in the right NCM (Remage-Healey et al., 2010).

A more recent study of this group revealed an age-dependent lateralisation of estradiol action on auditory processing in male zebra finches. More specifically, estradiol decreased auditory responsiveness in both hemispheres during the sensory phase of song learning, but estradiol increased auditory response in the right NCM and still decreased it in the left NCM during the sensorimotor phase (Vahaba et al., 2017). Given that previous studies in adult birds showed that inhibition of estrogens production in the left NCM suppresses burst firing of auditory neurons and interferes with proper song discrimination (Remage-Healey et al., 2010), it appears that there is an age-dependent shift in auditory processing and in its lateralized sensitivity to estrogens that would deserve further investigation. Taken together, all these data indicate that estrogens modulate auditory perception on songbirds by acting both at the level of the inner ear and in the telencephalic auditory area NCM.

4. Functional Magnetic Resonance Imaging for non-invasive studies of brain activity and its (hormonal) modulations

To obtain a comprehensive understanding of the neuromodulatory impact of hormones, a careful selection of appropriate research strategies and techniques is crucial. As outlined above, most research on neuronal activity and its modulation by hormones in songbirds was so far performed using electrophysiology or immediate early gene techniques (ZENK, fos). These techniques are highly sensitive and specific, but they are invasive and/or constrained to a specific region of the brain. Therefore, they do not allow (1) to link changes in time of behavior with possible alterations in brain structure and function in the same bird and (2) to discern comprehensive neural network modulations over time within the same bird. In addition, it has been clearly demonstrated that any kind of brain injury induces expression of glial aromatase in both the songbird and mammalian brain (Garcia-Segura et al., 1999; Peterson et al., 2001; Saldanha et al., 2005). The simple fact of lowering an electrode or dialysis probe into the brain thus induces expression of the estrogen-synthesizing enzyme, which potentially confounds the results of studies analyzing actions of neuroestrogens. In contrast, *in vivo* imaging techniques and in particular fMRI is capable of overcoming these limitations

and was proven appropriate and very promising for observing and further unraveling fast hormone actions in the brain.

We will now briefly introduce the principles and the applicability of *in vivo* MRI as a novel tool that was first applied to the study of songbirds by our team in 1998 (Van der Linden et al., 1998). This has since then resulted in an extensive list of publications based on *in vivo* MRI studies of songbirds from our team often in collaboration with other songbird neuroscientists. These studies are dedicated to topics such as MRI atlases (De Groof et al., 2016; Gunturkun et al., 2013; Poirier et al., 2008; Vellema et al., 2011), or seasonal and hormone-driven neuroplasticity in canaries (Tindemans et al., 2003; Van der Linden et al., 2002) or starlings (De Groof et al., 2009; De Groof et al., 2008; Van der Linden et al., 2004; Van der Linden et al., 2002; Van Meir et al., 2004).

These anatomical studies were followed by the first functional MRI studies of auditory processing in songbirds that were performed in starlings (Van Meir et al., 2005; De Groof et al., 2017; De Groof et al., 2013b) and in zebra finches (Boumans et al., 2008a; Boumans et al., 2007; Boumans et al., 2008b; Poirier et al., 2011; Poirier et al., 2010; van der Kant et al., 2013; Van Ruijssevelt et al., 2018; Van Ruijssevelt et al., 2017a; Van Ruijssevelt et al., 2017b; Vignal et al., 2008)(For reviews see: (Hamaide et al., 2016; Van der Linden et al., 2009; Van Ruijssevelt et al., 2013b)). Auditory processing in zebra finches was further investigated using functional MRI by Voss et al. (Voss et al., 2007), focusing on modulations in auditory responses in a model for stuttering (Voss et al., 2010) and in song-deprived males and females (Maul et al., 2010).

In principle, MRI is a non-invasive imaging technique used both in clinical and research settings to investigate all types of soft tissue including the brain. MRI allows the acquisition of virtual slices in any direction throughout the entire organism or any part of it such as the brain. Its success mainly resides in its high spatial resolution (e.g., 150 microns or even less in small animals), superior tissue contrast and non-invasive nature, which allows repeated imaging of the same subject. This permits longitudinal studies where for each subject the outcome can be compared to its own baseline measurement along the course of a pathology, over different training sessions or before and after neuromodulatory interventions of any kind (For more details on the MRI method, we refer the reader to the following books: (McRobbie et al., 2006; Westbrook et al., 2005)).

After image acquisition, data can be processed by assumption-free data-driven strategies enabling detection of changes throughout the entire brain instead of using

Regions Of Interest (ROI)-based processing techniques in a hypothesis-driven approach. Furthermore, the MRI signal can be sensitized to a wide range of biological phenomena by varying the MRI acquisition protocols. This implies that using MRI one can explore - even within the same experiment and in 3D - anatomy, microstructural tissue characteristics, blood perfusion and, if studying the brain, even neuronal activation in response to any type of stimulation.

This latter approach is called functional MRI (fMRI) and uses neuronal activity-induced changes in the local blood perfusion (the so-called hemodynamic response) as readout for brain activity. The responsible contrast mechanism is the Blood-Oxygenation Level-Dependent (BOLD) contrast that relies on subtle differences in the magnetic properties of oxygenated (diamagnetic) versus deoxygenated (paramagnetic, disturbing the local magnetic field) hemoglobin. When challenged by a specific task or stimulation, specific neuronal populations will metabolize more glucose and thus use more oxygen. This will induce a hemodynamic response resulting in an increase of the blood flow and blood volume in that area within a few seconds. This will also result in a local change of the ratio of oxygenated versus deoxygenated hemoglobin evoking a local BOLD contrast (Logothetis, 2002, 2008; Logothetis et al., 2001). Subjects must remain immobilized throughout the scanning procedure for fMRI to be successful. For animals, this usually requires the use of anesthesia, which limits the type of experiments or stimulation paradigms that can be applied (see also (Van der Linden et al., 2007)). However, a number of studies have explored possible ways of performing fMRI studies by implementing training protocols to teach animals to remain immobile in combination with restraining. Examples of awake fMRI are found in rodents (Ferris et al., 2011; Jonckers et al., 2014; King et al., 2005) but also in pigeons (De Groof et al., 2013a) and zebra finches (Van Ruijssevelt et al., 2017a).

Exact details on how fMRI can be performed in songbirds are provided in a review paper (Poirier et al., 2010) but also in a video available in JoVe (Van Ruijssevelt et al., 2013a). In the next chapter, we now summarize a study that we recently published (De Groof et al., 2017) to illustrate the use of fMRI in studying the spatial extend of rapid effects of aromatase inhibition on auditory processing in the telencephalon/brain of male starlings over different seasons.

5. Functional MRI visualizes fast effects of brain estrogens depletion following acute aromatase inhibition on auditory processing in a seasonal songbird

We initially established using fMRI that auditory processing by the caudomedial nidopallium (NCM) of male European starlings (*Sturnus vulgaris*) of species-specific aspects of songs (whistles and warblings i.e. high-pitched trills coming from the end of the warbling that are found in the repertoire of all male starlings; SPEC) significantly differs between breeding and non-breeding seasons, while processing of individual-specific aspects of songs (individual motifs taken from the initial part of the warbling that are specific to each individual; INDIV) and pure-tones (control) remain unaffected (De Groof et al., 2013b) (see (Hausberger et al., 1997) for detailed description of these vocalizations). Seasonal songbirds are exposed to extremely different concentrations of testosterone (T) and its brain metabolite estradiol (E2) in the breeding and non-breeding seasons (Fusani et al., 2000; Riters et al., 2001; Soma et al., 2003). In follow-up studies, we therefore asked whether these seasonal differences in activity of auditory regions are controlled by changes in steroid concentrations. Furthermore, since E2 has been shown to rapidly modulate neuronal activity in the auditory cortex of zebra finches (Remage-Healey et al., 2010; Tremere and Pinaud, 2011), we asked whether rapid changes in brain estrogens concentrations would also affect auditory processing and which brain regions would be involved. Availability of estrogens in the brain was affected in these experiments by acutely blocking aromatase activity by an i.p. injection of the aromatase inhibitor Vorozole™ (VOR).

Finally, because studies in a variety of models showed that rapid behavioral effects of E2 are modulated by the photoperiod (in California mice, *Peromyscus californicus*; (Trainor et al., 2008)) or by seasons (in a songbird, the song sparrow, *Melospiza melodia*; (Heimovics et al., 2012; Heimovics et al., 2015)), we wondered whether rapid effects of manipulations of E2 availability would be similar at different times of the year. Therefore, fMRI imaging sessions assessing acute effects of estrogen depletion were performed at three different times of the year, specifically in December (birds exposed to a 8 h light/16 h dark cycle), in early March (11 h light/13 h dark) and again in May/June (16 h light/8 h dark). Because we wanted to assess the effect of seasonal changes on rapid effects of E2 depletion without the confounding effect of the seasonal changes in circulating testosterone (T) concentrations, males were chronically treated with T for 3 weeks before each imaging session.

In each set of studies, fMRI was used to assess auditory brain responses to the presentation of songs stimuli conveying either species-specific (SPEC songs) or individual information (INDIV songs)(see (De Groof et al., 2017)) or to synthetic pure tones (PT). fMRI responses were first quantified during a control session and then 10 min after an intraperitoneal injection of the aromatase inhibitor Vorozole (6-[(4-chlorophenyl)(1*H*-1,2,4-triazol-1-yl)methyl]-1-methyl-1*H*-benzotriazole; 30 mg/kg) (VOR; (Wouters et al., 1994)) (See Figure 1).

Insert figure 1 about here

fMRI data were acquired in a Magnetic Resonance system (Bruker Biospin) as previously described (De Groof et al., 2013b). Subjects were anesthetized throughout data acquisition and their body temperature was constantly monitored and maintained at 41.5±0.5 °C by a feedback heating system, a critical feature to ensure reliability and reproducibility of measures of brain activity. Anesthesia was first induced by an intramuscular injection of a standard amount of medetomidine and ketamine and then maintained during the entire period of image acquisition by continuous infusion of this mixture at doses that were adapted to maintain a stable respiration rate and amplitude. fMRI data were acquired using a T_2-weighted fast-spin echo sequence.. In addition, anatomical three-dimensional (3D) images were obtained using a RARE T_2-weighted sequence to provide accurate localization of functional data.

Each imaging session was based on an ON/OFF block design alternating periods of auditory stimulation (ON blocks) with resting periods (OFF blocks). Each block (ON or OFF) lasted 16 s, which allowed enough time for the acquisition of two images (8 s are needed to acquired an image of the whole brain). Each stimulus type was presented 42 times, thus resulting in the acquisition of 84 images per stimulus. The order of stimuli presentation was counterbalanced within and between subjects and each starling was presented with the same set of three stimuli during each fMRI session in each season.

5.1. Localization by fMRI of the acute effects of aromatase inhibition on auditory responses in the starling brain over seasons

fMRI data sets of the entire brain were obtained repeatedly from the same bird over three different seasons and before and after an i.p. injection of Vorozole™ (VOR) (see figure 1). All fMRI images from each subject were then co-registered to each individual anatomical 3D dataset and spatially normalized (SPM12) with a high-resolution *ex vivo* starling MRI atlas (De Groof et al., 2016). Subsequently, the entire set of data was analyzed by a three way ANOVA including the two treatments (Control vs. VOR), the different stimuli (SPEC, INDIV and PT) and the three seasons (December, March, May/June) as factors. Main effects of the factors and of their interactions were subsequently explored looking selectively at voxels that demonstrated a significant BOLD response to any of the stimuli at any time point.

This global analysis identified a significant overall effect of stimuli in a bilateral cluster and a significant overall effect of treatments in a cluster specifically located on the left side of the brain. There was also a nearly significant season by treatment interaction.

The area demonstrating an overall effect of stimuli is illustrated in figure 2. The voxels displaying a significant effect overlap with NCM, Field L and CMM on both sides of the brain. Additional analyses showed that this global effect resulted from a higher BOLD response to INDIV as compared to PT and to SPEC as compared to PT.

Insert figure 2 about here

The overall effect of treatment (VOR vs. Control) concerned an area overlapping with the rostral NCM and Field L but was quite interestingly located exclusively in the left hemisphere (see figure 3).

Insert figure 3 about here

The acute inhibition of aromatase by VOR thus triggered a rapid and marked decrease in BOLD responses to conspecific songs in auditory brain regions. Additional analyses revealed that the overall effect was essentially triggered by the largest effect present in March and this is actually the only season when post-hoc tests indicated a significant effect of VOR. Although there was no overall effect of seasons, the post-hoc tests also

indicated a decrease in response in the control condition in May/June as compared to March.

Finally, the season by treatment interaction specifically concerned the caudal NCM and was again exclusively located in the left hemisphere (see figure 4).

Insert figure 4 about here

The post-hoc analyses demonstrated that the interaction was due to the presence of a significant treatment effect in March but not in the two other seasons and also from a lower response in control conditions in May/June as compared to the two other seasons.

5.2. Lateralization of responses

A number of brain activations were thus observed in the left but not the right hemisphere. This however did not by itself demonstrate the existence of a lateralization. Voxels might just be significant on one side and be just above the significance threshold on the other side but not be statistically different between the left and right side. In order to determine whether there were statistically significant differences in responses between the two hemispheres, we directly compared brain activation in the regions of interest in the left side defined by the analyses described above and in equivalent areas on the right side. These analyses thus concerned the rostral NCM/Field L where an effect of treatment had been identified on the left side and the caudal NCM that was affected by the treatment by season interaction. The corresponding results are summarized in figure 5.

Insert figure 5 about here

Analysis of these data by two separate two-way ANOVA (2 repeated factors: hemispheres and seasons) identified in both cases a significant season by hemisphere interaction that was in both cases driven by a larger response on the left compared to the right side observed selectively in March but not in December nor May/June. This confirmed therefore the presence of a true lateralization of responses but this lateralization was particularly prominent or exclusively observed in March during the reproductive season.

5.3. Song stimulus specificity of aromatase inhibition effects on auditory responses

We also wondered whether the treatment by season interaction would differentially affect the brain activation induced by the different stimuli. The effects of VOR were therefore analyzed for each stimulus during each season separately (figure 6).

Insert figure 6 about here

In December, aromatase inhibition rapidly reduced BOLD responses to INDIV songs but not to other stimuli in the left rostral NCM/Field L. In March, VOR also decreased - auditory-evoked BOLD responses in left NCM/Field L, but in this case, the effect was observed for each of the three types of stimuli including pure tones. Finally, in May/June estrogen depletion decreased auditory-evoked BOLD responses in left rostral NCM/Field L and post-hoc analysis revealed the effect was significant only for SPEC songs.

6. Discussion

6.1. Rapid changes in brain auditory activity are revealed by fMRI

Together, these data demonstrate that the estrogen synthase inhibitor Vorozole™ rapidly (within 10 to 45 min) affects brain auditory activity in a region- and season-specific manner. These effects are in full agreement with the rapid changes in neuronal activity previously observed by electrophysiology in the zebra finch NCM following aromatase inhibition (Remage-Healey et al., 2010; Tremere and Pinaud, 2011). Analysis of changes in brain activity by assumption-free methods additionally allowed describing the full neuroanatomical extent of the effects. These acute changes in activity differentially affect the rostral versus caudal NCM as a function of seasons: there was a major inhibition of the BOLD response by VOR in the caudal NCM in March but not in December nor in May/June. Interestingly, this region of the caudal NCM seems to correspond in parasagittal view to the region that shows a dense expression of aromatase mRNA in two separate species of songbirds, zebra finches (Pinaud et al., 2006) and black redstarts, *Phoenicurus ochuros* (Apfelbeck et al., 2013). This region also contains dense populations of neurons expressing the aromatase protein as revealed by immunohistochemistry (Balthazart et al., 1996; Saldanha et al., 2000).

These effects are presumably too fast to be mediated by the classical mechanism of steroid action that involves the binding to intracellular receptors and modulation of the transcription of specific genes. The effects probably result from membrane-initiated effects as already demonstrated in electrophysiological studies using estradiol bound to a compound that prevents its entrance into brain cells (e.g. bovine serum albumin or biotin; (Remage-Healey et al., 2012)). Recent research also demonstrated that the membrane GPER1 plays a critical sexually differentiated role in the control of the electrophysiological activity underlying auditory processing in zebra finches. Activation of GPER1 did not, however, mimic all effects of estradiol indicating that other types of membrane estrogen receptors must be implicated in the modulation of rapid effects of estrogens on auditory processing (Krentzel et al., 2018). Similar approaches using agonists and antagonists of membrane estrogen receptors or estrogenic compounds that cannot pass the cell membrane could be used in the future to confirm that effects described here by fMRI are similarly mediated by membrane estrogen receptors.

Interestingly, many of the observed effects were specifically lateralized to the left side of the brain. This lateralization is reminiscent of behavioral studies demonstrating that aromatase inhibition in the left but not the right NCM decreases the preference of zebra finches for their own song (Remage-Healey et al., 2010). The mechanism underlying this lateralization remains unclear at this time. Radioenzymatic assays did not detect a systematic difference in aromatase activity between the left and right NCM or CMM (De Groof et al., 2017) but this does not exclude that rapid changes in enzymatic activity, such as those triggered by activity-dependent phosphorylations (Charlier et al., 2011) could be lateralized and explain the differential response to VOR. Any other aspect of estrogens actions (density of receptors, their coupling with the downstream signaling cascades, …) could of course also be lateralized and this topic should be further investigated.

These fMRI studies also revealed that estrogens bioavailability seem to differentially affect the auditory BOLD responses to specific stimuli in a season-specific manner. This finding is reminiscent of our previously identified differences between birds studied during and outside the breeding season that showed a decreased BOLD response in caudal NCM to species-specific signals in the breeding season while responses to individual-specific signals remained unaltered (De Groof et al., 2013b). Note that responses to all auditory stimuli were on average decreased following VOR

injection at all three time points. The magnitude of the effects was however variable so that some decreases reached statistical significance while others did not. It would be biologically relevant to have more prominent effects of estrogens depletion during the breeding season than outside this season but additional work should be performed to test the reliability of these apparent seasonal changes in stimuli specificity. The final answer to this question should also guide future work investigating the anatomical site where aromatase inhibition produces these effects: more general effects would be expected following inhibition of aromatase in the ear or in initials relays of the auditory pathway (e.g. Field L) than following inhibition in NCM.

6.2. Cautions when applying (auditory) fMRI in songbirds

Anesthesia affects the outcome of functional imaging by influencing specific neurotransmitter systems, physiological parameters such as body temperature and vascular tone. These changes potentially impact the neurovascular coupling (Richards, 2002) and the BOLD signal (Vanhoutte et al., 2006). Boumans and colleagues compared in adult male zebra finches the auditory fMRI responses in the primary and secondary auditory cortices acquired under different types of anesthesia including isoflurane, medetomidine and urethane. They found that song stimulation under isoflurane and urethane anesthesia elicit larger areas of activation compared to medetomidine (Boumans et al., 2007). Moreover, the effects of anesthesia are considered to be region-specific, as demonstrated by different neural responses in HVC in awake versus urethane-anesthetized zebra finches (Vallentin et al., 2016). Based on electrophysiological recordings in the primary auditory area of female starlings, Karino et al. demonstrated that anesthesia (1.82 mg/kg of medetomidine and 4.55 mg/kg of ketamine) modulates neuronal responses to biologically irrelevant and relevant sounds (Karino et al., 2016). A recent publication however by Van Ruijssevelt et al. compared fMRI results in zebra finches that were either awake or lightly anesthetized with either isoflurane or a combination of medetomidine and isoflurane. They demonstrated that isoflurane alone (1.2%) appeared to be the most promising anesthesia given the high success rate, non-invasive induction, quick recovery and limited effects on selective BOLD responses to natural versus synthetic sounds in zebra finches (Van Ruijssevelt et al., 2017a).

Although anesthesia constrains the results that can be obtained by fMRI, awake experiments, which have recently shown to be feasible (De Groof et al., 2013a; Van Ruijssevelt et al., 2017a) also come with limitations. They require a very long and tedious training of the subjects, which limits the applicability of this approach when larger groups of animals are needed, as is the case for auditory fMRI studies (usually n= 10 to 15). The two awake studies we carried out also taught us that maintaining a constant level of alertness of the trained animal during scanning poses an additional challenge. In this respect, auditory fMRI under light anesthesia offers a well-controlled alternative provided sufficient validation studies comparing awake and anesthetized outcomes are performed.

The air cavities in the skull of songbirds induce marked artifacts on susceptibility weighted images, gradient echo or T2*-weighted images commonly used to perform BOLD fMRI. This T2* contrast increases with higher field strengths that are needed to obtain sufficient contrast to noise ratios in small voxels as required for fMRI on the very tiny songbird brain. To overcome this problem of susceptibility artifacts, Poirier et al. proposed using spin echo (SE) or T2-weighted, instead of gradient echo (GE) sequences for bird brain fMRI (Poirier et al., 2010). SE sequences, although suffering from a lower BOLD contrast-to-noise ratio, are still sufficiently sensitive to detect differential BOLD responses to different kinds of auditory stimuli such as avian songs and without substantial artefacts as shown by all our subsequent fMRI studies in starlings and zebra finches (Poirier et al., 2011; van der Kant et al., 2013; Van Ruijssevelt et al., 2018; Van Ruijssevelt et al., 2017a; Van Ruijssevelt et al., 2017b). Because one of the most important assets of fMRI remains its *in vivo* entire brain approach, allowing repeated measures and subsequent voxel-based analyses, the obtained images should be artefact-free to allow their accurate realignment in space and time. The use of SE images with fewer artefacts allowed us to obtain accurate voxel-based image analysis leading to assumption-free discoveries of zebra finch brain areas that are involved in own song recognition such as the dorso-lateral mesencephalic nucleus Mld (Poirier et al., 2009) and uncover a novel region involved in auditory perceptual decision-making in a sensory integrative region, the caudocentral nidopallium (NCC), outside the traditionally studied auditory forebrain pathways (Van Ruijssevelt et al., 2018). In relation to the present review, this approach led to the discovery of seasonal changes and hormone-induced fast modulations of auditory processing of specific vocalization in specific

regions of the auditory system (NCM) in starlings (De Groof et al., 2017; De Groof et al., 2013b).

The switching of magnetic gradients in the magnet during image acquisition produces substantial noise (80dB), which also poses specific challenges for auditory fMRI and auditory stimulation inside the MRI bore. Furthermore, this noise aggravates at higher field strengths, which could make it impossible for (anesthetized) birds to distinguish scanner noise from the auditory stimulation. To overcome this problem, long gradient ramp times can be implemented as they result in less acoustic noise produced by gradient switching, although they increase the acquisition time of one slice and thus limit the number of slices one can acquire (Poirier et al., 2010). Fortunately, BOLD responses to continuous tone stimulation (such as gradient noise) in the avian secondary auditory system (NCM) have a very low amplitude, while sounds with more complex acoustic features, such as songs, elicit a more sustained response (Van Meir et al., 2005) so that auditory fMRI experiments assessing brain activity in response to songs remain feasible. Therefore, we have never been forced to reduce the effect of gradient by noise cancellation headphones or similar procedures. Furthermore, and maybe more surprisingly, the scanner magnet enhances specific frequencies of sound stimuli. To compensate for this artificial enhancement, equalizer functions are usually applied and always validated in play back experiments with behavioral readouts (Boumans et al., 2008a).

6.3. Other in vivo imaging modalities used in (song)birds: PET

Other *in vivo* imaging methods, such as Positron Emission Tomography (PET), were introduced very recently in songbirds. Gold et al. (2016) studied the entire brain 18F-fluorodeoxyglucose (18F-FDG) uptake associated with flight in starlings (Gold et al., 2016) and several studies reported (18F-FDG) PET data on corvids visualizing the neural substrates that support different cognitive processes (Cross et al., 2013; Marzluff et al., 2012; Morell, 2013). Importantly with this technique, the cognitive tasks could take place in awake birds who had received an 18F-FDG injection before performing the task. Subsequently, the 18F-FDG PET data were acquired under anesthesia -for proper immobilization- and superimposed on anatomical MRI data for more accurate definition of the regions accumulating 18F-FDG in the (lower resolution) PET images. More relevant to the topic of this review, Lattin et al. using *in vivo* (18F-FDG) PET examined

the long-term effects of estradiol on male song processing in female house sparrows (*Passer domesticus*)(Lattin et al., 2017). They demonstrated that large scale brain activity in females exposed to heterospecific songs immediately prior to imaging are significantly reduced in females with estradiol implants.

As compared to BOLD fMRI, the limitations of PET are the lower spatial resolution (800 microns) and obligatory timed radiotracer injection, which limits the number of stimuli that can be individually assessed in a single experiment but also limits the time window during which neuromodulation can be detected. However, recently Villien and colleagues developed the so-called fPET-FDG technique that can be used in an analysis pipeline similar to fMRI to define within-session differential metabolic responses (Villien et al., 2014). PET on the other hand can provide excellent information complementing fMRI such as the localization and density of specific hormone or neurotransmitter receptors as shown for estrogen receptors in rats (Khayum et al., 2014). Using female zebra finches, a very nice recent study used a delayed [11C]raclopride-PET technique to detect changes in striatal dopamine neurotransmission after hearing song in awake female zebra finches (Tokarev et al., 2017)

Combining both imaging modalities, one could use PET to obtain information on the localization and quantification of specific receptors even in awake conditions (while performing particular tasks or being exposed to specific stimuli), while fMRI could provide accurate spatio-temporal information (about 150 versus 800 microns resolution) on differential brain responses to several sensory stimuli within a single experiment. Such multimodal imaging studies combining the best of two worlds have started to emerge in humans (Wei et al., 2017) and should be developed in animal models.

6.4. Advantages of using functional Magnetic Resonance Imaging in songbirds

Contrary to what happened in neuroscience research on rodent models, MRI came as a quite late player in the songbird neuroscience community. Since then the long list of publications from our team and others have proven that the major drawbacks associated with the use of this technique in songbird (see section 6.2 before) can be overcome. Functional MRI has proven to stand out as an excellent complementary tool with specific advantages as compared to the other more conventional methods

recognized by this field. In relation to the topic of the current paper, we show here that fMRI provides an outstanding tool to assess rapid effects of sex steroids on auditory processing and potentially on other brain functions that can be studied in anesthetized subjects. It has limitations compared to electrophysiology or to the immediate early gene approach but also has several advantages. One of the limitations relates to the recently publicized problem of false positive results (Cox et al., 2017; Eklund et al., 2016) and lack of reproducibility of fMRI (Glasser et al., 2016; Munafo et al., 2017; Poldrack et al., 2017). One should be fully aware that any fMRI outcome can be biased by (1) the nature of the data acquisition (spatial and temporal resolution, stability MR system); (2) the preprocessing algorithms to correct for motion, drift and normalization of the data to a template; (3) the methodology of data analysis and subsequent statistical analysis. The complexity of these processes might indeed lead to reproducibility problems but these can be minimized by carefully controlling all aspects in the procedure and standardizing them across experiments and laboratories. Ultimately, the reliability of the results will depend on whether they can be reproduced or not. The fact that two of our studies (De Groof et al., 2017; De Groof et al., 2013b) produced closely related results is certainly encouraging in this respect.

As opposed to electrophysiological approach, the fMRI technique allowed us to precisely define in an assumption-free manner the brain areas where changes in auditory processing occur and to delineate their extent. These changes in brain activity involve all secondary auditory brain regions when responses to specific stimuli are concerned but the season by treatment interaction only pointed out to the caudal NCM. This suggests that estrogen effects might be preferentially located in this subregion that displays a high aromatase expression and also potentially rapid fluctuations of E2 concentrations (Remage-Healey et al., 2008). Clearly the fMRI outcome could stimulate further molecular characterization of the observed changes.

In addition, as opposed to ZENK and fos data, the fMRI approach allows us to investigate the neuromodulatory impact of hormones on the neural response to multiple auditory stimuli in the same bird, which represents a major advantage compared to the approach based on immediate early genes detection in which response to only a single stimulus can usually be analyzed, unless different genes are detected by double or triple label immunohistochemistry or *in situ* hybridization following exposure at different time points to different stimuli (e.g. (Vazdarjanova and Guzowski, 2004; Zhang et al., 2018)).

However, this procedure is complex, technically challenging and potentially associated with interpretation problems. Moreover, the *in vivo* nature of the fMRI method allows linking the observed neuronal modulations to behavioral changes in the same individual.

The current review clearly shows the added value of fMRI but at the same time illustrates that still many opportunities and possibilities to investigate the brain responses to fast hormonal changes using fMRI remain unexplored. We hope that this review and the study presented as an example convinced the reader that functional MRI offers a clear assumption-free image-guided research strategy in which the spatio-temporal profile of fast steroid-induced changes in brain function can be uncovered and linked to specific functional responses. This should identify new mechanisms and areas of research that could then be further targeted with more localized invasive methods to help understanding underlying mechanisms down to the molecular level.

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Figure legends

Figure 1. *Experimental design used to test the effect of acute aromatase blockade by Vorozole™ (VOR; 30 mg/kg). The down pointing arrow indicates when VOR was injected. Each fMRI session lasted for 35 min and VOR injection was done 10 min before the second fMRI session. The figure illustrates the ON-OFF block paradigm diagram used to present the different auditory stimuli during acquisition of fMRI data. These imaging sessions were repeated in the same birds at three different time points across the year each time before and after an injection of the aromatase inhibitor VOR. SPEC= Species specific songs, INDIV=Individual songs (allowing individual recognition, most important during breeding season), PT=Pure tone (control).*

Figure 2. *Brain regions demonstrating an overall main effect of stimuli. The left column (INDIV>PT) identifies regions showing enhanced responses during individual songs versus pure tones. The right column (SPEC>PT) illustrates regions showing enhanced responses during species-specific songs versus pure tones. The region overlaps with NCM, CMM and Field L. The statistical threshold was set at p <0.05. Redrawn from data in (De Groof et al., 2017).*

Figure 3. *Statistical maps of the brain regions demonstrating (by t-tests) an overall main effect of treatment (Vorozole™ vs. Control). This region overlaps with the rostral NCM and Field L but is located exclusively on the left side of the brain. No statistically significant voxel was detected in the right hemisphere. T-test values are color-coded according to the scale displayed in panel A. The threshold for significance was set at $p_{uncorrected} < 0.01$. Panels A, B and C respectively represent the sagittal, coronal and horizontal view of the positive cluster of voxels. Panel D shows an enlargement of the positive area in sagittal view. The bar graph in panel E summarizes the relative response amplitude (+SEM) of the peak voxel in the left rostral NCM/Field L cluster. The baseline (zero) level corresponds to mean activation during rest periods. ***$p_{FWE} < 0.05$. Redrawn from data in (De Groof et al., 2017).*

Figure 4. *Brain region demonstrating an interaction between season and treatment. This region overlaps with the caudal NCM but is located exclusively on the left side of the brain. No statistically significant voxel was detected in the right hemisphere. T-test values are*

*color-coded according to the scale displayed in panel A. The threshold for significance was set at $p_{uncorrected} < 0.01$. Panels A, B and C respectively represent the sagittal, coronal and horizontal view of the positive cluster of voxels. Panel D shows an enlargement of the positive area in sagittal view. The bar graph in panel E summarizes the relative response amplitude (+SEM) of the peak voxel in the left caudal NCM cluster. The baseline (zero) level corresponds to mean activation during rest periods. ***$p_{FWE} < 0.05$; *$p_{uncorrected} < 0.01$. Redrawn from data in (De Groof et al., 2017).*

Figure 5: *Statistical analysis of the lateralization of Vorozole™ effects on brain responses to pooled auditory stimuli (INDIV and SPEC and PT). Errors bars represent the SEM of data in all subjects. Results of post-hoc tests performed after detecting of a significant hemisphere by season interaction are indicated by stars as follows: **=p<0.01. Redrawn from data in (De Groof et al., 2017).*

Figure 6. *Statistical map of regions displaying a significant main effect of treatment analyzed for each season separately. Reductions of brain responses after Vorozole™ were observed during each season in the left NCM/Field L but the stimuli and specific areas concerned changed seasonally. In March response to all types of stimuli were affected and these effects also concerned the caudal NCM, whereas only INDIV were concerned in December and SPEC in May/June and responses were located more rostrally. T-values are color-coded according to the scales displayed. The threshold was set at $p_{uncorrected} < 0.05$. Bar graphs represents plots of relative response amplitude (+SEM) of the local peak voxel at each season. Zero level corresponds to mean activation during rest periods. Stars indicate significant differences between control and VOR conditions as follows: ***$p_{FWE} < 0.05$; **$p_{uncorrected} < 0.001$; $p_{uncorrected} < 0.01$). Redrawn from data in (De Groof et al., 2017).*

References

Acharya, K.D., Veney, S.L., 2012. Characterization of the G-protein-coupled membrane-bound estrogen receptor GPR30 in the zebra finch brain reveals a sex difference in gene and protein expression. Developmental neurobiology 72, 1433-1446.

Amandusson, A., Blomqvist, A., 2013. Estrogenic influences in pain processing. Frontiers in neuroendocrinology 34, 329-349.

Apfelbeck, B., Mortega, K., Kiefer, S., Kipper, S., Vellema, M., Villavicencio, C.P., Gahr, M., Goymann, W., 2013. Associated and disassociated patterns in hormones, song, behavior and brain receptor expression between life-cycle stages in male black redstarts, Phoenicurus ochruros. General and comparative endocrinology 184, 93-102.

Au, A., Feher, A., McPhee, L., Jessa, A., Oh, S., Einstein, G., 2016. Estrogens, inflammation and cognition. Frontiers in neuroendocrinology 40, 87-100.

Ball, G.F., Balthazart, J., 2009. Endocrinology of Animal Communication: Behavioral, in: Squire, L.R. (Ed.), Encyclopedia of Neuroscience. Academic Press, Oxford, pp. 981-989.

Balthazart, J., 2013. Sex steroid modulation of sensory processing. Frontiers in neuroendocrinology 34, 253-254.

Balthazart, J., 2017. Steroid metabolism in the brain: From bird watching to molecular biology, a personal journey. Horm Behav 93, 137-150.

Balthazart, J., Absil, P., Foidart, A., Houbart, M., Harada, N., Ball, G.F., 1996. Distribution of aromatase-immunoreactive cells in the forebrain of zebra finches (Taeniopygia guttata): Implications for the neural action of steroids and nuclear definition in the avian hypothalamus. Journal of Neurobiology 31, 129-148.

Balthazart, J., Ball, G.F., 2013. Brain Aromatase, estrogens and behavior. Oxford University Press, New York.

Balthazart, J., Remage-Healey, L., Choleris, E., 2018. Steroid and the brain: 50 years of research, conceptual shifts and the ascent of non-classical and membrane-initiated actions. Horm Behav Epub ahead of print, https://doi.org/10.1016/j.yhbeh.2018.01.002.

Bass, A.H., 2008. Steroid-dependent plasticity of vocal motor systems: novel insights from teleost fish. Brain research reviews 57, 299-308.

Bass, A.H., Zakon, H.H., 2005. Sonic and electric fish: at the crossroads of neuroethology and behavioral neuroendocrinology. Horm Behav 48, 360-372.

Baum, M.J., Bakker, J., 2013. Roles of sex and gonadal steroids in mammalian pheromonal communication. Frontiers in neuroendocrinology 34, 268-284.

Boumans, T., Gobes, S.M.H., Theunissen, F.E., Vandersmissen, L., Pintjens, W., Verhoye, M., Bolhuis, J.J., Van Der Linden, A., 2008a. Functional MRI of Auditory Responses in the Zebra Finch Forebrain Reveals a Hierarchical Organisation Based on Response Strength but not Selectivity. PloS one 3 (9), e3184.

Boumans, T., Theunissen, F.E., Poirier, C., Van Der Linden, A., 2007. Neural representation of spectral and temporal features of song in the auditory forebrain of zebra finches as revealed by functional MRI. The European journal of neuroscience 26, 2613-2626.

Boumans, T., Vignal, C., Smolders, A., Sijbers, J., Verhoye, M., Van Audekerke, J., Mathevon, N., Van der Linden, A., 2008b. Functional magnetic resonance imaging in zebra finch

discerns the neural substrate involved in segregation of conspecific song from background noise. Journal of neurophysiology 99, 931-938.
Brenowitz, E.A., 2004. Plasticity of the adult avian song control system. Annals of the New York Academy of Sciences 1016, 560-585.
Brenowitz, E.A., 2008. Plasticity of the song control system in adult birds, in: Zeigler, H.P., Marler, P. (Eds.), Neuroscience of birdsong. Cambridge University Press, Cambridge, pp. 332-349.
Caras, M.L., 2013. Estrogenic modulation of auditory processing: a vertebrate comparison. Frontiers in neuroendocrinology 34, 285-299.
Caras, M.L., Brenowitz, E., Rubel, E.W., 2010. Peripheral auditory processing changes seasonally in Gambel's white-crowned sparrow. Journal of comparative physiology. A, Neuroethology, sensory, neural, and behavioral physiology 196, 581-599.
Caras, M.L., O'Brien, M., Brenowitz, E.A., Rubel, E.W., 2012. Estradiol selectively enhances auditory function in avian forebrain neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 32, 17597-17611.
Chariditi, K., Canlon, B., 2010. Estrogen receptors in the central auditory system of male and female mice. Neuroscience 165, 923-933.
Charlier, T.D., Harada, N., Balthazart, J., Cornil, C.A., 2011. Human and quail aromatase activity is rapidly and reversibly inhibited by phosphorylating conditions. Endocrinology 152, 4199-4210.
Cornil, C.A., Ball, G.F., Balthazart, J., 2012. Rapid control of male typical behaviors by brain-derived estrogens. Frontiers in neuroendocrinology 33, 425-446.
Cornil, C.A., Ball, G.F., Balthazart, J., 2015. The dual action of estrogen hypothesis. Trends Neurosci 38, 408-416.
Cox, R.W., Chen, G., Glen, D.R., Reynolds, R.C., Taylor, P.A., 2017. FMRI Clustering in AFNI: False-Positive Rates Redux. Brain connectivity 7, 152-171.
Cross, D.J., Marzluff, J.M., Palmquist, I., Minoshima, S., Shimizu, T., Miyaoka, R., 2013. Distinct neural circuits underlie assessment of a diversity of natural dangers by American crows. Proc Biol Sci 280, 20131046.
Cross, E., Roselli, C.E., 1999. 17beta-estradiol rapidly facilitates chemoinvestigation and mounting in castrated male rats. The American journal of physiology 276, R1346-1350.
De Groof, G., Balthazart, J., Cornil, C.A., Van der Linden, A., 2017. Topography and Lateralized Effect of Acute Aromatase Inhibition on Auditory Processing in a Seasonal Songbird. The Journal of neuroscience : the official journal of the Society for Neuroscience 37, 4243-4254.
De Groof, G., George, I., Touj, S., Stacho, M., Jonckers, E., Cousillas, H., Hausberger, M., Gunturkun, O., Van der Linden, A., 2016. A three-dimensional digital atlas of the starling brain. Brain structure & function 221, 1899-1909.
De Groof, G., Jonckers, E., Gunturkun, O., Denolf, P., Van Auderkerke, J., Van der Linden, A., 2013a. Functional MRI and functional connectivity of the visual system of awake pigeons. Behavioural brain research 239, 43-50.
De Groof, G., Poirier, C., George, I., Hausberger, M., Van der Linden, A., 2013b. Functional changes between seasons in the male songbird auditory forebrain. Frontiers in behavioral neuroscience 7, 196.
De Groof, G., Verhoye, M., Poirier, C., Leemans, A., Eens, M., Darras, V.M., Van der Linden, A., 2009. Structural changes between seasons in the songbird auditory forebrain. The Journal of neuroscience : the official journal of the Society for Neuroscience 29, 13557-13565.

De Groof, G., Verhoye, M., Van Meir, V., Balthazart, J., Van der Linden, A., 2008. Seasonal rewiring of the songbird brain: an in vivo MRI study. The European journal of neuroscience 28, 2475-2485; discussion 2474.

Del Negro, C., Edeline, J.M., 2002. Sex and season influence the proportion of thin spike cells in the canary HVc. Neuroreport 13, 2005-2009.

Del Negro, C., Kreutzer, M., Gahr, M., 2000. Sexually stimulating signals of canary (Serinus canaria) songs: Evidence for a female-specific auditory representation in the HVc nucleus during the breeding season. Behav.Neurosci. 114, 526-542.

Del Negro, C., Lehongre, K., Edeline, J.M., 2005. Selectivity of canary HVC neurons for the bird's own song: modulation by photoperiodic conditions. The Journal of neuroscience : the official journal of the Society for Neuroscience 25, 4952-4963.

Eklund, A., Nichols, T.E., Knutsson, H., 2016. Cluster failure: Why fMRI inferences for spatial extent have inflated false-positive rates. Proceedings of the National Academy of Sciences of the United States of America 113, 7900-7905.

Ervin, K.S., Lymer, J.M., Matta, R., Clipperton-Allen, A.E., Kavaliers, M., Choleris, E., 2015. Estrogen involvement in social behavior in rodents: Rapid and long-term actions. Horm Behav 74, 53-76.

Etgen, A.M., Pfaff, D.W., 2009. Molecular mechanisms of hormone actions on behavior. Elsevier, Amsterdam.

Ferris, C.F., Smerkers, B., Kulkarni, P., Caffrey, M., Afacan, O., Toddes, S., Stolberg, T., Febo, M., 2011. Functional magnetic resonance imaging in awake animals. Reviews in the neurosciences 22, 665-674.

Foradori, C.D., Weiser, M.J., Handa, R.J., 2008. Non-genomic actions of androgens. Frontiers in neuroendocrinology 29, 169-181.

Fortress, A.M., Frick, K.M., 2014. Epigenetic regulation of estrogen-dependent memory. Frontiers in neuroendocrinology 35, 530-549.

Fusani, L., Van't Hof, T., Hutchison, J.B., Gahr, M., 2000. Seasonal expression of androgen receptors, estrogen receptors, and aromatase in the canary brain in relation to circulating androgens and estrogens. J.Neurobiol. 43, 254-268.

Garcia-Segura, L.M., Wozniak, A., Azcoitia, I., Rodriguez, J.R., Hutchison, R.E., Hutchison, J.B., 1999. Aromatase expression by astrocytes after brain injury: Implications for local estrogen formation in brain repair. Neurosci. 89, 567-578.

Gentner, T.Q., Hulse, S.H., Duffy, D., Ball, G.F., 2001. Response biases in auditory forebrain regions of female songbirds following exposure to sexually relevant variation in male song. J.Neurobiol. 46, 48-58.

Gintzler, A.R., Liu, N.J., 2012. Importance of sex to pain and its amelioration; relevance of spinal estrogens and its membrane receptors. Frontiers in neuroendocrinology 33, 412-424.

Glasser, M.F., Smith, S.M., Marcus, D.S., Andersson, J.L., Auerbach, E.J., Behrens, T.E., Coalson, T.S., Harms, M.P., Jenkinson, M., Moeller, S., Robinson, E.C., Sotiropoulos, S.N., Xu, J., Yacoub, E., Ugurbil, K., Van Essen, D.C., 2016. The Human Connectome Project's neuroimaging approach. Nat Neurosci 19, 1175-1187.

Gold, M.E., Schulz, D., Budassi, M., Gignac, P.M., Vaska, P., Norell, M.A., 2016. Flying starlings, PET and the evolution of volant dinosaurs. Current biology : CB 26, R265-267.

Gunturkun, O., Verhoye, M., De Groof, G., Van der Linden, A., 2013. A 3-dimensional digital atlas of the ascending sensory and the descending motor systems in the pigeon brain. Brain structure & function 218, 269-281.

Gupta, P.D., Johar, K., Sr., Nagpal, K., Vasavada, A.R., 2005. Sex hormone receptors in the human eye. Survey of ophthalmology 50, 274-284.
Hamaide, J., De Groof, G., Van der Linden, A., 2016. Neuroplasticity and MRI: A perfect match. Neuroimage 131, 13-28.
Hausberger, M., Forasté, M.A., Richard-Yris, C.N., 1997. Differential response of female starlings to shared and nonshared song types. Etologia 5, 31-38.
Heimovics, S.A., Prior, N.H., Maddison, C.J., Soma, K.K., 2012. Rapid and widespread effects of 17beta-estradiol on intracellular signaling in the male songbird brain: a seasonal comparison. Endocrinology 153, 1364-1376.
Heimovics, S.A., Trainor, B.C., Soma, K.K., 2015. Rapid Effects of Estradiol on Aggression in Birds and Mice: The Fast and the Furious. Integrative and comparative biology 55, 281-293.
Henry, K.S., Lucas, J.R., 2009. Vocally correlated seasonal auditory variation in the house sparrow (Passer domesticus). The Journal of experimental biology 212, 3817-3822.
Hutchinson, C.V., Walker, J.A., Davidson, C., 2014. Oestrogen, ocular function and low-level vision: a review. The Journal of endocrinology 223, R9-18.
Jensen, E.V., Suzuki, T., Kawasima, T., Stumpf, W.E., Jungblut, P.W., De Sombre, E.R., 1968. A two-step mechanism for the interaction of estradiol with rat uterus. Proc.Natl.Acad.Sci.USA 59, 632-638.
Jeong, J.K., Burrows, K., Tremere, L.A., Pinaud, R., 2011. Neurochemical organization and experience-dependent activation of estrogen-associated circuits in the songbird auditory forebrain. The European journal of neuroscience 34, 283-291.
Jonckers, E., Delgado y Palacios, R., Shah, D., Guglielmetti, C., Verhoye, M., Van der Linden, A., 2014. Different anesthesia regimes modulate the functional connectivity outcome in mice. Magnetic resonance in medicine 72, 1103-1112.
Karino, G., George, I., Loison, L., Heyraud, C., De Groof, G., Hausberger, M., Cousillas, H., 2016. Anesthesia and brain sensory processing: impact on neuronal responses in a female songbird. Scientific reports 6, 39143.
Kelly, M.J., Moss, R.L., Dudley, C.A., 1976. Differential sensitivity of preoptic-septal neurons to microelectrophoresed estrogen during the estrous cycle. Brain Res 114, 152-157.
Khayum, M.A., de Vries, E.F., Glaudemans, A.W., Dierckx, R.A., Doorduin, J., 2014. In vivo imaging of brain estrogen receptors in rats: a 16alpha-18F-fluoro-17beta-estradiol PET study. Journal of nuclear medicine : official publication, Society of Nuclear Medicine 55, 481-487.
King, J.A., Garelick, T.S., Brevard, M.E., Chen, W., Messenger, T.L., Duong, T.Q., Ferris, C.F., 2005. Procedure for minimizing stress for fMRI studies in conscious rats. Journal of neuroscience methods 148, 154-160.
Krentzel, A.A., Macedo-Lima, M., Ikeda, M.Z., Remage-Healey, L., 2018. A Membrane G-Protein-Coupled Estrogen Receptor Is Necessary but Not Sufficient for Sex Differences in Zebra Finch Auditory Coding. Endocrinology 159, 1360-1376.
Krentzel, A.A., Remage-Healey, L., 2015. Sex differences and rapid estrogen signaling: A look at songbird audition. Frontiers in neuroendocrinology 38, 37-49.
Lattin, C.R., Stabile, F.A., Carson, R.E., 2017. Estradiol modulates neural response to conspecific and heterospecific song in female house sparrows: An in vivo positron emission tomography study. PloS one 12, e0182875.
Leitner, S., Voigt, C., Metzdorf, R., Catchpole, C.K., 2005. Immediate early gene (ZENK, Arc) expression in the auditory forebrain of female canaries varies in response to male song quality. J Neurobiol 64, 275-284.

Little, A.C., 2013. The influence of steroid sex hormones on the cognitive and emotional processing of visual stimuli in humans. Frontiers in neuroendocrinology 34, 315-328.
Logothetis, N.K., 2002. The neural basis of the blood-oxygen-level-dependent functional magnetic resonance imaging signal. Philosophical transactions of the Royal Society of London. Series B, Biological sciences 357, 1003-1037.
Logothetis, N.K., 2008. What we can do and what we cannot do with fMRI. Nature 453, 869-878.
Logothetis, N.K., Pauls, J., Augath, M., Trinath, T., Oeltermann, A., 2001. Neurophysiological investigation of the basis of the fMRI signal. Nature 412, 150-157.
Lord, L.D., Bond, J., Thompson, R.R., 2009. Rapid steroid influences on visually guided sexual behavior in male goldfish. Horm Behav 56, 519-526.
Lucas, J.R., Freeberg, T.M., Krishnan, A., Long, G.R., 2002. A comparative study of avian auditory brainstem responses: correlations with phylogeny and vocal complexity, and seasonal effects. Journal of comparative physiology. A, Neuroethology, sensory, neural, and behavioral physiology 188, 981-992.
Lucas, J.R., Freeberg, T.M., Long, G.R., Krishnan, A., 2007. Seasonal variation in avian auditory evoked responses to tones: a comparative analysis of Carolina chickadees, tufted titmice, and white-breasted nuthatches. Journal of comparative physiology. A, Neuroethology, sensory, neural, and behavioral physiology 193, 201-215.
Luine, V., Frankfurt, M., 2013. Interactions between estradiol, BDNF and dendritic spines in promoting memory. Neuroscience 239, 34-45.
Maney, D.L., Cho, E., Goode, C.T., 2006. Estrogen-dependent selectivity of genomic responses to birdsong. The European journal of neuroscience 23, 1523-1529.
Maney, D.L., Pinaud, R., 2011. Estradiol-dependent modulation of auditory processing and selectivity in songbirds. Frontiers in neuroendocrinology 32, 287-302.
Mangiamele, L.A., Gomez, J.R., Curtis, N.J., Thompson, R.R., 2017. GPER/GPR30, a membrane estrogen receptor, is expressed in the brain and retina of a social fish (Carassius auratus) and colocalizes with isotocin. The Journal of comparative neurology 525, 252-270.
Marler, P., 2004. Science and birdsong: the good old days, in: Marler, P., Slabbekoorn, H. (Eds.), Nature's music. The sicnece of birdsong. Elsevier, Amsterdam, pp. 1-38.
Marrocco, J., McEwen, B.S., 2016. Sex in the brain: hormones and sex differences. Dialogues in clinical neuroscience 18, 373-383.
Marzluff, J.M., Miyaoka, R., Minoshima, S., Cross, D.J., 2012. Brain imaging reveals neuronal circuitry underlying the crow's perception of human faces. Proceedings of the National Academy of Sciences of the United States of America 109, 15912-15917.
Maul, K.K., Voss, H.U., Parra, L.C., Salgado-Commissariat, D., Ballon, D., Tchernichovski, O., Helekar, S.A., 2010. The development of stimulus-specific auditory responses requires song exposure in male but not female zebra finches. Developmental neurobiology 70, 28-40.
McEwen, B.S., 1994. Steroid hormone actions on the brain: When is the genome involved? Horm.Behav. 28, 396-405.
McEwen, B.S., Alves, S.E., 1999. Estrogen actions in the central nervous system. Endocr.Rev. 20, 279-307.
McRobbie, D.W., Moore, E.A., Graves, M.J., Prince, M.R., 2006. MRI from picture to proton, 2nd Ed. Cambridge University Press, Cambridge.

Mello, C.V., Clayton, D.F., 1994. Song-induced ZENK gene expression in auditory pathways of songbird brain and its relation to the song control system. J.Neurosci. 14, 6652-6666.
Mello, C.V., Ribeiro, S., 1998. ZENK protein regulation by song in the brain of songbirds. J.Comp.Neurol. 393, 426-438.
Mello, C.V., Velho, T.A., Pinaud, R., 2004. Song-induced gene expression: a window on song auditory processing and perception. Annals of the New York Academy of Sciences 1016, 263-281.
Metzdorf, R., Gahr, M., Fusani, L., 1999. Distribution of aromatase, estrogen receptor, and androgen receptor mRNA in the forebrain of songbirds and nonsongbirds. The Journal of comparative neurology 407, 115-129.
Miranda, J.A., Liu, R.C., 2009. Dissecting natural sensory plasticity: hormones and experience in a maternal context. Hearing research 252, 21-28.
Monbureau, M., Barker, J.M., Leboucher, G., Balthazart, J., 2015. Male song quality modulates c-Fos expression in the auditory forebrain of the female canary. Physiology & behavior 147, 7-15.
Morell, V., 2013. Into the minds of birds. Science 341, 22-25.
Munafo, M.R., Nosek, B.A., Bishop, D.V.M., Button, K.S., Chambers, C.D., Percie du Sert, N., Simonsohn, U., Wagenmakers, E.-J., Ware, J.J., Ioannidis, J.P.A., 2017. A manifesto for reproducible science. Nature Human Behaviour 1, 0021. doi:0010.1038/s41562-41016-40021.
Munaut, C., Lambert, V., Noel, A., Frankenne, F., Deprez, M., Foidart, J.M., Rakic, J.M., 2001. Presence of oestrogen receptor type beta in human retina. The British journal of ophthalmology 85, 877-882.
Noirot, I.C., Adler, H.J., Cornil, C.A., Harada, N., Dooling, R.J., Balthazart, J., Ball, G.F., 2009. Presence of aromatase and estrogen receptor alpha in the inner ear of zebra finches. Hearing research 252, 49-55.
Nottebohm, F., 1980. Brain pathways for vocal learning in birds: A review of the first 10 years, in: Sprague, J.M., Epstein, A.N. (Eds.), Progress in Psychobiology and Physiological Psychology, Vol 9, 0 ed. Academic Press, New York, pp. 85-214.
Nottebohm, F., Stokes, T.M., Leonard, C.M., 1976. Central control of song in the canary, Serinus canarius. J.Comp.Neurol. 165, 457-486.
Pawlisch, B.A., Remage-Healey, L., 2015. Neuroestrogen signaling in the songbird auditory cortex propagates into a sensorimotor network via an 'interface' nucleus. Neuroscience 284, 522-535.
Peterson, R.S., Saldanha, C.J., Schlinger, B.A., 2001. Rapid upregulation of aromatase mRNA and protein following neural injury in the zebra finch (Taeniopygia guttata). J Neuroendocrinol 13, 317-323.
Peterson, R.S., Yarram, L., Schlinger, B.A., Saldanha, C.J., 2005. Aromatase is pre-synaptic and sexually dimorphic in the adult zebra finch brain. Proc Biol Sci 272, 2089-2096.
Petrulis, A., 2013. Chemosignals and hormones in the neural control of mammalian sexual behavior. Frontiers in neuroendocrinology 34, 255-267.
Pfaff, D.W., Pfaffmann, C., 1969. Olfactory and hormonal influences on the basal forebrain of the male rat. Brain Research 15, 137-156.
Phillmore, L.S., Veysey, A.S., Roach, S.P., 2011. Zenk expression in auditory regions changes with breeding condition in male Black-capped chickadees (Poecile atricapillus). Behavioural brain research 225, 464-472.

Pinaud, R., Fortes, A.F., Lovell, P., Mello, C.V., 2006. Calbindin-positive neurons reveal a sexual dimorphism within the songbird analogue of the mammalian auditory cortex. J Neurobiol 66, 182-195.

Poirier, C., Boumans, T., Vellema, M., De Groof, G., Charlier, T.D., Verhoye, M., Van der Linden, A., Balthazart, J., 2011. Own song selectivity in the songbird auditory pathway: suppression by norepinephrine. PloS one 6, e20131.

Poirier, C., Boumans, T., Verhoye, M., Balthazart, J., Van der Linden, A., 2009. Own-song recognition in the songbird auditory pathway: selectivity and lateralization. The Journal of neuroscience : the official journal of the Society for Neuroscience 29, 2252-2258.

Poirier, C., Vellema, M., Verhoye, M., Van Meir, V., Wild, J.M., Balthazart, J., Van Der Linden, A., 2008. A three-dimensional MRI atlas of the zebra finch brain in stereotaxic coordinates. Neuroimage 41, 1-6.

Poirier, C., Verhoye, M., Boumans, T., Van der Linden, A., 2010. Implementation of spin-echo blood oxygen level-dependent (BOLD) functional MRI in birds. NMR Biomed 23, 1027-1032.

Poldrack, R.A., Baker, C.I., Durnez, J., Gorgolewski, K.J., Matthews, P.M., Munafo, M.R., Nichols, T.E., Poline, J.B., Vul, E., Yarkoni, T., 2017. Scanning the horizon: towards transparent and reproducible neuroimaging research. Nature reviews. Neuroscience 18, 115-126.

Remage-Healey, L., 2012. Brain estrogen signaling effects acute modulation of acoustic communication behaviors: A working hypothesis. BioEssays : news and reviews in molecular, cellular and developmental biology 34, 1009-1016.

Remage-Healey, L., 2013. Acute synthesis and action of estrogens in communication circuits, in: Balthazart, J., Ball, G.F. (Eds.), Brain aromatase, estrogens, and behavior. Oxford University press, New York, pp. 474-496.

Remage-Healey, L., Coleman, M.J., Oyama, R.K., Schlinger, B.A., 2010. Brain estrogens rapidly strengthen auditory encoding and guide song preference in a songbird. Proceedings of the National Academy of Sciences of the United States of America 107, 3852-3857.

Remage-Healey, L., Dong, S.M., Chao, A., Schlinger, B.A., 2012. Sex-specific, rapid neuroestrogen fluctuations and neurophysiological actions in the songbird auditory forebrain. Journal of neurophysiology 107, 1621-1631.

Remage-Healey, L., Joshi, N.R., 2012. Changing neuroestrogens within the auditory forebrain rapidly transform stimulus selectivity in a downstream sensorimotor nucleus. The Journal of neuroscience : the official journal of the Society for Neuroscience 32, 8231-8241.

Remage-Healey, L., Maidment, N.T., Schlinger, B.A., 2008. Forebrain steroid levels fluctuate rapidly during social interactions. Nat Neurosci 11, 1327-1334.

Richards, C.D., 2002. Anaesthetic modulation of synaptic transmission in the mammalian CNS. British journal of anaesthesia 89, 79-90.

Riters, L.V., Baillien, M., Eens, M., Pinxten, R., Foidart, A., Ball, G.F., Balthazart, J., 2001. Seasonal variation in androgen-metabolizing enzymes in the diencephalon and telencephalon of the male European starling (Sturnus vulgaris). Journal of Neuroendocrinology 13, 985-997.

Ronnekleiv, O.K., Kelly, M.J., 2017. Membrane-Initiated Effects of Estradiol in the Central Nervous System, in: Pfaff, D.W., Joels, M. (Eds.), Hormones, Brain and Behavior. Elsevier Inc, Amsterdam, pp. 1-22.

Rudolph, L.M., Cornil, C.A., Mittelman-Smith, M.A., Rainville, J.R., Remage-Healey, L., Sinchak, K., Micevych, P.E., 2016. Actions of Steroids: New Neurotransmitters. The Journal of neuroscience : the official journal of the Society for Neuroscience 36, 11449-11458.

Saldanha, C.J., Coomaralingam, L., 2005. Overlap and co-expression of estrogen synthetic and responsive neurons in the songbird brain--a double-label immunocytochemical study. General and comparative endocrinology 141, 66-75.

Saldanha, C.J., Remage-Healey, L., Schlinger, B.A., 2011. Synaptocrine signaling: steroid synthesis and action at the synapse. Endocrine reviews 32, 532-549.

Saldanha, C.J., Rohmann, K.N., Coomaralingam, L., Wynne, R.D., 2005. Estrogen provision by reactive glia decreases apoptosis in the zebra finch (Taeniopygia guttata). J Neurobiol 64, 192-201.

Saldanha, C.J., Tuerk, M.J., Kim, Y.H., Fernandes, A.O., Arnold, A.P., Schlinger, B.A., 2000. Distribution and regulation of telencephalic aromatase expression in the zebra finch revealed with a specific antibody. J.Comp.Neurol. 423, 619-630.

Sanford, S.E., Lange, H.S., Maney, D.L., 2010. Topography of estradiol-modulated genomic responses in the songbird auditory forebrain. Developmental neurobiology 70, 73-86.

Schumacher, M., 1990. Rapid membrane effects of steroid hormones: an emerging concept in neuroendocrinology. Trends Neurosci 13, 359-362.

Sisneros, J.A., 2009. Steroid-dependent auditory plasticity for the enhancement of acoustic communication: recent insights from a vocal teleost fish. Hearing research 252, 9-14.

Soma, K.K., Schlinger, B.A., Wingfield, J.C., Saldanha, C.J., 2003. Brain aromatase, 5alpha-reductase, and 5beta-reductase change seasonally in wild male song sparrows: Relationship to aggressive and sexual behavior. J Neurobiol 56, 209-221.

Srivastava, D.P., Woolfrey, K.M., Penzes, P., 2013. Insights into rapid modulation of neuroplasticity by brain estrogens. Pharmacological reviews 65, 1318-1350.

Theunissen, F.E., Amin, N., Shaevitz, S.S., Woolley, S.M., Fremouw, T., Hauber, M.E., 2008. Song selectivity and the songbird brain, in: Ziegler, P.H., Marler, P. (Eds.), Neuroscience of birdsong. Cambridge Univerity press, Cambridge UK, pp. 157-173.

Thorpe, W.H., 1954. The process of song-learning in the Chaffinch as studied by means of sound spectrograph. Nature 173, 465-469.

Tindemans, I., Verhoye, M., Balthazart, J., Van Der Linden, A., 2003. In vivo dynamic ME-MRI reveals differential functional responses of RA- and area X-projecting neurons in the HVC of canaries exposed to conspecific song. European Journal of Neuroscience 18, 3352-3360.

Tokarev, K., Hyland Bruno, J., Ljubicic, I., Kothari, P.J., Helekar, S.A., Tchernichovski, O., Voss, H.U., 2017. Sexual dimorphism in striatal dopaminergic responses promotes monogamy in social songbirds. eLife 6.

Trainor, B.C., Finy, M.S., Nelson, R.J., 2008. Rapid effects of estradiol on male aggression depend on photoperiod in reproductively non-responsive mice. Horm Behav 53, 192-199.

Traub, R.J., Ji, Y., 2013. Sex differences and hormonal modulation of deep tissue pain. Frontiers in neuroendocrinology 34, 350-366.

Tremere, L.A., Pinaud, R., 2011. Brain-generated estradiol drives long-term optimization of auditory coding to enhance the discrimination of communication signals. The Journal of neuroscience : the official journal of the Society for Neuroscience 31, 3271-3289.

Vahaba, D.M., Macedo-Lima, M., Remage-Healey, L., 2017. Sensory Coding and Sensitivity to Local Estrogens Shift during Critical Period Milestones in the Auditory Cortex of Male Songbirds. eNeuro 4.

Vallentin, D., Kosche, G., Lipkind, D., Long, M.A., 2016. Neural circuits. Inhibition protects acquired song segments during vocal learning in zebra finches. Science 351, 267-271.

van der Kant, A., Deregnaucourt, S., Gahr, M., Van der Linden, A., Poirier, C., 2013. Representation of early sensory experience in the adult auditory midbrain: implications for vocal learning. PloS one 8, e61764.

Van der Linden, A., Van Camp, N., Ramos-Cabrer, P., Hoehn, M., 2007. Current status of functional MRI on small animals: application to physiology, pathophysiology, and cognition. NMR Biomed 20, 522-545.

Van der Linden, A., Van Meir, V., Boumans, T., Poirier, C., Balthazart, J., 2009. MRI in small brains displaying extensive plasticity. Trends in Neurosciences 32, 257-266.

Van der Linden, A., Van Meir, V., Tindemans, I., Verhoye, M., Balthazart, J., 2004. Applications of manganese-enhanced magnetic resonance imaging (MEMRI) to image brain plasticity in song birds. NMR in Biomedicine 17, 602-612.

Van der Linden, A., Verhoye, M., Van Audekerke, J., Peeters, R., Eens, M., Newman, S.W., Smulders, T., Balthazart, J., DeVoogd, T.J., 1998. Non invasive in vivo anatomical studies of the oscine brain by high resolution MRI microscopy. J.Neurosci.Methods 81, 45-52.

Van der Linden, A., Verhoye, M., Van Meir, V., Tindemans, I., Eens, M., Absil, P., Balthazart, J., 2002. In vivo manganese-enhanced magnetic resonance imaging reveals connections and functional properties of the songbird vocal control system. Neuroscience 112, 467-474.

Van Meir, V., Boumans, T., De Groof, G., Van Audekerke, J., Smolders, A., Scheunders, P., Sijbers, J., Verhoye, M., Balthazart, J., Van Der Linden, A., 2005. Spatiotemporal properties of the BOLD response in the songbirds' auditory circuit during a variety of listening tasks. NeuroImage 25, 1242-1255.

Van Meir, V., Verhoye, M., Absil, P., Eens, M., Balthazart, J., Van Der Linden, A., 2004. Differential effects of testosterone on neuronal populations and their connections in a sensorimotor brain nucleus controlling song production in songbirds: A manganese enhanced-magnetic resonance imaging study. NeuroImage 21, 914-923.

Van Ruijssevelt, L., Chen, Y., von Eugen, K., Hamaide, J., De Groof, G., Verhoye, M., Güntürkün, O., Woolley, S.C., Van der Linden, A., 2018. fMRI reveals a novel region for evaluating acoustic information for mate choice in a female songbird. Current Biology 28, 711-721..

Van Ruijssevelt, L., De Groof, G., Van der Kant, A., Poirier, C., Van Audekerke, J., Verhoye, M., Van der Linden, A., 2013a. Functional magnetic resonance imaging (FMRI) with auditory stimulation in songbirds. Journal of visualized experiments : JoVE 76.

Van Ruijssevelt, L., Hamaide, J., Van Gurp, M.T., Verhoye, M., Van der Linden, A., 2017a. Auditory evoked BOLD responses in awake compared to lightly anaesthetized zebra finches. Scientific reports 7, 13563.

Van Ruijssevelt, L., Van der Kant, A., De Groof, G., Van der Linden, A., 2013b. Current state-of-the-art of auditory functional MRI (fMRI) on zebra finches: technique and scientific achievements. Journal of physiology, Paris 107, 156-169.

Van Ruijssevelt, L., Washington, S.D., Hamaide, J., Verhoye, M., Keliris, G.A., Van der Linden, A., 2017b. Song Processing in the Zebra Finch Auditory Forebrain Reflects

Asymmetric Sensitivity to Temporal and Spectral Structure. Frontiers in neuroscience 11, 549.
Vanhoutte, G., Verhoye, M., Van der Linden, A., 2006. Changing body temperature affects the T2* signal in the rat brain and reveals hypothalamic activity. Magnetic resonance in medicine 55, 1006-1012.
Vasudevan, N., Pfaff, D.W., 2008. Non-genomic actions of estrogens and their interaction with genomic actions in the brain. Frontiers in neuroendocrinology 29, 238-257.
Vazdarjanova, A., Guzowski, J.F., 2004. Differences in hippocampal neuronal population responses to modifications of an environmental context: evidence for distinct, yet complementary, functions of CA3 and CA1 ensembles. The Journal of neuroscience : the official journal of the Society for Neuroscience 24, 6489-6496.
Vellema, M., Verschueren, J., Van Meir, V., Van der Linden, A., 2011. A customizable 3-dimensional digital atlas of the canary brain in multiple modalities. Neuroimage 57, 352-361.
Vignal, C., Boumans, T., Montcel, B., Ramstein, S., Verhoye, M., Van Audekerke, J., Mathevon, N., Van der Linden, A., Mottin, S., 2008. Measuring brain hemodynamic changes in a songbird: responses to hypercapnia measured with functional MRI and near-infrared spectroscopy. Physics in medicine and biology 53, 2457-2470.
Villien, M., Wey, H.Y., Mandeville, J.B., Catana, C., Polimeni, J.R., Sander, C.Y., Zurcher, N.R., Chonde, D.B., Fowler, J.S., Rosen, B.R., Hooker, J.M., 2014. Dynamic functional imaging of brain glucose utilization using fPET-FDG. Neuroimage 100, 192-199.
Voss, H.U., Salgado-Commissariat, D., Helekar, S.A., 2010. Altered auditory BOLD response to conspecific birdsong in zebra finches with stuttered syllables. PloS one 5, e14415.
Voss, H.U., Tabelow, K., Polzehl, J., Tchernichovski, O., Maul, K.K., Salgado-Commissariat, D., Ballon, D., Helekar, S.A., 2007. Functional MRI of the zebra finch brain during song stimulation suggests a lateralized response topography. Proceedings of the National Academy of Sciences of the United States of America 104, 10667-10672.
Wei, S.M., Baller, E.B., Kohn, P.D., Kippenhan, J.S., Kolachana, B., Soldin, S.J., Rubinow, D.R., Schmidt, P.J., Berman, K.F., 2017. Brain-derived neurotrophic factor Val(66)Met genotype and ovarian steroids interactively modulate working memory-related hippocampal function in women: a multimodal neuroimaging study. Molecular psychiatry.
Westbrook, C., Kaut Roth, C., Talbot, J., 2005. MRI in practice, 4th Ed. Blackwell publishing, Oxford UK.
Wilczynski, W., Allison, J.D., Marler, C.A., 1993. Sensory pathways linking social and environmental cues to endocrine control regions of amphibian forebrains. Brain, behavior and evolution 42, 252-264.
Wood, R.I., Newman, S.W., 1995. Hormonal influence on neurons of the mating behavior pathway in male hamsters, in: Micevych, P.E., Hammer, R.P.J. (Eds.), Neurobiological effects of sex steroid hormones, 0 ed. Cambridge University press, Cambridge, pp. 3-39.
Wouters, W., Snoeck, E., De Coster, R., 1994. Vorozole, a specific non-steroidal aromatase inhibitor. Breast Cancer Res.Treat. 30, 89-94.
Yue, S., Wadia, V., Sekula, N., Dickinson, P.S., Thompson, R.R., 2018. Acute effects of sex steroids on visual processing in male goldfish. Journal of comparative physiology. A, Neuroethology, sensory, neural, and behavioral physiology 204, 17-29.
Zhang, Q., He, Q., Wang, J., Fu, C., Hu, H., 2018. Use of TAI-FISH to visualize neural ensembles activated by multiple stimuli. Nature protocols 13, 118-133.

Zornik, E., Kelley, D.B., 2011. A neuroendocrine basis for the hierarchical control of frog courtship vocalizations. Frontiers in neuroendocrinology 32, 353-366.