Critical Review

Knotting Nets: Molecular Junctions of Interconnecting Endocrine Axes Identified by Application of the Adverse Outcome Pathway Concept

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Abstract: To be defined as an endocrine disruptor, a substance has to meet several criteria, including the induction of specific adverse effects, a specific endocrine mode of action, and a plausible link between both. The latter criterion in particular might not always be unequivocally determined, especially because the endocrine system consists of diverse endocrine axes. The axes closely interact with each other, and manipulation of one triggers effects on the other. The present review aimed to identify some of the many interconnections between these axes. The focus was on fish, but data obtained in studies on amphibians and mammals were considered if they assisted in closing data gaps, because most of the endocrine mechanisms are evolutionarily conserved. The review includes data both from ecotoxicological studies and on physiological processes and gives information on hormone/hormone receptor interactions or gene transcription regulation. The key events and key event relationships identified provide explanations for unexpected effects on one axis, exerted by substances suspected to act specifically on another axis. Based on these data, several adverse outcome pathway (AOP) segments are identified, describing connections between the hypothalamic–pituitary–gonadal (HPG) and hypothalamic–pituitary–thyroid (HPT) axes, the HPG and hypothalamic–pituitary–adrenal/interrenal (HPA/I) axes, and the HPT and HPA/I axes. Central key events identified across axes were altered aromatase activity as well as altered expression and function of the proteins 11β-hydroxysteroid dehydrogenase (11β-HSD) and steroidogenic acute regulatory (StAR) protein. Substance classes that act on more than one endocrine axis were, for example, goitrogens or aromatase inhibitors. Despite the wealth of information gathered, the present review only provides a few insights into the molecular nets of endocrine axes, demonstrating the complexity of their interconnections. Environ Toxicol Chem 2018;37:318–328. © 2017 The Authors. Environmental Toxicology and Chemistry published by Wiley Periodicals, Inc. on behalf of SETAC.

Keywords: Adverse outcome pathway; Molecular junctions; Interconnection; Cross-talk; Endocrine disruption; Mode of action

INTRODUCTION

Over the past 2 decades, there has been increasing evidence that both humans and the environment, particularly aquatic environments, are exposed to hormone-like substances. Correlations between increasing exposure to these substances and increasing numbers of hormonal disorders in the human population or disturbed growth and reduced reproduction in the environment (Jobling et al. 2006; Matthiessen 2013). Scientific and public awareness of these endocrine-disrupting chemicals (EDCs) triggered a series of regulatory actions from the 1990s on. In most of the current regulatory chemical frameworks, EDCs are identified following the World Health Organization/International Programme on Chemical Safety (WHO/IPCS) definition (Damstra et al. 2002). According to recently developed criteria for EDCs, the following basic requirements would identify a substance as an EDC. The substance should have an endocrine mode of action and provoke adverse effects. A plausible link between adverse effects and endocrine mode of action should be established. However, a clear discrimination between primary and secondary
effects on the endocrine system is necessary, because most substances act secondarily on the stress axis if applied in sufficiently high concentrations. The initial mode of action of these stressors is not related to any endocrine-disrupting effect. This aspect has to be kept in mind for the discussion in the following sections on the adverse effects of stressor-induced regulation of cortisol release.

The mode of action as well as a plausible link between it and an adverse effect cannot always be determined unequivocally. Some substances could interact with the hormonal system in a versatile and ambiguous manner, even though, from a regulatory point of view, the relevant effect distinctly points to a specific endocrine mode of action. Endocrine substances are able to influence more than one signaling cascade. For example, the effects of thyroid hormones on the development of testes in nonmammalian vertebrates are discussed by Castaneda Cortes et al. (2014) and Duarte-Guterman et al. (2014).

The endocrine axes (hypothalamic–pituitary–gonadal [HPG], hypothalamic–pituitary–thyroid [HPT], and hypothalamic–pituitary–adrenal/interrenal [HPA/I]) do not act independently of each other. Effects exceeding the borders of one endocrine axis could be explained by the physiological overlap between axes. They share common regions of hormone synthesis (i.e., the hypothalamus and the pituitary), as well as some hormones that regulate signaling along the axes. Each axis, however, still regulates different processes via axis-specific hormones. The HPG axis mainly controls and regulates reproductive processes via steroid hormones, whereas the HPT axis coordinates energy metabolism and development via thyroid hormones. The HPA axis regulates bodily responses to stress via glucocorticoids like cortisol or corticosterone, the main stress hormone in vertebrate species (Castaneda Cortes et al. 2014). Even though the cross-talk between the HPT and the HPG axis has been established, specific key events of the interactions have not yet been identified.

The present review aims to present a deeper insight into the relationship and connections of the endocrine axes. We applied the recently developed adverse outcome pathway (AOP) concept (Ankley et al. 2010), which links the initial interaction of a substance at the molecular level (molecular initiating event [MIE]) to an adverse effect at the organism or population level (the adverse outcome [AO]), which is in general an apical endpoint of regulatory interest. For endocrine disruptors, the plausible link between the test endpoints and population-level effects is often uncertain. To address this uncertainty, a 2016 Society of Environmental Toxicology and Chemistry (SETAC) Pellston Workshop evaluated the population relevance of toxicity endpoints from case studies with specific endocrine disruptors (Marty et al. 2017; Matthiessen et al. 2017). Based on the conclusions of this workshop, the AOs at the individual level described in the present review are extrapolated to the population level.

The AOPs are designed in a modular structure and comprise a sequence of key events and key event relationships, that is, the causal connection between the 2 adjacent key events (Villeneuve et al. 2014a, 2014b). The key events, which are necessarily essential, represent a measurable change in biological status. However, they are not necessarily sufficient for the progression of an AOP. Realistically, no AOP stands on its own but is rather involved in a larger network of AOPs, which share key events and key event relationships as well as AOs (Groh et al. 2015a, 2015b).

An AOP is not substance specific but is rather applicable to a whole class of substances that are related by a mode of action. It can be conserved across species, classes, and even phyla of the animal kingdom. However, the propagation of signaling cascades from terrestrial to aquatic organisms in particular has to be considered with caution, as the adverse substances are taken up by different exposure routes. Whereas the exposure route for terrestrial organisms is mostly via food, which must pass through the gastrointestinal tract and its metabolic processes, aquatic organisms are directly exposed to a substance, and the uptake via gills and skin results in direct distribution within the bloodstream. Depending on its specificity, a substance can induce different MIEs in the exposed organs. One and the same substance is thus able to result in 2 AOs affecting 2 endocrine axes in terrestrial and aquatic organisms, even though the endocrine axes themselves are identical in these groups of organisms.

Several publications have already discussed the cross-talk among the HPG, HPT, and HPA/I axes (Castaneda Cortes et al. 2014; Duarte-Guterman et al. 2014; Flood et al. 2013). However, these reviews mainly describe the basic molecular concepts resulting in these interactions. The new aspect of the literature search in the present review is the application of the AOP concept, with the intention of defining key events and their relationships, allowing us to attribute substances acting on the HPT axis, for example, to endpoints normally ascribed to another axis, such as the HPG. However, because of the complex network of interconnections, we cannot claim that our compilation of data describes all AOPs across all endocrine axes.

The basis for the present review was an extensive literature search for data on single endocrine axes as well as cross-talk, which allowed identification of different signaling pathways and interactions, as well as key events and key event relationships. First, to be able to identify potential cross-talk between the axes and thus common key events and key event relationships, the routes and points of interactions were identified. The focus was on fish studies. Amphibians and mammals were considered if data gaps observed in fish required evidence from other taxonomical levels. Ecotoxicological and toxicological studies considering the mode of action of specific substances as well as basic research demonstrating the interaction of individual biological elements such as hormone/hormone receptor interactions or gene transcription regulation were integrated, providing significant information on key events and key event relationships.

**Review approach**

We primarily used public databases of peer-reviewed journal articles and reports, mostly the science-specific web search engines PubMed, ScienceDirect, Scopus, and Web of Science. Because several AOPs for endocrine functions (e.g., aromatase inhibition [AOP: 25], androgen receptor agonism [AOP: 23], thyroperoxidase inhibition [AOP: 159]) have already been described on AOPWiki (https://aopwiki.org/wiki/index.php/Main_Page), relevant references from that site were also
considered. The initial search focused on the last 5 yr and included reviews addressing related topics that cited primary literature summarizing the state of knowledge. Earlier literature determined to be relevant for identification of potential cross-talk between axes was likewise included.

The search was structured according to search criteria and the key information to be extracted from the publications. As noted, the search initially focused on fish, with amphibians and mammals included when data gaps were identified. An article was deemed relevant if it included one or more of the following pieces of information: 1) descriptions of the different endocrine axes and their interactions; 2) the influence of substances described as endocrine disruptors on one or more endocrine axes; 3) connections among the endocrine axes and the developmentally and population relevant effects after treatment with the endocrine disruptor; and 4) identification of key events relevant for more than one endocrine axis.

The search criteria were mainly combinations of terms including one or several terms indicating that the endocrine axes were involved (e.g., “steroid hormone,” “peptide hormone,” “thyroid,” “estrogen,” “androgen,” “aromatase,” “stress,” “cortisol,” “HPG,” “HPT,” “HPA”); we also used “cross-talk” or “interaction.” Furthermore, the term “fish” was included if the search resulted in too many publications. For example, a search in PubMed for “thyroid” and “fish” and “endocrine” and “cross-talk” resulted in 6 publications, of which 4 included relevant information.

If a substance specific for one endocrine axis was identified, the name of the substance was used in combination with the search terms “HPG,” “HPT,” and “HPA” to identify any connections between the axes. For example, bisphenol A (BPA) is a known endocrine disruptor acting on the HPG axis. A combination of the search terms “BPA” and “endocrine” and “cross-talk” resulted in 6 publications, of which 4 included relevant information.

To obtain previous reports on key events, key event relationships, and AOPs for the endocrine axes, the search term “AOP” was used in combination with terms like “steroid hormone” or “thyroid hormone.” The combination “thyroid hormone” and “AOP” resulted in 4 hits, 2 of which we included.

**CROSS-TALK AMONG 3 ENDOCRINE AXES IN NONMAMMALIAN VERTEBRATES**

**Cross-talk between the HPG and HPA/I axes**

The HPG and HPA/I axes share a common precursor of their final hormones. Both the sex steroids and the glucocorticoid cortisol are derived from cholesterol, which is processed via the steroid biosynthesis pathway. Thus enzymes involved in steroid biosynthesis are of special interest for investigating interactions between the HPG and HPA/I axes. Several enzymes are directly involved in the synthesis of steroids as well as glucocorticoid hormones, for example, the steroidogenic acute regulatory protein (STAR) or 11β-hydroxysteroid dehydrogenase (11β-HSD). Furthermore, several studies point to the steriodogenic enzyme aromatase as a potential link between the HPG and HPA/I axes.

The interplay between the HPG and the HPA/I axes is demonstrated if stress is induced in fish, for example, by increased temperature (Abozaid et al. 2012; Fernandino et al. 2012, 2013; Hattori et al. 2009; Hayashi et al. 2010; Jin et al. 2011; Kitano et al. 2012; Lee et al. 2014; Okuzawa and Gen 2013; Uchida et al. 2004; Yamaguchi and Kitano 2012) or blue background color (Mankiewicz et al. 2013). Both stressors induce secondary endocrine effects. Furthermore, endocrine effects are mediated by direct cortisol treatment (Kitano et al. 2012; Mandiki et al. 2017). Several studies report on an increased synthesis of cortisol induced by stress, resulting in a male-biased sex ratio. Kitano et al. (2012), for example, noted that in medaka (Onyza latipes), cortisol or high temperature inhibits expression of the proliferation of female-type germ cells as well as expression of the ovarian aromatase (cyp19a1), which usually converts C19 androgens to C18 estrogens. The inhibition of aromatase activity thus results in an increased amount of 11-keto-testosterone (11-KT), the main male sex steroid in teleosts. Evidence that the observed effect on the sex ratio was indeed mediated by the inhibition of aromatase activity, and thus a decreased estrogen concentration, was demonstrated by a “rescue experiment” co-treating fish exposed to high temperatures with 17β-estradiol. This co-treatment completely abolished the effects on the sex ratio. Thus exposure during gonadal sex differentiation to high temperature or cortisol (stimulators normally attributed to the HPA/I axis) consequently results in a male-biased population in susceptible fish species, an effect clearly assigned to the HPG axis.

Mandiki et al. (2017) reported a massive decrease in aromatase activity in tissue cultures derived from Eurasian perch (Perca fluviatilis) ovaries after treatment with cortisol or 11-deoxycorticosterone (a cortisol precursor), also pointing to a downstream effect of glucocorticoids like cortisol on the HPG axis. Other studies demonstrated increased formation of 11-KT and cortisol at high temperature in pejerrey (Odontesthes bonariensis; Fernandino et al. 2012; Hattori et al. 2009). Hattori et al. (2009) also demonstrated decreased cyp19a1a gene expression and consequently a decreased aromatase activity. The increased levels of 11-KT observed in pejerrey might thus be ascribed to similar mechanisms as described for medaka, influencing aromatase activity. Male-biased sex ratios were also described for other fish species, for example, zebrafish (Danio rerio; Abozaid et al. 2012; Uchida et al. 2004). Even though aromatase activity was not measured in these studies, similar mechanisms can be postulated. Uchida et al. (2004) found similar phenotypes when exposing zebrafish to the specific aromatase inhibitor fadrozole or to high temperature, assuming that similar mechanisms were responsible for the same AO. In addition to high temperature, a male-biased sex ratio can also be induced by a blue background color (Mankiewicz et al. 2013). In that study, rearing of test fish in blue tanks, in comparison with black or gray tanks, resulted in 95% males in juvenile flounders (Paralichthys lethostigma). In addition, the cortisol levels were significantly increased, demonstrating the cross-talk between axes. Whether this color effect is restricted to flatfish species remains to be elucidated.

There is evidence that the inhibition of aromatase activity by increased cortisol levels (by high temperature, blue background, or any other cortisol-manipulating substance) is not monodirectional.
Liu et al. (2011) reported a down-regulation in zebrafish of corticotropin-releasing hormone (also known as corticotropin-releasing factor) after treatment with prochloraz, a substance known to inhibit aromatase activity. In consequence, reduced cortisol concentrations were observed, thus leading to an altered stress response, for example, altered immune status of the fish. The authors postulated that the decreased expression of corticotropin-releasing hormone was a result of decreased estradiol levels, and was thus in a direct line with the aromatase inhibition. As increased cortisol levels are supposed to inhibit aromatase activity, this might be a compensatory mechanism to counterbalance the cortisol response. However, as this assumption is based only on a single study, there is not enough evidence to describe an AOP for this cross-connection.

A central role for 11β-HSD during cross-talk between the HPA and HPA/I axes has also been postulated. In fish, 11β-HSD has crucial functions in both axes; it catalyzes the transformation of cortisol to cortisone and thus inactivates the stress hormone, and it converts testosterone to 11-KT. A connection was demonstrated by Fernandino et al. (2012), who exposed pejerrey to high temperatures at which increased hsd11b2 gene expression concomitant with an up-regulation of androgen (ar1, ar2) and glucocorticoid receptor (gr1, gr2) gene expression was observed. In addition, isolated testis tissue from adult males was exposed to cortisol at different concentrations, and a significant up-regulation of hsd11b2 gene expression and 11-KT levels was observed. Thus the hypothesis was generated that cortisol promotes 11-KT production during high temperature by modulation of hsd11b2 gene transcription (and thus 11β-HSD protein), which finally results in a male-biased sex ratio.

The StAR protein catalyzes the transport of cholesterol across the mitochondrial membrane, which is the rate-limiting step during steroid biosynthesis (Strauss et al. 1999). The mode of action and regulation of StAR proteins was first observed in mammals (Kallen et al. 1998; Stocco 2001) and only later was it investigated in nonmammals such as fish (Castillo et al. 2008). In both mammals and nonmammals, StAR regulates a rate-limiting step during steroid biosynthesis. In mammals, it is directly controlled by adrenocorticotropic hormone, which is regulated by the HPA axis (Arukwe et al. 2016). Thus the induction of StAR is identified as a shared key event in steroid and glucocorticoid synthesis, which leads to 2 different AOs.

The involvement of StAR in regulation of both endocrine axes has been demonstrated in studies with the endocrine disruptor BPA and analogues. Bisphenols are known to activate estrogen receptors (reviewed in Crain et al. 2007) and have been found to modulate StAR gene expression in gonads (Liu et al. 2012; Shi et al. 2015), perhaps because of their interaction with the estrogen receptor. Treatment with BPA resulted in a reduced fertilization rate of the parental and impaired embryonic development of the filial generation in zebrafish (Segner et al. 2003; Shi et al. 2015). In addition, reduction of cortisol levels and StAR gene expression were observed in a study with rainbow trout (Oncorhynchus mykiss) eggs, which were injected with increasing concentrations of BPA mimicking maternal transfer (Birceanu et al. 2015). Thus a bisphenol-mediated effect on the HPA/I axis by disruption of steroid biosynthesis, demonstrated by altered StAR gene expression, was also assumed.

It has been further demonstrated in fish that BPA influences corticoid receptor gene expression in addition to its well-described effect on the HPG axis (Terrien et al. 2011). Therefore BPA was identified as a substance disrupting both the HPA/I and HPG axes. This effect of BPA can also be observed in mammals. For example, in male and female pubescent rats low but chronic exposure to BPA leads to higher concentrations of basal corticosterone and lower concentrations of hypothalamic glucocorticoid receptors (Panagiotidou et al. 2014).

Three possible AOP segments can thus be proposed for the interaction between the HPA/I and HPG axes. The first AOP, shown in Figure 1A, is entitled “An unknown MIE resulting in increased cortisol release leads to masculinization in fish.” By a not yet described MIE, high temperature or other stress events result in increased cortisol production. Increased cortisol levels lead to aromatase inhibition. It remains elusive, however, whether this key event relationship is direct (e.g., by a direct interaction of glucocorticoid receptors with responsive elements on the promoter region of the aromatase gene) or indirect (e.g., by a down-regulation of aromatase activity because of decreased estrogen levels). This key event relationship bridges the HPA/I and HPG axes. Decreased aromatase activity leads to increased 11-KT levels (Hattori et al. 2009), and consequently to a male-biased sex ratio, the AO at the individual level. The AO at the population level would be a declining population trajectory because of a reduced number of females. Alternatively, a second, related AOP can be defined, also with an unknown MIE and masculinization as the AO, but with a different key event at the HPG axis. This key event at the HPG axis is described as an 11β-hsd gene expression up-regulation (and increased 11β-HSD enzyme levels), which further leads to increased 11-KT levels (Fernandino et al. 2012), and finally, to a male-biased sex ratio (Figure 1B). However, following the AOP conventions (Vileneuve et al. 2014a, 2014b), the 2 AOP segments have to be similarly titled.

Substances influencing cortisol release, like the fungicide methyl-parathion (Koakoski et al. 2014) are thus potential candidates verifying the proposed interconnections and the AOP network between the HPA/I and HPG axes. In addition, da Rosa et al. (2016) found a methyl-parathion–induced regulation of StAR gene expression in zebrifish, pointing to a correlation between cortisol and steroid biosynthesis. Furthermore, Hayashi et al. (2010) rescued medaka exposed to high temperature from masculinization by treatment with metyrapone, a cortisol synthesis inhibitor, supporting the AOP proposed in Figure 1A and B. However, it was not investigated whether metyrapone treatment alone results in a female-biased sex ratio.

A third AOP was described for estrogen-mimicking substances like bisphenols (see BPA in Figure 1C). The MIE at the HPG axis is an induced estrogen-receptor activation. This activation down-regulates the expression of StAR, which represents the connection between the 2 axes. As StAR activity and the cholesterol transport across the mitochondrial membrane is the rate-limiting step during steroid biosynthesis, the synthesis of cortisol as well as the sex steroid hormones is
influenced. Thus AOs related to both axes are suspected, that is, altered stress response for the HPA/I axis and reduced fertilization rate, resulting in a declining population trajectory for the HPG axis. An altered stress response could influence the immune status of the fish and therefore effects on survival are seen, especially of larval fish (Hartig et al. 2016). Therefore the AOP could be named “Estrogen receptor activation leads to reduced reproduction and thus to a declining population trajectory, as well as to an altered immune status of fish, resulting in effects on survival.”

Cross-talk between the HPG and HPT axes

The connection between the HPG and HPT axes is not as obvious as that between the HPG and HPA/I axes. Proteins that are involved in progression of one axis, such as hormone receptors or specific enzymes, are present in the hormone-producing organs of the other axis. For example, it has been reported that deiodinases, the enzymes catalyzing the conversion of triiodothyronine (T3) to thyroxine (T4) and vice versa, and thyroid receptors are present in gonadal tissue in a sex-specific manner (Flood et al. 2013). Accordingly, androgen receptors are found in the thyroid gland. Flood et al. (2013) also performed a promoter analysis and identified several thyroid receptor–responsive elements in genes expressed in gonadal tissue and androgen receptor–responsive elements in genes expressed in thyroid tissue. Thus, the interaction between the HPG and HPT axes is likely at the transcriptional level, by activation of genes assigned to the other endocrine axis, rather than by common synthesis pathways of their main hormones, as observed for the HPG and HPA/I axes.

Evidence for this hypothesis comes from several studies, which were reviewed by Castaneda Cortes et al. (2014) and Flood et al. (2013). The first prerequisite for thyroid hormone–mediated effects on the HPG axis is the expression of the respective receptors in gonadal tissue. This was shown by Morais et al. (2013), who performed in situ hybridization studies in zebrafish testis Sertoli and Leydig cells. This approach verified the expression of messenger ribonucleic acids (mRNAs) coding for 2 thyroid hormone receptors, \( \text{Thra} \) and \( \text{Thrb} \), indicating that thyroid hormone receptors are expressed in gonadal tissue. Because thyroid hormone receptors, like other receptors, act as transcription factors, their effect is likely a regulation of thyroid hormone receptor–regulated genes possessing a thyroid receptor–responsive element in their promoter region. Regulation of genes possessing thyroid receptor–responsive elements

![FIGURE 1: Adverse outcome pathway (AOP) segments crossing the hypothalamic-pituitary-adrenal/interrenal (HPA/I) axis and the hypothalamic-pituitary-gonadal (HPG) axis. (A) and (B) describe 2 AOP segments, both named “An unknown molecular initiating event (MIE) resulting in increased cortisol release leads to masculinization in fish.” This description applies to both AOP segments related to stress induction. The AOP segments differ in one key event. (A) includes aromatase inhibition as a key event, and (B) includes increased 11b-hydroxysteroid dehydrogenase (11b-HSD) levels. Both finally result in masculinization. (C) describes the AOP segment “Estrogen receptor activation leads to reduced reproduction and thus to a declined population trajectory, as well as to an altered immune status of fish, resulting in effects on survival.” This AOP segment is triggered by substances like bisphenol, with an estrogenic mode of action and effects on steroid biosynthesis. Thus AOs on both axes, such as a reduced fertilization rate and an impaired immune status, are described. Examples for substances/triggers are in gray squares, MIE in green squares, key events in blue squares, and AOs in orange squares. The key event relationships are shown as arrows. HPA/I = hypothalamic-pituitary-adrenal/interrenal axis; 11-kT = 11-keto-testosterone; NIS = sodium iodide symporter; StAR = steroidogenic acute regulatory protein.](image-url)
has been shown to ultimately result in an up-regulation of 11-KT levels in isolated zebrafish testis tissue. Accordingly, Flood et al. (2013) performed a promoter analysis of several genes related to the androgen pathway. They identified the presence of thyroid receptor-responsive elements in genes encoding the androgen receptors, in 11β-hsd2, and in steroid 5α-reductase (sdr5α), which is involved in biosynthesis of 5α-dihydrotestosterone (5α-DHT), the more active androgen in nonfish vertebrates across species and taxa (Mus musculus, Silurana tropicalis, Oryzias latipes). However, that study needs further experimental verification, because it only represents an in silico approach.

The antithyroid substance perchlorate exerts specific effects on fish and amphibians usually assigned to the HPT axis (Duarte-Guteman et al. 2014; Hu et al. 2006; Mukhi et al. 2005). It is known to act on thyroid hormone synthesis by interfering with iodide accumulation in the thyroid via a block of the sodium iodide symporter. The resulting decreased T3/T4 levels exert histopathological effects on the thyroid gland. In amphibians, decreased levels of thyroid hormones lead to disturbances in metamorphosis, as well as growth and developmental retardation. Furthermore, the reduced T3/T4 levels induced a feminizing effect on amphibians as well as on zebrafish (Bulaeva et al. 2015; Duarte-Guteman et al. 2014; Mukhi et al. 2007; Sharma and Patino 2013). In contrast, Bernhardt et al. (2006) reported a masculinizing effect on 3-spined sticklebacks (Gasterosteus aculeatus). Even though a connection to the HPG axis is thus unequivocally demonstrated, the mechanisms exerting this connection need to be clarified.

Sharma and Patino (2013) reared zebrafish larvae in the presence of the goitrogens sodium perchlorate and methimazole or in the presence of T4, which is converted to the more active thyroid hormone T3. Sodium perchlorate and methimazole both block thyroid hormone synthesis, but by different modes of action. Irrespective of the mode of action, goitrogen treatment resulted in a female-biased sex ratio. Similar effects were also reported by Mukhi et al. (2007) after perchlorate treatment. However, Sharma and Patino (2013) reported that the manipulation must persist until sexual development has finished, because the effect could be rescued if thyroid hormone levels return to normal during this phase. Interestingly, treatment with T4, in contrast to treatment with perchlorate, resulted in a male-biased sex ratio. As inhibition of thyroid hormone synthesis as well as thyroid hormone treatment results in a skewed sex ratio and in opposite directions, as supposed, it is likely that the connection of the HPT to the HPG axis is via the thyroid hormone levels and their regulation. This assumption is further supported by the finding that increased levels of the thyroid hormone T3 in adult goldfish (Carassius auratus) caused a down-regulation of aromatase activity as well as estrogen receptor expression (Nelson et al. 2010), thus exerting effects on reproduction.

Effects on gene expression levels of steriodogenic genes as a result of altered thyroid hormone levels were also observed earlier by Rasheeda et al. (2005). The authors treated adult catfish (Clarias gariepinus) with thiourea, a substance that inhibits thyroid hormone synthesis, and found regulation of several steriodogenic enzymes at the transcription level. In males, decrease of 11β-hsd gene expression was observed. This gene is also involved in the cross-talk between the HPG and the HPA/I axes. These authors reported that increased 11β-hsd2 transcription levels resulted in increased 11-KT levels, and thus a male-biased sex ratio. Even though we found no study noting that decreased 11β-hsd transcription levels led to decreased 11-KT hormone levels, it could be assumed that when the formation of 11-KT in fish is inhibited, a female-biased effect of a block of thyroid hormone signaling would be seen. In females, Rasheeda et al. (2005) observed an increase in aromatase transcript levels, whereas transcription levels of other genes involved in steroid biosynthesis remained unaffected. Even though changes in the sex ratio were not observed in that study, the effect on aromatase gene expression in females points to a direct effect on aromatase activity and thus on sex steroid balance.

A recent study by Sharma et al. (2016) investigated the effects of thyroid hormone treatment (T4), of goitrogen treatment (methimazole), and of a combination of both on the sex ratio of zebrafish after sexual differentiation. Furthermore, expression changes of genes involved in regulation of the HPG axis were analyzed. The goitrogen methimazole is usually used in hyperthyroidism therapy, through inhibition of thyroid peroxidase, finally resulting in decreased thyroid hormone levels. It was observed that treatment with this substance resulted in a female-biased sex ratio, treatment with T4 resulted in a male-biased sex ratio, and a combination of both treatments resulted in a sex ratio more skewed toward males, but to a lower degree, supporting the results of earlier studies (Mukhi et al. 2007; Sharma and Patino 2013). Concomitantly, gene expression of aromatase (cyp19a1a) and of the estrogen receptors (esr1, esr2a, esr2b) was found to be down-regulated after T4 treatment. Goitrogen treatment resulted in up-regulation of estrogen receptor gene expression. A slight decrease in aromatase gene expression was observed. Androgen receptor gene expression showed an opposing regulation to estrogen receptor gene expression, supporting the assumption that thyroid receptor signaling is involved in sex ratio regulation. A connection between thyroid hormone treatment and aromatase gene expression was also demonstrated by a study with the northern leopard frog (Rana pipiens), which was exposed to T3 during metamorphosis for 48 h (Hogan et al. 2007). That study demonstrated decreased aromatase mRNA levels in the developing amphibian brain after T3 treatment. Direct evidence for an influence of T3 treatment on aromatase activity was also found in older studies in primary cell cultures from immature mouse granulosa cells (Cecconi et al. 1999), and in follicle-stimulating hormonestimulated Sertoli cells (Panno et al. 1994; Ulisse et al. 1994): a dose- and time-related inhibition of aromatase activity was reported after T3 treatment. These studies support the evidence of cross-talk between the HPT and the HPG axes across taxa.

Exposure of fish (e.g., Chinese rare minnows [Gobiocypris rarus] or zebrafish) to chloroacetamide herbicides like acetochlor, butachlor, and pretiachlor resulted in expression changes of genes related to the HPG as well as to the HPT axes (Jiang et al. 2015, 2016; Zhu et al. 2014). An up-regulation of genes encoding for aromatase or StAR, along with other genes involved in steroid biosynthesis, was found in female rare minnows after exposure to butachlor (Zhu et al. 2014). Furthermore, transcript levels of the gene encoding the biomarker vitellogenin (VTG; vtg1, vtg2) were found to be significantly up-regulated in the liver of females. However,
transcript levels and protein levels did not correspond in that study. Whereas results of the gene expression analyses would suggest an up-regulation of estrogens and plasma VTG levels in females as well as a down-regulation of 11-KT levels in males, a decrease in plasma VTG levels in females as well as an increase in 11-KT levels in males was observed. This effect was assumed to be a feedback mechanism compensating for excess estrogenic signaling, consequently resulting in reduced VTG levels. Exposure to acetochlor resulted in an up-regulation of aromatase and vtg gene expression in zebrafish larvae, whereas a down-regulation of the estrogen receptor B1 (esr1b) was observed (Jiang et al. 2015). These authors thus claimed estrogenic activity for acetochlor, as similar effects of other estrogenic substances were described earlier, suggesting a feedback mechanism in the decreased estrogen receptor expression, compensating for the increased estrogen activity. Similar results were obtained by pretilachlor treatment (Jiang et al. 2016).

Chloroacetamide herbicide treatment also results in changes of the transcription levels of genes annotated to the HPT axis. For example, in the study by Zhu et al. (2014) noted in the preceding paragraph, deiodinase type 1 (Dio1) and deiodinase type 3 (Dio3) were found to be up- and down-regulated, respectively, at the transcriptional level, finally resulting in increased plasma T4 levels. As T4 is normally metabolized to the inactive rT3 by Dio3, it accumulates as a result of butachlor-mediated Dio3 inactivation. This butachlor-induced accumulation might be the reason for the rather masculinizing effect of butachlor (indicated by increased 11-KT levels), even though the effects on HPG-related genes indicate estrogenic activity. Similar results were described for zebrafish embryos treated with pretilachlor for 96 h (Jiang et al. 2016). Effects on VTG, aromatase (both aromatase isoforms cyp19a1a and cyp19a1b), and T3/T4 levels were observed. Analogous to the study with butachlor, an estrogenic effect could be assumed for pretilachlor, because VTG and aromatase were found to be up-regulated, even at the protein level. The increased thyroid hormone levels, however, point to the opposite direction, because increased thyroid hormone levels have been described as resulting in a masculinizing phenotype. The study performed with acetochlor did not investigate protein levels of VTG, aromatase, and the thyroid hormones, so no conclusion could be drawn regarding the effects of thyroid hormone levels on the sex ratio. Nevertheless, studies performed with chloroacetamide herbicides describe similar results, which provide evidence for an interaction of the HPG and the HPT axes. However, in these cases, the connection is not as straightforward as for other goitrogens, such as perchlorate or thiourea, and might involve feedback mechanisms that need to be clarified to obtain a reliable picture of this interaction.

Even more complicated is the cross-talk between the HPT and HPG axes after exposure to polybrominated diphenyl ethers (PBDEs), extensively reviewed by Yu et al. (2015). Even though influences on both axes were described, there is no consistent picture showing, for example, whether thyroid hormones are up- or down-regulated or whether effects on the HPG-axis, such as transcriptional changes of steroidalogenic genes, are a primary or secondary effect as a result of regulation of the HPT axis. The authors attributed this inconsistency to different reasons: the different developmental stages of fish in the studies performed with PBDEs, for example, or the mixture exhibiting different toxicokinetic properties. Thus no AOP was derived.

Finally, 5 AOP segments were derived from these studies, describing key events shared by the HPT and HPG axes. The first AOP segment, with different MIEs depending on the goitrogen used (Figure 2A), is entitled “Different MIEs resulting in decreased thyroid hormone levels lead to feminization, and finally, to a declining population trajectory.” The different MIEs are induced by treatment with goitrogens like perchlorate or methimazole. As described earlier, the MIE for perchlorate is the inhibition of the sodium iodide symporter, whereas methimazole inhibits thyroid peroxidase, both events finally leading to decreased thyroid hormone levels but by different mechanisms. These key events are also shared by the second AOP segment entitled “Thyroid receptor activation by increased thyroid hormone levels leads to masculinization.”

The following key event of the first AOP segment, which is located along the HPG axis, is increased aromatase levels at the transcriptional level as well as at the enzyme level. Increased aromatase levels result in increased estrogen levels, leading to a female-biased sex ratio, which represents the AO at the individual level (Mukhi et al. 2007; Sharma and Patino 2013; Sharma et al. 2016). Changed T3/T4 levels result in decreased 11b-HSD levels, which is the intermediated key event for the second AOP segment. Decreased 11b-HSD levels likely result in decreased 11-KT levels (Rasheeda et al. 2005; compare Figure 1A) and thus again in a female-biased sex ratio (Figure 1B). Correspondingly, treatment with thyroid hormones (i.e., thyroid receptor activation as the MIE) results in decreased aromatase levels as the key event and a masculinizing effect as the AO at the individual level (Mukhi et al. 2007; Sharma and Patino 2013). This AOP is called “Thyroid receptor activation by increased thyroid hormone levels leads to masculinization” (Figure 2C).

The next AOP (Figure 2D) describes a connection between the HPT and HPG axes via a deoxyribonucleic acid (DNA)-encoded mechanism. It is entitled “Thyroid hormone receptor activation of genes possessing a thyroid receptor–responsive element results in masculinization.” The MIE is an increased activation of thyroid hormone receptors by increased thyroid hormone levels. The activated receptors function as transcription factors for the regulation of genes possessing a thyroid receptor–responsive element in their promoter region representing the next key event in this AOP. These genes include 11β-hsd, shfr5α, and androgen receptors, expression of which is likely to be induced by thyroid hormone receptor activation. The resulting enzymatic change leads to increased levels of active androgens (either 11-KT in fish or 5α-DHT in nonfish vertebrates) and in masculinization as the AO. However, as explained previously, the findings underlying this AOP are only theoretically described and need further experimental verification, for example, by analyses of gene expression levels as well as enzymatic assays at the protein level.
Another AOP segment cross-talk between the HPT and HPG axes describes the effects of MIEs induced by substances such as chloroacetamide herbicides (Figure 2E). The effects of substances with this MIE are more appropriately described by separate AOP segments, with a shared AO at the organism level, that is, a skewed sex ratio toward males (Figure 2E). Only one AOP segment describes a cross-talk between the axes. However, for both segments, the precise MIE is unknown. Thus the first segment is entitled “An unknown MIE leading to estrogen receptor activation results in masculinization.” The first key event is located at the HPG axis and is probably estrogen receptor activation leading directly to an increase in aromatase (i.e., cyp19a1b) and vtg gene transcription, likely induced by increased estrogen receptor...
activation through an estrogen-receptor–responsive element in their promoter regions (Pellegrini et al. 2005). In consequence, a down-regulation of esr1/J gene expression as a compensatory mechanism (Jiang et al. 2015) is described, which finally results in down-regulation of aromatase activity. Decreased aromatase activity results in masculinization, which has been described by Muth-Kohnne et al. (2016). Thus the final AO at the individual level is dependent on the concentration of substances like the chloroacetamide herbicides.

The other AOP segment also includes an unknown MIE, which results in a transcriptional regulation of genes related to the HPT axis, the deiodinases (i.e., down-regulation of dio3). The following key event is an accumulation of T4 (because of reduced levels of Dio3, which normally converts T4 to the inactive rT3). If the AOP described in Figure 2A is taken into consideration, increased thyroid hormone levels lead to decreased aromatase activity and finally to increased 11-KT levels, resulting in a male-biased sex differentiation. At this point there might be a connection to the AOP segment described in the previous paragraph, as the down-regulation of estrogen receptor gene expression (and in consequence, of estrogen receptor signaling) might also lead to a masculinizing effect in fish. The resulting AOP segment is entitled “An unknown MIE leading to transcriptional regulation of deiodinases results in masculinization.” However, the final steps of these AOPs lack experimental verification. Furthermore, the interpretation of these AOPs remains difficult. The AOPs comprise increased as well as decreased aromatase activity and might be dependent, for example, on substance concentration and a nonmonotonous concentration–response relationship.

Cross-talk between the HPA/I and HPT axes

More than a decade ago, De Groof et al. (2006) had already reported regulation of thyroid-stimulating hormone secretion by corticotropin-releasing hormone signaling, mediated through different types of corticotropin-releasing hormone receptors in chicken (Gallus gallus). Similar results have also been reported for amphibians and fish (Castaneda Cortes et al. 2014). As described in the previous paragraph, both the HPA/I and the HPT axes act on the HPG axis, a connection that might ultimately result in regulation of sexual development and reproduction. However, as this connection is based on only a few studies, no AOP segment was defined.

CONCLUSIONS

It was possible to define potential AOP segments cross-linking the HPG and HPA/I axes, and the HPG and HPT axes. For the connection between the HPT and HPA/I axes, only a few studies were found, which were thus not translated into an AOP segment.

The following AOP segments were defined for fish, supported, if possible, by data on amphibians and mammals: 1) an unknown MIE resulting in increased cortisol release leads to masculinization in fish (for the 2 related AOP segments described in Figure 1); 2) estrogen receptor activation leads to reduced reproduction and thus to a declining population trajectory, as well as to an altered immune status of fish, resulting in effects on survival; 3) different MIEs resulting in decreased thyroid hormone levels lead to feminization, and finally to a declining population trajectory; 4) thyroid receptor activation by increased thyroid hormone levels leads to masculinization; 5) thyroid hormone receptor activation of genes possessing a thyroid receptor–responsive element results in masculinization; 6) an unknown MIE leading to estrogen receptor activation results in masculinization; and 7) an unknown MIE leading to transcriptional regulation of deiodinases results in masculinization.

The HPA/I and HPG axes share common precursors of their steroids. One important key event for their cross-talk is the inhibition of aromatase activity, which ultimately leads to a male-biased sex ratio. Furthermore, the enzymes StAR and 11β-HSD have functions that are involved in both axes. Their regulation is considered an important key event shared by the 2 axes.

Most important for the cross-talk between the HPT and the HPG axes is the level of the thyroid hormones T3 and T4. One key event in this context is again the regulation of aromatase activity. Another important key event seems to be the expression of genes ascribed to the HPG axis possessing a thyroid receptor–responsive element in their promoter region.

For the connection between the HPA/I and HPT axes, it was not possible to define an AOP segment, even though a cross-talk is likely. However, there is still too much uncertainty about the key events at the different organizational levels.

In general, more information needs to be gathered to increase the plausibility and reliability of the developed AOP segments. For several AOP segments, a specific MIE is missing, as studies often describe only key events at either the molecular level or at the organism level. The specific mode of action resulting in the AO often remains elusive (AOP segments 1, 6, and 7). For other substances (e.g., the goitrogens with a known mode of action), intermediate key events and key event relationships bridging between the endocrine axes are unknown (AOP segment 3). Furthermore, there are also key event relationships for which no empirical data exist, for example, in silico promoter analyses identifying the thyroid receptor–responsive elements in the promoter region of HPG-related genes (AOP segment 5). Determination of linear AOP segments is quite difficult if substances likely induce more than one MIE. In addition, increased substance concentrations result in compensatory feedback mechanisms, as has been described, for example, for chloroacetamide herbicides (AOP segments 6 and 7).

These uncertainties and data gaps need to be resolved and closed to validate the AOPs. Only then can AOPs be applied to determine the potential of substances to act on more than one endocrine axis.

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