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The progestin norethisterone affects thyroid hormone-dependent metamorphosis of *Xenopus laevis* tadpoles at environmentally relevant concentrations

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ABSTRACT

Previously, levonorgestrel (LNG) has been shown to be an endocrine disruptor of the amphibian thyroid system. In the present study, we investigated whether anti-thyroidal effects are a common property of progestins other than LNG. Premetamorphic *Xenopus laevis* tadpoles were exposed to norethisterone (NET) and dienogest DIE (each at 0.1–10 nM) and LNG (10 nM) until completion of metamorphosis. LNG and NET at all concentrations caused a significant developmental retardation whereas DIE did not impair time to metamorphosis. In LNG and 10 nM NET exposed animals, *tsh* mRNA levels increased considerably later than the developmental delay occurred and thyroid histopathology showed no signs of TSH-hyperstimulation. Instead, thyroid glands from these treatments appeared inactive in producing thyroid hormones. Thyroidal transcript levels of *dio2* and *dio3* were increased by treatments with LNG and NET at 1 nM and 10 nM, whereas *iyd* mRNA was reduced by LNG and 10 nM NET. Expression of *slc5a5* was not changed by any treatment. Effects of DIE differed from those induced by LNG and NET. No developmental delay was measurable; however, *tshβ* and *dio2* mRNAs were increased in pituitary glands of tadpoles exposed to 1.0 nM and 10 nM DIE. Thyroid histopathology displayed no abnormalities and thyroidal mRNA expression of the genes analyzed (*slc5a5, iyd, dio2, dio3*) was not changed by DIE. Overall, our results provide evidence that the anti-thyroidal effects already known from LNG are also present in another progestin, namely NET, even at environmentally relevant concentrations. In conclusion we suggest that progestins do not only pose an environmental risk in terms of their impact on reproductive success of aquatic vertebrates, but also with respect to their anti-thyroidal properties affecting amphibian metamorphosis.

1. Introduction

At present, progestins receive a lot of attention within ecotoxicological research. Substances that have been monitored, such as levonorgestrel (LNG), norethisterone (NET) or medroxyprogesterone acetate, have been detected in surface waters in a range of a few to tens ng/L (Fent, 2015; Kumar et al., 2015). However, there is only little information on environmental concentrations since there is still a lack of analytical methods for the detection of the majority of progestins. There are numerous reports dealing with the endocrine disrupting effects of progestins in aquatic (in)vertebrates, most of them focusing on impacts on the reproductive system in fish (Fent, 2015; Kumar et al., 2015; Capello et al., 2017). Compared to the considerable number of reports on progestin effects in fish, there are only few ecotoxicological studies using amphibians (*Xenopus laevis* and *X. tropicalis*) as target organisms (Kvarnryd et al., 2011; Lorenz et al., 2011a, 2011b; Hoffmann and Kloas, 2012; Säfholm et al., 2012, 2014, 2016; Zikova et al., 2017). All of these studies used levonorgestrel (LNG) or norethisterone (NET), two testosterone derivatives with well-known androgenic activity (Africander et al., 2011; Kumar et al., 2015). Consistent with results from studies on fish, the progestins tested were shown to adversely affect reproductive function in amphibians as well (Lorenz et al., 2011b; Kvarnryd et al., 2011; Säfholm et al., 2012). Examining the present literature dealing with progestin effects on aquatic organisms, a broad range of lowest observed effect concentrations can be noted (Kumar et al., 2015). This indicates varying sensibilities of the test species used but also varying potencies of the different progestins to act as endocrine disruptors. The term “progestin” comprises a group of about 20 synthetic steroidogenic compounds that bind to the progesterone receptor (PR), thereby mediating actions similar to those of progesterone (P4; Kumar et al., 2015). This progestogenic property is utilized in human medicine, most prominently in hormonal contraception and hormone replacement therapy (Sitruk-Ware, 2004). All progestins are derived from P4, testosterone or

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spironolactone. Besides their common progestogenic effects they possess varying selectivities to steroid receptors other than the PR, such as the androgen receptor, estrogen receptor, glucocorticoid receptor and mineralocorticoid receptor (Sitruk-Ware, 2004; Africander et al., 2011). Consequently, a progestin can display (anti-)androgenic, (anti-)estrogenic, (anti-)glucocorticoid and/ or (anti-)mineralocorticoid activities (Besse and Garric, 2009; Kumar et al., 2015) and may possess additional effects on (non-)target organisms. In clinical trials examining the effects of progestins on parameters related to the thyroid system, no or only minor effects on thyroid hormone (TH) plasma levels have been reported. It was concluded that progestins have no or only minor effects on the thyroid system (Croxatto et al., 1983; Biswas et al., 2000; Kivela et al., 2001; Wiegratz et al., 2003). In a previous study, we demonstrated for the first time thyroid disrupting effects of a progestin, namely LNG, when administered chronically during larval development of *X. laevis* tadpoles (Lorenz et al., 2011a). Amphibian metamorphosis is a well-established model for the detection of chemicals with thyroid disrupting properties (Kloas et al., 2009; Miyata and Ose, 2012; Sachs and Buchholz, 2017). The transition of a larval tadpole into a juvenile frog comprises numerous developmental processes which are coordinated spatiotemporally and under control of TH (Brown and Cai, 2007). On this basis, thyroid disrupting effects by exogenous contaminants can be identified just by observing and recording the developmental progress of exposed tadpoles. The examination of further significant biomarkers such as gene expression patterns, thyroid histopathology or plasma TH levels can give insight into the contaminant´s mode of action (Opitz et al., 2006; Miyata and Ose, 2012; Hornung et al., 2015). A thyroid system disruption is thereby typically reflected by (1) a metamorphic delay, (2) a significant increase of thyroid stimulating hormone (β-subunit, *tshβ*) mRNA and (3) thyroid gland hypertrophy including cell hypertrophy and hyperplasia (Degitz et al., 2005; Opitz et al., 2006; Coady et al., 2010; Grim et al., 2009; Miyata and Ose, 2012). The effect pattern in LNG exposed tadpoles clearly differed from the commonly-accepted consequences of a treatment with classical goitrogens: notwithstanding that metamorphosis was severely impaired by LNG indicating a hypothyroid state, *tshβ* mRNA levels were not elevated or if, only after very long chronic LNG treatment (Lorenz et al., 2011a). Consistently, thyroid gland histology revealed no signs of hypothyroidism. Taken together, these results indicated that the antithyroidal effects of LNG include a disruption of the negative feedback regulation of TH synthesis via TSH. The mechanisms of action causing the observed effect pattern in LNG treated tadpoles are not understood so far. In a first attempt to identify target sites of LNG, a subsequent *ex vivo* approach found that LNG directly affects gene expression in the pituitary and thyroid gland (Lorenz et al., 2016). In the thyroid gland, LNG directly reduced expression of *slc5a5* (encoding for sodium-iodide symporter (NIS)) and *iyd* (encoding for iodotyrosine dehalogenase (IYD)) (Lorenz et al., 2016). The NIS mediated iodide uptake in the thyrocytes is the first and rate limiting step of TH synthesis (Dohan et al., 2003). Deiodination of monoiodotyrosine and diiodotyrosine residues by IYD is essential for the rescue and recycling of iodide in order to reuse it for TH synthesis. Defects of NIS and IYD activity cause an iodine deficiency and are known to result in various forms of hypothyroidism (Iglesias et al., 2014; Targovnik et al., 2017). In addition to effects on *slc5a5* and *iyd*, LNG-exposure provoked also significant changes of *dio2* and *dio3* (encoding for iodothyronine deiodinases (DIO2 and 3)) mRNA levels (Lorenz et al., 2016). Inner and outer ring deiodination of iodothyronines, respectively, are substantial mechanisms of the local regulation of TH bioavailability and action (Brown, 2005; Darras et al., 2015). THs are mainly synthesized as tetraiodothyronine (T_4) and the bioactive form triiodothyronine (T_3) is generated peripherally via outer ring deiodination catalyzed by DIO2 (Gereben et al., 2008). Inner ring deiodination of T_4, mediated by DIO3, produces reverse T_3, which is biologically inactive (Gereben et al., 2008). In thyroid glands of hypothyroid tadpoles, mRNA expressions of *dio2* and *dio3* have been shown to be up-/ down-regulated, respectively, suggesting an adjustment of TH status (Opitz et al., 2009). It was therefore a notable finding that *in vivo* as well as *ex vivo* treatment with LNG caused a concurrent increase of thyroidal *dio2* and *dio3* gene expression (Lorenz et al., 2016). Summarizing the results from previous experiments, LNG provokes a unique constellation of molecular and histological markers that is clearly different to the classical signature of a goitrogen-induced hypothyroidism (Opitz et al., 2009). In the present study, we investigated whether these anti-thyroidal effects are unique to LNG or are a common property of more progestins. To address this issue, two further progestins, namely norethisterone (NET) and dienogest (DIE), were selected on the basis of their structural derivation from testosterone, and with consideration of their additional biological activities. In humans, NET and DIE have not only progestogenic, but also androgenic and anti-androgenic properties, respectively (Besse and Garric, 2009; Africander et al., 2011; Kumar et al., 2015). With the goal to identify potential structural and/ or functional properties of progestins contributing to (anti-)thyroidal actions, we treated premetamorphic *X. laevis* tadpoles over the period of larval development with the selected substances. Three concentrations were tested including the environmentally relevant range. In order to assess the comparability of the study with our previous results, LNG was used as positive control. Time to complete metamorphosis was determined, histological appearance of thyroid glands was assessed and mRNA levels of classical markers of thyroid function (*tshβ*, *slc5a5*, *dio2*, *dio3*) were analyzed.

2. Material and methods

2.1. Chemicals

Chemicals for preparation of stock solutions and analytical standards, respectively, were obtained from Sigma-Aldrich (Steinheim, Germany; D(-)-Norgestrel (LNG, 99%), 19-Norethisterone (NET, ≥ 98%)) and Santa Cruz Biotechnology (Heidelberg, Germany; Dienogest (DIE, ≥ 99%)). Ethanol (EtOH, ≥ 99.5%, Roth, Karlsruhe, Germany) was used as solvent. EtOH concentration in the stock solutions was 0.1%.

2.2. Animals and husbandry

Adult *Xenopus laevis* were taken from the breeding stock of the Leibniz-Institute of Freshwater Ecology and Inland Fisheries. Spawning was induced by injection of human chorionic gonadotropin (Sigma-Aldrich, Steinheim, Germany) into the dorsal lymph sac. Fertilized eggs and tadpoles, respectively, were reared in 50 L tanks under defined conditions as described by Urbatzka et al. (2010). Seven days post fertilisation (dpf) tadpoles were transferred into 7 L glass aquaria into a flow-through culture system and were maintained into tap water according to ASTM standards (Lutz et al., 2008) until the beginning of exposure.

2.3. Experimental conditions

A long-term exposure test was performed with NET and DIE, as well as LNG as positive control. A flow-through culture system with two replicate tanks per treatment was used to maintain constant conditions over the period of the experiment. Premetamorphic tadpoles at developmental stage 48 (according to Nieuwkoop and Faber (NF); Nieuwkoop and Faber, 1994) were treated with NET and DIE at 0.1 nM, 1.0 nM and 10 nM, respectively; 10 nM LNG and a solvent control (SC; 0.0005% EtOH). Each replicate tank contained 22 tadpoles resulting in a total of 44 tadpoles per group. The flow rate of test media was adjusted to 25 mL/min per tank, which was permanently aerated with glass pipets. Temperature was set to 22 ± 1 °C and photoperiod was maintained at 12:12 h (light:dark). Tadpoles were fed with Sera Micron® (Sera GmbH, Heinsberg, Germany) according to Lutz et al.