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# Implication of thyroid hormone signaling in neural crest cells migration: Evidence from thyroid hormone receptor beta knockdown and NH3 antagonist studies

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ABSTRACT

Thyroid hormones (TH) have been mainly associated with post-embryonic development and adult homeostasis but few studies report direct experimental evidence for TH function at very early phases of embryogenesis.

We assessed the outcome of altered TH signaling on early embryogenesis using the amphibian *Xenopus* as a model system. Precocious exposure to the TH antagonist NH-3 or impaired thyroid receptor beta function led to severe malformations related to neurocristopathies. These include pathologies with a broad spectrum of organ dysplasias arising from defects in embryonic neural crest cell (NCC) development. We identified a specific temporal window of sensitivity that encompasses the emergence of NCCs. Although the initial steps in NCC ontogenesis appeared unaffected, their migration properties were severely compromised both *in vivo* and *in vitro*. Our data describe a role for TH signaling in NCCs migration ability and suggest severe consequences of altered TH signaling during early phases of embryonic development.



## 1. Introduction

The pleiotropic roles played by thyroid hormones (TH) during vertebrate post-embryonic development and in adult physiology have been extensively studied (Cheng et al., 2010; Tata, 1968; Yen, 2001). In contrast, the relevance of TH signaling during early vertebrate embryogenesis has received less attention, perhaps as a consequence of discordant data and little direct experimental evidence.

Most effects of TH are thought to be mediated by thyroid hormone receptors (THRs), which are nuclear receptors, although non-genomic actions of TH are also recognized (Cheng et al., 2010; Davis and Davis, 1996). THRs exist as different isoforms encoded by two genes: *THRA* (*NR1A1*) and *THRB* (*NR1A2*). So far, genetic approaches

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in the mouse have not been used to specifically address the roles played by THR during embryogenesis (Forrest et al., 1996a, 1996b; Gauthier et al., 2001, 1999). Different mouse models, either THR deficient or expressing dominant negative THRs have been generated. However, since almost no obvious defects were observed at birth, THRs were thought to be dispensable for mouse embryonic development and studies have focused on postnatal stages (Gauthier et al., 1999; Göthe et al., 1999). This non-essential characteristic of THRs during embryogenesis somewhat conflicts with other studies (Flamant and Samarut, 2003). For example, the viability of THR mutants contrasts with a complete TH deficiency. Knockout mice for the *pax8* gene lack thyroid follicles and do not survive very long after birth, but this lethality can be rescued by complete disruption of the *THRA* gene (Flamant et al., 2002; Mansouri et al., 1998). Furthermore, *THRA1* (*THRA* isoform 1) disruption prevents cerebellum defects induced by hypothyroidism (Morte et al., 2002). Thus, unliganded THRs exert developmental functions that remain largely unknown (Chassande, 2003). In fact, unliganded THRs have been implicated in heart (Mai et al., 2004) and eye development (Havis et al., 2006).

In parallel to unliganded THR activity, fine-tuning of TH signaling is functionally important for the developing embryo in mammals, long before its thyroid gland becomes functional (Cao et al., 1994; Lischinsky et al., 2016; Momotani et al., 1984; Porterfield and Hendrich, 1993; Rovet, 2014). A number of epidemiological and clinical studies now raise the possibility that a broad range of early developmental processes might be TH-dependent. Alterations in maternal or fetal TH supply during pregnancy, notably during the first trimester, such as those produced by inadequate iodine intake and thyroid dysfunctions (cretinism, hypothyroidism and hyperthyroidism) can result in major neurologic impairments and severe malformations in the offspring, in particular in craniofacial structures (de Escobar et al., 2004; de Lima et al., 1999; Gamborino et al., 2001; Hirano et al., 1995; Israel et al., 1983; Lazarus, 2015; Moog et al., 2015; Rovet, 2014; Zimmermann, 2011). However, the developmental processes involved remain unclear and evidence from mammalian and human studies for the relevance of TH signaling in early development is sparse. In fact, it is difficult to determine the relationship between anomalies and impaired TH signaling during embryogenesis.

Addressing the function of TH signaling experimentally in early mammalian embryos is challenging due to complications associated with uterus-enclosed embryos and maternal-fetal exchanges, making TH level manipulations difficult. This highlights the need to improve tools and *in vivo* animal models to assess the outcomes of modulating TH signaling during early development. To this end, experimental studies that take advantage of species developing as free-living embryos, such as fish and amphibian, appear best suited.

Amphibian development, and in particular that of *Xenopus* species, provides an excellent model to study TH function *in vivo* (Sachs, 2004; Shi et al., 1996; Tata, 1993). Since TH triggers metamorphosis, THRs have been primarily implicated in processes that occur during post-embryonic development. During this developmental period, a correlation was established between THRs expression and tissue responsiveness to TH signaling. However, transcripts and proteins are also detected during early embryogenesis both as maternal and zygotic products (Baker and Tata, 1990; Banker et al., 1991; Cossette and Drysdale, 2004; Duarte-Guterman et al., 2010; Eliceiri and Brown, 1994; Havis et al., 2006; Kawahara et al., 1991; Oofusa et al., 2001; Yaoita and Brown, 1990a, 1990b). Interestingly, the presence of TH in amphibian eggs and embryos and the expression of TH signaling components (such as deiodinases) during embryogenesis is now well documented (Duarte-Guterman et al., 2010; Fini et al., 2012; Morvan-Dubois et al., 2006; Tindall et al., 2007; Weber et al., 1994). Thus, we know that *Xenopus* embryos express both THRs and TH signaling activities at early developmental stages and therefore they represent a good experimental system to assess the relevance of TH signaling during early vertebrate embryogenesis.

It has been shown that early *Xenopus* neurogenesis can be affected by TH signaling and is sensitive to endocrine disruption (Fini et al., 2012). These data are supported by additional experiments performed in the zebrafish model in which pharmacological and genetic TH signaling alterations also lead to severe developmental abnormalities (Bagci et al., 2015; Bohnsack and Kahana, 2013; Campinho et al., 2014; De Vrieze et al., 2014; Fetter et al., 2015; Heijlen et al., 2014). In particular, these data point to the implication of TH signaling in craniofacial formation, as previously suggested by human epidemiological and clinical studies. However, equivalent data do not exist for *Xenopus*.

Finally, the early function of THRs in both zebrafish and *Xenopus* remains unclear. In zebrafish, a *thraa* knockdown or the expression of THRs dominant negative forms result at 24hpf in severe developmental abnormalities and for the latter in manifestations of resistance to thyroid hormone (RTH) at later stages of development (Bohnsack and Kahana, 2013; Marelli et al., 2016). In *Xenopus*, a THRA knockout does no appear to affect early embryogenesis (Choi et al., 2015; Wen and Shi, 2015). In both species, the outcome of a THRB knockout or knockdown remains unknown.

Taken together, these studies suggest that TH signaling could exert more functions during embryogenesis than initially anticipated. So far, few studies have been conducted on early developmental stages in a model system adapted to perform both precise TH level manipulations and direct phenotypic evaluation. In this study, we provide experimental evidence in the amphibian *Xenopus* that altered TH signaling impairs early developmental processes leading to major malformations, including craniofacial defects, through direct exposure to pharmacological compound in the rearing water. We highlight a specific temporal window of sensitivity during embryogenesis encompassing the emergence of neural crest cells (NCCs). During this process, we show that NCCs induction and specification were not altered but their migration properties were severely compromised. Finally, we show that a THRB knockdown could phenocopy the effects produced by a pharmacological treatment suggesting that THRB could be a major molecular actor of the TH signaling pathway during early embryogenesis.

## 2. Materials and methods

### 2.1. Animals, treatments and microinjections

*Xenopus tropicalis* and *Xenopus laevis* were bred in our facility (TGA strain). Embryos were obtained and microinjected as previously described (Ymlahi-Ouazzani et al., 2010). Developmental stages were determined according to Nieuwkoop and Faber (NF; Nieuwkoop and Faber, 1994). For NH-3 treatments, 50 embryos were maintained in 10 ml of Marc's Modified Ringer (MMR) 1/20× for *X. tropicalis* and MMR 1/10× for *X. laevis* with 2 μM NH-3 unless otherwise stated. NH-3 was synthesized as previously described (Nguyen et al., 2002) and extemporaneously diluted from a 5 mM stock solution in ethanol. An equivalent amount of compound diluents (ethanol) was added to the media for control experiments (untreated embryos: Untreat.). For microinjections experiments, 0.1 ng of locked nucleic acid (LNA) oligonucleotides was injected at the one cell stage (Lennox et al., 2006):

Control LNA directed against GFP sequence, CO-LNA (+precedes a LNA residue): 5′-C + TTCAGC + TCA + TG + CGG + TT-3′.

THRB-LNA directed against the initiation codon region of the *Xenopus tropicalis THRB* (Fig. 6A, vertical bar): 5′-