A mixture of chemicals found in human amniotic fluid disrupts brain gene expression and behavior in *Xenopus laevis*

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Abstract

Thyroid hormones (THs) are essential for normal brain development, influencing neural cell differentiation, migration, and synaptogenesis. Multiple endocrine-disrupting chemicals (EDCs) are found in the environment, raising concern for their potential effects on thyroid hormone signaling and the consequences on neurodevelopment and behavior. While most research on EDCs investigates the effects of individual chemicals, human health may be adversely affected by a mixture of chemicals. Many compounds belonging to a wide range of chemical classes have been identified as EDCs, notably those affecting thyroid hormone signaling.

We hypothesized that embryonic exposure to a mixture of chemicals (containing phenols, phthalates, pesticides, heavy metals, perfluorinated -, polychlorinated, and polybrominated compound) commonly found in the human amniotic fluid could lead to altered brain development to assess its effect on thyroid hormone signaling and neurodevelopment in an amphibian model (*Xenopus laevis),* highly sensitive to thyroid disruption.

Newly hatched tadpoles were exposed for eight days to either TH (thyroxine, T_4 10nM) or the amniotic mixture (1x concentration) and gene expression was analyzed in the brains of exposed tadpoles using both RT-qPCR and RNA sequencing. Results indicate that whilst some overlap on TH-dependent genes exist, T_4 and the mixture have different gene signatures. Immunohistochemistry showed increased proliferation in the brains of T_4-treated animals whereas no difference was observed for the amniotic mixture. Further, we demonstrated diminished tadpoles' motility in response to T_4 and mixture exposure. As the individual chemicals composing the mixture are considered safe, these results highlight the importance of examining the effects of mixtures to improve risk assessment.

Key words: Thyroid hormones, Endocrine disruption, Neurodevelopment, *Xenopus laevis*

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Introduction

In 1894, two clinical studies demonstrated that some features of mental retardation characteristic of cretinism could be improved by treating young patients with thyroid extract (Railton 1894; Smith 1894). Since then, the role of thyroid hormone (TH) in brain maturation has been documented in detail (Bernal 2007). Most research focuses on the rapid period of brain growth, *i.e.* the perinatal period in mammals (Nunez et al. 2008; Bernal 2007). However, recent epidemiological and clinical studies have suggested that the early stages of neurogenesis are also TH-dependent (Pharoah et al. 1976; Pop et al. 1999; Levie et al. 2019). The main idea arising from these studies is that before the development of the fetal thyroid (which is not functional before the 16th week of gestation in humans (Obregon et al. 2007)), maternal TH levels are critical in determining post-natal neuro-motor development of the child. Various studies have shown that a child's IQ is correlated with their mother's thyroid status during early pregnancy (Korevaar et al. 2016; Pop et al. 1999).

Experimental studies on the role of functional thyroid signaling during early embryogenesis are more easily addressed in free-living embryos that do not require dissection from the mother. In the *Xenopus laevis* embryo, the thyroid gland is observed at NF (Nieuwkoop and Faber) 43 (Honda, Ogawa, and Taniguchi 1993), which is at about one week of age in standard rearing conditions. Analyzing morphological and biochemical data as well as thyroid receptor alpha (*thra)* mRNA profiles led to the proposition that *Xenopus laevis* embryos start to show competence to respond to T_3 treatment between stages NF40-44 (Tata 1968; Baker and Tata 1990; Shi et al. 1996; Nunez et al. 2008). Our research team previously demonstrated that exposing tadpoles to TH at stage NF 37 for 24 hours induced TH-regulated gene response in brain tissue at stage NF 41 (Fini et al. 2012).

Multiple studies have documented significant contamination of human populations and wildlife by multiple anthropogenic chemicals (Woodruff et al. 2011; Saaristo et al. 2018). About 30 anthropogenic chemicals are present in all American women, with 15 being ubiquitous, including in pregnant women (Woodruff et al. 2011). Most of these chemicals are demonstrated or suspected thyroid hormone disruptors (Boas et al. 2012; Crofton 2008), raising the question of whether current exposure to ubiquitous chemicals affects thyroid signalling and thereby early brain development. Even though certain xenobiotics have been investigated for their actions on specific endocrine axes, few studies have addressed their

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combined or 'cocktail' effects. We previously examined the consequences of a 3-day exposure to a mixture made of 15 ubiquitous molecules (Fini et al. 2017). Results suggested a T_3-like effect on most of the endpoints measured. However, some gene expression patterns in brain tissue revealed the opposite effects when comparing T_3 or mixture-induced effects.

A growing body of evidence demonstrates that the entire duration of pregnancy is a sensitive window for toxicant exposure. Therefore, we investigated a more extended exposure period (8 days) to the mixture (see Table 1), as reported in Fini et al. (2017), at environmentally relevant concentrations, during embryogenesis. After exposure, gene expression analysis was conducted employing RT-qPCR and RNA sequencing on the brains of *Xenopus laevis* tadpoles exposed during the embryonic period. Additionally, immunohistochemistry (IHC) was performed on brain samples to unravel possible aberrant molecular mechanisms of neurodevelopment after mixture exposure. Furthermore, the behavior of tadpoles was analyzed, as altered behavior could be a possible marker of abnormal neurodevelopment. In all the experiments, a positive control, thyroid hormone (T_4, 10 nM)) was included.

Results

Exposure to the amniotic mixture or THs alters the expression of genes essential in the thyroid hormone signaling pathway and affects neuronal developmental genes

A schematic representation of the conducted exposure study is depicted in Figure 1. Embryonic mixture and thyroid hormone (TH) exposure (T_3 5 nM and T_4 10 nM) were tested to evaluate their effects on gene expression in brain tissue. First, a selection of genes was elected, to be measured with RT-qPCR, based on their involvement in the thyroid hormone signaling pathway and their essential role in normal brain development.

The expression of TH-dependent transcription factors, kruppel-like factor (*klf9)* (Fig. 2, E), and thyroid hormone receptor beta (*thrb)* (Fig. 2, J) was induced after the T_3, T_4, and amnios mixture exposure. The thyroid hormone receptor alpha (*thra)* (Fig. 2, I) was only upregulated under mixture exposure. Further, the expression of membrane transporters allowing specific TH transport *mct8* (Fig. 2, G) and *oatp1c1* (Fig. F) was investigated. Interestingly, while both *mct8* and *oatp1c1* were upregulated in mixture exposed brains, the expression of *mct8* was downregulated by T3 and T4, showing opposite expression pattern. Under both T_3 and T_4

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exposure, the expression of deiodinase 1 (*dio1)* was downregulated and the expression of deiodinase 3 (*dio3)* upregulated to maintain TH homeostasis. Under mixture exposure, deiodinase 2 (*dio2)* was induced, similarly to the T_4 exposure. Furthermore, the effects on genes that are dysregulated in human ASD patients were investigated (e.g. *sin3a, bdnf, mbp, mecp2).* Strikingly, all neurodevelopmental genes were found to be induced after mixture exposure and found to be reduced after TH exposure (Figure 2, D, H, K, L).

Taken together, a remarkable impact of the amniotic mixture on brain gene expression was observed by RT-qPCR and, promoted us to investigate possible effects on a much broader range of genes. Given the multiple interesting opposite TH effects compared to the mixture effect, we performed RNA sequencing on samples of both TH (T_4 10 nM) and amniotic-treated animals.

A principal component analysis (PCA) was conducted for all the sequenced brain samples using their respective gene expression profiles for their representation on a two-dimensional graphic (Figure 3, A). Each dot in the PCA graphic represents the gene expression profile of a brain sample (each containing a pool of two brains), and the distance between two dots is proportional to the extent of similarity between the gene expression profiles. The PCA plot containing a projection on the first two principal components, which together explain 60.24% (49.70% + 10.54%) of the total variance, illustrated that the exposed samples clustered apart from the control group. The RNA sequencing analysis revealed that 587 transcripts were significantly altered in the group exposed to the amniotic mixture compared to the controls; whereas 6552 were significantly altered after exposure to T_4 10nM. 210 genes were commonly found to be affected by both TH or mixture exposure (Figure 3, B). Of the differentially expressed genes (DEGs) from the mixture exposure, more than 70% appear to be down-regulated (Fig 3, C). In the case of TH exposure, many genes were significantly down- or up-regulated (Fig 3, C, D).

KEGG pathway analysis indicated that the predicted targets of mixture exposure are involved in biological processes such as the MAPK signaling pathway (Table 2). This pathway is connected with the cell cycle and particularly the G1/S switch (Zhang and Liu 2002). The second pathway we found to be enriched by the amniotic mixture in the embryonic brain is lysosomal activation. This pathway is considered to be a milestone in several cell death

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pathways including apoptosis (Kavčič et al. 2017). Another target of TH exposure was the interaction between various neuro-active ligands and receptors (Table 2).

Given the 35% overlap of DEGs between mixture treated and TH-treated genes, we further investigated the commonality between these two exposure conditions by crossing them with a dataset containing TH-responsive genes obtained from (Chatonnet et al., 2015). Twelve genes are at the intersection of these three lists (DEGs amnios, DEGs T_4, and TH-responsive genes) (Fig. 4, A), 9 of which are regulated in the same direction. Surprisingly, *tshb*, a gene encoding for thyrotropin, a thyroid-stimulating hormone that induces the thyroid gland to produce T4 is up-regulated in the amniotic mixture.

The amniotic exposure affected the MAPK signaling pathway and given the importance of this signaling pathway in learning and memory and its implication in neurodevelopmental disorders (Rosina et al. 2019; Ryu and Lee 2016; Vithayathil, Pucilowska, and Landreth 2018; Faridar et al. 2014) we crossed the DEGs of amniotic exposure with the SFARI dataset, an online database of autism genes. Eighteen genes are at the intersection; half of them are also shared with the T_4 DEGs. Altered neurodevelopmental important RNAseq transcripts were confirmed employing RT-qPCR (Figure 5).

Exposure to TH but not amniotic mixture induces both apoptosis and proliferation

Given the crucial role of TH in both proliferation and apoptosis and the predicted effects of the amniotic mixture on both the MAPK signaling pathway and lysosomal activation, we investigated the proliferation/cell death ratios. Following an 8-day exposure, we fixed the tadpoles and performed immunohistochemistry on cryosections, using anti-phosphorylated histone H3 (P-H3), a mitotic marker, and anti-caspase 3 as an apoptotic marker. We measured an increase in proliferating cells (PH3+ cells) after T4 exposure but not with the amniotic mixture. No significant difference was detected for the apoptotic marker (Figure 6).

Exposure to an amniotic mixture alters the tadpole behavior

Next, we addressed the phenotypic consequences of early exposure since motor behavior changes can imply an alteration in the neural circuitry controlling movement (Straka and Simmers 2012). For this, we used a video tracking system and recorded the total distance traveled by individual tadpoles with 30 secs alternating light and dark cycles for a total of 10 minutes.

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The distance traveled decreased with the mixture- and TH-exposure in the light periods (Fig 7 A & B).

Figures

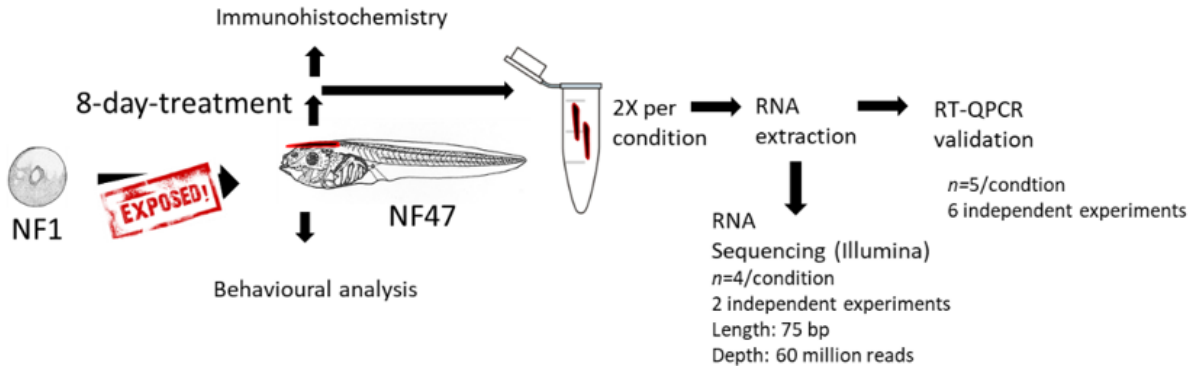

Figure 1 Schematic representation of the conducted exposure study. Fifteen *X. laevis* tadpoles per group were exposed for 8 days starting just after fertilization (NF1) to stage NF 47 before gene expression analysis, mobility assays, and brain immunohistochemistry.

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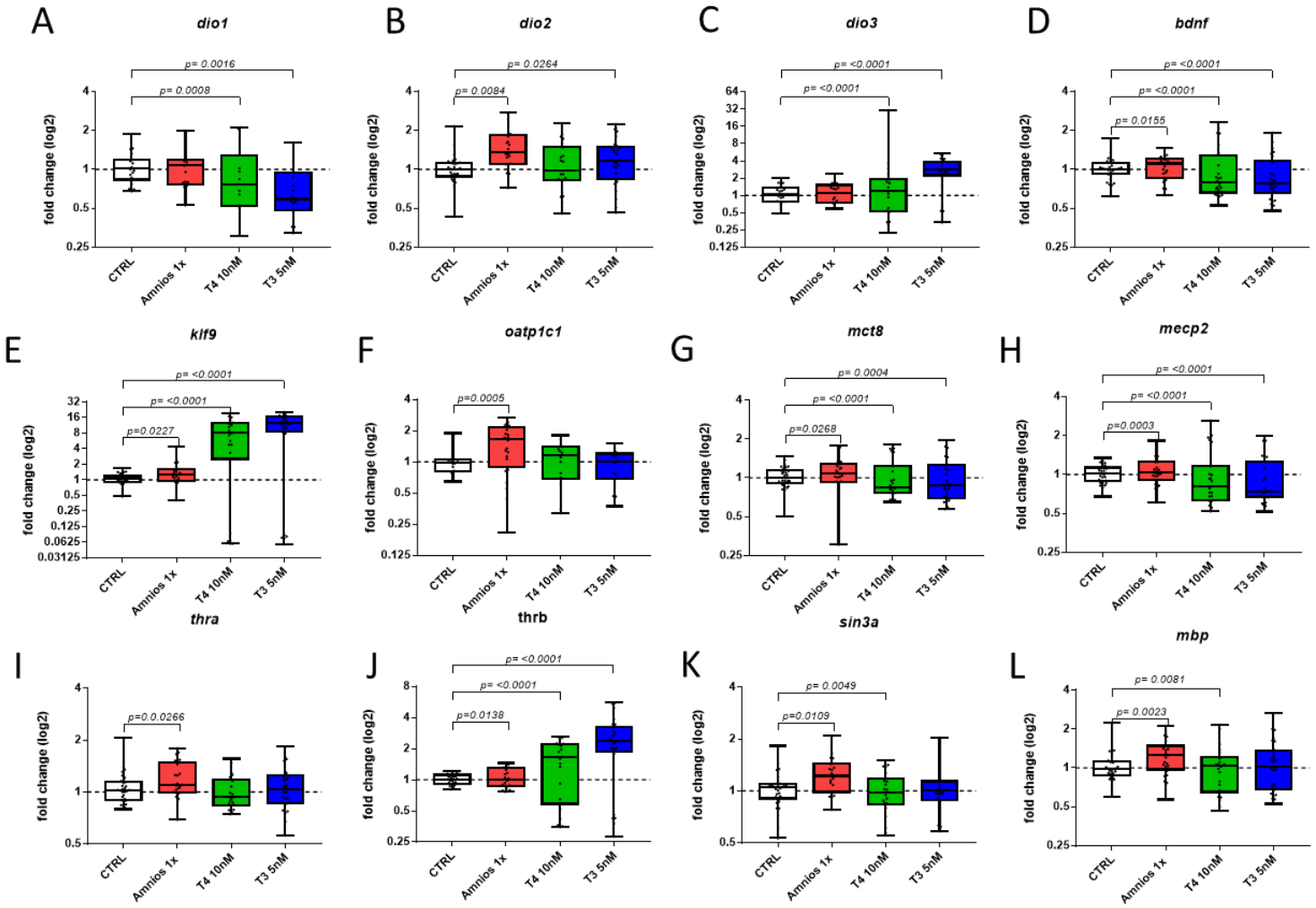

Figure 2 Gene expression after embryonic exposure to the amniotic mixture and thyroid hormones.

Embryos (NF 1 – NF 47) were exposed to DMSO (CTRL), amniotic mixture at 1x concentration (Amnios 1x), T_4 (10 nanomolar), and T_3 (5 nanomolar). After brain dissection at stage NF 47, RNA extraction and RT-qPCR were conducted on genes involved in the TH signaling pathway: A. *dio1*, B. *dio2*, C. *dio3*, E. *klf9*, F. *oatp1c1*, G. *mct8*, I. *thra*, J. *thrb*, and brain development: D. *bdnf*, H. *mecp2*, K. *sin3a*, L. *mbp.* Results are normalized to the geometric mean of the expression levels of the genes *ube2m* and *ralb*. Results are a pool of 6 independent replicates with n=5 by group by replicate. The line in each box represents the median.

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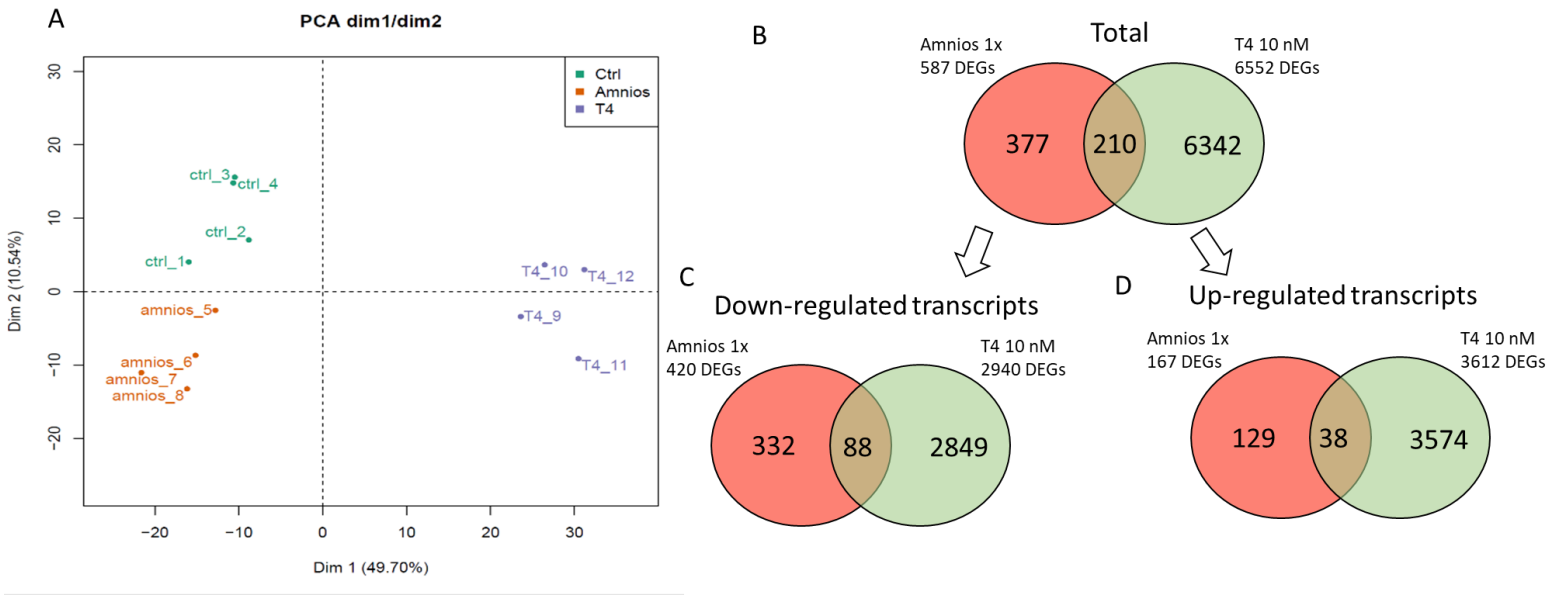

Figure 3 Principal component analysis plot of gene expression profiles from brain samples of exposed Xenopus tadpoles and Venn diagram from differentially expressed transcripts. A. The profiles from the amniotic mixture (amnios (n=4)) cluster separately to clusters representative of T_4 (T_4 10 nM (n=4)) or control exposures (control (n=4)). B. Venn diagram showing the differentially expressed genes in each group compared to the control. C. Venn diagrams showing either down- or D. up-regulated genes compared to the controls.

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n°	Family	Molecule	Concentration 1X
1	Phenol	Bisphenol A	0.2 10-8M
2	Phenol	Triclosan	0.7 10-7M
3	Phenol	Benzophenone-3	0.86 10-7M
4	Phthalate	Dibutyl phthalate	0.24 10-6M
5	Phthalate	Di-2-ethylhexylphthalate	0.1 10-6M
6	Organochlorine pesticide	Hexachlorobenzen	0.8 10-11M
7	Organochlorine pesticide	Dichlorodiphenyldichloroethylene	0.66 10-9M
8	Perfluorinated compound	Perfluorooctanoic acid	0.43 10-8M
9	Perfluorinated compound	Perfluorrooctanyl sulfonate	0.8 10-8M
10	Poly aromatic hydroxylated compound	2-napthol	0.5 10-8M
11	Polychlorinated compound	Sodium perchlorate monohydrate	0.3 10-8M
12	Polybrominated compound	Decabromodiphenylether	0.63 10-9M
13	Polychlorinated compound	2,2',4,4',5,5'-Hexachlorbiphenyl	0.2 10-8M
14	Heavy metal	Methyl mercury	0.5 10-7M
15	Heavy metal	Lead (II) chloride	0.21 10-8M

Table 1 – Composition of chemical mixture.

Condition	Gene	Full name	Log2 Foldchange	p-value adjusted	Pathway
Down-regulated genes					
T4 (10nM)	adcyap1.L	adenylate cyclase activating polypeptide 1 (pituitary) L homeolog	-0.257	0.001	neuroactive ligand-receptor interaction
T4 (10nM)	aplnr.L	apelin receptor L homeolog	-0.331	0.025	neuroactive ligand-receptor interaction
T4 (10nM)	c3.L	complement component 3 L homeolog	-2.044	0.036	neuroactive ligand-receptor interaction
T4 (10nM)	calcr.S	calcitonin receptor S homeolog	-0.542	0.006	neuroactive ligand-receptor interaction
T4 (10nM)	cga.S	glycoprotein hormones, alpha polypeptide S homeolog	-0.752	0.000	neuroactive ligand-receptor interaction
T4 (10nM)	chrna7.S	cholinergic receptor, nicotinic alpha 7 S homeolog	-0.280	0.019	neuroactive ligand-receptor interaction
T4 (10nM)	crhr2.S	corticotropin releasing hormone receptor 2 S homeolog	-0.362	0.035	neuroactive ligand-receptor interaction
T4 (10nM)	drd1.S	dopamine receptor D1 S homeolog	-0.425	0.004	neuroactive ligand-receptor interaction
T4 (10nM)	drd2.L	dopamine receptor D2 L homeolog	-0.548	0.002	neuroactive ligand-receptor interaction
T4 (10nM)	drd2.S	dopamine receptor D2 S homeolog	-0.430	0.015	neuroactive ligand-receptor interaction
T4 (10nM)	gabbr1.S	gamma-aminobutyric acid (GABA) B receptor, 1 S homeolog	-0.213	0.018	neuroactive ligand-receptor interaction
T4 (10nM)	gabrd.L	gamma-aminobutyric acid (GABA) A receptor, delta L homeolog	-0.585	0.000	neuroactive ligand-receptor interaction
T4 (10nM)	galr3.L	galanin receptor 3 L homeolog	-0.680	0.000	neuroactive ligand-receptor interaction
T4 (10nM)	galr3.S	galanin receptor 3 S homeolog	-0.466	0.011	neuroactive ligand-receptor interaction
T4 (10nM)	ghr.L	growth hormone receptor L homeolog	-0.536	0.008	neuroactive ligand-receptor interaction
T4 (10nM)	glrb.L	glycine receptor beta L homeolog	-0.300	0.000	neuroactive ligand-receptor interaction
T4 (10nM)	glrb.S	glycine receptor beta S homeolog	-0.301	0.002	neuroactive ligand-receptor interaction
T4 (10nM)	gnrh2.L	gonadotropin releasing hormone 2 L homeolog	-0.397	0.017	neuroactive ligand-receptor interaction
T4 (10nM)	gpr83.2.L	G protein-coupled receptor 83 L homeolog	-0.331	0.001	neuroactive ligand-receptor interaction
T4 (10nM)	gria1.L	glutamate receptor, ionotropic, AMPA 1 L homeolog	-0.356	0.013	neuroactive ligand-receptor interaction
T4 (10nM)	gria2.S	glutamate receptor, ionotropic, AMPA 2 S homeolog	-0.325	0.014	neuroactive ligand-receptor interaction
T4 (10nM)	grik2	glutamate receptor, ionotropic, kainate 2	-0.368	0.000	neuroactive ligand-receptor interaction
T4 (10nM)	grpr.L	gastrin releasing peptide receptor L homeolog	-0.375	0.018	neuroactive ligand-receptor interaction
T4 (10nM)	htr2c.L	5-hydroxytryptamine (serotonin) receptor 2C, G protein-coupled L homeolog	-0.397	0.017	neuroactive ligand-receptor interaction
T4 (10nM)	htr5a.L	5-hydroxytryptamine (serotonin) receptor 5A, G protein-coupled L homeolog	-0.419	0.047	neuroactive ligand-receptor interaction
T4 (10nM)	kiss1.S	kisspeptin S homeolog	-0.515	0.030	neuroactive ligand-receptor interaction
T4 (10nM)	mc5r.L	melanocortin 5 receptor L homeolog	-0.565	0.020	neuroactive ligand-receptor interaction
T4 (10nM)	nmur1.L	neuromedin U receptor 1 L homeolog	-0.529	0.020	neuroactive ligand-receptor interaction
T4 (10nM)	nts.L	neurotensin L homeolog	-0.433	0.004	neuroactive ligand-receptor interaction
T4 (10nM)	pdyn.L	prodynorphin L homeolog	-0.486	0.001	neuroactive ligand-receptor interaction
T4 (10nM)	pdyn.S	prodynorphin S homeolog	-0.544	0.012	neuroactive ligand-receptor interaction
T4 (10nM)	penk.L	proenkephalin L homeolog	-0.338	0.010	neuroactive ligand-receptor interaction
T4 (10nM)	pth2r.L	parathyroid hormone 2 receptor L homeolog	-0.456	0.001	neuroactive ligand-receptor interaction
T4 (10nM)	pth2r.S	nociceptin receptor-like	-0.525	0.016	neuroactive ligand-receptor interaction
T4 (10nM)	pyy.S	peptide YY S homeolog	-0.566	0.003	neuroactive ligand-receptor interaction
T4 (10nM)	sstr5.S	somatostatin receptor 5 S homeolog	-0.479	0.012	neuroactive ligand-receptor interaction
T4 (10nM)	tac1.L	tachykinin precursor 1 L homeolog	-0.235	0.032	neuroactive ligand-receptor interaction
T4 (10nM)	tac1.S	tachykinin precursor 1 S homeolog	-0.289	0.050	neuroactive ligand-receptor interaction
T4 (10nM)	tacr3.L	tachykinin receptor 3 L homeolog	-0.425	0.001	neuroactive ligand-receptor interaction
T4 (10nM)	trh.L	thyrotropin-releasing hormone L homeolog	-0.194	0.042	neuroactive ligand-receptor interaction
T4 (10nM)	tshb.L	thyroid stimulating hormone, beta L homeolog	-0.848	0.002	neuroactive ligand-receptor interaction
T4 (10nM)	tshb.S	thyroid stimulating hormone, beta S homeolog	-2.056	0.000	neuroactive ligand-receptor interaction
T4 (10nM)	vip.S	vasoactive intestinal peptide S homeolog	-0.145	0.111	neuroactive ligand-receptor interaction
Up-regulated genes					
T4 (10nM)	adm.S	adrenomedullin S homeolog	0.862	0.025	neuroactive ligand-receptor interaction
T4 (10nM)	avpr1a.L	arginine vasopressin receptor 1A L homeolog	0.346	0.009	neuroactive ligand-receptor interaction
T4 (10nM)	cga.L	glycoprotein hormones, alpha polypeptide L homeolog	0.183	0.039	neuroactive ligand-receptor interaction
T4 (10nM)	ednrb2.S	endothelin receptor B subtype 2 S homeolog	0.765	0.033	neuroactive ligand-receptor interaction
T4 (10nM)	gh1.L	growth hormone 1 L homeolog	0.677	0.000	neuroactive ligand-receptor interaction
T4 (10nM)	lpar1.L	lysophosphatidic acid receptor 1 L homeolog	0.849	0.026	neuroactive ligand-receptor interaction
T4 (10nM)	lpar1.S	lysophosphatidic acid receptor 1 S homeolog	1.443	0.000	neuroactive ligand-receptor interaction
T4 (10nM)	p2rx5.L	purinergic receptor P2X, ligand gated ion channel, 5 L homeolog	0.776	0.017	neuroactive ligand-receptor interaction
T4 (10nM)	prl.1.S	prolactin, gene 1 S homeolog	2.876	0.000	neuroactive ligand-receptor interaction
T4 (10nM)	s1pr1.L	sphingosine-1-phosphate receptor 1 L homeolog	0.558	0.001	neuroactive ligand-receptor interaction
T4 (10nM)	s1pr5.L	sphingosine-1-phosphate receptor 5 L homeolog	0.346	0.009	neuroactive ligand-receptor interaction
T4 (10nM)	thrb.L	thyroid hormone receptor, beta L homeolog	1.274	0.000	neuroactive ligand-receptor interaction

Condition	Gene	Full name	Log2 Foldchange	p-value adjusted	Pathway
Down-regulated genes					
Amnios 1x	irak4.L	interleukin 1 receptor associated kinase 4 L homeolog	-0.410	0.015	MAPK signaling pathway
Amnios 1x	myd88.S	myeloid differentiation primary response 88 S homeolog	-0.815	0.008	MAPK signaling pathway
Amnios 1x	nfkb2.S	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100) S homeolog	-0.392	0.049	MAPK signaling pathway
Up-regulated genes					
Amnios 1x	cacnb1.S	calcium channel, voltage-dependent, beta 1 subunit S homeolog	0.328	0.022	MAPK signaling pathway
Amnios 1x	fos.S	FBJ murine osteosarcoma viral oncogene homolog S homeolog	0.358	0.033	MAPK signaling pathway
Amnios 1x	hspa8.L	heat shock protein family A (Hsp70) member 8 L homeolog	0.238	0.023	MAPK signaling pathway
Amnios 1x	myc.S	v-myc avian myelocytomatosis viral oncogene homolog S homeolog	0.474	0.014	MAPK signaling pathway

Condition	Gene	Full name	Log2 Foldchange	p-value adjusted	Pathway
Down-regulated genes					
Amnios 1x	ap3s2.S	adaptor related protein complex 3 sigma 2 subunit S homeolog	-0.446	0.018	lysosome
Amnios 1x	arsa.1.S	arylsulfatase A, gene 1 S homeolog	-0.554	0.000	lysosome
Amnios 1x	ctns.L	cystinosin, lysosomal cystine transporter L homeolog	-0.386	0.042	lysosome
Amnios 1x	galc.L	galactosylceramidase L homeolog	-0.333	0.033	lysosome
Amnios 1x	glb1l.L	galactosidase beta 1 like L homeolog	-0.367	0.011	lysosome
Amnios 1x	gm2a.L	GM2 ganglioside activator L homeolog	-0.355	0.035	lysosome
Amnios 1x	mfsd8.L	major facilitator superfamily domain containing 8 L homeolog	-0.552	0.010	lysosome
Amnios 1x	naga.L	N-acetylgalactosaminidase, alpha- L homeolog	-0.632	0.008	lysosome

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Table 2 KEGG pathway analysis of target genes after mixture and TH exposure. Target genes up-regulated or down-regulated that were annotated for neuroactive ligand-receptor interaction under T_4 (10nM) exposure, and the MAPK signaling pathway and lysosome activity under mixture exposure (Amnios 1x).

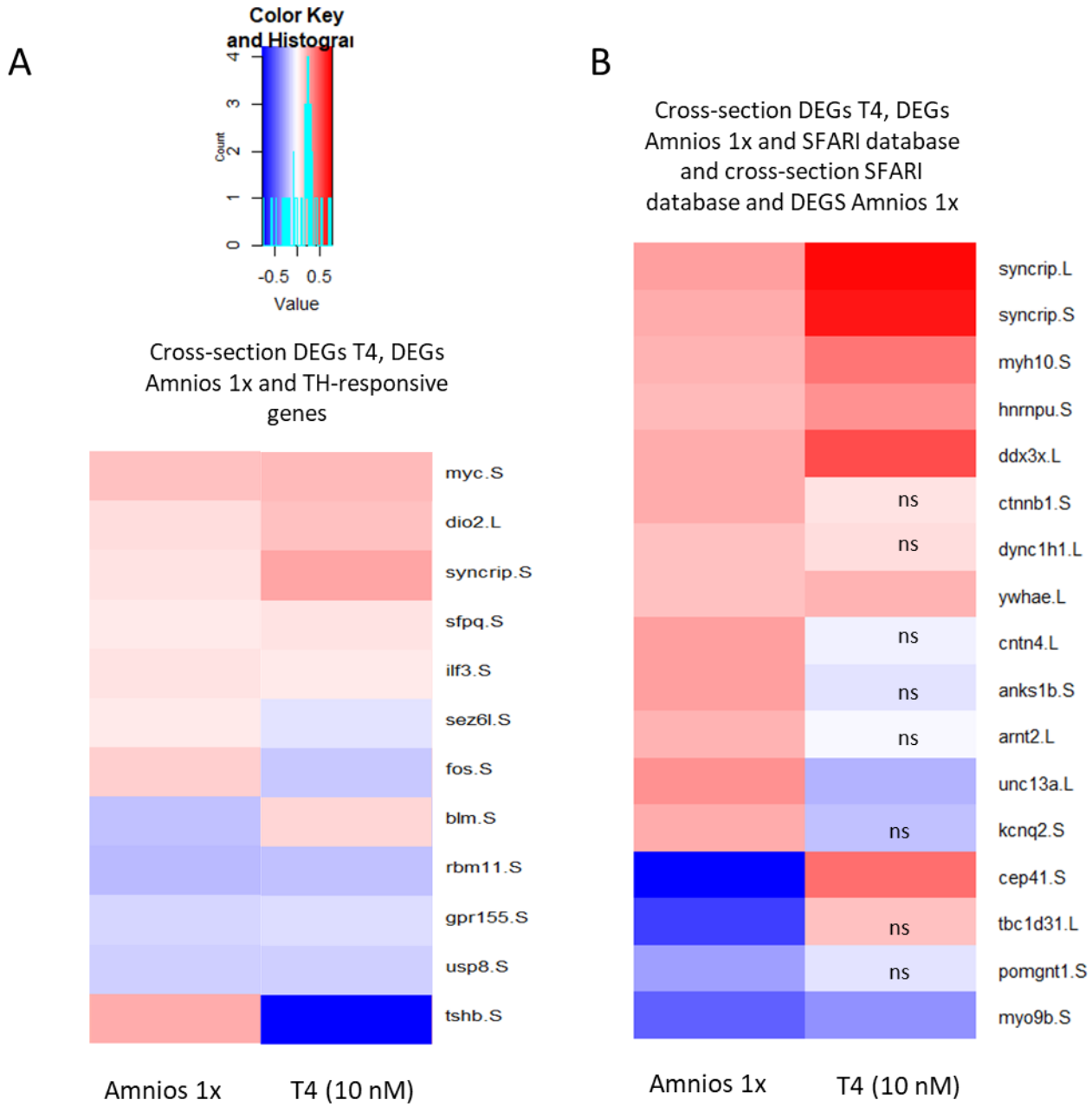

Figure 4 Changes in expression levels of genes specifically affected by amniotic mixture or T_4 treatment. Heatmap depicts the average change in the expression level of genes affected by either amniotic mixture (Amnios 1x) or T_4 (10nM). Gene names are shown at the right of the

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heatmap. The color bar represents log2 differences from the control for each treatment. A. DEGs that are in common between T_4 and amniotic treatment as well as TH-responsive genes (Chatonnet et al., 2015) B. DEGs that are common between either amniotic mixture and SFARI gene list and DEGs in common between Amnios 1x, T_4 (10nM) and SFARI gene list. ns is standing for non-significant; these genes do not belong to the DEG list.

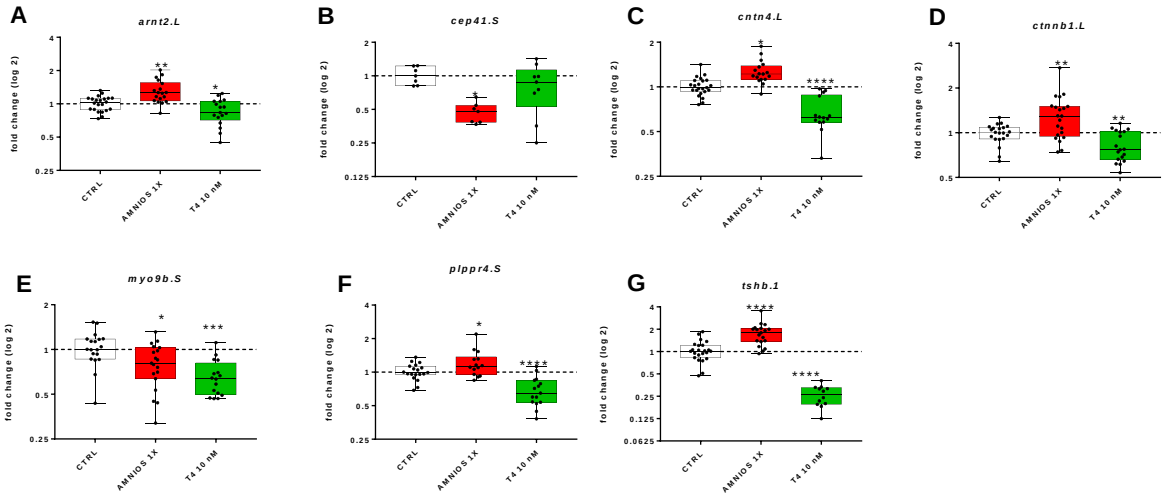

Figure 5 Gene expression after embryonic exposure to the amniotic mixture and thyroid hormones. Embryos (NF 1 – NF 47) were exposed to DMSO (CTRL), amniotic mixture at 1x concentration (Amnios 1x), and T_4 (10 nanomolar). After brain dissection at stage NF 47, RNA extraction and RT-qPCR were conducted on genes involved in thyroid hormone signaling or autism-related genes.

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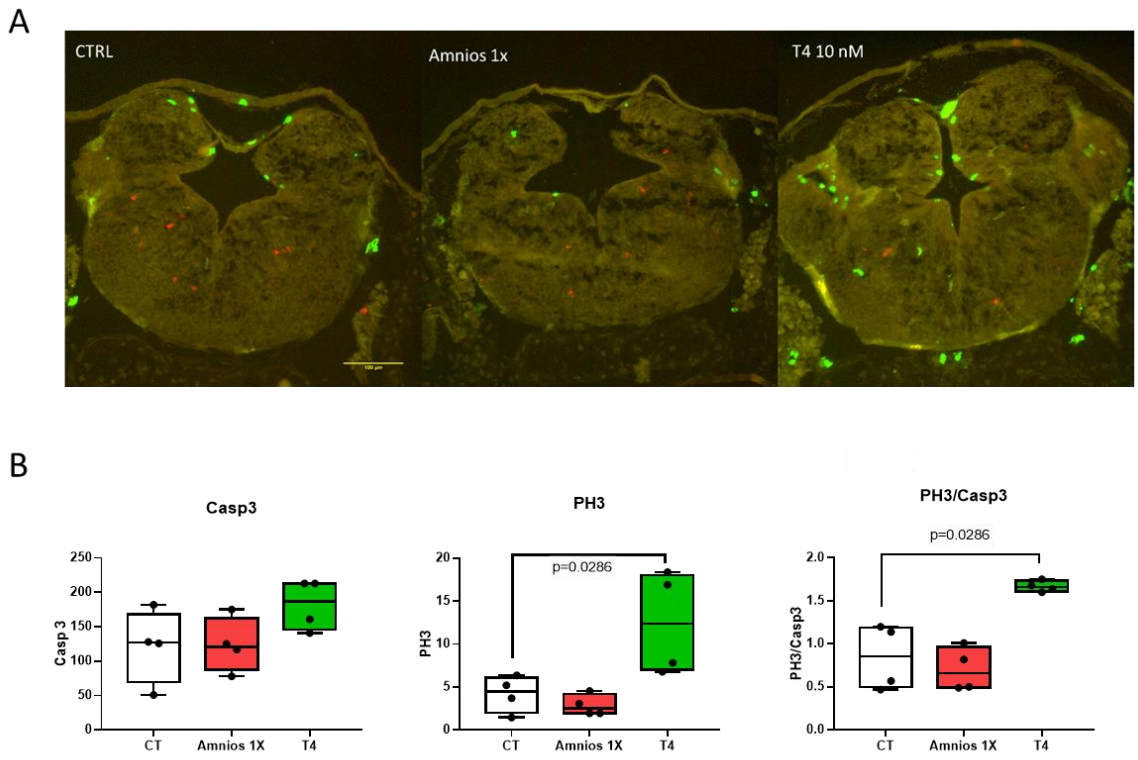

Figure 6 Effect of Amnios 1X and T4 on proliferation and apoptosis in tadpole brains. For each treatment group, 2 tadpoles from 3 females were analyzed (A) Example cross-sectional images of brains following immunochemistry: apoptosis marker in red (caspase3), proliferation marker in green (PH3). Scale bar, 100µm (B). Average number of positive cell nuclei per section for Caspase3 and PH3, and the ratio of proliferative/apoptotic cells.

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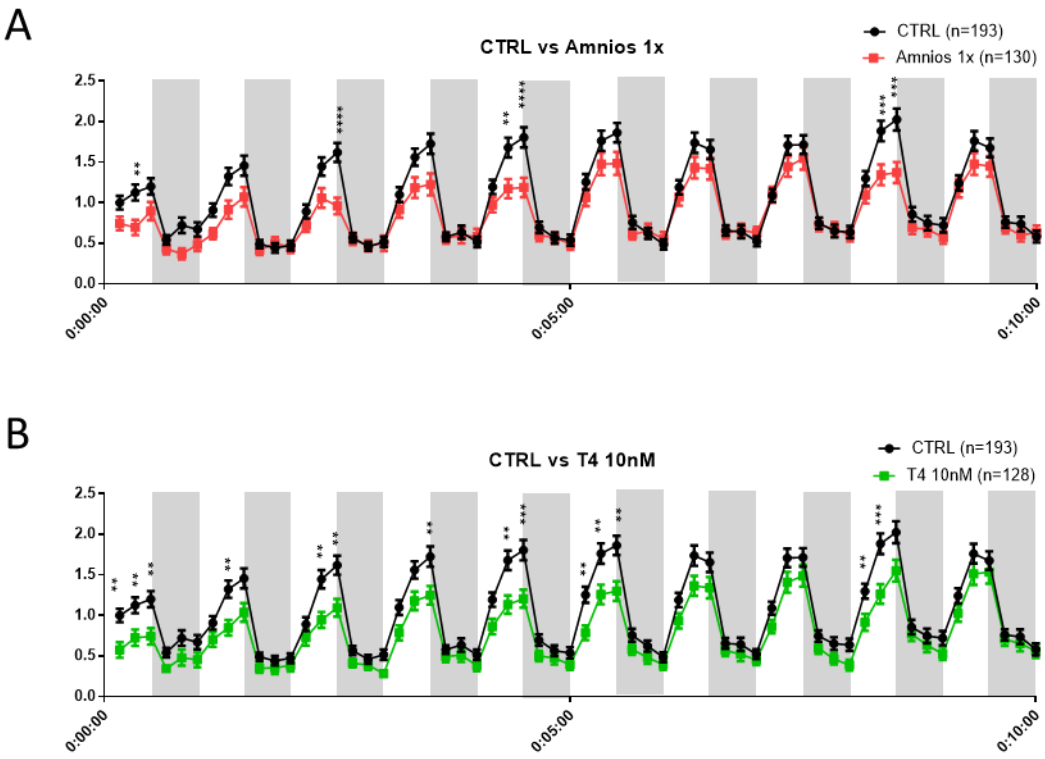

Figure 7 Behavioral study of tadpoles exposed to amniotic mixture and T_4 (10nM). The normalized distance was measured during 10-minute trials with 30 sec light/30-sec dark alternation using Video tracking Noldus Ethovision system. NF47 tadpoles, directly after 8-day exposure to either the amniotic mixture(A) or T_4 (10nM) were used to investigate traveled distance. Graph: mean +/- SEM., multiple t-tests with FDR approach, FDR (Q)= 5%, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Discussion

The dependence of amphibian metamorphosis on T_3 (Leloup J and Buscaglia M. 1977) has led to the wide use of *X. laevis* for analyzing TH action and interference with TH signaling. Given the central role of TH in orchestrating metamorphosis, initial work on TH levels and amphibian development focused on measuring components of the TH signaling pathways from NF54 (prometamorphosis) onwards (Leloup et al., 1977; Regard et al., 1978). However, more recent work has shown that eggs and embryos of fish reptiles, birds (Prati et al., 1992; McNabb and Darras 2014; Ruuskanen et al. 2016) and amphibians (Flickinger 1964; Fini et al. 2012) contain

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both tetraiodothyronine, T_4, and the more biologically active form of the hormone, triiodothyronine, T_3. We questioned the impact of external exposure to TH, from fertilized eggs up until the developing larvae (NF1-NF47). In the series of experiments that we did not present in this manuscript, we discovered that survival was affected by all TH antagonists tested, i.e. NH3 (10^{-7}M), iopanoic acid (IOP 10^{-6}M), methimazole (MMI, 10^{-4}M) either during the 7 day lasting treatment or the four days after it. All animals treated with NH3 died, and only 5% of the animals exposed to MMI could reach metamorphosis. Interestingly T_3 5nM treatment also induced mortality just a few days after the end of the treatment.

We questioned whether "less-toxic" treatments such as T_4 10nM (a concentration equivalent to those found during metamorphosis) or an amniotic mixture replicated from a mixture of 15 ubiquitous compounds at concentrations measured in human amniotic fluid (Woodruff et al. 2011), would modify brain gene expression in tadpoles exposed from fertilization through embryogenesis. Examining effects on well-characterized direct T_3 target genes, notably *klf9* and *TRβ*, we show that T_4 and amniotic mix exhibited similar effects suggesting a pro-thyroid effect. In our previous study using the same mixture (Fini et al., 2017) during a shorter exposure time (3 days) at 1-week post-fertilisation (NF 45 – NF 47), we observed increased GFP signaling using the Xenopus Eleutheroembryonic Thyroid Assay (XETA). Results included a loss of mobility and increased proliferation in neurogenic zones suggesting also a T3-like effect, at least in some cells. However, in this manuscript, we used a non-biased approach for candidate gene research using RNA sequencing after embryonic exposure (NF 1 – NF 47).

KEGG pathway analysis identified upregulated or down-regulated DEGs in numerous pathways following T_4 treatment, including a gene set of neuroactive ligand-receptor interactions (Table 2). This list contains a series of altered genes affecting a myriad of downstream target genes and physiological functions, through direct pathways or via cross-talk between these genes coding for various neuropeptides, hormones, and neurotransmitters (Denef 2008). While predicted KEGG pathways affected by the amniotic mixture suggested possible effects on apoptosis and proliferation, immunohistochemistry only revealed an increase in proliferative cells in the region analyzed (the midbrain) in the group exposed to T_4. However, this does not exclude the possibility of effects on proliferation and/or apoptosis in more specific regions of the brain.

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Interestingly we found that the *thsb* gene, encoding the pituitary hormone thyrotropin, was significantly deregulated, in opposite ways, by treatment of T_4 or the amniotic mixture. Circulating TSH activates the synthesis of THs in the thyroid and represses TRH at the hypothalamus level (CRH in amphibians, Licht and Denver 1990). This circulating hormone activates the synthesis of TH in the thyroid gland and, is measured at birth in humans for blood spot tests to analyze both TSH levels and different aspects of TH regulation. Higher TSH levels are indicative of problems in the thyroid axis, including the fact that a normal postnatal peak of TH has not occurred (Büyükgebiz 2013). Here we observe a very strong reduction of the expression of *tshb* after treatment of T_4 at 10nM, suggesting a centrally compensated hyperthyroid state. In zebrafish, Tonyushkina et al. (2014) observed that *tshb* transcription was effective 96 hours post fertilization. They also showed that *tshb* and *dio2* were co-expressed in thyrotrope cells and that their numbers were reduced after T_4 treatment. Here we observe that T_4 treatment reduced expression of *tshb,* increased *dio3* expression, and decreased *mct8* expression, suggesting a feedback mechanism in response to an excess of TH in the brain. These findings demonstrate that even when the thyroid glans is not yet synthetizing thyroid hormones, brain cells are able to cope with an excess of T4.

Strikingly, the treatment with the amniotic mixture leads to an opposite response, with overexpression of the *tshb* gene, increased expressed of TH transporters *oatp1c1* and *mct8*, and increased *dio2* expression suggesting a hypothyroid state being compensated centrally. The increase in *dio2* expression observed after T_4 treatment in RNA seq and not in qPCR is not well understood. *Dio2* converts T_4 into T_3 and therefore contributes to the TH excess, and it cannot be ruled out that *dio2* overexpression could also convert T_3 into T_2, documented as less active than T_3 (Zucchi et al. 2019).

Two things stand out from this result: firstly, the mixture has an anti-thyroid effect as well as some T_3-like effects, even though some parameters point in the same direction (notably behavior), and secondly, tadpoles can be affected by hypothyroidism at the stage when the thyroid gland is formed but only just beginning to synthesize TH. This highlights that peripheral mechanisms of deiodination are crucial during early development. Activating and inactivating deiodinases are intimately involved in determining T_3 availability in specific tissues and cell types, and inter ring deiodination (IRD) and outer ring deiodination (ORD) are also present at these early embryonic stages mostly in the head region (Tindall et al. 2007; Dubois et al. 2006).

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Our findings illustrate the concept that too little or too much TH during critical phases of development can cause adverse effects. Notably, results from human epidemiology show that low or high maternal TH, or low iodine during early pregnancy is associated with a loss of IQ and impacts cortical thickness (Korevaar et al. 2016). It is even more striking that Korevaars' results showed that the variation in maternal TH were within normal levels.

To conclude, we have shown that early exposure to a common mixture of EDCs may induce adverse effects as T_4 treatment. This latter condition is equivalent to maternal hyperthyroidism in humans. It is remarkable that both treatments disrupt *tshb*, brain gene expression, and result in altered behavior. Specifically, even though exposure to T_4 or the amniotic mixture may result in opposite thyroid status, effects appear identical. From a molecular point of view, the majority of the common DEGs are regulated in the same direction, and from a macroscopic point of view, a loss of mobility is measured in both cases. These findings highlight the need of specific endpoint markers of thyroid hormone disruption. The obtained results call urgent attention to the necessity of enhanced protection of humans and the environment from EDCs, particularly those that affect the thyroid axis.

Material and Methods

Chemical exposure

The 10 000x exposure solution was prepared, according to Fini et al. (2017). 1x exposure solution was prepared by adding 1µL of 10 000x concentrated mixture to 10 mL of Evian. In all experiments, stage NF1 *Xenopus laevis* eggs (dejellied by cysteine) were placed into 6-well plates (15 per well). 8mL of previously prepared 1x exposure solution was added into the corresponding well after the removal of any excess liquid. The final concentration of DMSO was 0.01% in all groups, including the control. Multi-well plates were kept in the dark incubator at 23°C for eight days. The renewal was daily, exposure solutions being prepared extemporaneously for eight days.

Gene expression analysis

RNA extraction

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At the end of the chemical exposure described above, tadpoles were anesthetized in 0.01% MS-222, and their brains were dissected under sterile conditions. Two brains were placed in 1.5 ml tubes containing 100µL of lysis solution from an RNAqueous –micro kit (Thermofisher) and flash-frozen in liquid nitrogen followed by storage at − 80 °C. In total, five tubes containing two brains each per exposure group and per replicate were collected. RNA extraction was performed using the RNAqueous –micro kit following the manufacturer's instructions. RNA concentrations were measured by a NanoDrop spectrophotometer (ThermoScientific, Rockford, IL) and RNA quality was verified by BioAnalyzer (Agilent). Only samples with a RIN >7 were selected for further study. cDNA was synthesized using Reverse Transcription Master Mix (Fluidigm).

RT-qPCR

cDNA was diluted 1/20 in nuclease-free water. A quantitative PCR reaction was performed in 384 well-plates, with a standard reaction containing 1 µl of cDNA and 5 µl of the mix (3 µL of Power SYBR master mix, 1.7 µL of nuclease-free water, 0.15 µL of reverse primer (10 pM) and 0.15 µL of forward primer (10 pM) per well). The measurement was carried out by the QuantStudio 6 Flex (Life technologies) device. The 2−ΔΔCt method was used to calculate the relative concentrations of cDNA for the analysis of relative changes in gene expression (see (Spirhanzlova et al. 2018) for a detailed description). The geometric mean of endogenous controls *ralb* and *ube2m* was used for normalization.

Data are represented as fold change (2−ΔΔCt) using a log (base2) scale plotted as a traditional box, and whisker plot by Tukey where the bottom and top of the box represent the 25th lower and 75th percentile, and the median is the horizontal bar in the box. Statistical analyses were performed on delta Cts using non-parametric Mann-Whitney in case of non-normal distributed data. In the case of a normal distribution, a student t-test was conducted. Significance was determined at $p < 0.05$(*), $p < 0.01$ (**) and $p < 0.001$(***).

RNA-sequencing

Two sets of experiments were realized at two different point times. The transcriptome libraries were prepared from total RNA using Illumina TruSeq Stranded mRNA Sample Preparation kits (Illumina Inc., USA). The libraries were sequenced on the Illumina NextSeq device using the 75bp single-end sequencing strategy with the TruSeq kit (Illumina Inc., USA).

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Raw reads were first cleaned by removing PCR bias conserving one copy per cluster of duplicated reads (python script). Then, reads were trimmed for the 11 first nucleotides (fastx-trimmer from fastx-toolkit version 0.0.13.2) to remove adapter remants. 64bp-long reads were specifically selected (cutadapt version 1.15). The global quality of the reads was checked using the FastQC (version 0.11.2). Bowtie2 (v2.2.4) was used to map the clean reads against the Xenopus laevis genome (release 9.2) downloaded from XenBase (ftp://ftp.xenbase.org/pub/Genomics/JGI/Xenla9.2/XL9_2.fa) only conserving uniquely mapped reads. Reads were counted on gene annotations was accomplished by HTSeq-count (v0.9.1) in union mode against the annotation of Xenopus laevis genome downloaded from XenBase (ftp://ftp.xenbase.org/pub/Genomics/JGI/Xenla9.2/XENLA_9.2_Xenbase.gff3).
Raw read counts were normalized by the variance stabilization transformation of DESeq (R package – version 1.16.1) and used to check the global behavior of the libraries by a Principal Components Analysis (R package FactoMineR – version 1.39). This revealed a batch effect between samples on component 2 of the PCA. We have chosen to remove the complete impact of component 2 by recalculating a denoised table of raw read counts (R scripts). This consists in calculating the variance stabilizing matrix from the raw read counts table, performing the principal components analysis with the package prcomp, initializing the variable loading of interest (component 2) at zero, and reconstructing the denoised raw read counts matrix. Then, this new denoised table was used by DESeq for the differential expression analysis.

DAVID

Differentially expressed genes were submitted to DAVID database (https://david.ncifcrf.gov/) for systematically extracting biological meaning for them by retrieving pathway maps from the Kyoto Encyclopedia of Genes and Genomes (KEGG).

Mobility

After the eight days of chemical exposure from NF 1 to NF 47, tadpoles were rinsed, placed separately into a 12-well plate containing 4 mL of Evian water in each well, and left to accommodate for 15 minutes. The behavior of the tadpoles was recorded by DanioVision (Noldus) behavior analysis system during a 10-minute trial composed of ten 30s light on/30s

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light off intervals. During the light-on phase, maximal light stimulus (5 K Lux) was set. The total distance traveled by each tadpole was calculated by EthoVision software XT (11.5). The mean distance traveled by the control group in the 0s-10s period of the first light-on interval was used to normalize all data. Statistics were conducted using multiple t-tests with FDR approach, FDR (Q)= 5%, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Immunohistochemistry

Tadpoles were euthanized in MS-222 1 g/l and whole tadpoles were fixed in 4% paraformaldehyde overnight at 4 ˚C and transferred for storage in PFA 0.4%. The night before embedding in optimal cutting temperature compound, samples were cryoprotected in sucrose 15% (in PBS 1x) at 4 ˚C. Coronal cryosections (12µm thickness) of whole tadpoles spanned the region containing lateral ventricles to the mid-hindbrain region. For immunohistochemical investigation of sections on slides, the following antibodies were used: 1) primary antibodies: anti-Ser10 phosphorylated on Histone H3 mouse (05–806 Millipore) at 1/300 dilution, and anti-caspase3 rabbit (Ab 3022 –Abcam) at 1/200 dilution, 2) secondary antibodies: Alexa Fluor 488 anti-mouse (A11029 Invitrogen) and Alexa Fluor 594 (A11012 Invitrogen) at 1/500 dilution. Image analysis was conducted in the midbrain (delimited by the end of lateral ventricles and the end of the optic tectum). All positive nuclei were manually counted from 2 independent experiments with n = 2 tadpoles per experiment, and statistical analyses were performed using the non-parametric Mann-Whitney test compared to the control group.

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