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Effects of endocrine disruptors on *Phallusia mammillata* embryonic development

Isa Gomes

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Thèse de doctorat en Biologie du Développement

Université Pierre et Marie Curie

Ecole Doctorale Complexité du Vivant (ED515)

Laboratoire de Biologie du Développement de Villefranche-sur-mer (UMR7009)

Ascidian BioCell group

Effects of endocrine disruptors on *Phallusia mammillata* embryonic development

Par Isa Gomes

La thèse sera présentée et soutenue le 23 novembre 2018 à Villefranche-sur-mer

Devant un jury composé de:

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- Dr. Stéphanie Bertrand
- Dr. Laura Canesi
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*Porque eu sou do tamanho do que vejo
E não, do tamanho da minha altura...*

Fernando Pessoa

*La mer,
une fois qu'elle vous a jeté son sort,
vous tient dans son filet pour toujours.*

Jacques-Yves Cousteau

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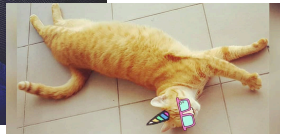


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Résumé

Les plastiques sont produits et utilisés à grande échelle dans le monde. Par conséquent, ils se sont accumulés dans les océans, où ils affectent les écosystèmes de nombreuses manières. Le bisphénol A (BPA) est une molécule dérivée du plastique, et est aujourd'hui considérée comme un perturbateur endocrinien (PE). Il altère les systèmes hormonaux en se liant aux récepteurs nucléaires (RN) et a été associé à des troubles neurodéveloppementaux, probablement en raison de la présence de certains RN dans le cerveau des vertébrés. Les PEs peuvent également affecter les invertébrés marins tels que les ascidies, mais leur mode d'action reste largement inconnu.

Mon projet de thèse a pour but d'étudier la toxicité du BPA sur le développement embryonnaire de l'ascidie *Phallusia mammillata* (Tunicata). Tout d'abord, j'ai évalué l'expression des RNs de *P. mammillata* et j'ai trouvé que 5 d'entre eux (COUP, ERR, PPAR, PXR/VDR α) sont exprimés dans le cerveau de l'ascidie (également appelé vésicule sensorielle) ou à proximité (TR). La plupart d'entre eux correspondent aux orthologues humains connus pour lier le BPA. Deuxièmement, j'ai évalué la toxicité du BPA au cours du développement et j'ai constaté que le BPA est toxique d'une façon dose-dépendante. En effet, de faibles doses de BPA induisent une toxicité neurodéveloppementale en affectant la différenciation de l'organe sensoriel pigmenté de l'ascidie. J'ai montré que ce phénotype est spécifique aux bisphénols. Enfin, l'étude d'agonistes et d'antagonistes de l'*estrogen-related receptor* (ERR) a montré des phénotypes correspondant à ceux obtenus avec le BPA. J'ai trouvé que *Pm-ERR* est exprimé dans la vésicule sensorielle de la larve, autour de l'organe sensoriel pigmenté. Il semble donc que *Pm-ERR* est impliqué dans ce phénotype.

Toutefois, la présence des autres RNs que j'ai identifiés dans le cerveau des larves d'ascidie soulève la possibilité de leur implication dans son développement, en médiant la toxicité de PEs. Ils ne doivent donc pas être négligés.

Mots-clés: Bisphénol A, *Estrogen-related receptor*, organe sensoriel pigmenté, récepteurs nucléaires, vésicule sensorielle

Abstract

Plastics are produced and used on a large scale worldwide. Consequently, they have been accumulating in our oceans in the last years, affecting the ecosystems in various ways. Bisphenol A (BPA) is a plastic-derived molecule that is now considered an endocrine disruptor (ED). BPA impairs hormonal systems *via* binding to nuclear receptors (NRs) and it has been linked to neurodevelopmental disorders, possibly due to the presence of some NRs in the vertebrate brain. BPA has been reported to affect marine invertebrates such as ascidians, but how BPA acts is not known.

My PhD project is aimed at deciphering the toxicity of BPA on embryonic development of the marine invertebrate chordate *Phallusia mammillata* (Tunicata). Firstly, I assessed the embryonic expression of *P. mammillata* NRs and found that 5 are expressed within the ascidian brain (also called sensory vesicle, SV) (COUP, ERR, PPAR, PXR/VDR α) or nearby (TR). Interestingly, the human orthologues of most of these NRs are known to bind BPA. Secondly, I assessed BPA toxicity during *P. mammillata* embryonic development and found that BPA is toxic in a dose-dependent manner. I also show that at micromolar doses BPA induces neurodevelopmental toxicity by impairing differentiation of the ascidian pigmented sensory organ (PSO). I further show that this phenotype is specific to bisphenols. Finally, estrogen-related receptor (ERR) agonists and antagonists partially phenocopied BPA phenotype. Interestingly, I found that *Pm*-ERR is expressed in the larval SV close to the ascidian PSO, thus suggesting an involvement of *Pm*-ERR in the BPA phenotype.

Furthermore, the complex pleiotropic action of BPA together with the presence of other NRs in the ascidian larval brain raises the possibility that these NRs are involved both in ascidian brain development and EDs toxicity, thus they should not be overlooked.

Keywords: Ascidian pigmented sensory organ, Bisphenol A, Estrogen-related receptor, Nuclear receptors, Sensory vesicle

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List of abbreviations

2,2 DPP: 2,2 Diphenylpropane

4-OHT: 4-Hydroxytamoxifen

ADHD: Attention-Deficit Hyperactivity Disorder

AF (domains): Activation Function

AR: Androgen Receptor

ASD: Autism Spectrum Disorder

BPA: Bisphenol A

BPE: Bisphenol E

BPF: Bisphenol F

CAR: Constitutive Androstane Receptor

CAS: Chemical Abstract Service

CBP/p300: CREB-binding protein/histone acetyltransferase p300

CNS: Central Nervous System

COUP: Chicken Orphan Upstream Promoter

DBD: DNA Binding Domain

DES: Diethylstilbestrol

DMSO: Dimethylsulfoxide

E2B: 17 β Estradiol 3-benzoate

EC50: half Effective Concentration

ECHA: European Chemicals Agency

EDC: Endocrine-Disrupting Chemical

EGFR: Epidermal Growth-Factor Receptor

EPA: Environmental Protection Agency

ER: Estrogen Receptor

ERK: Extracellular signal-Regulated Kinases

ERR: Estrogen-Related Receptor

FXR: Farnesoid X Receptor

GCNF: Germ Cell Nuclear Factor

GR: Glucocorticoid Receptor

HNF4: Hepatocyte Nuclear Factor 4

Hpf: hours post-fertilization

HRE: Hormone Response Element

LBD: Ligand Binding Domain

LC50: half Lethal Concentration

LXR: Liver X Receptor

MAPK: Mitogen-Activated Protein Kinases

MED1/TRAP220: mediator complex subunit 1/thyroid hormone receptor-associated protein 220 kDa component

MoA: mode-of-action

NCOA: Nuclear receptor Coactivator

NCOR: Nuclear receptor Corepressor

ND: Neurohypophysial duct

NR: Nuclear Receptor

Oc: Ocellus

OECD: Organization for Economic Co-operation and Development

Ot: Otolith

PC: Pigment cell

PGC1: Peroxisome proliferator-activated receptor Gamma Coactivator 1

PNS: Peripheral Nervous System

PPAR: Peroxisome Proliferator-Activated Receptor

PR: Progesterone Receptor

PSO: Pigmented Sensory Organ

PXR: Pregnane X Receptor

RAR: Retinoic Acid Receptor

Rev-ERB: reverse ERB

ROR: RAR-related Orphan Receptor

RXR: Retinoid X Receptor

SR: Steroid Receptor

SV: Sensory Vesicle

SVI: Sensory Vesicle lumen

TBBPA: tetrabromobisphenol-A

TCBPA: tetrachlorobisphenol- A

TF: Transcription Factor

TR: Thyroid Receptor

TR2/4: Testicular Receptor 2/4

TSCA: Toxic Substances Control Act

UNESCO: United Nations Educational, Scientific and Cultural Organization

VDR: Vitamin D Receptor

WHO: World Health Organization

Preface

I arrived in the Villefranche-sur-mer Developmental Biology laboratory for my PhD project on October 2015. The McDougall team is studying ascidian embryology for the last 20 years, with a special focus on the cell cycle (<http://lbdv.obs-vlfr.fr/fr/ascidian-biocell-group.html>). A new scientific interest emerged in the team 4 years ago, with the main aim of using the ascidian embryo as a model in toxicology research. In my PhD, I studied how brain development of the ascidian larva is affected by known endocrine-disrupting chemicals such as bisphenol A. I, thus, hope this manuscript will convince the scientific community that ascidian larvae are a great system to study brain malformations induced by endocrine disrupting chemicals. My thesis manuscript structure is summarized below.

In this manuscript, I will first describe the main aims of my project and sum up the main results obtained. Then I will give a brief **Introduction** of my project. For this, I will explain how plastic pollution is a large source of toxic molecules for human. I will then introduce the field of environmental toxicology and depict how the term “endocrine-disrupting chemicals” (“EDCs”) was introduced, the main EDCs modes-of-action (MoA) and conclude this section with the example of bisphenol A (BPA). I will finish the introduction by a brief description of ascidians and how they can be a useful model for developmental neurotoxicology studies. The introduction (together with the Results part I) will be published as a review in the *Molecular Reproduction and Developmental* journal (Special Issue “Effects of ecosystem degradation/stress on reproduction and development”).

In the **Results**, I will first describe *Phallusia mammillata* nuclear receptors (**part I**), and secondly I will describe the phenotype induced by bisphenols and, in an attempt to find a MoA, I will assess whether the nuclear receptor (NR) estrogen-related receptor (ERR) could be involved (**part II**). This part II corresponds to the scientific manuscript that is now going under the last modifications to soon submit to *Open Biology*, *PLOS One*, *Aquatic Toxicology* or *Ecotoxicology and Environmental Safety* journals.

Finally, I will discuss my findings in the **General Discussion & Perspectives** section, where I propose a model to explain the potential pleiotropic action of BPA in the ascidian brain, and summarizing the conclusions of my project.

Aim of thesis

Nuclear receptors are key transcription factors mediating important signals in many organisms, from simple cnidarians (Gauchat et al., 2004) up to humans (Sever and Glass, 2013), as I will review in my introduction. An important increase on studies linking nuclear receptors to different type of cancers, fertility problems and neurodevelopmental disorders has been noticed and countries are working together to better identify daily-life products as potential endocrine disruptors.

My thesis is part of a toxicology project called MarineEmbryoTox (ANR-14-OHRI-009) that aims at developing new tools to identify potential endocrine-disrupting chemicals (EDCs) insult on the marine environment, by using ascidian embryos as model system. EDCs, and particularly Bisphenol A (BPA), a plasticizer molecule recently considered as an endocrine disruptor by the European Commission, have been related to several brain malformations in vertebrates, and to some extent, to neurodevelopmental disorders such as attention-deficit hyperactivity disorder (ADHD) and autism spectrum disorder (ASD). However, the clear mechanisms behind EDC neurodevelopmental toxicity are still blurry and more studies are needed to understand which key factor might be responsible. Therefore, the main aim of my thesis is to evaluate the neurodevelopmental effects of BPA on embryonic development of the ascidian *Phallusia mammillata* and assess the potential involvement of nuclear receptors (NRs) in the BPA-induced phenotype. The different aims of my project are the following:

- **Aim nr. 1:**

The first aim is to describe in detail the expression of *P. mammillata* NRs during embryonic development. For this, I cloned 14 NRs within the 17 present in the genome and assessed their expression patterns by *in situ* hybridization technique. I compared the obtained expression data with the transcriptomic data available in the ascidian database *Aniseed*. I found that 4 NRs (ERR, PPAR, PXR/VDR α) are expressed within the ascidian brain (also called sensory vesicle), or nearby (TR), which their human orthologues are known to bind BPA. Moreover, genome analysis revealed the presence of 3 coactivators and 1 corepressor in the ascidian genome, confirmed by transcriptomic data available at *Aniseed*.

- **Aim nr. 2:**

Another aim of my study is to describe the toxicity of BPA to *P. mammillata* embryos. For this, I exposed embryos to different concentrations of BPA at the beginning of the embryonic development, as well as at different embryonic developmental stages. I found out that BPA is toxic to the embryos in a dose-dependent manner. Moreover, I found that micromolar concentrations BPA induces neurodevelopmental toxicity by disrupting the differentiation of the pigmented cells of the ascidian pigmented sensory organ (PSO), present within the ascidian brain (also called sensory vesicle). More precisely, three main phenotypes were observed:

impaired pigmentation, reduced distance between the two pigmented cells and reduced sensory vesicle lumen.

- **Aim nr. 3:**

After describing BPA phenotype in the ascidian brain, I wondered if this phenotype is bisphenol-specific. For this, I exposed *P. mammillata* embryos to three molecules similar to BPA, bisphenols E and F (BPE, BPF) and 2,2 diphenylpropane (2,2DPP). The three main observed phenotypes for BPA were also observed in embryos exposed to BPE and BPF, but not to 2,2DPP, suggesting a bisphenol-specific phenotype.

- **Aim nr. 4:**

Because previous studies suggested the role of Estrogen-Related Receptor (ERR) in zebrafish otolith development, I decided to apply a pharmacological approach and assess the phenotypes of different ER/ERR agonists/antagonists. Embryos were exposed to different concentrations of E2B, GSK4716, DES and 4-OHT, and three endpoints were measured: the pigmentation area, the distance between the two pigmented cells and the trunk length/width ratio. The three endpoints were significantly reduced, with the distance between pigment cells the most severely affected, showing that ERR agonist and antagonists partially phenocopy BPA phenotype and suggesting that ERR might be involved in BPA neurodevelopmental toxicity.

- **Aim nr. 5:**

For the time being, only one study explored an ascidian ERR (*Halocynthia roretzi*), showing that *Hr-ERR* is mainly expressed during the first stages of development (egg up to gastrula), contrasting with the expression pattern obtained in my study during aim 1. I thus decided to carefully characterize *P. mammillata* ERR (*Pm-ERR*). For this, I cloned the promoter region of *Pm-ERR* into a H2B::Venus reporter to follow ERR promoter activity (pERR>H2B::Venus). I also characterized ERR protein levels during embryonic development using a specific antibody raised against recombinant *Pm-ERR* protein. I found that *Pm-ERR* is expressed in the ascidian sensory vesicle at stage 26.

1. INTRODUCTION

1.1. Plastic pollution in oceans

Life on our planet depends on the oceans, based on countless ecosystems, which are sources of wealth, opportunity and abundance. About 71% of the surface of the planet is covered by salt water. Marine ecosystems provide us food, energy, water and sustain directly the livelihoods of hundreds of millions of people and are the main highway for international trade as well as the main stabilizer of the world's climate. However, as the former UNESCO Director-General Federico Mayor Zaragoza said "Whatever we do, the ocean will survive in one way or another. What is more problematic is whether we shall preserve it in a state that ensures humanity's survival and well-being" (Zaragoza, 1998).

Human activity is the main reason for the world's biological diversity decline and marine ecosystems are no exception, being increasingly subject to a multitude of anthropogenic contaminants (Islam and Tanaka, 2004). Most advances of human society over the past century have been facilitated by the use of plastics. Plastics are composed by a network of molecular monomers bound together to form macromolecules of infinite use in human society. Plastic production has increased exponentially from 2.3 million tons in 1950 to 448 million by 2015. Today, there are more than 20 different major types of plastics in use worldwide (North and Halden, 2014).

Reports of plastic pollution in the ocean first appeared in the scientific literature in the early 1970s. In 2010, about 2.5 billion tons of municipal solid waste was generated by 6.4 billion people living in coastal countries (corresponding to 93% of the global population). Within these 2.5 billion, approximately 275 million tons was plastic, with 8 million tons (18 billion pounds, figure 1) estimated to be mismanaged plastic waste entering the ocean (Jambeck et al., 2015).

Figure 1. *National Geographic* magazine cover of last June 2018 reporting the presence of plastics in the oceans.



Plastics in the marine environment are of increasing concern because of their persistence and their effects on the marine wildlife, and, directly or indirectly, on humans. Once in the ocean, microplastics can be ingested by different trophic-level marine organisms, from copepods (Heindler et al., 2017) up to fishes (Baalkhuyur et al., 2018) and turtles (Pham et al., 2017). And macroplastics can be ingested by big mammals, if we remember the whale found in Thailand in May 2018 with 80 plastic shopping bags and other debris in its stomach (Zachos, 2018). While macroplastics can be easily spotted and efforts are underway to develop devices aimed at eliminating them from the marine environment (Slat, 2013), micro/nanoplastics on the other hand are untraceable to their source and extremely difficult to remove from open ocean environments (Wright et al., 2013).

When confronting people to the serious issue of plastic pollution, most of them consider to not be concerned as they think they are not exposed to plastics. The reality is that we are more exposed to plastics and their associated molecules than we think. On the one hand, ingested plastic by marine biota can leach plastic-associated molecules and be easily transferred and bioaccumulated in the tissues of marine organisms, meaning a human exposure to these molecules if seafood is part of our diet (Seltenrich, 2015). On the other hand, humans are surrounded by plastic-based products every day, from water plastic bottles and food containers, to personal care products (Calafat et al., 2015; Frye et al., 2012). High concentrations of plastic-associated molecules, such as Diethylhexyl Phthalate (DEHP) and Bisphenol A (BPA), have been found in both aquatic systems (surface waters, sediments, groundwater, see review (Flint et al., 2012)) and humans (urine, fetal serum, blood, see review (Rochester, 2013)), and are today a real concern due to their endocrine-disrupting potential (Zoeller et al., 2012). In the following section I will explain how molecules end up in the environment and the efforts that have been done to set up safe exposure thresholds in organisms.

1.2. Environmental toxicology

1.2.1. Definition of environmental toxicology

The systematic study of the effect of toxic substances on ecosystems is essentially a twentieth-century phenomenon. Although the science of environmental toxicology is a relatively new one, it has its roots in the study of human classical toxicology. The best known toxicologist is the Swiss physician Paracelsus (1499–1541) who first formulated the notion of dose-response, including the idea that any chemical could be toxic at higher doses – *The dose makes the poison* – today considered as “the father of toxicology”. During the latter half of the nineteenth century, along with the rapid expansion of the chemical manufacturing industry, chemicals were tested with a main focus on human health. However, an awareness that increasing industrialization was having an adverse effect on species other than *Homo sapiens* was beginning to take shape (Wright and Welbourn, 2002). Around World War II and into the 1960s several pollution events, like the methyl mercury wastewater released from Chisso Corporation’s industrial into Minamata Bay and the Shiranui Sea (1956), or the huge oil spill by the crude oil tanker Amoco Cadiz after it ran aground on Portsall Rocks (1978), lead to huge environmental consequences. But how to deal with such issues? It was thus essential for society to understand and make decisions about environmental contaminants, and this is how the science of environmental toxicology was born.

Environmental toxicology emerged from the science of toxicology, when some scientists had begun to consider the effects of toxic substances that had been released into the environment. However, awareness of environmental pollutants did not increase markedly until the publication of “*Silent Spring*” in 1962, from the biologist Rachel Carson, where she exposed the effects of uncontrolled pesticide use. Although it is based on toxicology, environmental toxicology draws heavily on principles and techniques from other fields, including biochemistry, cell biology, developmental biology, and genetics.

Environmental toxicology subject matter spans a wide range of biological levels (figure 2). Issues associated with higher levels are based on biogeochemical parameters, populations/communities

observation, and take time to evaluate. Levels drawing heavily on classic toxicology extend from the biomolecular to the individual, and their response is usually very fast. Assessing the effects of chemicals at the biomolecular level allow us to find the mode-of-action of these molecules and better understand pollution events and most importantly animals and human associated diseases.

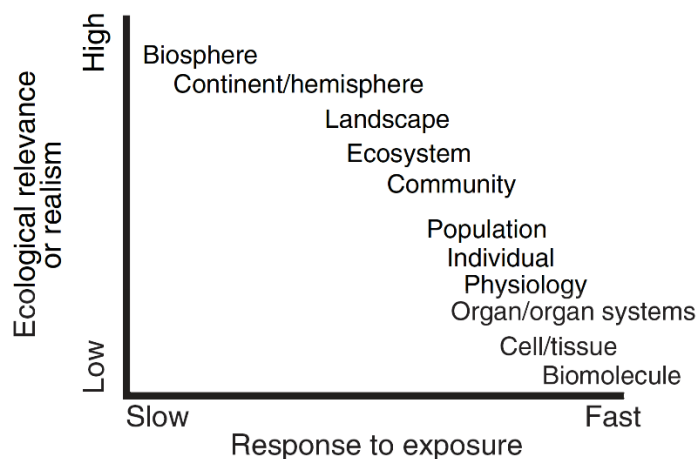


Figure 2. Schematic diagram of the hierarchical relationship between toxicological responses measured at different levels of biological organization (adapted from (Newman, 2008)).

Mankind has found and developed over 100 million unique chemical substances, registered at the *Chemical Abstracts Service* (known as CAS REGISTRYSM). The rate of discovery is rapidly increasing, but without a corresponding increase in knowledge of their environmental and health implications. Within these 100 million chemical substances, only around 85,000 are listed in the Toxic Substances Control Act (TSCA) inventory (from the Environmental Protection Agency (EPA)). Scarcely, only around 150 have recommended water quality guidelines in our surface waters, and only 40 of these compounds with legally enforceable limits. But how are these guidelines and limits defined?

1.2.2. Aims of environmental toxicology

To assess the potentially detrimental effects of a chemical (or other agent) on biota, it is necessary to establish a reproducible quantitative relationship between chemical exposure and some measure of damage to the organism or group of organisms under investigation. Such measurements are established as biomarkers, which by definition refers to *“any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction”* (WHO). Because they are assessed at the first levels of biological organization, biomarkers allow the early detection of biological changes, which may result in long-term physiological disturbances. Hypotheses concerning the toxicity mechanism of a specific chemical or group of chemicals are typically tested at the cellular or molecular level on the basis of which investigators often make assumptions about how a particular biochemical or physiological change may affect the overall fitness of the organism (Wright and Welbourn, 2002).

A fundamental aspect of toxicological investigation is the relationship between the amount of chemical exposure and the degree of toxic response. Common measures are median *lethal* (LC₅₀) and *effective* (EC₅₀) concentrations, for chemicals that kill or affect the development of half of the population, respectively. However chemicals do not always follow a typical dose-response curve, i.e. with the increase of the dose the higher is the response (also referred as a monotonic response). In some particular cases, molecules can have a U-shaped response effect (also known as non-monotonic response), meaning they can induce a response at low and high but not intermediate doses. Hormones are a clear example of non-monotonic response (Vandenberg et al., 2012). This difficulties the task of institutions to set up safe chemical dose thresholds to human health.

Established in 1948, the intergovernmental organization known as Organization for Economic Co-operation and Development (OECD) first started to focus on specific chemicals known to pose health or environmental problems, such as mercury or chlorofluorocarbons (CFCs), with the main purpose of sharing information about these chemicals with member countries and to act jointly to reduce risks. With the increase of produced chemicals by the industries, the OECD began developing common tools that countries could use to test and assess the potential risks of new chemicals before they were manufactured and marketed. Since 1981, the OECD has been developing *Guidelines for the Testing of Chemicals* to determine their physical and chemical properties (e.g. water solubility), effects on human health and wildlife (e.g. short and long-term toxicity) and environmental fate and behavior. Test Guidelines are prepared with input from experts working in governments, academia, industry and other non-governmental organizations such as environmental groups and the animal welfare community. For example, concerning endocrine-disrupting chemicals (EDCs), in 1996 an Advisory Group on *Endocrine Disrupters Testing and Assessment (EDTA)* was set up at the OECD to develop new or update existing OECD Test Guidelines to identify chemicals with endocrine disrupting properties. Because of the high production rate of chemical substances but the limited available data concerning their potential endocrine-disrupting capacities, there is an emergent need to assess EDC activity in both marine environment and human.

1.3. Endocrine-disrupting chemicals: trick or threat?

In the early 1970's, ecologists began noticing unexpected patterns in animals, providing the first clues that certain chemicals were causing damage to wildlife through endocrine disruption. Such observations began spreading beyond the field of ecology: if chemicals could have such effects in wildlife, what might they do in humans? The medical tragedy involving diethylstilbestrol (DES) provided clues. DES is an artificial estrogen and it was prescribed from 1940–1971 to women during pregnancy to reduce miscarriage. Sadly, DES was later shown to have serious health consequences, such as cancers and reproductive system malformations, and confirmed as a transplacental carcinogen in mice (McLachlan et al., 1980). With the increase of studies showing the potential of some molecules to interfere with the endocrine system, there was a need to clarify what were these molecules, and it was in 1991, at the Wingspread Conference Center in Wisconsin (USA), that the term *endocrine-disrupting chemical* (EDC) was

firstly used and described by Theo Colborn, the pioneer in the field of EDCs. The paper by Theo Colborn and colleagues in 1993 was one of the earliest papers about this phenomenon, where the authors referred EDCs as exogenous substances that can interfere with the endocrine system and then lead to a range of developmental, reproductive, immune, neurological, or metabolic diseases in human and animals (Colborn et al., 1993).

With the development of *in vitro* screens, the list of EDCs rapidly increased, from a few pesticides and industrial chemicals, to plastics and personal care products, due to their estrogenic activity. By 2000, the research had demonstrated that EDCs can interfere with biological processes by mimicking hormones due to their similar structures (figure 3A), activating or blocking the body's hormone receptors, disrupting the synthesis of hormones, or altering their degradation (Schug et al., 2016). After a lot of debate about the most adequate definition for EDC, the Endocrine Society defined an EDC as "an exogenous chemical, or mixture of chemicals, that can interfere with any aspect of hormone action" (Zoeller et al., 2012).

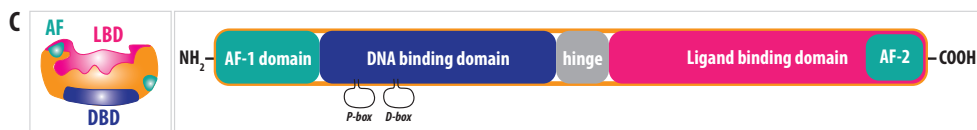
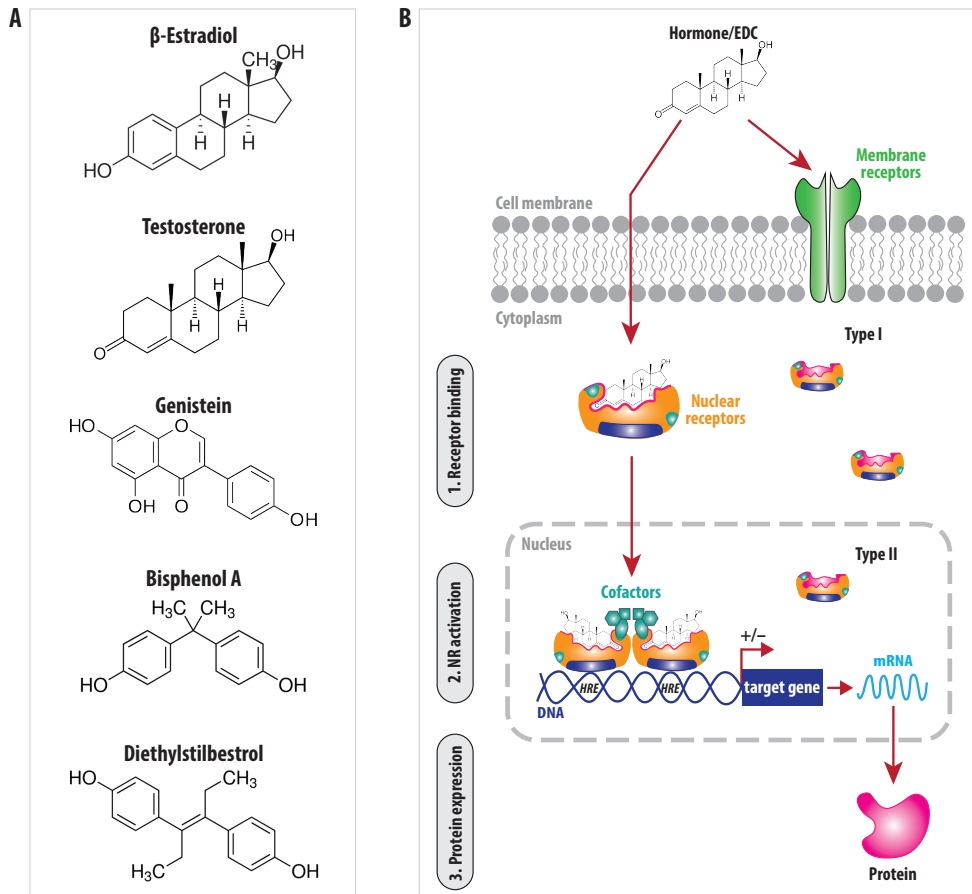
One of the main ways by which EDCs interact in organisms is via binding to nuclear receptors (NRs), such as Estrogen Receptor (ER), Thyroid Receptor (TR), among others. Briefly, EDC binds to the NR present in the cytoplasm (type I) or in the nucleus (type II), the resulting nuclear receptor-ligand complex binds, with the help of cofactors, to hormone response elements (HRE) in the DNA and activates or inhibits transcription of target genes (figure 3B) (Schug et al., 2013). While human and zebrafish genomes possess 48 and 71 NRs, respectively (Bertrand et al., 2007), the marine invertebrates ascidians possess only 17 (Satoh, 2003; Yagi et al., 2003).

Alternatively, some studies revealed that estrogens could act independently of nuclear ER to trigger rapid estrogen signaling, via binding to the membrane receptor G protein-coupled receptor 30 (GPR30), a rhodopsin-like GPCR, today renamed G Protein-coupled Estrogen Receptor (GPER) (Barton et al., 2018; Owman et al., 1996) (figure 3B) (Lee et al., 2013; Schug et al., 2013). Just as estrogens, studies suggested that EDCs like BPA can bind to GPER to activate ERK and EGF pathways, to stimulate tumor proliferation and migration in human breast and lung cancer cells (Dong et al., 2011; Sauer et al., 2017; Zhang et al., 2014). Nevertheless, no data concerning a GPER-like protein in the ascidian nor in the cephalochordate genomes can be found, suggesting they are an innovation of vertebrates. Therefore, I will not describe GPCRs further.

Additionally, there is a non-conventional Estrogen Receptor called *mER* (for *membrane Estrogen Receptor*), due to its presence in the cell membrane, thought to be a result of alternative splicing variant of the mammalian nuclear receptor *ER* (Soltysik and Czekaj, 2013). However, as for GPER, this *mER* seems to be specific to mammalian species and I will not describe it further.

Finally, EDCs may also affect epigenetic regulation (DNA methylation, histone modification) (Tran and Miyake, 2017) but the conservation with invertebrates of these mechanisms is largely unknown.

Altogether, I thus decided to focus on EDC toxicity in ascidians via NRs. In the next sections I will describe EDCs mode-of-action via NRs and the associated human diseases, with a special focus on Bisphenol A (BPA).



E

<p>NR1</p> <p>Thyroid Receptors (TR, NR1A1/2) Retinoic-Acid Receptors (RAR, NR1B1/2/3) Peroxisome Proliferator-Activated Receptors (PPAR, NR1C1/2/3) Rev-erb (NR1D1/2) RAR-related Orphan Receptors (ROR, NR1F1/2/3) Liver X Receptors (LXR, NR1H2/3) Farnesoid X Receptor (FXR, NR1H4) Vitamin D Receptor (VDR, NR1I1) Pregnane X Receptor (PXR, NR1I2) Constitutive Androstane Receptor (CAR, NR1I3)</p>	<p>NR3</p> <p>Estrogen Receptors (ER, NR3A1/2) Estrogen Related Receptors (ERR, NR3B1/2/3) Glucocorticoid Receptor (GR, NR3B) Mineralocorticoid Receptor (MR, NR3B) Progesterone Receptor (PR, NR3B) Androgen Receptor (AR, NR3B)</p>
<p>NR2</p> <p>Hepatocyte Nuclear Factor 4 (HNF4, NR2A1/2) Retinoic X Receptors (RXR, NR2B1/2/3) Testicular Receptors (TR, NR2C1/2) Tailless (TLX, NR2E2) Photoreceptor Nuclear Receptor (PNR, NR2E3) Chicken Ovalbumin Upstream Promoter (COUP, NR2F1/2) EAR Receptor (EAR-2, NR2F6)</p>	<p>NR4</p> <p>Nerve Growth Factor I-B (NGFI-B, NR4A1) Nuclear Receptor Related 1 (NURR1, NR4A2) Orphan Nuclear Receptor 1 (NOR1, NR4A3)</p>
	<p>NR5</p> <p>Steroid Factor 1 (SF1, NR5A1) Liver Receptor Homolog-1 (LRH-1, NR5A2)</p>
	<p>NR6</p> <p>Germ Cell Nuclear Receptor (GCNF, NR61)</p>
	<p>NR0</p> <p>DAX-1 (NR0B1) Small Heterodimer Partner (SHP, NR0B2)</p>

*Orphan Receptors

Figure 3. Endocrine disrupting chemicals (EDCs) and nuclear receptors (NRs). (A) Similarity between molecular structures of naturally occurring molecules (female hormone β -estradiol, male hormone testosterone and phytoestrogen genistein) and some known EDCs (Bisphenol A and Diethylstilbestrol). (B) EDCs mode-of-action occurs primarily via NRs: EDC binds to the NR present in the cytoplasm (type I) or in the nucleus (type II) (1), the nuclear receptor-ligand complex binds, with the help of cofactors, to hormone response elements (HRE) in the DNA of the cell (2) and activates or inhibits (+/-) transcription of target genes and protein translation (3). EDCs might also act through membrane receptors. (C) NRs are characterized by three main domains: the DNA-binding domain (DBD), the ligand-binding domain (LBD) and the activation-function domains 1 and 2 (AF-1/AF-2) present in the N-terminal and C-terminal, respectively. (D) NRs can function as monomers, homodimers or heterodimers. (E) NRs are divided in 7 main subfamilies, from 0 to 6.

1.3.1. EDCs mode-of-action via nuclear receptors

Nuclear receptors (NRs) are the most abundant class of transcription factors in metazoans, present from marine sponges up to vertebrates (Holzer et al., 2017; Markov et al., 2008). The first NRs were discovered in the 70s-80s by endocrinologists searching for the receptors of major human hormones such as steroids and thyroid hormones (Jensen and Desombre, 1973; Spurr et al., 1984), which made scientists think that the NR superfamily was primarily a family of hormone receptors. However, with the finding of retinoic acid receptors (RARs), identified soon after the steroid and thyroid receptors, the concept of *nuclear hormone receptors* changed, since RAR ligands (retinoic acids) are locally diffusing growth factors rather than hormones.

Most of them being activated by ligand binding, nuclear receptors can sense environmental changes (such as chemicals concentration) and translate them into gene expression modulation (Bertrand et al., 2011; Sever and Glass, 2013). NRs have a very conserved structure of functional domains: a N-terminal domain which harbors a ligand-independent activation function (AF-1), a DNA-binding domain (DBD) which comprises *P-box* and *D-box* regions responsible for DNA binding specificity to hormone response elements (HREs), a hinge region and a C-terminal ligand-binding domain (LBD) hosting a ligand-dependent transcriptional activation function (AF-2) (figure 3C) (Kumar et al., 2004).

Activity of most NRs is controlled not only by hormones but also by other important lipophilic molecules such as retinoic acids and fatty acids that can diffuse through the cell membrane and bind to nuclear receptors. However, while some NRs have known ligands (e.g. retinoic acid receptor that binds retinoic acid), other NRs have no known ligand, they are thus called orphan receptors. In their mode-of-action, NRs can bind to the DNA either as monomers, as dimers with themselves (homodimers) or with other NRs (heterodimers) (figure 3D). While monomer action is pretty rare, the most reported mode-of-action is NR heterodimer with Retinoid X Receptor (RXR). Moreover, cofactors (that can be either coactivators or corepressors) can also interact with these receptors to regulate gene expression (Sever and Glass, 2013). Up to now, NRs have been classified into seven subfamilies (figure 3E) (Bertrand et al., 2007).

These receptors and their natural ligands are essential throughout life, as they regulate gene networks involved in various physiological processes including development, differentiation,

reproduction, metabolism, metamorphosis and homeostasis. Yet, in some situations NR activity can also be inappropriately modulated by xenobiotics like EDCs. Xenobiotics can be defined as any foreign molecule within an organism that is not naturally produced or expected to be present in the organism, such as drugs, pollutants, as well as some food additives and cosmetics (Schug *et al.*, 2013). All NR family members, including the orphan receptors, can thus represent potential targets of environmental contaminants as they all possess LBD that can be targeted by different types of molecules (Delfosse *et al.*, 2015b). Consequently, studies have showed that recurrent exposure to environmental pollutants may lead to reproductive pathologies like hormonal cancers (mainly acting *via* ER/AR/GR/PR), metabolic diseases like obesity or diabetes (PPAR/ERR), thyroid disorders (TR) and neurodevelopmental disorders such as attention-deficit hyperactivity disorder (ADHD) and autism spectrum disorder (ASD) (TR, ERR) (Braun, 2017; Delfosse *et al.*, 2014).

1.3.2. EDCs and developmental neurotoxicity

The occurrence of EDCs in our environment and our bodies represents a significant global public health challenge. The majority of epidemiological studies about EDCs focused essentially on reproductive effects, thyroid-related disorders, hormone-dependent cancers, immune diseases and metabolic and bone disorders in humans. In the early 90's a few studies started to address the potential link between EDCs and neurodevelopment, and it was only in the last few years that studies showing "neuroendocrine disruption" have dramatically increased (Leon-Olea *et al.*, 2014).

In humans, neurodevelopment begins as early as three weeks into gestation and continues through the neonatal period and puberty, and, to some extent even spans into adulthood (Rock and Patisaul, 2018). But the developing nervous system has a unique sensitivity to interference by exogenous agents, and the gestation period has been considered as the most critical window of susceptibility to chemicals, since it is in this period that rudimentary structures of both central nervous system (CNS) and peripheral nervous system (PNS) are formed and organized (Braun, 2017; Heyer and Meredith, 2017; Schug *et al.*, 2015).

According to the United States Environmental Protection Agency (US EPA), around 1000 chemical substances were reported to have endocrine effects, and only 200 chemicals are currently characterized as "neurotoxic" to humans, but even less is known about which of these chemicals are *developmentally neurotoxic*, in other words, chemicals that can disrupt the formation of the CNS. Neurotoxicity is defined by US EPA as an adverse change in structure or function of the CNS and/or PNS, culminating in cell death and a degree of quantifiable pathology (Rock and Patisaul, 2018). Known examples of neurotoxic molecules can be pesticides such as chlorpyrifos and methyl parathion. However, EDCs do not fit in the classical definition of neurotoxicant because they do not typically result in overt cell death and neural pathology, but rather have subtle yet profound effects on development, physiology, and behavior (Rock and Patisaul, 2018). They are thus considered to exert *neurodevelopmental toxicity*.

As a consequence, nervous system disruption by EDCs may have severe and long-lasting consequences on brain structure and function, potentially resulting in neurodevelopmental disorders. While some disturbances underlying neurodevelopmental disorders are genetic in origin, it is increasingly

evident that such disturbances can either be created or exacerbated by environmental factors (Heyer and Meredith, 2017). The most extensively discussed neurodevelopmental disorders in the literature, regarding environmental toxicology and early exposure, has been autism, Attention Deficit/Hyperactivity Disorder (ADHD) and schizophrenia. And according to the published literature, most of the environmental toxicants are particularly dangerous during gestation and first years after birth when they are more likely to be associated with neurodevelopmental disorders (please refer to review from (Heyer and Meredith, 2017)).

For more than 20 years, laboratory animals and epidemiological studies have highlighted the pivotal role of NRs in transducing several adverse effects of EDCs (Delfosse et al., 2015b). Interestingly, within 48 human nuclear receptors, some are expressed in the brain: ER (Whalen and Maurer, 1969), AR (Attardi and Ohno, 1976), TR (Bernal and Nunez, 1995), ERR (Hong et al., 1999), PPAR and RXR (Cullingford et al., 1998), suggesting their potential involvement in mediating EDC developmental neurotoxicity in humans. If we take the example of ERR, besides humans, it has been described to be expressed in the brain of other species such as zebrafish (Bertrand et al., 2007) and amphioxus (Bardet et al., 2005). Thus, there are more NRs expressed in the brain than previously thought, meaning that all these brain NRs are candidate mediators of neurodevelopmental disorders. Plus, the fact that invertebrates might also have NRs expressed in their brains raises the concern that aquatic invertebrates are potential targets of EDCs toxicity.

The OECD has been developing *Guidelines for the Testing of Chemicals* with a potential endocrine-disrupting activity (figure 4). Published assays are based on fish species (fathead minnow, zebrafish, medaka, stickleback), amphibian (*Xenopus laevis*), avian (Japanese quail), mammals (rats), and invertebrates (copepods, *Daphnia magna*, chironomids, and snails). *In silico* and docking models for estrogenic and androgenic chemicals have also been developed to complement the *in vivo* and *in vitro* assays. It is important to note that although these assays are mostly based on estrogen, androgen, thyroid or steroidogenesis pathways, nevertheless endocrine disruption may occur through other effectors such as ERR-, PXR- or PPAR-related pathways. For example, some EDCs such as organotins and halogenated bisphenols are considered as “obesogens”, due to their potential to disrupt metabolic signaling pathways and consequently leading to fat accumulation and obesity, and PPARs are suspected to be the main mediators of such metabolic disorders (Darbre, 2017). If we consider that any molecule capable to affect any NR is an EDC, then this means that endocrine disruption can occur through other NRs than the estrogen, androgen, thyroid or steroid ones.

Moreover, most assays are based on terrestrial vertebrates or aquatic vertebrates, but no OECD guidelines able to assess EDCs activity using marine invertebrates are proposed, revealing a gap in the available tools to assess environmental toxicology in marine ecosystems. Developing a test on marine organisms such as ascidians will provide us information about marine fauna environmental exposure to EDCs. However, scarce data is available concerning basic knowledge on invertebrate NRs and therefore there is a need to gather more information about their role during development.

Test Guidelines Number and Name*	Pathway Addressed			
	Oestrogen	Androgen	Thyroid	Steroidogenesis
TG 493: <i>In Vitro</i> Oestrogen Receptor Binding Assay	X			
TG 455: <i>In Vitro</i> Oestrogen Receptor Transactivation Assay	X			
TG 458: <i>In Vitro</i> Androgen Receptor Transactivation Assay		X		
TG 456: H295R Steroidogenesis Assay	X	X		X
TG 440: Uterotrophic Bioassay	X			
TG 441: Hershberger Bioassay		X		
TG 229: Fish Short-Term Reproduction Test	X	X		X
TG 230: Fish Screening Assay	X	X		X
TG 231: Amphibian Metamorphosis Assay			X	
TG 407: 28-day Repeated Dose Toxicity Study in Rodents			X	X
TG 408: 90-day Repeated Dose Toxicity Study in Rodents			X	X
TG 414: Prenatal Developmental Toxicity Study	X	X	X	X
TG 421: Reproduction/Developmental Toxicity Screening Test	X	X	X	X
TG 422: Combined Repeated Dose Reproduction/Developmental Toxicity Screening Test	X	X	X	X
TG 426: Developmental Neurotoxicity Study	X	X	X	X
TG 451-3: Combined Chronic Toxicity/Carcinogenicity Study	X	X	X	X
TG 234: Fish Sexual Development Test	X	X		X
TG 241: Larval Amphibian Growth and Development Assay			X	
TG 443: Extended One-Generation Reproductive Toxicity Study	X	X	X	X
TG 240: Medaka Extended One-Generation Reproductive Toxicity Study	X	X	X	X
TG 416: Two Generation Reproduction Toxicity Study	X	X	X	X

*Test guidelines may evolve following best practices and emerging science.



Figure 4. Relevant OECD Test Guidelines for the detection of endocrine-disrupting chemicals (from OECD, 2018).

1.3.3. The EDC Bisphenol A

One of the most known EDC is Bisphenol A (BPA), a carbon-based synthetic compound with two hydroxyphenyl groups (figure 3A). It has been in commercial use since 1957, being employed to make certain plastics and epoxy resins. BPA-based plastic is clear and tough, and is made into a variety of common consumer goods, such as water bottles, sports equipment, CDs, and DVDs. Epoxy resins containing BPA are also used to line water pipes, as coatings on the inside of many food and beverage cans (Rochester, 2013). Less than 1% of environmental BPA is thought to occur in the atmosphere, yet it is estimated that the largest environmental compartments of BPA are abiotic and are associated with water and suspended solids (~53%), soil (~25%), or sediments (~23%), due to continuous release in the environment during chemical manufacturing, transport, and processing. Post-consumer releases are primarily via effluent discharge from municipal wastewater treatment plants, leaching from landfills, combustion of domestic waste, and the natural breakdown of plastics in the environment (Flint et al., 2012).

Based on reported median effective (EC_{50}) and median lethal (LC_{50}) concentrations that range from 1.0 to 10 mg/L (equivalent to 4.38 and 43.8 μ M), BPA is classified as “moderately toxic” and “toxic” to aquatic biota by the European Commission and the US EPA, respectively. However, studies of BPA effects on wildlife indicate that the compound may be harmful even at environmentally relevant concentrations, which has been defined as 12 μ g/L (~0.05 μ M) or lower (Flint et al., 2012). The highest concentration found in natural surface waters was 21 μ g/L (~0.1 μ M) in Netherlands, but landfill leachates from municipal wastes in Japan registered values of BPA around 17.200 μ g/L (~ 75 μ M) (Flint et al., 2012). In humans, detectable levels of BPA were found in 93% of urine samples taken from US people above the age of six (Calafat et al., 2008). Even if several studies from the early 2000’s demonstrate the high potential of BPA

to interact with human endocrine system, it is only in 2015 that countries like France decide to ban all BPA-associated products, and only in June 2017 that the European Chemicals Agency (ECHA) considered BPA as an endocrine disruptor (ECHA, 2017).

BPA, its analogues or its halogenated derivatives have been shown to interact with several nuclear receptors (NRs). In the following paragraphs I will shortly talk about the NRs known to interact with BPA.

Human ER α and ER β bind the sex hormone, 17 β -estradiol (E2), which play important roles in the growth and maintenance of a diverse range of tissues. BPA has been shown to bind both ERs but with a very low affinity when compared to the endogenous hormone (1000-fold lower than that of E2), which makes BPA a weak environmental estrogen. A similar affinity binding has been shown between BPA and androgen receptor (AR), a receptor involved in physiological processes like development and differentiation of the male embryo and spermatogenesis initiation and maintenance. While BPA is considered to act as an ER agonist, several studies suggests that BPA acts as an AR antagonist (Mackay and Abizaid, 2018).

Thyroid hormones, tetraiodothyronine (thyroxine or T4) and triiodothyronine (T3), are essential for the normal development, growth, and metabolism of all vertebrates, playing a major role in metamorphosis, neurogenesis and brain function at all stages of development. The functions of thyroid hormones are mediated by several isoforms of nuclear thyroid receptors, TR α and TR β . Human TRs are relatively ubiquitous and their expression begins early in development. Several articles revealed that BPA and its halogenated derivatives can alter thyroid hormone action by inhibiting T3 binding to or by activating TRs in the 1–10 μ M concentration range (Delfosse et al., 2014). Moreover, halogenated BPAs such as tetrabromobisphenol-A (TBBPA) and tetrachlorobisphenol-A (TCBPA) were also suggested to inhibit TR at lower concentrations than BPA (Delfosse et al., 2014). A recent and very interesting study suggested that exposure of pregnant women to BPA during gestation impacts children's IQ by affecting thyroid hormones and TR function (Fini et al., 2017).

The glucocorticoid receptor (GR) is a vertebrate-specific nuclear receptor that is expressed in virtually every cell type throughout the body and it regulates pleiotropic steroid hormones called glucocorticoids. Cells transfected with a luciferase construct linked to the GR response element (GRE) showed a marked increase in GR-mediated luciferase expression when exposed to BPA at 1 μ M, even if it was not stronger than glucocorticoids in inducing adipogenesis and promoting lipid accumulation (Mackay and Abizaid, 2018).

The Peroxisome Proliferator-Activated Receptors (PPARs) are mostly known to act as lipid sensors and regulators of lipid metabolism in response to fatty acids and their derivatives, eicosanoids, and drugs used in the treatment of hyperlipidemia and diabetes. Besides adipose tissues, PPARs expression has been found in human and mouse brains and have shown anti-inflammatory and potentially neuroprotective effects, increasing the interest of using PPAR agonists for the treatment of neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's disease (Warden et al., 2016). Xenobiotic ligands of PPARs include the thiazolidinedione (TZD) class of anti-diabetic agents, certain herbicides, fungicides, phthalates, flame retardants, as well as industrial plasticizers (including BPA) which are all capable of binding to PPAR γ and modulate its transcriptional activity. Moreover, its halogenated derivatives TBBPA

and TCBPA were capable of partially activating human, zebrafish and *Xenopus* PPAR γ , suggesting that activation of PPAR γ depends on the halogenation degree of BPA analogues (Delfosse et al., 2014).

Pregnane X Receptor (PXR) is a broad-specificity sensor playing a critical role in the regulation of phase I (CYP), phase II (conjugating), and phase III (ABC family transporters) detoxifying enzymes. While liver and intestine express high levels of PXR, it has been recently suggested that other tissues express lower but significant levels of this nuclear receptor protein including kidney, ovary, stomach, and brain (Shen, 2017). Unlike most NRs that tend to be specialized to bind few ligands with structural homologies, PXR is able to bind a large number of structurally diverse ligands with a wide range of affinities. Human PXR binds a multitude of drugs such as the antibiotic rifampicin, the anticancer taxol, the anticholesterol SR12813, among others. Furthermore, numerous studies have focused on its ability to bind environmental compounds, such as pesticides, polychlorinated biphenyls, brominated flame retardants, antimicrobial triclosan, or natural and synthetic estrogens and, among them, BPA in the 1–10 μ M concentration range, as well as two other bisphenols, BPB and BPAF.

The ERR family includes three members, ERR α , ERR β , and ERR γ . ERR proteins are closely related to ERs, as they recognize the same DNA-binding elements, share common target genes, and are coexpressed in many tissues. However, ERRs cannot bind estrogens such as estradiol, and a natural ligand remains to be found, making them orphan receptors. Several lines of evidence suggest that ERRs play a central role in regulating energy metabolism, probably commanding such metabolism from the brain, as ERR γ is highly expressed in the mammalian brain during development and during adulthood. Interestingly, several studies have shown that this NR is constitutively active (Tohmé et al., 2014), suggesting that no ligand is needed for its activity and thus explaining the fact that ERRs are orphan receptors. Although being orphan, several synthetic ligands have been identified for ERR. The rise in the incidence of metabolic syndromes correlates with the rise in the use and distribution of industrial chemicals that may play a role in generating obesity, suggesting that EDCs and ERR γ may be linked to this epidemic crisis. Indeed, BPA, BPE and BPB have been shown to act as human ERR γ agonists, while diethylstilbestrol (DES) and 4-hydroxytamoxifen (4-OHT) act as antagonists. Moreover, the structure of ERR γ in complex with BPA has been solved, revealing an overall protein conformation indistinguishable from that of the unliganded receptor. Thermal stability studies revealed that BPA binding leads to global thermodynamic stabilization of ERR γ LBD, a phenomenon that could increase steady-state levels of the receptor and impact both its cellular half-life and biological activity.

In summary, there is a multitude of target receptors, mechanisms and pathways by which BPA can affect organisms (figure 5), particularly during very susceptible windows like embryonic development.

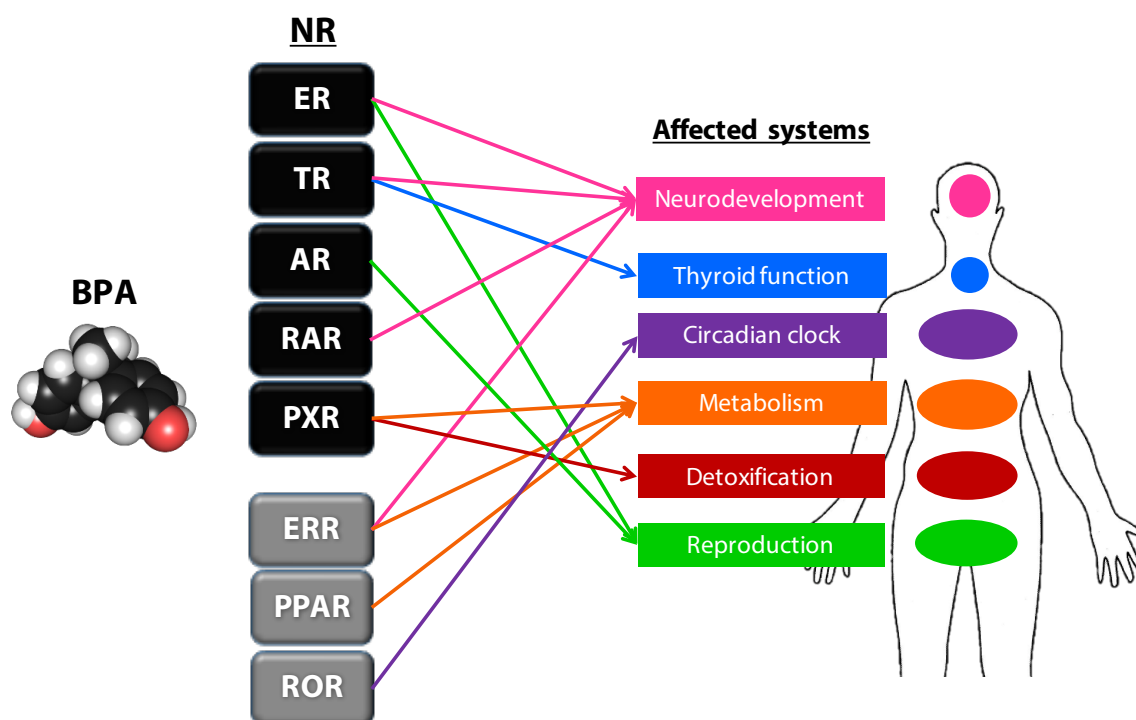


Figure 5. Main nuclear receptors targeted by BPA and respective systems affected in humans.

1.4. Ascidians as model system for neurodevelopmental toxicity by EDCs

Tunicates (also known as urochordates) are marine invertebrate chordates, found throughout the world. Tunicates are divided in five main orders: the Appendicularia, the Thaliacea, the Phlebobranchia, the Aplousobranchia and the Stolidobranchia (Delsuc et al., 2018). Ascidians are a group of tunicates composed by phlebobranchians, aplousobranchians and stolidobranchians. Tunicates can be solitary or colonial (several individuals that live closely associated with each other). In this section, I will focus on solitary ascidian species from Phlebobranchia order, like *Ciona robusta* and *Phallusia mammillata*.

Ascidians have been used in developmental studies since the beginning of 20th century, when the American zoologist Edwin Conklin described for the first time the embryonic development of ascidian eggs (Conklin, 1905). For years, scientists described the remarkable analogy between ascidian and vertebrate embryonic development, suspecting on the close relationship between the two clades. The recent genome sequencing of *Ciona robusta* (formerly *Ciona intestinalis* type A) allowed scientists to conclude that ascidians were indeed the closest invertebrate to vertebrates, together with cephalochordates (figure 6A) (Dehal, 2002).

Another great interest in using ascidians for developmental biology is because of their fast life cycle, characterized by 3 main periods: the embryonic development, the larva and the adult periods (figure 6B). *Ciona* embryonic development has been fully characterized (Hotta et al., 2007). Once fertilized, embryos quickly develop into a “vertebrate-like tadpole” (at 18°C, 18 hours post fertilization (hpf) for *Ciona robusta*, 22 hpf for *Phallusia mammillata*), characterized by a central and peripheral nervous systems (CNS and PNS, respectively), a neural tube and a notochord. This larval stage is a planktonic phase since the larvae need to swim until they find a substrate to settle. Once they settle, larvae undergo

metamorphosis, where they reorganize their body plan to reach their adult stage, achieved when they become sexually mature. The adults are hermaphrodite sessile filter feeders, which spawn their gametes via a light-dark dependent stimuli (Lambert and Brandt, 1967).

Furthermore, the simplicity of their larval body plan with only ~2.600 cells allows the investigation of the molecular mechanisms underlying cell-fate specification and differentiation during development (McDougall et al., 2012; Satoh, 2003). Additional characteristics such as high gametes content, transparent embryos (for some species such as *Phallusia mammillata*) and extensively developed transgenesis and genomic techniques (electroporation, mRNA injection, CRISPR/Cas9 genome editing, genome and transcriptome studies, ATAC-seq, among others) places ascidians in a very interesting position for modern developmental studies (Gandhi et al., 2017; McDougall et al., 2012; Stolfi and Christiaen, 2012). Today, several genome sequences have been assembled for additional ascidian species, widely used as models in comparative genomics and evo-devo (Lemaire, 2011), including *Ciona savignyi*, *Oikopleura dioica*, *Botryllus schlosseri*, *Molgula occidentalis*, *M. occulta*, and *M. oculata*, *Halocynthia roretzi*, *Phallusia fumigata* and *Phallusia mammillata*.

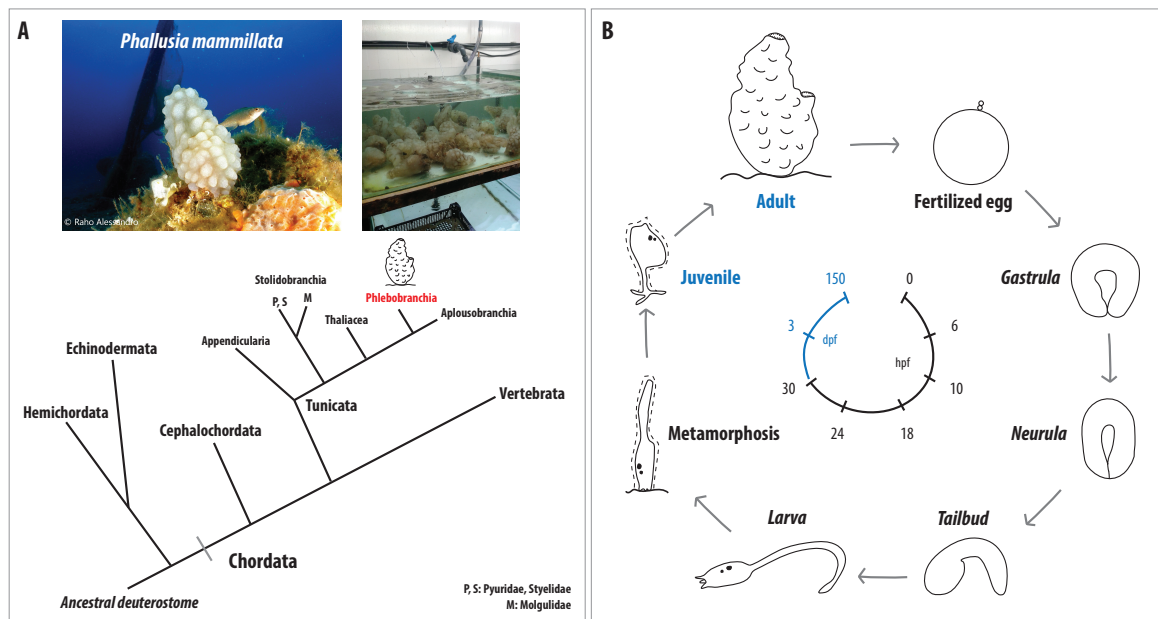


Figure 6. The marine invertebrate chordate ascidian. (A) Deuterostome phylogeny shows tunicates as the sister group of vertebrates. Phlebobranchian ascidians (in red), like *Ciona robusta* and *Phallusia mammillata*, are within the most used species in developmental biology. **(B) *Phallusia mammillata* life cycle** is characterized by an embryonic phase of 24 hours, reaching the planktonic phase characterized by a larva stage, until settlement and metamorphosis phase before giving rise to the adult stage.

Moreover, ascidians frequently encode a single gene for multigene families, making them a simplified system to study signaling pathways. For example, while human and zebrafish genomes possess 48 and 71 nuclear receptors respectively (Bertrand et al., 2007), ascidians possess only 17 (Satoh, 2003; Yagi et al., 2003). This reduced genetic redundancy found in the compact ascidian genome facilitates

functional analyses of homologous NRs in the context of the typical chordate body plan of embryos and larvae.

Studying the role of a specific nuclear receptor in humans or other vertebrates might be a challenging task, as most of these NRs have several copies (for example, there are 3 and 5 copies of ERR in the human and zebrafish genomes, respectively). Because of the potential role of vertebrate NRs in neurodevelopmental toxicity, in the next sections I will describe the ascidian brain and review the available information concerning ascidian NRs. I will discuss their potential as a model to assess not only the role of nuclear receptors but also to assess EDC toxicity and mode-of-action during chordate embryonic development.

1.4.1. Ascidian brain and the pigment sensory organ

Although the solitary adult ascidian is a sessile organism with little resemblance to the vertebrate, the ascidian larva is similar to a frog tadpole and shares basic body structures with vertebrates, with both CNS and PNS (Ryan et al., 2016). The CNS of the ascidian larvae is divided into three parts: an anterior brain sensory vesicle, a motor ganglion and a caudal nerve cord (figure 7A) (Hudson, 2016). The main block of the ascidian CNS is the sensory vesicle, a hollow cavity situated in the trunk of the larva. It contains different sensory receptor cells, including the anterior geotactic otolith (Dilly, 1962) and the posterior photoreceptive ocellus (Dilly, 1964), easily distinguishable one from the other based on their morphology and their specific type of pigmentation. The otolith is a gravity-sensory organ characterized by a single pigment cell, while the photo-sensing ocellus is a multicellular structure (Esposito et al., 2015; Ryan et al., 2016). Together, these structures constitute the pigment sensory organ (PSO). In addition, the lineages leading to the formation of these two pigmented cells have been well characterized in recent years (Racioppi et al., 2014).

The ascidian ocellus is a multicellular structure, composed by one pigment cell, three lens cells and 37 photoreceptors cells in *Ciona robusta* (Ryan et al., 2016), although the number of photoreceptor cells may vary among specimens (Horie et al., 2008). The pigmented ocellus contains several membrane-bound pigment granules (of 1-2 μ m in diameter). Besides the pigmented structures, the ocellus contains three distinct groups of photoreceptor cells: groups I and II are associated with the ocellus pigment cell and are evident in the right lateral view of *Ciona* larvae, while the group III photoreceptor cells are located at the left ventral part of the brain vesicle, in proximity to the otolith and apart from the ocellus (Horie et al., 2008). For clarity, hereinafter each time I refer to the ocellus I refer to the single pigmented cell and not the remaining structures. Ocellus role in light perception was shown in 2003, when ocellus-ablated larvae lost their photic response (Tsuda et al., 2003). Moreover, the authors revealed that melanin-deprivation experiments (either by inhibiting tyrosinase enzyme with phenylthiourea, either by *tyrosinase* gene knock-out) showed that melanin pigment itself was dispensable for photo-response behavior of the larvae, suggesting that the photoreceptor cells are the ones responsible for their photic response and swimming behavior (figure 7B) (Tsuda et al., 2003).

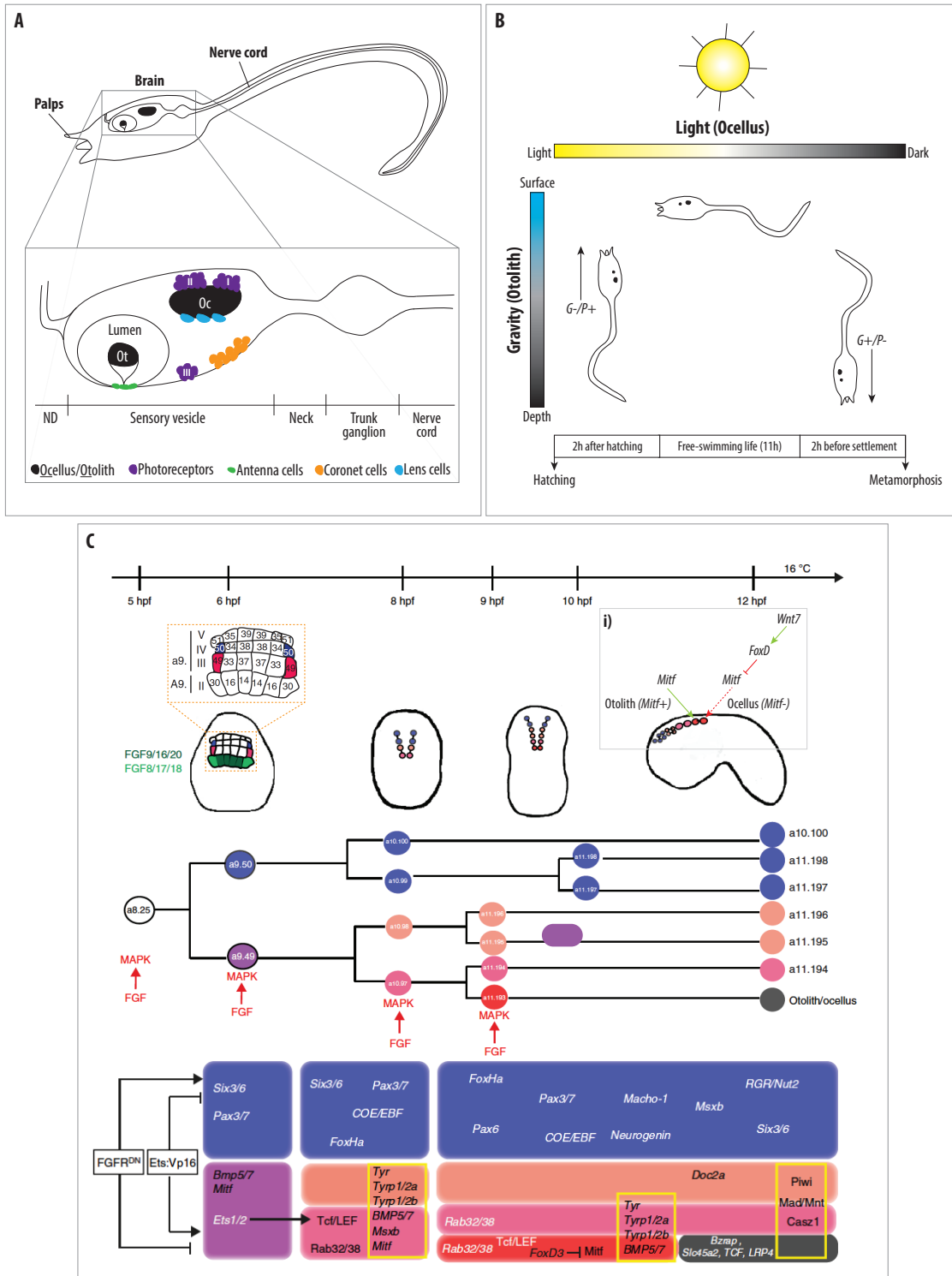


Figure 7. Ascidian central nervous system structure. (A) Ascidian CNS. The ascidian CNS is composed of a neurohypophyseal duct (ND), a sensory vesicle (SV), a neck region, a trunk ganglion (TG) and a nerve cord (NC). Within the sensory vesicle, the pigment sensory organ is visible. **(B) Phototactic and geotactic behavior of ascidian larvae.** The ocellus is responsible for light perception, while the otolith senses gravity. Two hours after hatching, larvae swim upwards (negatively geotactic, G-) in direction to the light (positively phototactic, P+). The larvae will freely swim for 11h, and 2h before metamorphosis they swim downwards (positively geotactic, G+), far from light (negatively phototactic, P-), to find a substrate where they settle and undergo metamorphosis to reach

the adult stage, ready to start a new life cycle (Tsuda et al. 2003). **(C) Ascidian pigment cell lineage.** Pigment cells are specified, through several *FGF/MAPK* signals, from a8.25 cells until they stop dividing at a11.193 lineage. Several melanogenic gene markers are expressed, such as *Mitf*, *Tyr*, *Tyrp*, *Msx*, *Rab32/38* (adapted from Racioppi et al., 2014). *Mitf* signal is crucial to determine pigment cell fate **(C inset)**.

The ascidian otolith, unlike the ocellus, is an unicellular structure that protrudes into the lumen of the brain sensory vesicle (Dilly, 1962) from a small group of cells in the ventral floor, recently described as antenna cells and otolith-associated ciliated cells (Dilly, 1962; Ryan et al., 2016). The body of this cell contains, besides the nucleus, a large, round-shaped melanin granule (10–15 μm in diameter), occupying about one-half of the volume of the cell. The otolith is connected by a narrow stalk that enlarges in an anchoring foot into the ventral floor of the sensory vesicle. Moreover, Sakurai and colleagues showed that two dendrites (synaptotagmin-positive structures) connect the otolith body to the brain sensory vesicle, and through their deformation, caused by the movement of pigment granule in the cell body, they supply gravity information to the larva (Sakurai et al., 2004). Indeed, the otolith-ablated larvae lost the upward swimming behavior, confirming that the otolith is responsible to react to gravity or inertia most likely through deformation of the dendrites (figure 7B) (Tsuda et al., 2003).

Pigment cells (PC) arise from the paired a8.25 blastomeres. During gastrulation, these blastomeres divide and form a9.49 and a9.50 cell pairs: the a9.50s (located in the row IV of the neural plate) are progenitor cells of the anterior brain, while the a9.49s (located in the row III) become fate restricted as pigment cell precursors (PCPs) (figure 7C). At the mid neurula stage (stage 15), bilateral PCPs divide, forming two cell pairs (a10.98s and a10.97s, figure 7C), and after a second division (a10.97 into a11.194 and a11.193), the two a11.193 cells finally give rise to either the otolith or the ocellus, depending on their anterior or posterior position (Abitua et al., 2012; Racioppi et al., 2014). It has been shown that several *FGF/MAPK* signals during embryonic development are essential for PC specification (figure 7C) (Racioppi et al., 2014). Furthermore, like vertebrate pigment cells, ascidian PC precursors express firstly *Mitf* (microphthalmia-associated transcription factor) from late gastrula (st. 13) and then Tyrosinase (*Tyr*), Tyrosinase-related (*Tyrp*) and *Rab32/38* from mid neurula (st. 15) (Racioppi et al., 2014). Later in development, both pigment cells express *Mitf* but while one of the two will give rise to the otolith (*Mitf*⁺), the other more posterior cell receives a *Wnt7* signal that activates *FoxD* which in turn inhibits *Mitf* expression (*Mitf*⁻), becoming the ocellus (figure 7C) (Abitua et al., 2012). Additionally, other genes have been reported to be crucial for ocellus specification like *Rx* (D'Aniello et al., 2006), *Opsin* (Kusakabe et al., 2001), *Arrestin* (Nakagawa et al., 2002), or for otolith specification like the $\beta\gamma$ -crystallin (Shimeld et al., 2005).

When it comes to the origin of ascidian PC, and their orthologues in vertebrates, there is still controversy. It has been suggested that the ascidian ocellus is homologous to the median eye (pineal gland) of vertebrates, based on their similarities in some aspects, like the shadow response of ascidian larvae that also occurs in young *Xenopus* larvae via the pineal eye (Kusakabe and Tsuda, 2007). However, while ocellus photoreceptor cells show morphological and electrophysiological properties that are similar to those of the vertebrate photoreceptor cells (Kusakabe and Tsuda, 2007), the same is not true for the

lens cells. Despite their name, ascidian lens cells are not homologous to the vertebrate ones, for several reasons as reviewed before (Esposito et al., 2015), one of them being the fact that the ascidian ortholog of $\beta\gamma$ -crystallin gene (in vertebrates coding for a highly stable protein typical of vertebrate lens) is expressed in the otolith and in the adhesive papillae, but not in the lens cells (Shimeld et al., 2005). The otolith gravity-sensing organ tempted researchers to propose a partial level of homology to the vertebrate inner ear. The vertebrate ear is the sense organ of hearing and balance, originated from the otic vesicle which in turn is believed to originate from both neural crest and cranial placodes (Sandell, 2014). The vertebrate ear is divided in three parts: the inner ear, the middle ear and the outer ear. While fish only possess the inner ear, amphibians possess both inner and middle ear, but only reptiles, birds and mammals possess all the three types, suggesting that their common ancestor was based on a basic inner ear structure. Interestingly, the ascidian otolith has as main function the detection of gravity in order to allow the good balance of the larvae while swimming. Hence, maybe the primitive ear functioned primarily as an organ to sense motion and balance (ascidian otolith), before evolving to a more complex hearing-balance system in vertebrates (vertebrate ear). Furthermore, recent evidences suggest an evolutionary relationship between ascidian pigment cells and the vertebrate neural crest (Abitua et al., 2012). The neural crest is a transient vertebrate cell type that arise between the neural and non-neural ectoderm, delaminate during neural tube formation, and migrate and differentiate into numerous derivatives, as diverse as cartilage, bone, peripheral neurons, glia and melanocytes (Green et al., 2015). Ascidian pigment cells arise at the neural plate border and express several neural plate border genes (Zic, Msx, Pax3/7), as well as some neural crest specification genes (Id, Snail, Ets, FoxD) (Green et al., 2015). Furthermore, the mechanism to specify neural crest-derived melanocytes also derives from a common bipotent Mitf1-expressing progenitor, suggesting that the PC lineage of *Ciona* represents a rudimentary neural crest (Abitua et al., 2012). Nevertheless, the ascidian PC lineage lacks some of the defining properties of cephalic neural crest, such as long-range migration and the potential to form ectomesenchyme derivatives (Abitua et al., 2012). During neural tube closure in vertebrates, not only neural crest cells are formed but also the cranial placodes (Feinberg and Mallatt, 2013). The cranial placodes are thickenings of the embryonic ectoderm that subsequently give rise to migrating cells and contribute sensory receptor cells, sensory neurons, secretory cells, and supporting cells to multiple cranial sense organs and ganglia (Schlosser, 2014). Within the different vertebrate placodes, there is the otic placode that gives rise to the otic vesicle, the precursor of the vertebrate ear. Thus, it would be worth to hypothesize that if the ascidian otolith is an ancestor of the vertebrate ear, it might have arisen from a rudimentary ascidian placode and not only necessarily from a neural crest-like cell. It would be thus more correct to propose that ascidian PC arise from the neural plate border (Stolfi et al., 2015) and that this ascidian neural plate border might be a mixture between both neural crest and cranial placodes, like recently suggested (Horie et al., 2018).

In vertebrates, melanogenesis (also referred as pigmentation) is the process of pigment melanin synthesis, the primary determinant of skin, hair, and eye color, within membrane-bound organelles termed melanosomes (Videira et al., 2013). It is regulated by the GPCR melanocyte-stimulating hormone receptor (MSHR) that stimulates MITF transcription. Then, MITF stimulates proteins such as Tyr, Tyrp and

Rab GTPases (Wasmeier et al., 2008) to produce and transport mature melanosomes (figure 8). Melanin synthesis, common for all melanin kinds, starts with hydroxylation of tyrosine to 3-4-dihydroxyphenylalanine (DOPA), and after some non-enzymatic and enzymatic processes, gives rise to yellow/red pheomelanin, or dark brown/black eumelanin (figure 8) (D’Mello et al., 2016; Videira et al., 2013). Because many cytotoxic agents are formed (e.g. hydrogen peroxide, quinones) during melanogenesis, the process of melanisation takes place in melanosomes, to avoid the interaction between them and other cytosolic components (Cichorek et al., 2013). Melanosomes, lysosome-related organelles, are the cellular site for synthesis, storage and transport of melanin granules that provide color to tissues and are involved in photoprotection. Melanosomes are present in mammalian skin melanocytes, in choroidal melanocytes and in retinal pigment epithelial (RPE) cells of the eye, and in chromatophores (pigment-containing cells) of amphibians, fish, reptiles, crustaceans and cephalopods (Wasmeier et al., 2008). There are four morphologically distinct stages of melanosome development: stage I pre-melanosomes are nonpigmented vacuoles; in stage II melanosomes acquire characteristic internal striations; in stage III melanin pigment is deposited onto the striations; and finally become mature fully melanised stage IV melanosomes (figure 8B) (Wasmeier et al., 2008).

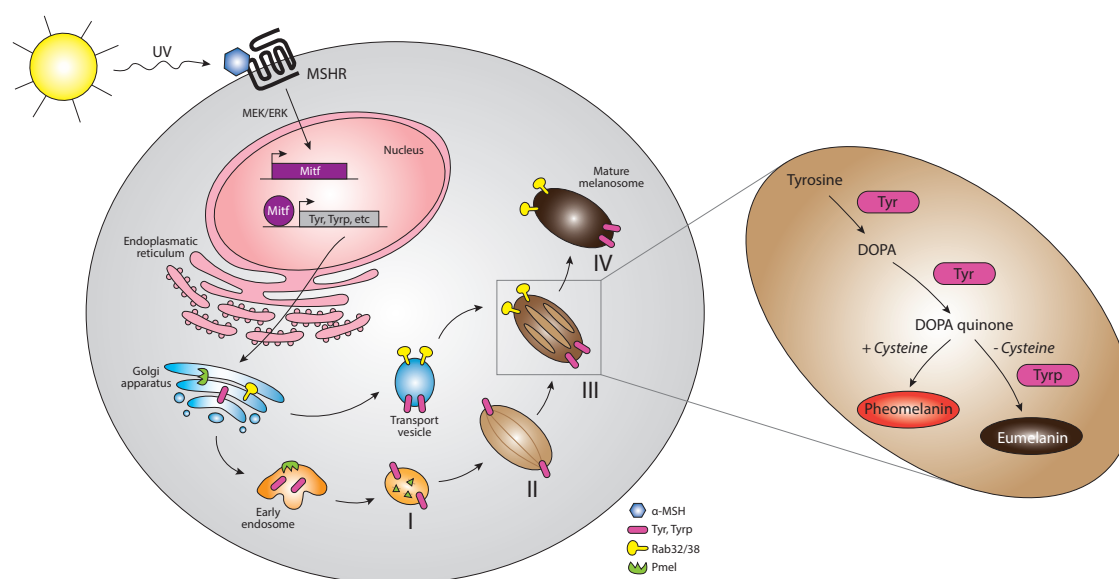


Figure 8. Vertebrate melanogenesis is regulated by the melanocyte-stimulating hormone receptor (MSHR).

Alpha melanocyte stimulating hormone (α -MSH) stimulates MSHR, a class A GPCR protein present in melanocyte membrane, to produce melanin. This will stimulate MITF transcription, which in turn activates transcription of melanogenesis key genes such as tyrosinase (Tyr), tyrosinase-related protein (Tyrp), Rab GTPase 32/38 (Rab32/38) and premelanosome protein (Pmel). The production of melanin occurs inside the melanosome, which undergo four stages (I-IV) until becoming totally mature (Wasmeier et al., 2008). Tyrosine undergoes hydroxylation to 3-4-dihydroxyphenylalanine (DOPA) and DOPA undergoes oxidation to DOPAquinone, both of which catalyzed by tyrosinase (Tyr), a crucial enzyme for the melanin biosynthetic pathway. The availability of substrates and the function of melanogenetic enzymes decide which kind of melanin is produced. DOPAquinone in the presence of cysteine give rise to yellow/red pheomelanin, or in the absence of cysteine DOPAquinone undergoes enzymatic processes, through tyrosine related proteins (Tyrp), to form dark brown/black eumelanin (Videira et al., 2013).

As I discussed above, ascidians, like vertebrates, possess key genes for melanogenesis such as *Mitf*, *Tyr*, *Tyrp* and *Rab32/38*, allowing the formation of ascidian pigment cells. However, while in vertebrates the MSHR is the key factor for melanin production in melanocytes, genome analysis showed that ascidians do not possess such receptors (Kamesh et al., 2008). If this is the case, which receptor is triggering ascidian melanogenesis? The transcriptional and post-transcriptional mechanisms governing melanogenic molecular pathways remain uncertain, but other pathways have been suggested to be crucial in vertebrate melanogenesis, such as the pathway *SCF-KIT receptor tyrosine kinase* (stem cell factor-kit receptor), *MAPK/ERK* signaling, or *Wnt* signaling. All these pathways activate *Mitf*, which in turn will trigger *Tyr/Tyrp* and *Rab* genes activation towards melanin production and transport (D'Mello et al., 2016).

1.4.2. Ascidian nuclear receptors: what is known?

It has been proposed that the gene duplications seen in vertebrates did not occur in cephalochordates and ascidians. Indeed, ascidians have a basic non-duplicated chordate-type genome (Satoh, 2003). One clear example are nuclear receptors (NRs), where we find only 17 NRs in *Ciona*, against 48 and 71 in human and zebrafish genomes, respectively (Bertrand et al., 2007; Yagi et al., 2003). More precisely, a single copy of *Ciona* NRs was evident in the groups of TRs, RARs, PPARs, RORs, Rev-erbs, RXRs, HNF4s, COUP-TFs, TR2/4, ERR, FTZF, GCNF and orphan receptors including NR41/42/43. Only in the group of VDR/PXR two *Ciona* proteins (α and β) were suggested. Surprisingly, genes encoding ER or other steroid hormone receptors (such as AR, MR, GR or PR) were not found in the ascidian genome (Yagi *et al.*, 2003), but were found in the amphioxus genome (ER and SR only), suggesting that the loss of steroid receptors in ascidians was an innovation and occurred independently of cephalochordates and vertebrates (Bertrand et al., 2011). In this section, I will review all the information known concerning nuclear receptors in ascidians and their roles in development, as well as their potential to bind EDCs.

1.4.2.1. Thyroid Receptor (TR)

The first study suggesting biogenesis of the thyroid hormones 3,5,3'-triiodothyronine (T3) and thyroxine (T4) in ascidians was published in 1962, with *Ciona* species (Roche et al., 1962). In 1993, Fredriksson and colleagues documented not only the presence of T3 and T4 in different *Phallusia mammillata* adult tissues (alimentary canal, pharynx and tunic), but also that T3 could bind to nuclear proteins present in the pharynx and alimentary canal, indicating the presence of a putative nuclear receptor for T3 and suggesting its potential physiological role in ascidians (Fredriksson et al., 1993). Later in 1998, Carosa and colleagues report the presence of a Thyroid Receptor (TR, previously named CiNR1), in the developing embryo and larva, but not in the analyzed adult tissues (endostyle, gonad and muscles) (Carosa et al., 1998). It was in 2001 when Patricolo and colleagues showed that exogenous T4 can accelerate *Ciona* larvae tail resorption, suggesting that thyroid hormones would trigger a specific nuclear signal involved in ascidian metamorphosis (Patricolo et al., 2001). This finding was within what has been found before in vertebrates, where thyroid hormones and TR were crucial for a correct metamorphosis in

Xenopus (Galton, 1992), and suggested later in *Ciona* (Staiano, 2011), raising the question about the presence of an endogenous ligand of ascidian TR. Even if it is known that TR is expressed in the embryo, its role is completely unknown.

1.4.2.2. Retinoic Acid and Retinoid X Receptors (RAR and RXR)

It is known that Retinoic acid (RA) regulates proliferation, differentiation and morphogenesis of various embryonic and adult cell types in vertebrates. RA signal is mediated by the heterodimer retinoic acid receptor/retinoid X receptor (RAR/RXR) (Botling et al., 1997; Chung and Cooney, 2003; Olivares et al., 2015; Rastinejad et al., 2000).

The first studies about this nuclear receptor were done in colonial ascidians, showing the crucial role of RAR in bud regeneration (Kamimura et al., 2000; Kawamura et al., 2013; Rinkevich et al., 2007). When it comes to solitary ascidians, it has been shown that *Ciona* RAR mRNA is expressed in the anterior ectoderm and endoderm during gastrulation, in the trunk endoderm and in the nerve cord in the tailbud embryo (Nagatomo et al., 2003). Exposing embryos to RA affected the closure of the neural tube and the formation of the adhesive papillae, but unlike vertebrates where RA upregulates RAR, significant upregulation of *Ciona* RAR was not observed in RA-treated embryos (Nagatomo et al., 2003). Nevertheless, RAR activation induces expression of *Hox1* in *Ciona* tailbud (and in the endostyle of juveniles) and of *Cyp26* (Ishibashi et al., 2003; Kanda et al., 2009), two key RAR target genes in vertebrates, indicating conservation in the GRN modulated by RAR.

In vertebrates, RXR α is predominantly expressed in the liver, RXR β is widely distributed and RXR γ is mostly restricted to the muscles and certain parts of the brain and the pituitary. *Halocynthia* RXR was highly expressed in the hepatopancreas and gills at adult stages (Maeng et al., 2012), but unlike human RXRs, *Halocynthia* RXR did not show retinoids-dependent transactivation. In embryos, both *Ciona* and *Halocynthia* RXRs were ubiquitously expressed throughout the development, with a stronger expression at early stages (Maeng et al., 2012; Nagatomo et al., 2003).

1.4.2.3. Liver X Receptor and Farnesoid X Receptor (LXR and FXR)

Liver X receptors (LXRs) are members of the NR1H subfamily, which includes Farnesoid X Receptors (FXRs) and Ecdysone Receptor (EcR, found exclusively in invertebrates). Whereas mammalian genomes possess two Liver X Receptors (LXR α and LXR β), non-mammalian vertebrates have only a single LXR gene, suggesting that the LXR gene was duplicated during mammalian evolution (Raslan et al., 2013). LXRs are key regulators of lipid and cholesterol metabolism. While LXR α is typically detected at high levels in adipose tissues, kidney, lung, and spleen, LXR β is ubiquitously expressed in vertebrate tissues. As for non-mammalian species, only one LXR copy is found in the ascidian genome. Transcriptomic data suggests high LXR expression in adults in gonadal tissue and neural complex, but lower expression in eggs, embryos and young adult animals, suggesting that LXR might be only crucial at adult stages (Reschly et al., 2008b). When studying LXR ligand specificity and evolution, Reschly and colleagues have shown that the *Ciona* LXR is not activated by the mammalian LXR agonists T-0901317 and GW3965, but by a limited number of oxysterols, as well as some androstane and pregnane steroids (Reschly et al., 2008b).

Farnesoid X Receptor (FXR) serves as the major transcriptional regulator of bile salt synthesis, in part by controlling the rate-limiting enzyme in bile salt synthesis. In vertebrates, FXR is typically expressed at high levels in the liver, intestine, kidney, and adrenal glands (Reschly et al., 2008a). While embryonic expression of *C. robusta* FXR (available at *Aniseed*) was very weak, adult animals showed expression in the digestive gland (Reschly et al., 2008a). The putative *Ciona* FXR was not activated either by any bile salts or by synthetic FXR agonists, also seen by docking analysis, which is perhaps not surprising given that invertebrates are not thought to produce bile salts. Screening of several compounds comprising steroid hormones, vitamins, and xenobiotics revealed that some sulfated steroids (pregnanolone sulfate, epipregnanolone sulfate and epiallopregnanolone sulfate) activated *Ciona* FXR. These findings suggest that the endogenous ligand(s) of *Ciona* FXR may be steroidal in nature. The homology model for *Ciona* FXR revealed a significantly smaller ligand binding pocket compared with that for human FXR (Reschly et al., 2008a). Hence, the sensitivity of *Ciona* FXR to sulfated steroids raises the possibility that an ancestral FXR, selective for invertebrate steroids or structurally related ligands, evolved to recognize bile salts during vertebrate evolution. This is an intriguing subject, since ascidian genomes do not possess any steroid-like receptors like ER, AR, GR or MR, in sharp contrast with cephalochordates, mollusks and annelids (Bertrand et al., 2011; Eick and Thornton, 2011). Moreover, it has been shown that primitive metazoans such as soft corals are able to produce sterols that antagonize human FXR (Putra et al., 2012). Some studies suggest that ascidians can produce steroids (Delrio et al., 1971; Imperatore et al., 2014) and FXR might be thus the receptor responsible to respond to them and to intervene in adult sexual maturation.

1.4.2.4. Pregnane X Receptor and Vitamin D Receptor (PXR and VDR)

As referred before, activity of most NRs is controlled not only by endogenous compounds, but also by xenobiotics. Vertebrate members of NR11 subfamily Pregnane X Receptor (PXR), Vitamin D Receptor (VDR) and Constitutive Androstane Receptor (CAR) are responsible to eliminate toxic endogenous and exogenous molecules (Reschly and Krasowski, 2006).

Pregnane X Receptors (PXR) are NRs responsible to sense toxic concentrations of a wide variety of endogenous and exogenous compounds and thus transcriptionally control detoxification pathways mainly in the liver and intestine. Because PXR binds different types of molecules such as bile salts, steroid hormones, vitamins and xenobiotics, in order to regulate their metabolism, transport, and excretion, they have a broad ligand pocket specificity. On the other hand, Vitamin D Receptor (VDR) bind 1,25-(OH)₂-vitamin D₃ (calcitriol) with high affinity and has been shown to influence a variety of physiological functions, as they are expressed in a wide range of organs/tissues that include brain, gut, heart, skeletal muscle, liver, and pancreas. Human CAR has a high constitutive activity in the absence of ligand, differing from PXR and VDR. Additionally, CAR genes have so far been detected only in vertebrates (Reschly and Krasowski, 2006).

Except for CAR, the presence of these NRs is quite diverse between vertebrates. Humans possess only one copy of both PXR and VDR, while some teleost fish have one copy of PXR but two VDR copies, and *Xenopus* possess two PXR copies but only one VDR (Bertrand et al., 2007; Reschly and Krasowski, 2006).

Intriguingly, cephalochordates do not possess any of these NR11 subfamily NRs (Lecroisey et al., 2012). Regarding the ascidian genome, two *Ciona* proteins were suggested in the group of PXR/VDRs (Yagi et al., 2003). Because the resolution within the clade was not high enough to deduce orthologous relationships among this group of proteins, it is possible that these two proteins are the ancestor of vertebrates CAR/PXR/VDR, and that these later evolved and diverged within vertebrates into distinct NRs. Since CAR are only found in vertebrates, *Ciona* proteins were therefore named as PXR/VDR α and PXR/VDR β .

It has been shown that *Ciona* PXR/VDR β does not respond to vitamin D ligands nor to bile salts, steroids and fat-soluble vitamins other than vitamin D (i.e., vitamins A, E, and K), but only by a small number of synthetic compounds including n-butyl-p-aminobenzoate, carbamazepine (CBZ) and 6-formylindolo-(3,2-b)-carbazole (Ekins et al., 2008). Richter and Fidler have recently shown that *Ciona* LBD of PXR/VDR α binds both natural marine biotoxins, like okadaic acid and pectenotoxin-2, and synthetic molecules such as the insecticide esfenvalerate, the pharmaceutical carbamazepine (CBZ) and the plasticizer bisphenol A (BPA), within the micromolar range (Fidler et al., 2012; Richter and Fidler, 2015). With CBZ being used in medicine for the treatment of epilepsy and neuropathic pain, and BPA being used as a plasticizer in several daily-life plastic materials, these chemicals can easily be found in wastewater effluents. Additionally, Imperatore and colleagues found that the sterols produced by *Phallusia fumigata* (phallusiasterols) were able to activate human PXR (Imperatore et al., 2014). This suggests that the ascidian PXR/VDR proteins are probably closer to vertebrate PXR than VDR in terms of function, since their ligand pocket seem to respond more to xenobiotics than vitamins, in an attempt of chemical defense from potential xenobiotics present in the marine environment, or suggesting that ascidian PXR/VDR acts as xenobiotic receptors.

1.4.2.5. Estrogen-Related Receptor (ERR)

It is important to note that on the 48 genes encoding for NRs in human, still half are considered orphan, meaning that no endogenous ligand is known for these receptors (figure 3E). Among them, there is the Estrogen-Related Receptor (ERR). ERRs are present throughout evolution, with three ERR copies in the human genome (ERR α , ERR β , and ERR γ), but with a single orthologue present in different invertebrate (fly, amphioxus and ascidians) genomes. In terms of sequence, ERRs are similar to the ERs in the DBD regions, and their target genes are often the same, but the lower conservation level of the LBDs is consistent with the incapacity of ERRs to bind estradiol (E2) (Coward et al., 2001; Horard and Vanacker, 2003).

Until now, ERRs were mostly studied for two main reasons: their potential involvement in cancer and their implication in the regulation of energy metabolism (Bardet et al., 2006), mainly due to their expression in several tissues like liver, skeletal muscle, immune cells, bones and heart (Huss et al., 2015). Additionally, recent studies also point out the presence of ERRs in the brains of a broad range of chordate species such as human, zebrafish (Bertrand et al., 2007) and amphioxus (Bardet et al., 2005), with suspected implication in neurodevelopment (Saito and Cui, 2018). Spatial ERR expression in ascidians is not known but during embryonic development expression is quite divergent: in the stolidobranch

Halocynthia roretzi, ERR was detected mainly in eggs and early stages of embryonic development (Park et al., 2009), while in another stolidobranch *Herdmania* ERR was ubiquitously expressed throughout embryonic development (Devine et al., 2002), and the phlebobranch *Ciona* transcriptomic data suggests a higher expression in eggs and larval stage but not inbetween. To this date, there is no data indicating ERR presence in the brain of ascidian larvae.

1.4.2.6. Other nuclear receptors

There are few other NRs present in ascidian genomes, such as PPAR, Rev-erb, ROR, HNF4, TR2/4, COUP, NR4A1/2/3, NR5A and GCNF, but there is no information about their expression and roles in ascidians. Nevertheless, I will briefly describe the known expression and roles in vertebrates for later discussion.

Peroxisome proliferator-activated receptors (**PPARs**) are NRs involved in lipid metabolism and energy homeostasis, being activated mainly by fatty acids, but also by eicosanoids and antidiabetic agents (Green, 1995; Michalik et al., 2002). Its expression in vertebrates is quite widespread, as humans possess three PPAR genes: PPAR α is expressed in adult liver, kidney, heart, large intestine and skeletal muscle; PPAR β shows a higher expression in the digestive tract and placenta; PPAR γ is abundantly expressed in adipose tissue (Michalik et al., 2002). Furthermore, human and mouse PPARs were recently shown to be expressed in brain tissues (Warden et al., 2016).

In humans, **Rev-erba** is transcribed from the opposite strand of the thyroid receptor α (*c-erbA α*) and hence its name is derived from “reverse strand of ERBA”. Both Rev-erba and Rev-erb β are widely expressed throughout the body, and have an atypical LBD that lacks the AF-2 region. **RORs** are RAR-related orphan receptors, with three copies available in the human genome (ROR α , ROR β , and ROR γ). While ROR α and ROR γ are widely expressed in many tissues (liver, thymus, skeletal muscle, skin, lung, adipose tissue and kidney), ROR β is found in regions of the CNS that are involved in the processing of sensory information, the retina and the pineal gland (Kojetin and Burris, 2014). There is considerable overlap in the DNA response elements that are recognized by Rev-erbs and RORs, and both receptors are often co-expressed in the same tissues. Because RORs constitutively activate transcription, whereas Rev-erbs repress transcription, the balance of ROR and Rev-erb activity is crucial for the dynamic regulation of target genes involved in circadian rhythm (Kojetin and Burris, 2014). Interestingly, an *in silico* study showed that ROR γ would probably bind BPA with high affinity (AC50: 0.89 μ M) (Montes-Grajales and Olivero-Verbel, 2013), and clock/circadian rhythmic pathway-associated genes from the mangrove killifish *Kryptolebias marmoratus* were affected after BPA exposure (Rhee et al., 2014), suggesting that EDCs might have an effect in circadian rhythm via ROR/Rev-erb NRs.

In humans, the Hepatocyte Nuclear Factor 4 alpha (**HNF4 α**) is mainly expressed at high levels in the liver and kidney. HNF4 α 's LBD interacts with endogenous fatty acids, such as linoleic acid, and regulates important hepatic functions such as glycolysis, gluconeogenesis, fatty acid metabolism, among others (Walesky and Apte, 2015). It appears to play a role on differentiation of visceral endoderm, and studies suggest that it can directly regulate PXR expression during fetal liver development and thus

regulate PXR responses to xenobiotics (Chung and Cooney, 2003). Today, the potential binding of EDCs to vertebrate HNF4 is not known, and no data concerning the function of the ascidian HNF4 is available.

The testicular nuclear receptors have two members in the human genome, **TR2** and **TR4**. TR2 is highly expressed in the testes as well as in several other tissues, including brain, retina, kidney, and intestine, with the highest peak of expression during embryonic time points. TR2 has the ability to act as a repressor of gene expression. TR4 is expressed in several tissues from the CNS, adrenal gland, spleen, and prostate gland with the highest expression observed in the skeleton and testes. Unfortunately, there is no information about these NRs in ascidians.

COUP-TFs (for Chicken Ovalbumin Upstream Promoter-Transcription Factors) genes bring a major contribution to neurogenesis and neurophysiology in vertebrates, and its orthologs have been found from the earliest branched phyla of metazoan evolution (ctenophores) up to deuterostomes (Gauchat et al., 2004). Besides neurogenesis, vertebrate COUP-TF has been shown to play a crucial role in eye development, since mutations of COUP-TF genes lead to coloboma and/or optic atrophy in both mouse and human (Tang et al., 2015).

The **NR4A** subfamily consists of three structurally related transcription factors: NR4A1, also called NGFIB (nerve growth factor IB) or Nur77; NR4A2 or NURR1 (Nuclear receptor related 1 protein); and NR4A3 or NOR1 (neuron-derived orphan receptor 1). These NRs regulate cell context dependent cell fate decisions in response to extracellular signals including inflammatory, genotoxic (property of a chemical to damage the genetic information within a cell causing mutations, which may lead to cancer), apoptotic, and mitogenic stimuli. NR4A1, NR4A2 and NR4A3 are orphan nuclear receptors and immediate early genes induced by multiple stressors. All three receptors bind the same genomic cis-elements; however, their distinct differences in activities are due, in part, to their more unique N- and C-terminal domains that differentially interact with various cofactors and ligands and their tissue-specific expression (Safe et al., 2016).

In humans, **NR5A** orphan receptors includes Steroidogenic Factor 1 (SF1, NR5A1) and Liver Receptor Homolog 1 (LRH1, NR5A2). SF-1 has a restricted pattern of expression (adrenal cortex, gonads, spleen, pituitary gonadotropes, hypothalamic VMH) and is required for the development of adrenal glands and gonads of both sexes. SF-1 was first described to regulate the expression of genes involved in steroidogenesis and reproductive function, but recent studies have shown its important role also in angiogenesis, cell adhesion, cytoskeleton dynamics, proliferation, apoptosis, transcriptional and post-transcriptional regulation of gene expression. LRH-1 is expressed at high levels in embryonic stem cells and is essential for embryonic development. In the adult vertebrate, LRH-1 has an essential function in ovulation and pregnancy. LRH-1 also has an important role in the gastrointestinal system by regulating bile acid biosynthesis, enterohepatic circulation and glucose sensing in the liver (Ruggiero et al., 2015).

In humans, germ cell nuclear receptor (**GCNF**) is highly expressed in germ cells, and is highly expressed during gametogenesis in adults (Wang and Cooney, 2013). GCNF has been shown to be a transcriptional repressor, which recruits various corepressor complexes to repress and silence gene transcription. Unlike most NR members, GCNF does not have a conserved AF-2 domain at the C terminal end of the LBD. GCNF is critical for repressing Oct4 gene activity as pluripotent stem cells differentiate

and thus play role in confining Oct4 expression to the germ line. Moreover, in vertebrates GCNF is essential for embryonic survival and it is required for normal posterior development, somitogenesis and neural tube closure (Chung et al., 2006; Gu et al., 2005; Wang and Cooney, 2013).

A few toxicological studies have shown that several known EDCs can affect brain development of the ascidian larvae (reviewed in Dumollard et al., 2017). However, modes of action have never been proposed and whether NRs participate in the expression of the phenotype is currently unknown.

2. METHODOLOGIES

2.1. Animals

Adult *Phallusia mammillata* were collected in Sète (Hérault, France), and kept at 17±1°C in circulating seawater aquaria. They were reared under constant light conditions to avoid uncontrolled spawning of eggs and sperm (Lambert and Brandt, 1967).

For exposure experiments, eggs and sperm were collected separately by dissecting the gonoducts of several hermaphrodite adults. Sperm was stored at 4°C, while eggs were placed in natural filtered (0.2 µm) seawater (FSW) supplemented with TAPS buffer (0.5 mM) and EDTA (0.1 mM) (Sardet et al., 2011). To allow fluorescent microscopy analysis, chorion was removed from eggs by incubating in a trypsin solution for 2 hours, and then washed 3 times before use (Sardet et al., 2011). Eggs were fertilized with a sperm dilution (1:100) for 10 min, then washed 3 times and left in seawater (FSW/TAPS/EDTA) at 18°C until use. Since *Phallusia mammillata* development is very similar to *Ciona*, all referred developmental stages are based on *Ciona* development (Hotta et. al 2009). The details of all protocols for embryos handling can be found online at <http://lbdv.obs-vlfr.fr/fr/ascidian-biocell-group.html>.

2.2. Chemical products

For embryo culture, TAPS buffer (CAS Nr. 29915-38-6) and EDTA (CAS Nr. 60-00-4) were used. For toxicity studies, the following chemicals were diluted in dimethyl sulfoxide (DMSO, CAS Nr. 67-68-5): Bisphenol A (BPA, CAS Nr. 80-05-7), Bisphenol E (BPE, CAS Nr 2081-08-5), Bisphenol F (BPF, CAS Nr 620-92-8), Diethylstilbestrol (DES, CAS Nr 56-53-1), GSK4716 (CAS Nr 101574-65-6), Phenylthiourea (PTU, CAS Nr. 103-85-5), UO126 (CAS Nr. 109511-58-2), β-Estradiol 3-benzoate (E2B, CAS Nr 50-50-0), 2,2-Diphenylpropane (2,2DPP, CAS Nr 778-22-3), 4-Hydroxytamoxifen (4-OHT, CAS Nr 68392-35-8). For F-actin and DNA staining, phalloidin-tetramethylrhodamine B isothiocyanate (Phalloidin-TRITC, Santa Cruz Biotechnology) and Hoechst (CAS Nr. 23491-52-3) were used, and samples conserved with Citifluor AF1 antifade mounting solution (Biovalley). All chemicals were purchased from Sigma Aldrich, unless stated otherwise.

2.3. Exposure experiments

Exposure experiments with BPA, BPE, BPF, 2,2 DPP, E2B, GSK4716, DES, 4-OHT and PTU were performed similarly. Chemicals were dissolved in DMSO to have a 0.5 M main stock solution. After fertilization, stage 1 embryos were transferred to chemical-containing seawater (FSW-TAPS-EDTA). Due to a possible confusion arising from differences in the concentrations of DMSO, each solution was diluted in DMSO to a concentration 10,000 times higher than required, and again diluted in filtered seawater (1:10000), giving the required concentration of chemical in an overall solution of 0.01% DMSO in seawater. As a control treatment, 0.01% DMSO seawater was used.

For the exposure experiment with UO126, the chemical was dissolved in a 0.04 M DMSO stock solution. Embryos were fertilized and left to develop at 18°C until stage 16. Embryos were then exposed at stages 16 and 18, at a final concentration of 4 µM defined from a previous study (Racioppi et al., 2014). A control treatment with 0.01% DMSO was performed.

At stage 26, larvae were fixed in 4% paraformaldehyde (4% PFA, 0.5 M NaCl, PBS; Sigma), washed 3 times in Phosphate Buffered Saline (PBS 1X) and imaged by light microscopy (*Zeiss Axiovert 200*) at 10x magnification. Each experiment was replicated at least 3 times, unless stated otherwise.

2.4. Embryo morphology analysis

For general morphology analysis, larvae (st. 26) were analyzed by scoring the percentage of normal (good general embryo morphology, with proper trunk and palps formation, as well as tail elongation), malformed (embryos with bent tail) and not developed embryos (with arrested development before gastrulation/tail extension) (see figure S1A). Statistical analysis was performed as described in section 2.11.

For trunk morphology analysis, the area of each PC (*Oc/Ot area*, μm^2), the distance between the two PCs (*Oc/Ot distance*, μm) and the length/width ratio of the trunk were measured (see figure 1A). The morphological analysis was performed using *Toxicosis8*, a software developed in our laboratory (software is deposited for protection by the SATT Lutech). The resulting data was normalized to each respective control treatment (100%) and plotted in radar charts for better comparison of phenotypes between chemicals. Statistical analysis was performed as described in section 2.11.

2.5. BPA time-window action

To assess the time-window action of BPA during *Phallusia mammillata* embryogenesis, embryos were incubated in BPA-containing seawater at different times during embryogenesis. In a first phase, embryos were exposed either 6 hours before fertilization (6 *hbf*), either 6 hours after fertilization (6 *hpf* is the time to reach gastrulation at stage 10) or both for a total of 12 hours exposure to BPA, and then washed and incubated in FSW-TAPS-EDTA up to stage 26. In a second phase, embryos were exposed at different stages (10, 12, 14, 16, 18, 20, 22 and 24) until stage 26. At stage 26, embryos from all treatments were fixed (4% PFA) for later analysis of pigment cell area (*Oc/Ot area*, μm^2). For a matter of technical feasibility, embryos were cultured at 14°C from fertilization to stage 16 and then placed at 18°C from stage 16 to stage 26. Statistical analysis was performed as described in section 2.11.

2.6. Immunofluorescence

To assess BPA potential effects on mitotic spindle formation, microtubule organization during the first mitosis was assessed. For this, eggs were fertilized and immediately exposed to 0.01% DMSO or BPA 40 μM . Sixty-four minutes after fertilization (during the first mitosis), embryos were fixed in cold 100% methanol overnight. They were then progressively rehydrated in PBS/0.02%Triton, permeabilized in PBS/0.25%Triton for 15 min at RT, and then incubated in primary and secondary antibodies in PBS/1%BSA overnight at 4°C (for each antibody incubation, fixed embryos were washed for 15 min for 4 times with PBS/0.1%Tween). Fixed embryos were stained for microtubules with primary antibody anti- α -tubulin (DM1A; Sigma-Aldrich 1:1000) and mouse FITC secondary antibody (Jackson) diluted 1:50. To stain DNA,

immunofluorescence labelled embryos were mounted in VectaShield that contains DAPI (Vector Laboratories) analyzed with epifluorescence microscopy (40X objective, Zeiss Axiovert 200).

Cilia of neurohypophysial duct and neural tube were assessed by immunostaining with anti-acetylated α -tubulin antibody according to previously published protocols (Sardet et al., 2011). Stained larvae were imaged by confocal microscopy (Leica, SP8).

2.7. Analysis of otolith movement by timelapse imaging

To follow otolith development, eggs were injected with reporter plasmids bearing promoter region for *Ciona* genes driving the expression of GFP based reporters. The promoters used are Tyrosinase-related protein (pTyrrp, kind gift from Filomena Ristoratore), β Crystallin (p β C, kind gift from Phil Abitua) and Muscle segment homeobox (pMsx, kind gift from Phil Abitua) to visualize pigment cell precursors. The GFP-based reporters were either Venus/Cherry (cytosolic), H2B::Venus/Cherry (DNA) or Lifeact::Venus/Cherry (actin-rich cell cortex). To visualize the plasma membrane of all cells mRNAs coding for PHdomain::Tomato was injected in unfertilized eggs as previously described (Sardet et al., 2011). Injected eggs were fertilized 2 hours after injection and left to develop at 16°C until stage 10 (gastrula). At stage 10, embryos were either transferred to 0.01% DMSO (figure 2A) or to BPA-containing seawater (10 μ M, figure 2B), and at stage 20 (early tailbud) embryos were mounted on a slide for time-lapse live imaging using confocal microscopy (Leica SP8).

2.8. Analysis of otolith foot by Phalloidin staining

To analyze the otolith foot structure, embryos were exposed to BPA analogues BPE, BPF and 2,2 DPP (section 2.3). Once at the desired stage (st. 26), embryos were fixed with 4% PFA overnight at 4°C. Embryos were washed twice with PBS 1X, incubated in PBS-BSA solution (3% BSA diluted in PBS 1X) for 1h and stained with Phalloidin-TRITC and Hoechst diluted 1:500 in PBS-BSA, for 3h at RT. Embryos were washed twice in PBS-BSA, once in PBS and stored in AF1 antifade mounting solution Citifluor (Biovalley) until analysis. Phenotypes were compared to a control treatment (0.01% DMSO) using confocal microscopy (Leica SP8). Please note the same final concentration was used for all molecules (10 μ M), with the exception of 2,2DPP (25 μ M), since no phenotype was visible at 10 μ M, 25 μ M nor 100 μ M.

2.9. mRNA expression pattern analysis by in situ hybridization

mRNA expression pattern of *Phallusia mammillata* nuclear receptors (NRs), as well as pigment cell (PC) and central nervous system (CNS) gene specification lineages were assessed by *in situ* hybridization (ISH) technique. For this, embryos were fixed overnight at 4°C (ISH fix: 4% formaldehyde, 100 mM MOPS, 0.5 M NaCl, pH 7.6), washed in PBS, dehydrated in ethanol and stored at -20°C until analysis. *Phallusia* ISH was performed as previously described (Paix et al., 2009).

ISH probes covering the entire cDNAs were designed based on *Aniseed* (<http://www.aniseed.cnrs.fr/>) and *Octopus* (<http://octopus.obs-vlfr.fr/index.php>) databases and were

obtained from the *Phallusia mammillata* cDNA library (pExpress-1 vector based) available in our laboratory. After amplification by PCR and confirmation by electrophoresis, PCR products were purified (Qiagen MinElute PCR purification kit, Qiagen) and DNA quantified. Digoxigenin-labeled antisense RNA probes (DIG RNA Labeling Mix, Roche) were then prepared and detection was based on NBT/BCIP (Roche) principle. Within the 17 NRs present in the ascidian genome, 3 were not successfully cloned due to absence of information in both *Aniseed* and *Octopus* databases. Gene ID information is available in table I of Annexes. The imaging of all embryos was performed using an optical microscope (*Zeiss Axiovert 200*) microscope.

2.10. *P. mammillata* estrogen-related receptor (ERR) analysis

The sequence for *Phallusia mammillata* ERR (*Pm-ERR*) is published on *Aniseed* database (gene ID: *Phamm.g00012306*) and was cloned from *Phallusia mammillata* cDNA library, as described in section 2.9.

2.10.1. *Pm-ERR* gene activity by promoter cloning

Gene activity was assessed by cloning the promoter region of ERR into a vector driving the expression of a GFP reporter. For this, a 1.7 kbp fragment (-1705 from the Transcription Start Site, *TSS*, figure 5C) was selected from ascidian genome available on *Aniseed*. This region, which is highly conserved with *Phallusia fumigata*, was amplified by PCR from *P. mammillata* genomic DNA and cloned upstream a H2B::Venus reporter by In-Fusion HD Cloning Plus kit (Takara Bio). The prepared construct (*pERR*>H2B::Venus) was microinjected in *P. mammillata* eggs and the transgenic embryos analyzed by confocal imaging (Leica TCS SP8).

2.10.2. *Pm-ERR* protein levels analysed by a specific *Pm-ERR* antibody

To determine protein presence by immunoblot, an antibody against the ERR protein was made by producing the protein and sending it to Covalab (France) to immunize mice. Four anti-serum were screened and results from the most reactive anti-serum are shown. The anti-serum was further validated as described in figure 5D. In order to confirm the antibody specificity, the antibody was tested against exogenous *Pm-ERR* (*Pm-ERR*::Venus mRNA injected larvae).

2.10.3. *Pm-ERR* in silico analysis

The sequence of *Pm-ERR* was analysed together with the three human ERRs (NCBI Protein accession numbers: AAH63795.2 for ERR α , EAW81258.1 for ERR β and AAQ93381.1 for ERR γ), four zebrafish ERRs (AAS66634.1 for ERR α , AAS66635.1 for ERR β , AAI63298.1 for ERR β - γ , AAS66636.1 for ERR γ and AAS66637.1 for ERR δ), two *Xenopus* ERRs (AAI60530.1 for ERR α and NP_001093680.1 for ERR γ), the single amphioxus ERR (AAU88063.1) and several ascidian ERRs such as *Ciona robusta* (*Aniseed*: *Cirobu.g00013110*), *Halocynthia roretzi* (*Aniseed*: *Harore.g00011080*), *Ciona savignyi* (*Aniseed*: *Cisavi.g00006479*) and *Molgula oculata* (*Aniseed*: *Moocul.g00008753*). Human Estrogen Receptors (AAG41359.1 for ER α and AAC05985.1 for ER β) were used as outgroup. Phylogenetic tree was generated

based on maximum likelihood analysis (PhyML-LG), with 1000 replicates bootstrap, using Seaview free software (supplementary figure 1A in Annexes). *Pm*-ERR aminoacid sequence was compared to human ERR γ , as well as with *C. robusta* and *H. roretzi*, and the most important domains were highlighted (supplementary figure 1B-C in Annexes).

2.11. Statistical analysis

All presented data show mean with error bars indicating standard deviation (mean \pm SD). The number of embryos (*n*) analysed for each graph is indicated in the figure legend. For figure 1C and figure 4, descriptive statistics is depicted in table I. For figure 1D, *n* and *P values* are indicated in the figure legend.

GraphPad Prism[®] software (GraphPad Software, Inc. CA, USA) was used to calculate median lethal (LC₅₀) and effective (EC₅₀) concentrations, as well as to perform analysis of variance ($\alpha=0,05$) to evaluate differences between treatments. Since data did not follow a Gaussian distribution (Shapiro-Wilk normality test), nonparametric Kruskal-Wallis test was performed and statistical differences between control and treatments were assessed by Dunnett's test.

3. RESULTS

I. Embryonic expression of *Phallusia mammillata* nuclear receptors

Context

As I described in my introduction, scarce data is available on ascidian nuclear receptors. Because my main aim is to study the potential involvement of nuclear receptors on bisphenol A neurodevelopmental toxicity in ascidian larvae, I first needed to study *Phallusia mammillata* nuclear receptors expression throughout embryonic development. For this, I analysed the spatial expression (by ISH technique) of the 17 nuclear receptors found in *P. mammillata* genome during development. Unfortunately, due to technical issues, I could not clone 3 nuclear receptors (FXR, NR4A1/2/3 and NR5A), thus my following results will only refer to 14 out of 17 *P. mammillata* nuclear receptors.

Results

The expression pattern of *Phallusia mammillata* nuclear receptors is presented in figure 9. In the following paragraphs I will describe the expression patterns obtained and discuss the implications of such expression patterns, since I will not discuss this part of my results in section 4.

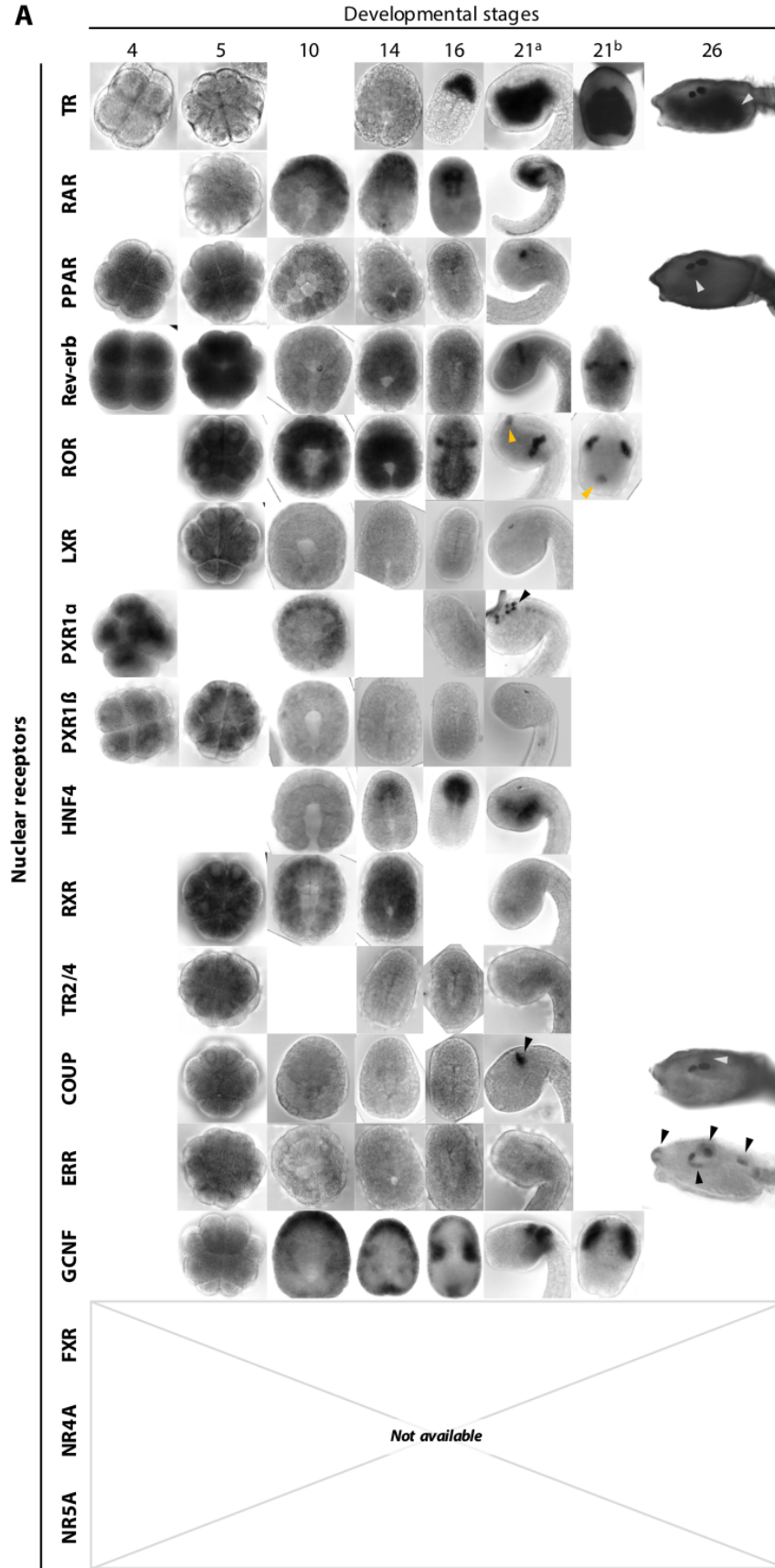
3.1.1. *Phallusia mammillata* nuclear receptors

Pm-TR mRNA is first detectable at late neurula stage, and is strongly remarkable in endodermal cells later at tailbud (stage 21) and larva (st. 26, white arrowhead) stages. Such expression is in agreement with the available transcriptomic data from *Aniseed* and is also similar to *Ciona* TR expression proposed by Carosa and colleagues (Carosa et al., 1998). *Ciona* TR was suggested to be important for larvae metamorphosis (Staiano, 2011). Because *Ciona* and *Phallusia* expressions are similar, we can suggest a role of TR in *Phallusia* metamorphosis that needs to be confirmed by functional studies. Furthermore, *Pm-TR* is expressed underneath the ascidian brain, it would thus be interesting to study whether *Pm-TR* is essential for ascidian brain development as it is for vertebrates (Bernal, 2007).

Pm-RAR is expressed in the anterior ectoderm and endoderm during gastrulation, and in the head endoderm and in the nerve cord in the tailbud embryo (st. 21). **Pm-RXR** is ubiquitously expressed throughout embryonic development with a more accentuated maternal expression. *Phallusia* RAR and RXR expression patterns were in concordance with the available transcriptomic data and with the described patterns in *Ciona* and *Halocynthia* (Maeng et al., 2012; Nagatomo et al., 2003). This suggests that RAR and RXR in different ascidian species might have similar roles, with RAR probably involved in neural tube closure, and RXR most likely an important partner in NR heterodimer binding to DNA, as shown in vertebrates (Kojetin et al., 2015).

Transcriptomic data of **Pm-PPAR** suggests a slight maternal expression which then decreases at gastrulation (st. 10-12), and slightly increases again at stages later stages (st. 22-26). My ISH data is in concordance with the transcriptomic data, interestingly with few PPAR positive cells at tailbud (st. 21) and larva (st. 26) within the ascidian brain (white arrowhead at st. 26). Nonetheless, no information about this NR role in ascidian embryonic development is known so far.

A



B

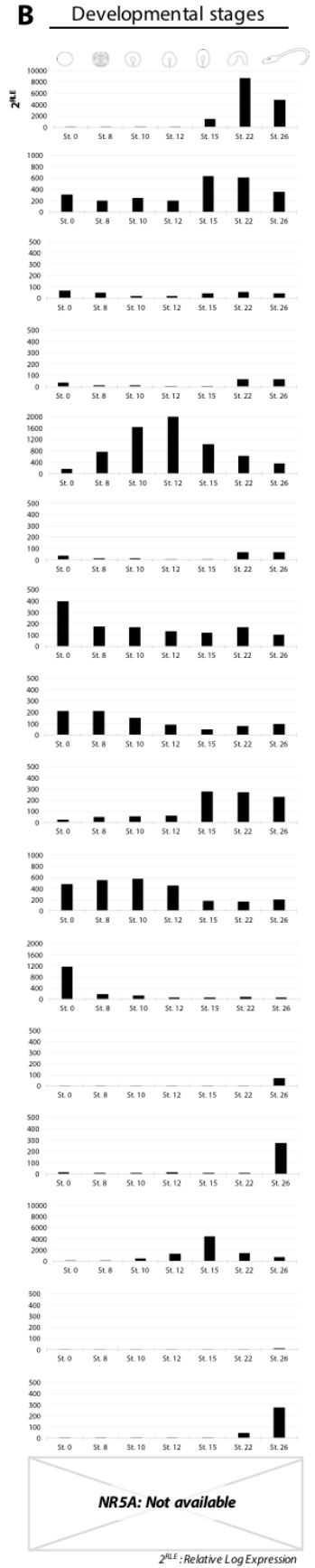


Figure 9. Embryonic expression of *Phallusia mammillata* nuclear receptors. (A) Spatial expression pattern of 14 *P. mammillata* NRs were assessed by *in situ* hybridization technique, at different developmental stages. Embryos from stage 5 to 16 are anterior to the top, while stages 21^a and 26 are anterior to the left. Stage 21^a denotes expression viewed from the lateral side, and stage 21^b denotes expression viewed from the dorsal side, anterior to the bottom. **(B) Transcriptomic data of *P. mammillata* NRs** available from the ascidian database *Aniseed*. Y axis denotes expression in 2^{RLE} for *Relative Log Expression*. X axis refers to the different developmental stages. Please note that Y axis of each NR was adjusted for better clarity.

Transcriptomic data suggests that ROR is overall more expressed than Rev-erb. ***Pm-ROR*** is ubiquitously expressed from 16-cell stage (st. 5) to tailbud (st. 21), very likely in trunk ventral cells (TVC). On the contrary, ***Pm-Rev-erb*** is ubiquitously expressed in 8/16-cell stages (st. 4 and 5) but not during gastrulation/neurulation, and then becomes expressed again at tailbud (st. 21), also likely in TVC. Both ROR and Rev-erb are known to actively participate in vertebrate circadian rhythm (Kojetin and Burris, 2014), and we might suggest a similar role in ascidians considering *Phallusia* ROR and Rev-erb ISH data. ISH at tailbud (st. 21) shows that both ROR and Rev-erb have partially overlapping expression in TVC. After tailbud stage, these cells divide in two different lineages, the heart and the atrial siphon muscle (ASM) (Tolkin and Christiaen, 2016). Moreover, ROR expression in few cells positioned anteriorly to the brain (yellow arrowhead at st. 21) seems to correspond to trunk lateral cells (TLC) that after some divisions give rise to oral siphon muscle (OSM), before becoming the future oral siphon in the adult stage (Tolkin and Christiaen, 2016; Veeman et al., 2010). Ascidian siphons contain photoreceptor and muscle cells, able to sense light and to contract respectively, allowing phlebobranchian ascidians to spawn in a light:dark dependent cycle (Eakin and Kuda, 1971). It is thus tempting to suggest that ascidian ROR/Rev-erb might also regulate the circadian rhythm in adult ascidians.

Transcriptomic data on ***Pm-LXR*** indicates a weak but ubiquitous expression throughout embryonic development, yet by ISH there is not a clear signal except a gentle expression at 16-cell stage (st. 5). Reschly and colleagues suggested that ascidian LXR might only be crucial in adult stage to respond to oxysterols (Reschly et al., 2008b), which would go along with both transcriptomic data and my obtained ISH data for embryonic stages.

The two ascidian PXR/VDR showed very distinct expression. ***Pm-PXR/VDR α*** showed a maternal expression, followed by no zygotic signal throughout development until tailbud (st. 21) where interestingly few cells within the sensory vesicle (black arrowhead at st. 21) were seen to express *Pm-PXR/VDR α* . Concerning ***PXR/VDR β*** , ISH data suggests a slight maternal signal but no expression in the remaining developmental stages. It is believed that ascidian PXR/VDR proteins are probably closer to vertebrate PXR than VDR in terms of function, probably acting as xenobiotic receptors. Indeed, some studies showed that *Ci-PXR/VDR α* could bind marine microalgal biotoxins and synthetic toxicants such as bisphenol A and carbamazepine (Fidler et al., 2012; Richter and Fidler, 2015).

Pm-HNF4 is expressed in the endoderm from late gastrula (st. 12) up to tailbud (st. 21), similarly to the available transcriptomic data and to vertebrate embryos. In mouse liver development, HNF4 α is expressed in the primary and extraembryonic visceral endoderm prior to gastrulation (Walesky and Apte,

2015). In adult vertebrates, HNF4 α is highly expressed in liver and kidney, and in the small intestine, colon, and pancreatic β cells in lower quantity, participating in important functions such as glycolysis, gluconeogenesis, fatty acid metabolism, among others (Bertrand et al., 2007; Walesky and Apte, 2015). Ascidian larval endodermal cells give rise to various endodermal organs, including endostyle, branchial sac, peribranchial epithelium, peripharyngeal band and digestive organs (Hirano and Nishida, 2000), suggesting that ascidian HNF4 might have a similar role in adult ascidians and vertebrates.

Pm-TR2/4 seems to be a maternal gene, with no expression during embryonic life, as visible in both ISH and transcriptomic data. Since scarce data about these testicular receptors is available in vertebrates, and no information is available for ascidians nor cephalochordates, it is very hard to suggest what the function of this NR might be in basal chordates.

Pm-COUP is not expressed until tailbud (black arrowhead at st. 21), with an interesting expression in the ascidian brain and very likely in the future ocellus (white arrowhead at st. 26). This data is in agreement with the available transcriptomic data. COUP has been shown to play a role in neurogenesis from cnidarians to vertebrates (Gauchat et al., 2004). More precisely, in vertebrates COUP has been associated with eye development (Tang et al., 2015). Ascidian larvae do not possess paired lateral eyes as vertebrates, and while some studies suggest the ascidian ocellus to be equivalent to a very primitive ancestor version of vertebrate pineal gland (Kusakabe and Tsuda, 2007), other studies suggest it to be a primitive eye. Hence, *Pm-COUP* would give us a hint that ascidian ocellus may be closer to the vertebrate eye than the pineal gland, however this is very hypothetical and more studies are needed to understand the origin and evolution of the eye of basal chordates. Nevertheless, like in other invertebrates, *Pm-COUP* is more likely involved in ascidian neurogenesis and neuronal differentiation.

Transcriptomic data in *Phallusia (Aniseed)*, figure 9) and *Ciona (Aniseed)* suggests that ERR is expressed only in larva (st. 26). ISH analysis of **Pm-ERR** confirms the transcriptomic data and shows its exclusive expression in the brain (black arrowheads at st. 26). This expression contradicts observations in two stolidobranch ascidians *Halocynthia roretzi* and *Herdmania*, where ERR transcripts are detected (by PCR) mainly in eggs and early stages (st. 1-13 for *H. roretzi* (Park et al., 2009)) or from 64-cell stage to larva (st. 8-26 for *Herdmania* (Devine et al., 2002)). The proposed implication of ERR in human neurodevelopmental disorders (Saito and Cui, 2018) and the ascidian ERR expression exclusively in the larval brain makes this NR an interesting candidate to mediate the effect of bisphenol A on brain malformation in ascidians (see section 3. Results - part II).

Pm-GCNF is highly expressed between gastrula and tailbud (st. 10 to 21), with a clear expression in what seems to be mesenchyme (Tokuoka et al., 2004), but not in the germ cell lineage. Time of expression of *Pm-GCNF* from gastrula to larva occurs similarly as in mouse and *Xenopus* embryos, but not in similar tissues, as mouse GCNF is expressed mainly in the neuroepithelium and *Xenopus* GCNF in the neural plate and neural crest (Wang and Cooney, 2013), suggesting a different role of GCNF between ascidians and vertebrates.

3.1.2. Co-factors

Along with NRs, co-factors are an important key in NR regulation, either in NR activation (co-activators) or in NR repression (co-repressors) (Perissi and Rosenfeld, 2005). For example, in vertebrates, ERR is known to interact with co-activators such as PGC1 α (peroxisome proliferator-activated receptor gamma coactivator 1 alpha) and NCOA/SRC1 (nuclear receptor coactivator/steroid receptor coactivator 1) (Hentschke et al., 2002).

After a quick *BLAST* analysis in *Aniseed* database (please refer to gene ID's in table S1 in Annexes), I found the potential presence of 3 coactivators and 1 corepressor in both *C. robusta* and *P. mammillata* genomes. The potential coactivators are NCOA, PGC1 and CBP/p300 (CREB-binding protein/histone acetyltransferase p300), and one single nuclear corepressor (NCOR). Additionally, in the same way as the cephalochordate genome (Schubert et al., 2008), there is a potential orthologue of the vertebrate MED1/TRAP220 (mediator complex subunit 1/thyroid hormone receptor-associated protein 220 kDa component). When assessing *P. mammillata* transcriptomic expression (2^{\wedge} LE *relative log expression* units, available at *Aniseed* database), *Pm*-NCOA shows a very low homogeneous expression (~10) throughout development. *Pm*-PGC1 shows an expression higher in eggs and at later stages 21-26 (~2,000). *Pm*-CBP/p300 is highly expressed in eggs (15,000) and still shows high homogeneous expression throughout development (5,000), and *C. robusta* ISH data shows expression in the CNS. Finally, *Pm*-NCOR and *Pm*-TRAP220 show a good homogeneous expression throughout development (~1,250 and ~2,000 respectively).

This is, to some extent, similar to cephalochordates, where vertebrate orthologues of 3 coactivators (NCOA, PGC1 and CBP/p300), 2 corepressors (NCOR and SHP (for Small Heterodimer Partner, or NR0B)) and one mediator complex (TRAP220) were identified. The main difference is that ascidians do not possess SHP (NR0B), like previously reported (Yagi et al., 2003).

Park and colleagues showed that transactivation of the ascidian *Halocynthia roretzi* ERR was strongly augmented by mammalian coactivators such as PGC-1 α and SRC-1, and decreased by the mammalian corepressors SHP and SMRT (Park et al., 2009), suggesting that ascidian ERR can function in mammalian cells and interact with mammalian NR cofactors. Because the ascidian orthologues of these regulators are present in the ascidian genome and expressed during embryonic development, it can be hypothesized that ascidian NR signaling is somewhat similar to vertebrates. However, because *BLAST* analysis revealed low levels of identity (low score/e-value), further studies are thus needed.

To sum up, the obtained expression patterns lead me to suggest that, within the 14 analysed NRs, 8 NRs (TR, RAR, RXR, Rev-erb, ROR, LXR, HNF4 and COUP) may have functions in the ascidian development in a similar way as vertebrates, while other NRs (PPAR, PXR/VDR α , TR2/4, ERR and GCNF) show a previously unknown and intriguing expression.

Interestingly, the vertebrate orthologues of the ascidian NRS expressed in the ascidian sensory vesicle (PPAR, PXR/VDR α and ERR) or nearby (TR), have been shown to bind known EDCs (some with high affinity like *Hs-ERR γ* (K_d BPA: 5nM)). Additionally, PXR/VDR α of the ascidian *Ciona robusta* has been shown (*in vitro*) to bind BPA (Richter and Fidler, 2015). Thus, these data strongly suggest that several classes of EDCs may exert neurodevelopmental toxicity to ascidian embryos.

3. RESULTS

II. Bisphenols disrupt differentiation of the ascidian pigmented sensory organ in the larval brain

Overview of the study, major findings and PhD contribution

In this study, I aimed to describe the phenotype induced by the endocrine-disrupting chemical bisphenol A (BPA) during *P. mammillata* embryonic development. Briefly, the findings presented in this manuscript are as follows:

- BPA induces embryo malformations between 5 μ M and 10 μ M (EC₅₀: 11.8 μ M) and is lethal for embryo development from 20 μ M (LC₅₀: 21.1 μ M)
- At 10 μ M, BPA induces neurodevelopmental toxicity to *P. mammillata* by disrupting pigment cell development, and more precisely by:
 - Reducing pigmentation
 - Reducing ocellus-otolith distance
 - Reducing sensory vesicle lumen
- BPA does not need to accumulate in the embryo to induce the pigment cell phenotype
- The pigment cell phenotype is also seen in the presence of other bisphenols (E and F) but not in the presence of a bisphenyl (2,2 diphenylpropane), suggesting bisphenol specificity
- Exposure of *P. mammillata* embryos to agonists and antagonists of the Estrogen-Related Receptor (ERR) partially phenocopies BPA phenotype, i.e., it reduces ocellus-otolith distance
- *Phallusia mammillata* possesses only one ERR in the genome, expressed around the pigment cells during their development (from stage 25).

The manuscript is going under the last modifications and the aim is to submit to *Open Biology*, *PLOS One*, *Aquatic Toxicology* or *Ecotoxicology and Environmental Safety* journals.

For the present manuscript, I performed the experiments that allowed me to accomplish the figures 1, 2, 3 and partially the figure 5. The experiments, as well as the analysis of the figure 4 were performed by a post-doc in the laboratory (Genia Gazo). In the figure 5, the *Phallusia* ERR antibody was produced by the technician of my host laboratory (Lydia Besnardeau), and the protein expression performed by Genia Gazo. Additionally, the supplementary figure 1B was performed by a master student (Dalileh Nabi).

Bisphenols disrupt differentiation of the ascidian pigmented sensory organ in the larval brain

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Abstract

Bisphenol A (BPA) is a widely employed molecule in polycarbonate plastics and food packages. BPA is thought to affect several hormone-dependent processes and is now considered to be an endocrine disruptor (ED). BPA impairs steroid and thyroid systems via binding to nuclear receptors (NRs), but in the last years BPA has been linked to neurodevelopmental disorders, probably because of the presence of some NRs in the vertebrate brain. Besides vertebrates, EDs can also affect marine invertebrates such as ascidians, molluscs or echinoderms but its mode of action in these organisms is largely unknown.

In this study, we assessed the effect of BPA on larval brain development of the ascidian *Phallusia mammillata*. We found that BPA is toxic to *P. mammillata* embryos in a dose-dependent manner. Furthermore, micromolar BPA induces neurodevelopmental toxicity by impairing differentiation of the ascidian pigmented sensory organ (PSO), within the larval brain. We further show that this phenotype is specific to bisphenols over biphenyls. Finally, estrogen-related receptor (ERR) agonists and antagonists partially phenocopied BPA phenotype suggesting an involvement of *Pm*-ERR. *Pm*-ERR was found to be expressed in the larval brain, adjacent to the differentiating PSO, making *Pm*-ERR a very likely target of BPA mediating neurodevelopmental toxicity in ascidians.

Keywords:

Endocrine disruptor, ascidian, neurodevelopment, estrogen-related receptor, bisphenol A,

Abbreviations:

BPA: bisphenol A; DES: diethylstilbestrol; ED: endocrine disruptor; ERR: estrogen-related receptor; E2B: 17 β -estradiol 3-benzoate; NR: nuclear receptor; PSO: pigment sensory organ; 4-OHT: 4-hydroxytamoxifen

1. Introduction

Bisphenol A (BPA) is a well-known molecule in commercial use since 1957 which is employed in manufacturing consumer products such as polycarbonate plastics, epoxy resins, and food-packaging. It was considered harmless to human health until Krishnan and colleagues found that it could easily leach from plastics into their yeast cultures (Krishnan et al., 1993). BPA was later found accidentally to be a potent meiotic aneugen in mouse females (Hunt et al., 2003). Because BPA is continuously released into the environment, human and wildlife exposure to BPA is ubiquitous despite its short half-life, making BPA a pseudo-persistent chemical (Flint et al., 2012). Ingestion, inhalation and dermal exposure are the main routes of exposure for humans, with more than 90% of human population showing measurable urinary levels of BPA (Matsumoto et al., 2003).

With such pervasive presence, concerns have been raised about BPA over the past decade due to its potential endocrine disrupting activities. Endocrine disruptors (EDs) are compounds able to mimic, antagonize, or modify normal hormonal activity, interfering with the endocrine system through two main pathways: the genomic pathway, *via* binding to nuclear receptors (NRs); or the non-genomic pathway, *via* binding to cell membrane receptors (Schug et al., 2013). BPA was shown to bind several NRs in vertebrates such as Estrogen Receptor (ER), Estrogen-Related Receptor (ERR), Peroxisome Proliferator-Activated Receptor (PPAR), Pregnane X Receptor (PXR) and Thyroid Receptor (TR) (Acconcia et al., 2015), which are all expressed in the brain of chordates during either embryonic or adult life. In the last few years, EDs like BPA have been linked to neurodevelopmental disorders such as increased anxiety, attention-deficit hyperactivity disorder (ADHD), autism spectrum disorder (ASD), among others (Inadera, 2015). Although the major studies focused on the roles of genetic factors, researchers suspect the environment as a strong contributor to the increased prevalence of this kind of neurodevelopmental disorders. Yet, apart from ER, AR and TR, the involvement of NRs in neurodevelopment has been overlooked even though some NRs are known to be expressed in the brain. Among these NRs, human ERR γ has the highest affinity for BPA (Liu et al., 2014) and it is thought to be responsible for the BPA-induced impairment of inner ear differentiation in zebrafish larvae (Tohmé et al., 2014). However, the mode of action (MoA) and the GRNs affected by BPA in the vertebrate brain are mostly unknown.

Besides impacting human health, BPA can also affect invertebrate development (reviewed in Dumollard, Gazo, et al. 2017; Flint et al. 2012). Based on reported median effective (EC₅₀) and median lethal (LC₅₀) concentrations that range from 1.0 to 10 mg/L (equivalent to 4.38 and 43.8 μ M), BPA is classified as “moderately toxic” and “toxic” to aquatic biota by the European Commission and the United States Environmental Protection Agency (US EPA), respectively. However, studies on the effects of BPA on wildlife indicate that the compound may be harmful even at environmentally relevant concentrations, which has been defined as 0.05 μ M or lower (Flint et al., 2012). The highest concentration found in natural surface waters was 0.1 μ M in Netherlands, but landfill leachates from municipal wastes in Japan registered values of BPA around 75 μ M (Flint et al., 2012). Despite several studies showing the potential effects of BPA on living organisms, there is a lack of knowledge concerning the effect of BPA on invertebrates’ neurodevelopment and its potential mode-of-action (Kaur et al., 2015; Mersha et al., 2015).

Ascidians (Tunicata) are marine invertebrate chordates. Their rapid embryonic development has been well characterized (Hotta et al., 2007) and results, 18 hours after fertilization (at 18°C), in a swimming larvae with a prototypical chordate body plan: a centralized brain and a dorsal nerve cord supporting beating of the tail (Hudson, 2016). Because the ascidian larva is composed of roughly 2600 cells (Hotta et al., 2007; Imai et al., 2004), it is a much simpler system than vertebrate embryos to follow cell behaviour (Dumollard et al., 2017). The CNS of the ascidian larva is divided into three parts: an anterior sensory vesicle (homologous to the vertebrate diencephalon), a trunk ganglion (homologous to the vertebrate hindbrain) and a caudal nerve cord (homologous to the vertebrate spinal cord) (Hudson, 2016). Within the sensory vesicle, two pigmented cells (PCs) are visible. The anterior pigment cell is the otolith (Ot), an unicellular structure which has a free part within the lumen of the sensory vesicle and a ventral foot part (Dilly, 1962; Esposito et al., 2015). The ascidian ocellus (Oc), present in the posterior dorsal part of the sensory vesicle, was described as a multicellular structure made up of three parts: one pigment cell, three lens cells and 37 photoreceptor cells (Dilly, 1964; Esposito et al., 2015; Ryan et al., 2016). Altogether, they form the ascidian pigmented sensory organ (PSO), responsible for gravity sensing (otolith) and light perception (ocellus) (Tsuda et al., 2003). The PCs forming this sensory organ have a common origin (a9.49 cell lineage) and are specified at late gastrula (stage 13) (Nishida and Satoh, 1989; Racioppi et al., 2014).

In this study, we assessed the effect of BPA on the development of the brain of the ascidian tadpole (*Phallusia mammillata*). We found that BPA is lethal to *P. mammillata* embryos at 30 μ M, whereas lower doses impair PC differentiation during brain development. We further show that this effect is specific to bisphenols over biphenyls. In order to evaluate the potential involvement of ERR in the neurodevelopmental phenotype of BPA, we characterized the embryonic expression of ERR in the ascidian embryo and screened known ERR agonists/antagonists. Strikingly we found that ERR is expressed in the sensory vesicle of the ascidian larva and found that some but not all aspects of BPA’s phenotype could be reproduced by ERR antagonists.

2. Material and Methods

2.1. Animals

Adult *Phallusia mammillata* were collected in Sète (Hérault, France), and kept at 17 \pm 1°C in circulating seawater aquaria. They were reared under constant light conditions to avoid uncontrolled spawning of eggs and sperm (Lambert and Brandt, 1967). The animals were kept and maintained by the aquariology services of the Centre de Ressources Biologiques Marines of the institute (CRBM - IMEV).

For exposure experiments, eggs and sperm were collected separately by dissecting the gonoducts of several hermaphrodite adults. Sperm was stored at 4°C, while eggs were placed in natural filtered (0.2 μ m) seawater (FSW) supplemented with TAPS buffer (0.5 mM) and EDTA (0.1 mM) (Sardet et al., 2011). To allow fluorescence microscopy analysis, chorion was removed from eggs by incubating in a trypsin solution for 2 hours, and then washed 3 times before use (Sardet et al., 2011). Eggs were fertilized by incubating them with a sperm dilution (1:100) for 10 min, then washed 3 times and left in filtered seawater (FSW/TAPS/EDTA) at 18°C until use. Since *Phallusia mammillata* development is very similar to *Ciona*, all referred developmental stages are based on

Ciona development (Hotta et al., 2007). The details of all protocols for embryos handling can be found online at [http://lbdv.obs-
vlfr.fr/fr/ascidian-biocell-group.html](http://lbdv.obs-vlfr.fr/fr/ascidian-biocell-group.html).

2.2. Chemical products

For embryo culture, TAPS buffer (CAS Nr. 29915-38-6) and EDTA (CAS Nr. 60-00-4) were used. For toxicity studies, the following chemicals were diluted in dimethyl sulfoxide (DMSO, CAS Nr. 67-68-5): Bisphenol A (BPA, CAS Nr. 80-05-7), Bisphenol E (BPE, CAS Nr. 2081-08-5), Bisphenol F (BPF, CAS Nr. 620-92-8), Diethylstilbestrol (DES, CAS Nr. 56-53-1), GSK4716 (CAS Nr. 101574-65-6), Phenylthiourea (PTU, CAS Nr. 103-85-5), UO126 (CAS Nr. 109511-58-2), β -Estradiol 3-benzoate (E2B, CAS Nr. 50-50-0), 2,2-Diphenylpropane (2,2DPP, CAS Nr. 778-22-3), 4-Hydroxytamoxifen (4-OHT, CAS Nr. 68392-35-8). For F-actin and DNA staining, phalloidin-tetramethylrhodamine B isothiocyanate (Phalloidin-TRITC, Santa Cruz Biotechnology) and Hoechst (CAS Nr. 23491-52-3) were used, and samples conserved with Citifluor AF1 antifade mounting solution (Biovalley). All chemicals were purchased from Sigma Aldrich, unless stated otherwise.

2.3. Exposure experiments

Exposure experiments with BPA, BPE, BPF, 2,2 DPP, E2B, GSK4716, DES, 4-OHT and PTU were performed in the same way. Chemicals were dissolved in DMSO to have a 0.5 M main stock solution. After fertilization, stage 1 embryos were transferred to chemical-containing seawater (FSW-TAPS-EDTA). Due to a possible confusion arising from differences in the concentrations of DMSO, each solution was diluted in DMSO to a concentration 10,000 times higher than required, and again diluted in filtered seawater (1:10000), giving the required concentration of chemical in an overall solution of 0.01% DMSO in seawater. As a control treatment, 0.01% DMSO seawater was used.

For the exposure experiment with UO126, the chemical was dissolved in a 0.04 M DMSO stock solution. Embryos were fertilized and left to develop at 18°C until stage 16. Embryos were then exposed at stages 16 and 18, at a final concentration of 4 μ M defined from a previous study (Racioppi et al., 2014). A control treatment with 0.01% DMSO was performed.

At stage 26, larvae were fixed in 4% paraformaldehyde (4% PFA, 0.5 M NaCl, PBS; Sigma), washed 3 times in Phosphate Buffered Saline (PBS 1X) and imaged by light microscopy (Zeiss Axiovert 200) at 10x magnification. Each experiment was replicated at least 3 times.

2.4. Embryo morphology analysis

For general morphology analysis, larvae (st. 26) were analyzed by scoring the percentage of normal (good general embryo morphology, with proper trunk and palps formation, as well as tail elongation), malformed (embryos with bent tail) and not developed embryos (with arrested development before gastrulation/tail extension) (see figure S1A). Statistical analysis was performed as described in section 2.11.

For trunk morphology analysis, the area of each PC (*Oc/Ot area*, μm^2), the distance between the two PCs (*Oc/Ot distance*, μm) and the length/width ratio of the trunk were measured (see figure 1A). The morphological analysis was performed using *Toxicosis8*, a software developed in our laboratory (software is deposited for protection by the SATT Lutech). The resulting data was normalized

to each respective control treatment (100%) and plotted in radar charts for better comparison of phenotypes between chemicals. Statistical analysis was performed as described in section 2.11.

2.5. BPA time-window action

To assess the time-window action of BPA during *Phallusia mammillata* embryogenesis, embryos were incubated in BPA-containing seawater at different times during embryogenesis. In a first phase, embryos were exposed in three different ways: 1) six hours before fertilization (6 *hbf*), 2) six hours after fertilization (6 *hpf*, time to reach gastrulation, i.e. stage 10), or 3) both before and after fertilization (from stage 0 to 10) for a total of 12 hours BPA exposure (please refer to diagram on figure 1D). Embryos were then washed and incubated in FSW/TAPS/EDTA up to stage 26. In a second phase, embryos were exposed at different stages (10, 12, 14, 16, 18, 20, 22 and 24) until stage 26. At stage 26, embryos from all treatments were fixed (4% PFA) for later analysis of pigment cell area (*Oc/Ot area*, μm^2). For a matter of technical feasibility, embryos were cultured at 14°C from fertilization to stage 16 and then placed at 18°C from stage 16 to stage 26. Statistical analysis was performed as described in section 2.11.

2.6. Immunofluorescence

To assess the effect of BPA on mitotic spindle formation, microtubule organization during the first mitosis was assessed. For this, eggs were fertilized and immediately exposed to 0.01% DMSO or BPA 40 μM . Sixty-four minutes after fertilization (during the first mitosis), embryos were fixed in cold 100% methanol overnight. They were then progressively rehydrated in PBS/0.02%Triton, permeabilized in PBS/0.25%Triton for 15 min at RT, and then incubated in primary and secondary antibodies in PBS/1%BSA overnight at 4°C (for each antibody incubation, fixed embryos were washed for 15 min for 4 times with PBS/0.1%Tween). Fixed embryos were stained for microtubules with primary antibody anti- α -tubulin (DM1A; Sigma-Aldrich 1:1000) and mouse FITC secondary antibody (Jackson) diluted 1:50. To stain DNA, immunofluorescence labelled embryos were mounted in VectaShield that contains DAPI (Vector Laboratories) analyzed with epifluorescence microscopy (40X objective, Zeiss Axiovert 200).

Cilia of neurohypophysial duct and neural tube were assessed by immunostaining with anti-acetylated α -tubulin antibody according to previously published protocols (Sardet et al., 2011). Stained larvae were imaged by confocal microscopy (Leica, SP8).

2.7. Analysis of otolith movement by timelapse imaging

To follow otolith development, eggs were injected with reporter plasmids bearing promoter region for *Ciona* genes driving the expression of GFP based reporters. The promoters used are Tyrosinase-related protein (pTyrrp, kind gift from Filomena Ristoratore), $\beta\gamma$ Crystallin (p $\beta\gamma$ C, kind gift from Phil Abitua) and Muscle segment homeobox (pMsx, kind gift from Phil Abitua) to visualize pigment cell precursors. The GFP-based reporters were either Venus/Cherry (cytosolic), H2B::Venus/Cherry (DNA) or Lifeact::Venus/Cherry (actin-rich cell cortex). To visualize the plasma membrane of all cells mRNAs coding for PHdomain::Tomato was injected in unfertilized eggs as previously described (Sardet et al., 2011). Injected eggs were fertilized 2 hours after injection and left to develop at 16°C until stage 10 (gastrula). At stage 10, embryos

were either transferred to 0.01% DMSO (figure 2A) or to BPA-containing seawater (10 μ M, figure 2B), and at stage 20 (early tailbud) embryos were mounted on a slide for time-lapse live imaging using confocal microscopy (Leica SP8).

2.8. Analysis of otolith foot by Phalloidin staining

To analyze the otolith foot structure, embryos were exposed to BPA analogues BPE, BPF and 2,2 DPP (section 2.3). Once at the desired stage (st. 26), embryos were fixed with 4% PFA overnight at 4°C. Embryos were washed twice with PBS 1X, incubated in PBS-BSA solution (3% BSA diluted in PBS 1X) for 1h and stained with Phalloidin-TRITC and Hoechst diluted 1:500 in PBS-BSA, for 3h at RT. Embryos were washed twice in PBS-BSA, once in PBS and stored in AF1 antifade mounting solution Citifluor (Biovalley) until analysis. Phenotypes were compared to a control treatment (0.01% DMSO) using confocal microscopy (Leica SP8). Please note the same final concentration was used for all molecules (10 μ M), with the exception of 2,2DPP (25 μ M), since no phenotype was visible at 10 μ M, 25 μ M nor 100 μ M.

2.9. mRNA expression pattern analysis by *in situ* hybridization (ISH)

mRNA expression patterns of *Phallusia mammillata* genes were assessed by *in situ* hybridization (ISH) technique. For this, embryos were fixed overnight at 4°C (ISH fix: 4% formaldehyde, 100 mM MOPS, 0.5 M NaCl, pH 7.6), washed in PBS, dehydrated in ethanol and stored at -20°C until analysis. *Phallusia* ISH was performed as previously described (Paix et al., 2009).

ISH probes covering the entire cDNAs were designed based on *Aniseed* (<http://www.aniseed.cnrs.fr/>) and *Octopus* (<http://octopus.obs-vlfr.fr/index.php>) databases and were obtained from the *Phallusia mammillata* cDNA library (pExpress-1 vector based) available in our laboratory. After amplification by PCR and confirmation by electrophoresis, PCR products were purified (Qiagen MinElute PCR purification kit, Qiagen) and DNA quantified. Digoxigenin-labeled antisense RNA probes (DIG RNA Labeling Mix, Roche) were then prepared and detection was based on NBT/BCIP (Roche) principle. The following genes were assessed: Tyr (gene ID: *Phamm.g00005364*), Rab32/38 (gene ID: *Phamm.g00006935*), Pax6 (gene ID: *Phamm.g00010911*), Cnga (gene ID: *Phamm.g00015101*), Coe (gene ID: *Phamm.g00012706*) and Islet (gene ID: *Phamm.g00000576*). The imaging of all embryos was performed using an optical microscope (Zeiss Axiovert 200) microscope.

2.10. Estrogen-Related Receptor embryonic expression

The sequence for *Phallusia mammillata* ERR (*Pm-ERR*) is published on *Aniseed* database (gene ID: *Phamm.g00012306*) and was cloned from *Phallusia mammillata* cDNA library, as described in section 2.9.

2.10.1. *Pm-ERR* gene activity by promoter cloning

Gene activity was assessed by cloning the promoter region of ERR into a vector driving the expression of a GFP reporter. For this, a 1.7 kbp fragment (-1705 from the Transcription Start Site, TSS, figure 5C) was selected from ascidian genome available on *Aniseed*. This region, which is highly conserved with *Phallusia fumigata*, was amplified by PCR from *P. mammillata* genomic DNA and cloned upstream a H2B::Venus reporter by In-Fusion HD Cloning Plus kit (Takara Bio). The prepared construct (pERR>H2B::Venus) was

microinjected in *P. mammillata* eggs and the transgenic embryos analyzed by confocal imaging (Leica TCS SP8).

2.10.2. *Pm-ERR* protein levels analysed by a specific *Pm-ERR* antibody

To determine protein presence by immunoblot, an antibody against the ERR protein was made by producing the protein and sending it to Covalab (France) to immunize mice. Four anti-serum were screened and results from the most reactive anti-serum are shown. The anti-serum was further validated as described in figure 5D. In order to confirm the antibody specificity, the antibody was tested against exogenous *Pm-ERR* (*Pm-ERR*::Venus mRNA injected larvae).

2.11. Statistical analysis

All presented data show mean with error bars indicating standard deviation (mean \pm SD). The number of embryos (n) analysed for each graph is indicated in the figure legend. For figure 1C and figure 4, descriptive statistics is depicted in table I. For figure 1D, n and P values are indicated in the figure legend.

GraphPad Prism® software (GraphPad Software, Inc. CA, USA) was used to calculate median lethal (LC₅₀) and effective (EC₅₀) concentrations, as well as to perform analysis of variance ($\alpha=0,05$) to evaluate differences between treatments. Since data did not follow a Gaussian distribution (Shapiro-Wilk normality test), nonparametric Kruskal-Wallis test was performed and statistical differences between control and treatments were assessed by Dunnett's test.

3. Results

3.1. Bisphenol A is toxic to *P. mammillata* embryos in a dose-dependent manner

Figure S1 shows the effect of BPA on the general morphology of *Phallusia* embryos after 24 hours of exposure. Embryos of *P. mammillata* were exposed to different concentrations of BPA (from 100 nM to 30 μ M) and a significant increase in malformed and not developed larvae was observed from 10 μ M BPA (compared to the control treatment 0.01% DMSO, ANOVA - Dunnett's Multiple Comparison Test: p<0.05, figure S1A). At 15 μ M and 20 μ M larvae were mostly malformed (60% and 65%, respectively), and at 25 and 30 μ M larvae were mostly not developed (95% and 100%, respectively). Observation of the mitotic spindle in one cell embryos exposed to 40 μ M BPA showed a severe disruption of mitotic spindle in BPA exposed embryos compared to controls (fig S1B) suggesting that, at this dose BPA acts as an aneugenic genotoxic. No visible effect was detected when embryos were exposed to nanomolar concentrations. BPA median effective (EC₅₀, malformed) and lethal (LC₅₀, not developed) concentrations for *P. mammillata* embryogenesis were 11.8 μ M and 21.1 μ M, respectively.

3.2. Bisphenol A induces neurodevelopmental toxicity in *P. mammillata* embryos

Careful observation revealed that at 10 μ M of BPA, within embryos showing normal overall morphology with proper trunk and tail elongation (figure S1A), the pigmented cells (PC) within the sensory vesicle were strongly affected. In order to better describe the BPA phenotype, formation of sensory vesicle was recorded by timelapse imaging of embryonic development between early tailbud (stage 21) and hatching larva (stage 26) (figure 1A, B), during

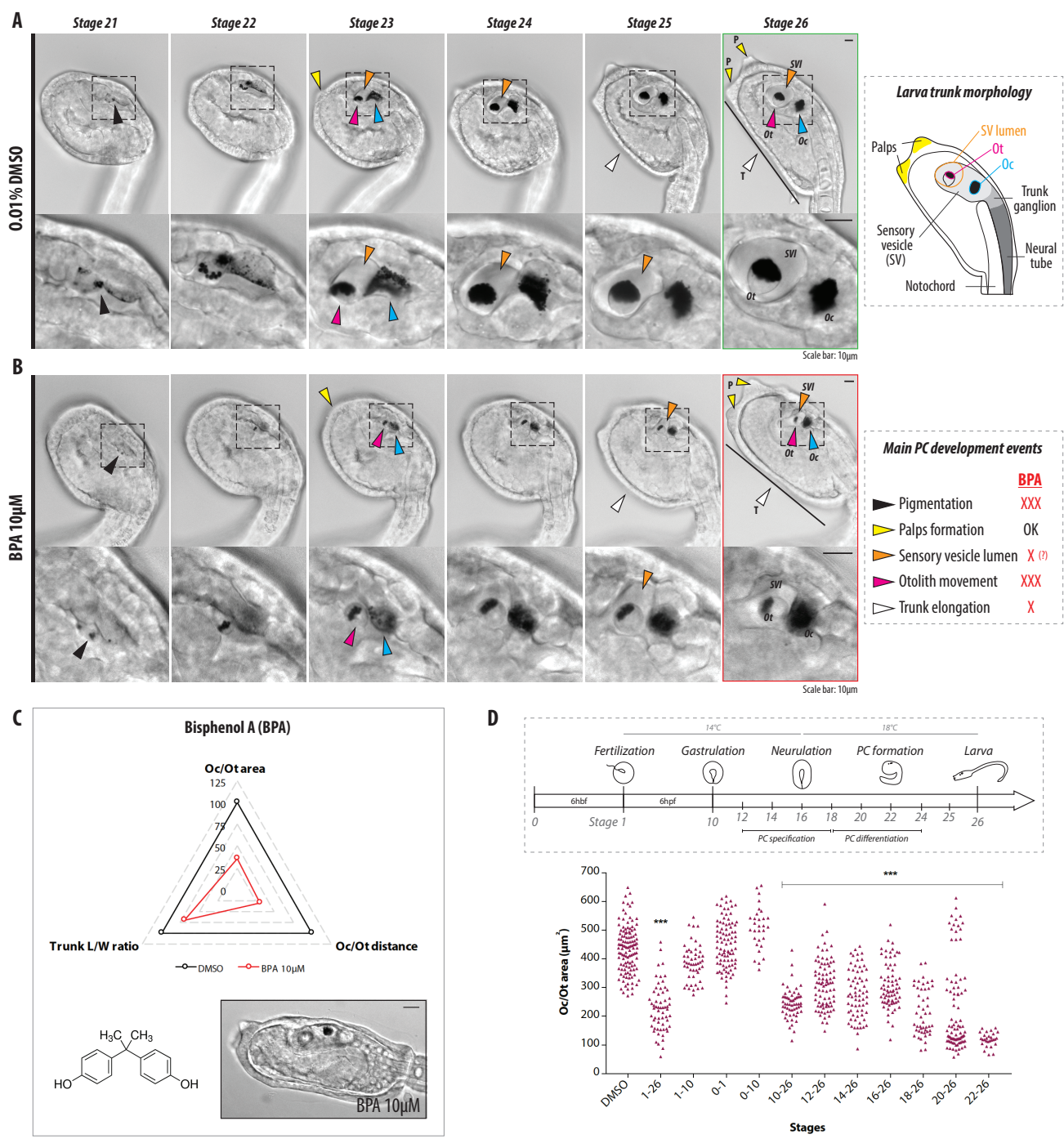


Figure 1. Bisphenol A disrupts pigment cell development in *Phallusia mammillata*. (A) Timecourse of pigment cell (PC) development in non-exposed embryos. The first pigments arrive at stage 21 (black arrowhead), with an increase of pigmentation at stage 22. At stage 23, two distinct pigment cells (PC) are visible, the otolith (Ot) and the ocellus (Oc) (pink and blue arrowheads, respectively). Sensory vesicle lumen (SVI) and adhesive palps (P) (orange and yellow arrowheads, respectively) are visible as well at stage 23. Strong pigmentation for both PC is visible at stage 24. At stage 25 the trunk (T) starts to elongate (white arrowhead). Finally, at stage 26 the trunk of the larvae is elongated and show two distinct pigmented cells, as well as adhesive palps. The otolith is now isolated within the sensory vesicle lumen. Scale bars: 10µm. (B) Timecourse of PC development in Bisphenol A (BPA) exposed embryos. Embryos were exposed to 10µM BPA from stage 1. BPA-exposed embryos showed severe reduction of pigmentation and an inhibition of otolith movement, resulting in a reduced distance between otolith and ocellus. Trunk elongation and sensory vesicle lumen also seem reduced. Scale bars: 10µm. (C) Effect of BPA in *P. mammillata* larvae. Embryos were exposed to BPA 10µM and three endpoints were quantified: the area of the pigment cells (Oc/Ot area), the distance between the pigment cells (Oc/Ot distance) and the length and width of the trunk (Trunk L/W ratio). Data was normalized to the control (DMSO) and plotted in a radar chart. All the three endpoints were significantly different from the control (0.01% DMSO; ANOVA - *Dunnnett's* test), with the Oc/Ot area and Oc/Ot distance being the most affected ones (reduced to 35% and 31%, respectively). Descriptive statistics are represented in table I. (D) Window of sensitivity of *P. mammillata* embryos to BPA. Embryos were exposed to BPA (10µM): 6 hours post fertilization (6hpf, from stage 1 to 10), 6 hours before fertilization (6hbf, from stage 0 to 1), before fertilization up to gastrula (from stage 0 to 10), and then at different developmental stages between stages 10 and 26. The Oc/Ot area was assessed at stage 26, when pigmentation process is finished. Exposure to BPA from stage 1 to 26, as well as all exposures between stage 10 and 26 were significantly different from the control (0.01% DMSO; ANOVA - *Dunnnett's* test; *** p-value<0,001). Each point represents one embryo (n≥50 embryos from three independent experiments).

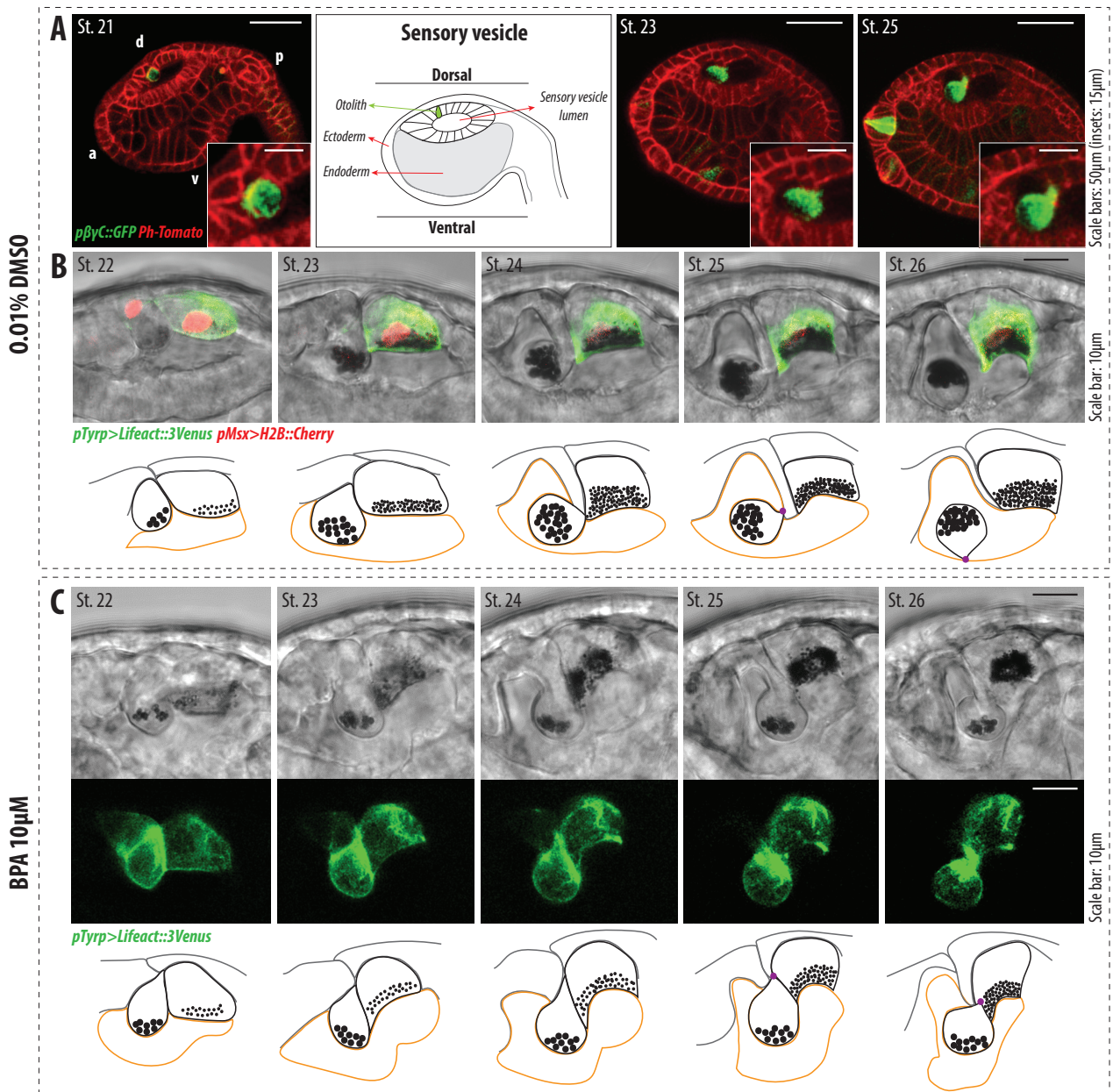


Figure 2. Bisphenol specifically disrupt otolith movement towards the ventral side of the sensory vesicle. (A) Pigment cells development within the sensory vesicle. At stage 21, otolith cell (followed by *βy-Crystallin* promoter *pβyC::GFP*) is visible within the sensory vesicle. The sensory vesicle surrounds the lumen that results from neural tube closure. At later stages (st. 23, 25), the otolith develops a foot that will allow the cell to move towards the ventral side of the brain sensory vesicle. **(B) Otolith movement towards the ventral side of the sensory vesicle lumen.** At the beginning of the pigmentation, otolith cell is next to ocellus cell (st. 22). Later, the epithelium invaginates towards the ventral side of the sensory vesicle lumen (st. 23-25), allowing the otolith to move away from the ocellus. At the end, the otolith foot is ventral and pigments are dorsal within the otolith cell (st. 26). **(C) Otolith movement is blocked by BPA.** In BPA-exposed embryos (BPA 10 μM), otolith cell is next to ocellus cell (st. 22) and the epithelium still invaginates (st. 23-25), however the otolith stays attached to the epithelium (st. 26) until the embryo metamorphoses (not shown). Besides reduced pigmentation and blocked otolith movement, the sensory vesicle lumen seems reduced as well when compared to the control.

which differentiation of the sensory vesicle and of pigmented sensory organs occur (Esposito et al., 2015).

In non-exposed embryos (0.01% DMSO, figure 1A), the first pigments appear at stage 21 (black arrowhead), increasing at stage 22. At stage 23 adhesive palps start to protrude (yellow arrowhead) and two distinct PCs are visible: the ocellus (*Oc*, blue arrowhead) and the otolith (*Ot*, pink arrowhead). At this stage, a lumen is first visible in the sensory vesicle (orange arrowhead) and this lumen expands until stage 26. At stage 25, the trunk begins to elongate and concomitantly the otolith moves towards the ventral side of the

sensory vesicle. Finally, at stage 26, larvae display two clearly separated PCs and palps, as well as an elongated trunk.

When comparing to non-exposed embryos, embryos exposed to BPA at stage 1 (BPA 10 μM) clearly showed a reduction of pigmentation and a blocked otolith movement towards the ventral side of the sensory vesicle (figure 1B). Additionally, both sensory vesicle lumen (*svl*) formation and trunk elongation seemed slightly reduced. Palps were the only well-formed structures when compared to non-exposed embryos. We then quantified the area of both PCs (*Oc/Ot* area), the distance between PCs (*Oc/Ot* distance)

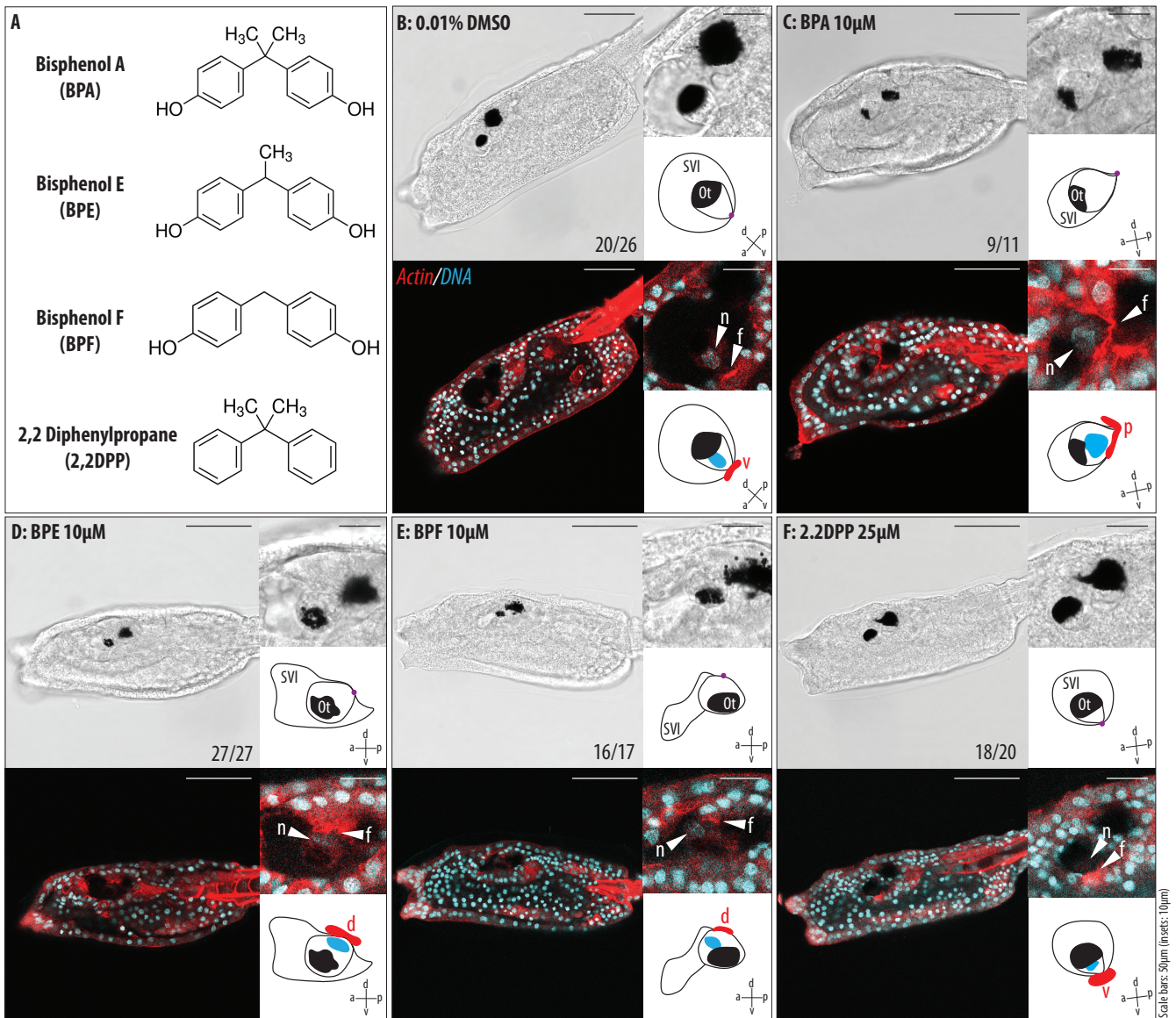


Figure 3. Bisphenols, but not bisphenyl, specifically disrupt pigmentation and otolith movement towards the ventral side of the sensory vesicle. (A) Molecular structure of BPA, BPE, BPF and 2,2DPP. (B-F) Comparison of otolith foot structure between non-exposed embryos (0.01% DMSO), and bisphenols and bisphenyl. In non-exposed larvae (**B: 0.01% DMSO**), the otolith foot (purple dot) is attached to the ventral side of the sensory vesicle, and an accumulation of actin is observed. A similar otolith foot phenotype was also visible in larvae exposed to 2,2 DPP (**F**). However, in larvae exposed to BPA (**C**), BPE (**D**) and BPF (**E**), the otolith foot is arrested on the posterior or even dorsal side of the sensory vesicle. For comparison, all larvae were exposed to 10µM of each drug with exception for 2,2 DPP that showed no otolith phenotype either at 10, 25 or 100 µM. Larvae were fixed and stained for actin and DNA (Phalloidin and Hoechst staining, respectively). *n*: nucleus, *f*: foot, *v*: ventral, *p*: posterior, *d*: dorsal.

and the trunk length/width ratio (Trunk L/W ratio). BPA-exposed embryos showed significant reduction for all the three endpoints (ANOVA - Dunnett's Multiple Comparison Test: p -value < 0.001; table I), with Oc/Ot area reduced to 35%, Oc/Ot distance reduced to 31% and Trunk L/W ratio reduced to 70% (figure 1C). Additionally, the sensory vesicle lumen seemed also reduced but this was not quantified.

In order to understand the window of action of BPA on PC formation, embryos were incubated at different times during embryogenesis (figure 1D). Reduction of Oc/Ot area was observed when BPA was present between stage 10 and stage 26 (ANOVA - Dunnett's Multiple Comparison Test: p -value < 0.001), and not when it was washed before stage 10. Strikingly, reduced pigmentation was observed when BPA was added as late as stage 22. We also exposed eggs to BPA before fertilization (stages 0-1 and 0-10) to assess whether bioaccumulation of BPA could influence the

phenotypes observed. However, when BPA was washed out at stage 10 no effect was observed even if BPA was allowed to accumulate for 6-12 hours. Together these observations suggest that window of action of BPA is between stages 22 and 26.

In *Ciona*, the formation of pigment cells necessitates tyrosinase activity for the pigmentation of melanosomes (Caracciolo et al., 1997) and FGF/MAPK signals up to stage 16 for specification (Racioppi et al., 2014). Accordingly, *Phallusia* embryos exposed to the tyrosinase enzyme inhibitor phenylthiourea (PTU, from 1-cell stage (Sakurai et al., 2004; Whittaker, 1966)) resulted in the complete lack of pigmented melanosomes in the PC, but the unpigmented otolith cell seemed well formed and ventrally placed in the sensory vesicle lumen (figure S2A). The phenotype obtained is thus clearly different from the phenotype induced by application of BPA at a similar timing (figure 1B).

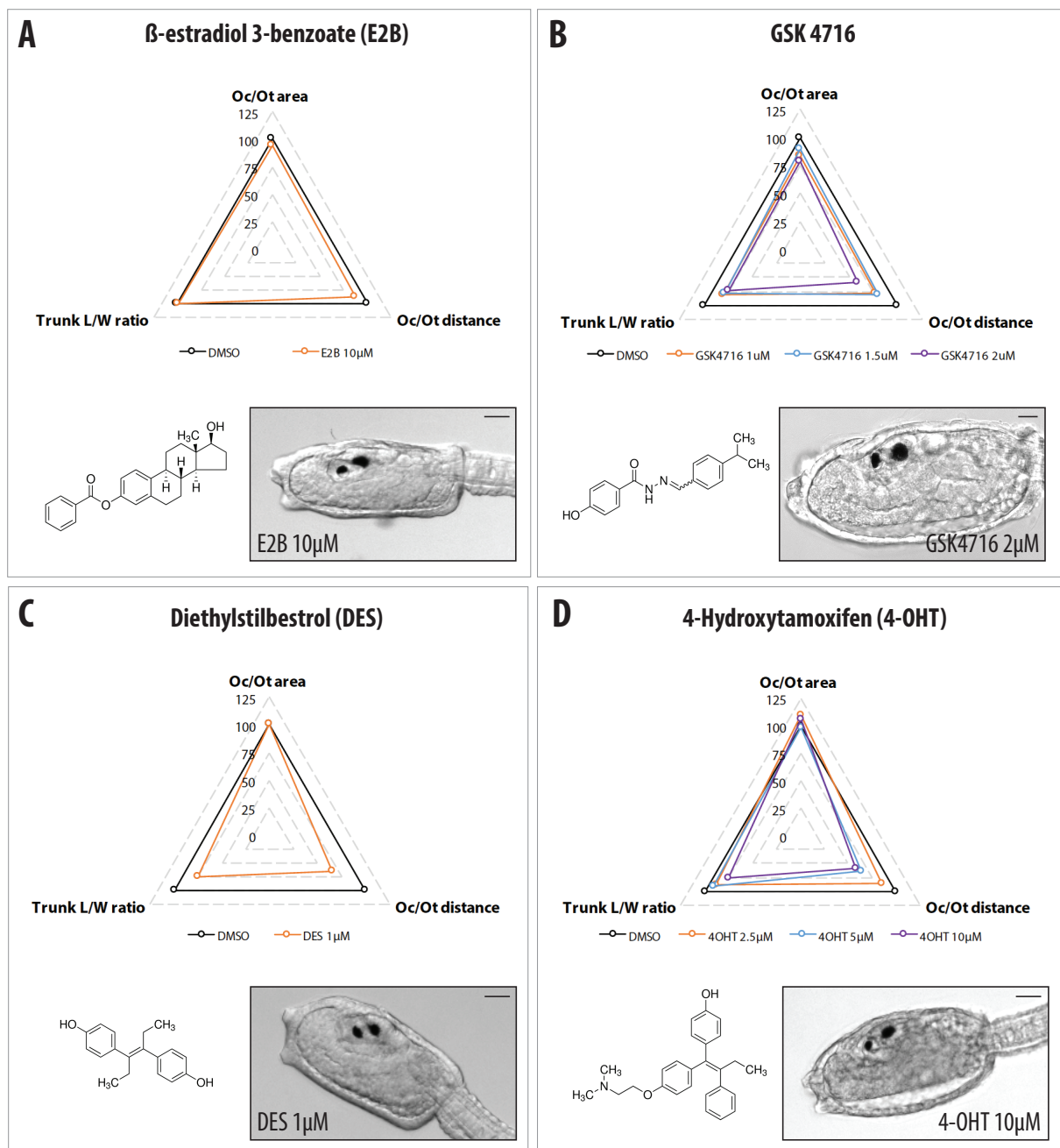


Figure 4. Estrogen-related receptor (ERR) agonist and antagonists, but not Estrogen Receptor (ER) agonist, partially phenocopied BPA neurodevelopmental toxicity. (A-D) Three endpoint-radar charts for comparison between BPA and ER/ERR agonists and antagonists. For phenotype comparison, the following endpoints were measured: the pigment cell area (Oc/Ot area), the distance between pigment cells (Oc/Ot distance) and the length and width of the trunk (Trunk L/W ratio). Graphs are normalized to the control (DMSO). Embryos exposed to the ER agonist estradiol β-benzoate (**E2B, A**) had no effect on development. Embryos exposed to the ERR agonist **GSK4716 (B)** showed the three endpoints significantly different from the control. Embryos exposed to the ER agonist/ERR antagonist diethylstilbestrol (**DES, C**) and the ER/ERR antagonist 4-hydroxytamoxifen (**4-OHT, D**) showed mostly affected Oc/Ot distance and trunk elongation. Respective molecular structures and representative phenotype pictures are provided (scale bars: 20 μm). Please refer to the table I for the descriptive statistics.

Moreover, embryos exposed to the MAPK inhibitor UO126 at stage 16 are completely devoid of PC and sensory vesicle lumen, whereas embryos treated with UO126 at stage 18 display two well separated PC and normal sensory vesicle lumen (figure S2A). Again, the phenotypes induced by MAPK inhibition (which blocks PC specification) are different from the BPA phenotype. Additional analysis of the expression of Tyr, Rab32/38, Pax6, Cnga, Coe and Islet genes, known to be necessary for PC and CNS specification, was performed by ISH (figure S2B). No differences in the spatial expression patterns between non-exposed (DMSO) and BPA-exposed embryos (BPA 10 μM) were observed, suggesting that BPA

does not affect PC nor CNS specification, and neither tyrosinase activity. Finally, differentiation of the neurohypophysial duct and neural tube (Konno et al., 2010) was not affected by BPA as shown in figure S2C, reinforcing the idea that only PC differentiation is affected by BPA.

3.3. Bisphenol A impairs otolith cell movement towards the ventral side of the sensory vesicle

We have observed so far that BPA affects processes intervening in sensory vesicle differentiation between stage 21 and 26. In order to characterize with more precision the processes affected by BPA

Table I. Descriptive statistics of radar charts. Raw data of each radar chart is provided. Data was analysed based on One-Way ANOVA. Because data did not follow a Gaussian distribution (normality test Shapiro-Wilk), data was analysed using Kruskal-Wallis test followed by Dunnett's multiple comparison test (Dunn's MCT). * p-value<0.05; ** p-value<0.01; *** p-value<0.001.

<i>Oc/Ot area</i>	n	Min	Max	Mean	Std	Dunn's MCT	% to the control	Adj p-value	Summary
DMSO	486	188	636	382	82,8	-	-	-	-
BPA 10µM	255	0	361	122	83,5	DMSO vs. BPA 10µM	35	<,001	***
E2B 10µM	162	138	602	360	100	DMSO vs. E2B 10µM	94	0,881	ns
GSK4716 1µM	144	0	554	321	97,4	DMSO vs. GSK4716 1µM	84	<,001	***
GSK4716 1.5µM	136	0	559	345	99,2	DMSO vs. GSK4716 1.5µM	90	0,009	**
GSK4716 2µM	122	0	571	303	115	DMSO vs. GSK4716 2µM	79	<,001	***
DES 1µM	144	171	557	384	68,3	DMSO vs. DES 1µM	101	>,999	ns
4-OHT 2.5µM	125	198	611	417	76,9	DMSO vs. 4-OHT 2.5µM	109	0,004	**
4-OHT 5µM	148	73,2	624	376	102	DMSO vs. 4-OHT 5µM	98	>,999	ns
4-OHT 10µM	105	150	672	398	107	DMSO vs. 4-OHT 10µM	105	>,999	ns

<i>Oc/Ot distance</i>	n	Min	Max	Mean	Std	Dunn's MCT	% to the control	Adj p-value	Summary
DMSO	485	0	41,5	24,1	8,52	-	-	-	-
BPA 10µM	278	0	32,3	7	10,1	DMSO vs. BPA 10µM	31	<,001	***
E2B 10µM	161	0	36,7	22,1	9,28	DMSO vs. E2B 10µM	88	0,15	ns
GSK4716 1µM	146	0	36,2	18,8	13,2	DMSO vs. GSK4716 1µM	77	0,009	**
GSK4716 1.5µM	136	0	38,2	19,4	12,8	DMSO vs. GSK4716 1.5µM	80	0,013	*
GSK4716 2µM	122	0	33,3	14,2	12,6	DMSO vs. GSK4716 2µM	59	<,001	***
DES 1µM	145	0	34,2	15,9	11,9	DMSO vs. DES 1µM	65	<,001	***
4-OHT 2.5µM	127	0	41,9	20,7	11,8	DMSO vs. 4-OHT 2.5µM	85	0,084	ns
4-OHT 5µM	149	0	40	15,3	13,1	DMSO vs. 4-OHT 5µM	63	<,001	***
4-OHT 10µM	105	0	40,6	13,9	13,6	DMSO vs. 4-OHT 10µM	58	<,001	***

<i>Trunk L/W ratio</i>	n	Min	Max	Mean	Std	Dunn's MCT	% to the control	Adj p-value	Summary
DMSO	486	1,01	4,34	2,53	0,52	-	-	-	-
BPA 10µM	279	1,01	2,89	1,72	0,41	DMSO vs. BPA 10µM	70	<,001	***
E2B 10µM	162	1,21	3,5	2,5	0,4	DMSO vs. E2B 10µM	98	>,999	ns
GSK4716 1µM	146	1,17	2,62	2,03	0,32	DMSO vs. GSK4716 1µM	80	<,001	***
GSK4716 1.5µM	136	1,15	2,59	1,99	0,27	DMSO vs. GSK4716 1.5µM	78	<,001	***
GSK4716 2µM	122	1,01	2,5	1,89	0,28	DMSO vs. GSK4716 2µM	74	<,001	***
DES 1µM	270	1,07	2,73	1,75	0,32	DMSO vs. DES 1µM	75	<,001	***
4-OHT 2.5µM	127	1,36	3,11	2,22	0,34	DMSO vs. 4-OHT 2.5µM	88	<,001	***
4-OHT 5µM	148	1,36	3,82	2,29	0,53	DMSO vs. 4-OHT 5µM	91	<,001	***
4-OHT 10µM	106	1,03	2,75	1,91	0,31	DMSO vs. 4-OHT 10µM	75	<,001	***

we performed time-lapse imaging of embryos expressing GFP reporters in the PC lineage. By using the otolith specific promoter ($\beta\gamma\text{C}:\text{GFP}$) we could clearly observe changes in the shape of the otolith between stage 21 when it is in the epithelium of the sensory vesicle and stage 25 when the otolith is bulging into the sensory vesicle lumen and contacts the sensory vesicle epithelium by a foot structure (figure 2A). The actin network of PC was specifically visualized with a PC-specific promoter ($\text{pTyrrp}>\text{LifeAct}::3\text{xVenus}$) together with BF imaging. Timelapse imaging between stage 22 and 26 shows that the otolith cell detaches from the dorsal sensory vesicle epithelium (and the ocellus) by pinching off its foot from the sensory vesicle epithelium, moving towards the ventral sensory vesicle epithelium (figure 2B and supplementary movie 1). Note that the sensory vesicle lumen also expands during these stages. In BPA-exposed embryos, the otolith cell still forms a foot but this foot cannot detach from the dorsal sensory vesicle epithelium and the otolith remains tethered to the ocellus. In addition, sensory vesicle lumen seems to expand less (figure 2C and supplementary movie 2).

We then assessed whether this phenotype is specific to bisphenol A by exposing embryos to BPE and BPF (two other bisphenols) and 2,2DDP (a biphenyl) (figure 3A). For this, exposed larvae (stage 26) were stained with Phalloidin and Hoechst to visualize both actin and DNA. The control larvae (0.01% DMSO, figure 3B) showed an accumulation of actin abutting the otolith foot on the ventral side of the sensory vesicle. In contrast BPA-exposed larvae displayed a dorsal/posterior otolith actin-rich foot

(figure 3C). Interestingly, both BPE and BPF exposures resulted in the presence of the otolith actin-rich foot in the dorsal side of the sensory vesicle (figures 3D and 3E), suggesting that otolith movement did not occur. Finally, exposure to 25µM 2,2 DPP did not affect the localization of the otolith foot which was still observed on the ventral side of the sensory vesicle (figure 2F). No phenotype was observed with either 10 or 100µM 2,2 DPP (data not shown). Even though we did not quantify it, BPE and BPF (but not 2,2 DPP) also seem to decrease PC pigmentation and svl expansion (compare the different larvae shown in figures 3B-F).

3.4. ERR agonists and antagonists also affect PC development

BPA is known to bind both ER and ERR γ in vertebrates (Takayanagi et al., 2006). We thus exposed embryos to known ER/ERR agonists and antagonists and assessed their effect on PC differentiation and trunk elongation. Figure 4 shows radar chart plotting of the relative differences in three measured endpoints (Oc/Ot area, Oc/Ot distance and Trunk L/W ratio) between control and exposed embryos, in order to compare the phenotypes induced by the different molecules. As expected (see Discussion), the ER agonist, estradiol-

benzoate (E2B) did not affect any endpoint measured (figure 4A, table I).

The Oc/Ot area was reduced only by the ERR agonist GSK4716 albeit to a lesser extent than BPA (down to 35% with 10 µM BPA versus 79% with 2 µM GSK4716). Oc/Ot distance was reduced by all drugs again to a lesser extent than BPA (BPA: 31%, GSK4716: 59%, DES: 65%, 4OHT: 58%). Finally, trunk elongation was affected by all inhibitors in similar proportions with BPA (BPA: 70%, GSK4716: 74%, DES: 75%, 4OHT: 75%) (figure 4, table I).

Our small pharmacological screen shows that ERR agonists and antagonists can partially reproduce the phenotype of BPA on PC differentiation whereas an ER agonist has no effect. We thus sought for ERR presence in *Phallusia* larvae.

3.5. Estrogen-Related Receptor (ERR) is expressed in *Phallusia* larvae

In order to substantiate the possibility that BPA targets ERR in the ascidian embryo, we analysed ERR transcripts and protein level during embryonic development (figure 5). Published transcriptomic data in the ascidian *Aniseed* database suggests that ERR transcripts are found at larvae (stage 26) but not before (figure 5A). ISH analysis revealed that, at stage 26, ERR is expressed within the sensory vesicle, more precisely above the ocellus, between otolith and ocellus and underneath the otolith, as well as in adhesive palps and trunk ganglion (figure 5B, black arrowheads). Since ISH staining using NBT/BCIP at such a late stage is made difficult by the secretion of the ascidian tunic (larvae become dark

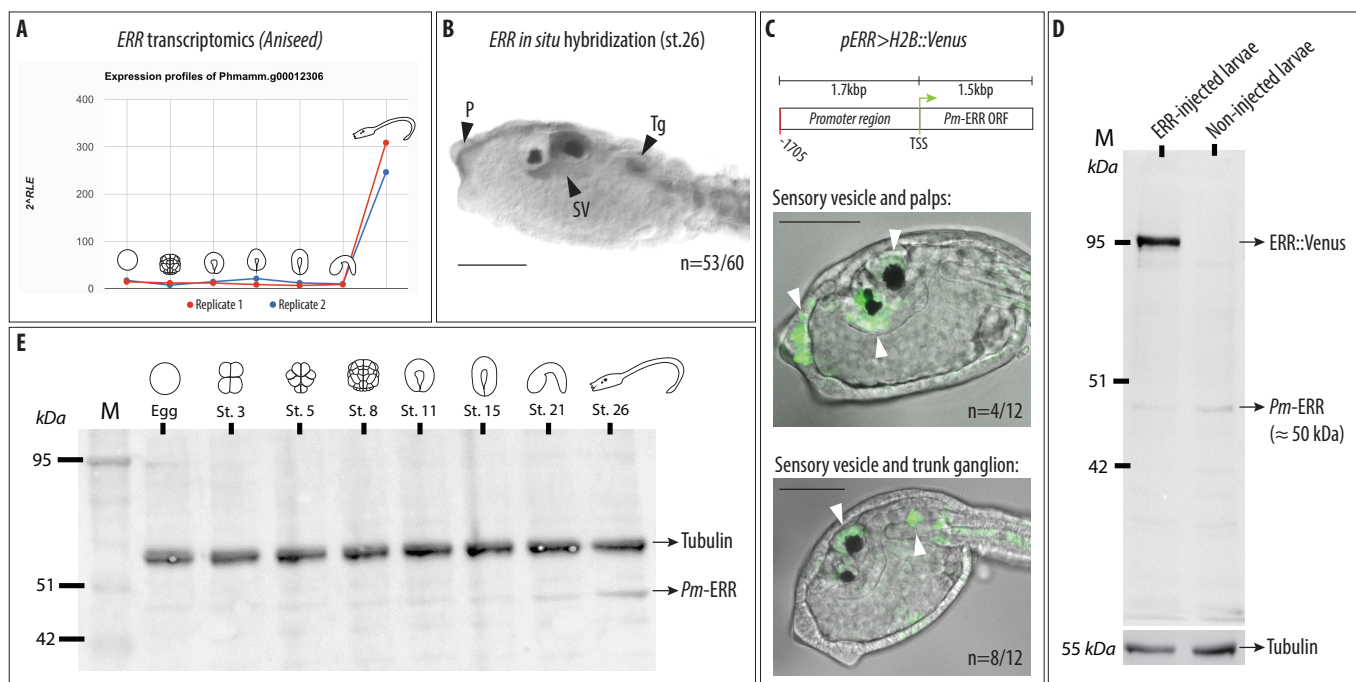


Figure 5. *Phallusia mammillata* express an Estrogen-Related Receptor (ERR) in the ascidian larval sensory vesicle. (A) Expression profile of *Pm-ERR* from transcriptomic data. According to transcriptomic data available at *Aniseed* database (<https://www.aniseed.cnrs.fr>), *Pm-ERR* (gene ID: g00012306) is poorly expressed throughout development until larva (stage 26), where its expression increases by 300 ($2^{\Delta RLE}$ stands for Relative Log Expression). **(B) *Pm-ERR* spatial expression.** *Pm-ERR* transcript was assessed by *in situ* hybridization and expression was only visible from stage 26. *Pm-ERR* mRNA is expressed within the sensory vesicle (SV), specifically below the otolith, above the ocellus and between both pigment cells, and also in the papillae neurons (Pn) and in the trunk ganglion (Tg) (black arrowheads). **(C) *Pm-ERR* promoter activity.** The promoter region of *Pm-ERR*, corresponding to 1.7kbp before the first ATG, was cloned into a *H2B::Venus* reporter plasmid and injected in *P. mammillata* eggs. Two types of expression were obtained: in the sensory vesicle and palps, or in the sensory vesicle and trunk ganglion (white arrowheads). **(D-E) *Pm-ERR* protein.** **(D)** In order to study protein expression, a *Pm-ERR* antibody was raised and validated by injecting eggs with exogenous ERR mRNA (ERR::Venus). **(E)** Protein expression was then assessed throughout *P. mammillata* embryonic development, showing its presence only from stage 26, in a similar way as mRNA and promoter results. Tubulin antibody was used as control. Scale bars: 50 μ m.

quickly), we further confirmed ERR zygotic expression by monitoring its promoter activity ($pERR>H2B::Venus$) in transgenic embryos (Stolfi and Christiaen, 2012) (figure 5C). On the 14 larvae analysed, all showed fluorescent nuclei (i.e. promoter activity) in the sensory vesicle and then 4 had additional cells in the adhesive palps, while the other 8 had additional cells in the trunk ganglion. Additionally, 2 larvae showed ectopic expression in the mesenchyme due to promoter leakiness previously reported in ascidians (Stolfi and Christiaen, 2012).

Using an antibody specific for *Pm-ERR* (figure 5D), ERR protein levels were measured during development by western blot analysis (figure 5E). As expected from RNA-seq, ISH and promoter analysis, a faint band was observed at stage 26 but not before.

4. Discussion

BPA has been shown to affect multiple targets and may have diverse and pleiotropic modes-of-action. Moreover, embryonic development has been shown as the most critical window of action for EDCs, particularly during neurodevelopment (Heyer and Meredith, 2017). In our study we show that BPA acts during a precise window of the ascidian embryonic development, disrupting pigment cell pigmentation and specifically blocking otolith movement within the sensory vesicle. A pharmacological approach revealed that the observed phenotype is specific to bisphenols (BPE, BPF), but not to biphenyls (2,2DPP). Finally, we hypothesize that BPA's effect on ascidian larval brain formation may be mediated by ERR as suggested by a screen of ERR agonists and

antagonists and the zygotic expression of ERR in a few cells of the sensory vesicle.

4.1. Bisphenols (but not biphenyls) specifically alter differentiation of the otolith in ascidians

Phallusia mammillata embryos display a dose-dependent sensitivity to BPA with a common monotonic dose-response curve. BPA was also shown to affect the pigmented sensory organ in another ascidian species, *Ciona intestinalis* (Matsushima et al., 2013). Concerning dose-response toxicity, our data indicates that *P. mammillata* is less sensitive to BPA (EC50: 11.8 μ M; LC50: 21 μ M) than other marine invertebrates like the ascidian *Ciona robusta* (EC50: 0.7 μ M; LC50: 5.4 μ M (Matsushima et al., 2013); LC50: 5.2 μ M (Messinetti et al., 2018)) or the sea urchin *P. lividus* (LC50: 3.1 μ M (Ozlem and Hatice, 2007)), but more sensitive than the vertebrate zebrafish (EC50: 25 μ M; LC50: 73.4 μ M (Tse et al., 2013)), even though all the referred EC50/LC50 values are within the micromolar range. Additionally, we observed that at high dose (40 μ M) BPA is an aneugen, as previously described in other invertebrate species like *C. elegans* (Allard and Colaiacovo, 2010) and sea urchin (2.2 μ M BPA (George et al., 2008)), and in mouse (20ng/g/day (Hunt et al., 2003)).

In addition, our study reveals that BPA exerts neurodevelopmental toxicity by disrupting the differentiation of the ascidian pigmented sensory organ. Neurodevelopmental defects induced by BPA have been found in vertebrates (mouse, fish, frog, reviewed in Inadera 2015; Nesan, Sewell, and Kurrasch 2017) and in invertebrates such as *Drosophila* (Kaur et al., 2015), *C.*

elegans (Mersha et al., 2015) and *Ciona* (Messinetti et al., 2018). Time-lapse live imaging of the transparent *Phallusia* embryos allowed us to describe in detail the pigment cell (PC) differentiation occurring in the ascidian brain (Esposito et al., 2015; Racioppi et al., 2014) and how it is affected by BPA.

One of the main events affected by BPA was Ot/Oc pigmentation. Several studies described pigmentation problems caused by BPA both in invertebrates and vertebrates. Concerning invertebrates, a similar phenotype was observed in other ascidian species, *C. robusta*, where both ocellus and otolith were severely affected by BPA, with an EC₅₀ of 0.16 μM (Messinetti et al., 2018). In zebrafish, more than 50% of the embryos exposed to 25 μM BPA displayed reduced otolith pigmentation (Gibert et al., 2011). In vertebrates, pigmentation is the process of melanin pigments production and storage in melanosomes, regulated by microphthalmia-associated transcription factor (MITF) and dependent on proteins such as tyrosinase (Tyr), tyrosinase-related proteins (Typr) and Rab GTPases (Wasmeier et al., 2008). In *Ciona*, PC specification is also dependent on Mitf, Tyr, Typr and Rab32/38 (Abitua et al., 2012; Racioppi et al., 2014), regulated under several FGF/MAPK signals from stage 13 up to stage 16 (Racioppi et al., 2014). In our study, the time-window of action of BPA (i.e. after stage 22) is after the final FGF-mediated induction of PC lineage (at stage 16), suggesting that BPA is affecting PC differentiation rather than PC specification. Several observations confirmed that PC specification is not affected by BPA. First, inhibiting PC specification by timed inhibition of MAPK signaling (using UO126) did not phenocopy BPA phenotype. Second, several marker genes for PC and CNS specification in ascidians (Tyr, Rab32/38, Pax6, Cnga, Coe and Islet) are not affected by BPA exposure. Finally, inhibiting tyrosinase enzyme activity (using PTU), once again, did not phenocopy BPA phenotype. Altogether, our data reinforces the idea that BPA is not acting at the PC specification level, but most likely during PC differentiation. However, the gene regulatory networks involved in ascidian PC differentiation are not known and thus more efforts are needed to assess the potential genes that are being targeted by BPA.

Another main affected event after BPA exposure was the distance between ocellus and otolith which was significantly reduced. By imaging transgenic *Phallusia* embryo expressing GFP markers in the PC lineage (*Typr*, *βCristallin* and *Msx*), it was possible to visualize that the otolith cell changes shape and protrudes into the sensory vesicle lumen, which is enclosed by the sensory vesicle epithelium. The otolith cell assumes a round shape at the beginning of PC differentiation, and then becomes a pigmented cup-shaped cell with a foot that moves towards the ventral side of the sensory vesicle while the ocellus remains embedded in the dorsal sensory vesicle epithelium. BPA did not inhibit foot formation but did prevent the detachment of the otolith from the dorsal sensory vesicle epithelium. Our data also suggests that the lower Oc/Ot distance observed in BPA-treated embryos may also be due to a reduced expansion of the sensory vesicle lumen. Indeed, even though it was not quantified, we noted that all bisphenols and ERR ligands also resulted in reduction of the size of the sensory vesicle lumen. Because the ocellus and the otolith are located on opposite sides of the sensory vesicle, lumen expansion should participate in the separation of the two cells from each other. It has been shown that the ammonium transporter AMT-1a (Marino et al., 2007) and the ions transporter SLC26a (Deng et al., 2013) proteins are involved

in sensory vesicle lumen expansion in ascidians. It would be thus interesting to assess if such genes are being targeted by BPA.

Exposure of embryos to BPA and, strikingly, its analogues BPE and BPF inhibited the otolith movement towards the ventral side of the sensory vesicle. Such otolith phenotype is specific to bisphenols since exposure to the biphenyl 2,2DPP had no effect on larvae sensory vesicle (neither pigmentation, neither otolith movement nor sensory vesicle lumen). Interestingly, Tohmé and colleagues showed a similar bisphenol-specificity effect in zebrafish otolith development, suggesting that bisphenols may share a similar mode of action in both ascidians and fishes (Tohmé et al., 2014). Because BPA, BPE and BPF can bind ERR and not 2,2 DDP (due to the lack of hydroxyl groups on its phenyl rings (Okada et al., 2008; Starovoytov et al., 2014)), our data indicate that, like in fishes, BPA may impair otolith development by affecting ERR activity.

4.2. BPA may target ERR during otolith differentiation in ascidian larvae

Recent evidence demonstrates that BPA is a weak ligand for estrogen receptor (ER) but high affinity ligand for estrogen-related receptor gamma (ERR γ) (Liu et al., 2014). Thus, we sought to explore further this possibility in the ascidian embryo. Ascidian genome have one gene coding for ERR, and no steroid receptors such as Estrogen nor Steroid Receptors (Yagi et al., 2003).

Transcriptomic data suggests ERR expression only at stage 26 for *Ciona robusta* and *Phallusia mammillata* (*Aniseed*). In other ascidian species PCR analysis showed that, in *Herdmania* ERR is expressed from stage 8 throughout development (Devine et al., 2002), while in *Halocynthia* ERR would be expressed only in eggs and up to stage 8 (Park et al., 2009). Our observations and RNA-seq data in *Ciona* and *Phallusia* (*Aniseed*) are not in agreement with these studies as we show here that ERR transcripts and protein are present only from stage 26. Strikingly we found that *Pm*-ERR is expressed within the sensory vesicle, specifically around the PC, around the time when the otolith moves towards the ventral side of the sensory vesicle. Additionally, most of the aminoacids known to be crucial for BPA binding to human ERR γ (Liu et al., 2014; Okada et al., 2008; Takayanagi et al., 2006) are conserved in *Pm*-ERR (data not shown), further supporting the hypothesis that *Pm*-ERR might bind BPA to impair PC differentiation.

Our pharmacological screen further suggest that altered ERR activity may participate in BPA mode of action in ascidians. Lack of effect of E2B is consistent with the fact that no ER-like or GPER-like proteins can be found in the ascidian genome (Kamesh et al., 2008; Yagi et al., 2003). However, we cannot be sure of the specificity or effect of these inhibitors. Proving the implication of *Pm*-ERR will take to directly show binding to BPA and to confirm that ERR is necessary for BPA phenotype.

Finally, even if ERR is involved, it is probably not the only target involved in ascidian larvae. It is important to note that BPA is affecting the Oc/Ot distance twice stronger than ERR agonists and antagonists (31% vs ~60%), suggesting that BPA may have a pleiotropic action by targeting other nuclear receptors. Pregnane X Receptor/Vitamin D Receptor alpha (PXR/VDR α), Thyroid Receptor (TR) and Peroxisome Proliferator-Activated Receptor (PPAR) are all potential BPA targets for several reasons. First, *P. mammillata* PXR/VDR α , TR and PPAR are expressed in the ascidian brain and during brain development (Gomes et al., in preparation). Second, human PXR, TRs and PPARs bind BPA (Ahmed and Atlas, 2016;

Delfosse et al., 2014; Moriyama et al., 2002; Sui et al., 2012). Third, ascidian PXR/VDR α has already been shown to bind BPA (*Ciona robusta*, K_d: 5 μ M) (Richter and Fidler, 2015), while ascidian TR and PPAR show a good LBD conservation with human orthologues (data not shown). We are now assessing the involvement of these NRs in the neurodevelopmental phenotype induced by BPA.

5. Conclusion

In this manuscript we show that BPA affects pigmentation and otolith movement in the brain of an invertebrate chordate species, the ascidian *Phallusia mammillata*. Because pigment cells constitute the brain of the ascidian larvae, we demonstrate here that BPA induces neurodevelopmental toxicity to *P. mammillata* embryos.

We describe here for the first time otolith formation and its movement towards the ventral side of the ascidian brain vesicle. The expression of ERR during *Phallusia mammillata* embryonic development supports the hypothesis that ERR is part of BPA's induced inhibition of otolith movement in brain vesicle during the stages 22-26. Furthermore, both ERR agonist and antagonist experiments had smaller effect on the pigmentation, suggesting that reduction of pigmentation induced by BPA is most likely controlled by other pathways in addition to the ERR pathway. The potential binding of BPA to ascidian ERR, as well as the involvement of other nuclear receptors, such as TR, PXR or PPAR, in BPA neurodevelopmental toxicity is now being investigated.

Supplementary data

Supplementary figures are provided after the manuscript. Supplementary videos can be visualized from the following link: <http://lbdv-local.obs-vlfr.fr/~igomes/>

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Supplementary figures

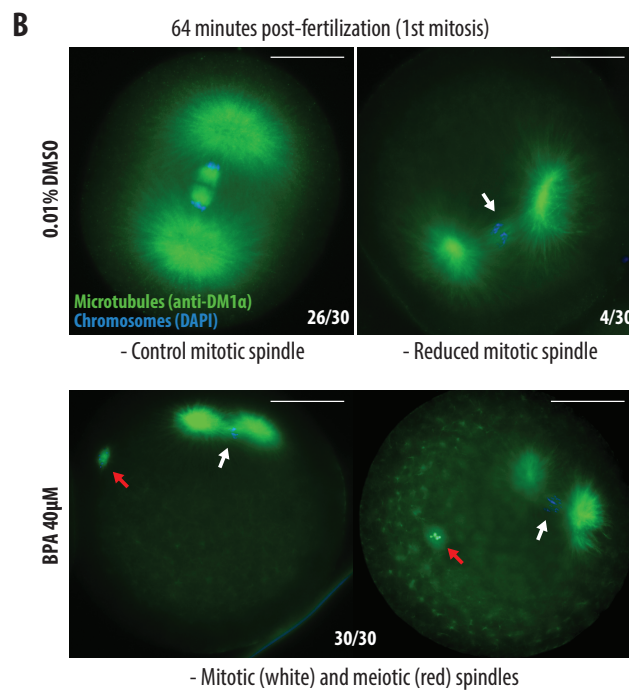
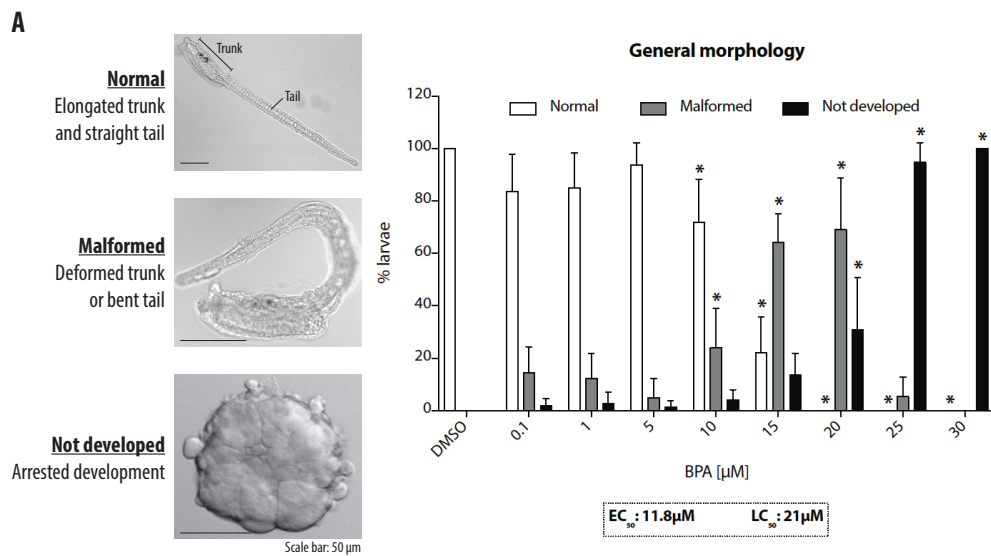


Figure S1. Bisphenol A is toxic to *Phallusia mammillata* embryos in a dose-dependent manner. (A) BPA effect on embryo general morphology. Embryos were exposed to different concentrations of Bisphenol A (BPA) from 1-cell stage, and three different phenotypes were assessed: *normal*, for larvae with elongated trunk and straight tail (white bars), *malformed* for larvae showing deformed trunk and/or bent tails (grey bars) and *not developed* for embryos displaying arrested development (black bars). Concentrations higher than 10 μ M significantly induced malformations. Concentrations higher than 20 μ M significantly arrested development. The median effective (EC₅₀) and lethal (LC₅₀) concentrations are shown. **(B) BPA induces genotoxicity at high doses.** *P. mammillata* eggs were fertilized and immediately exposed to BPA 40 μ M. Embryos were then fixed at the first mitosis (64min after fertilization) and stained for microtubules (anti-DM1 α , green) and DNA (DAPI, blue). While most of non-exposed embryos (0.01% DMSO) showed a well formed mitotic spindle, high dose of BPA blocked embryos to exit meiosis before mitosis, displaying both mitotic and meiotic spindles.

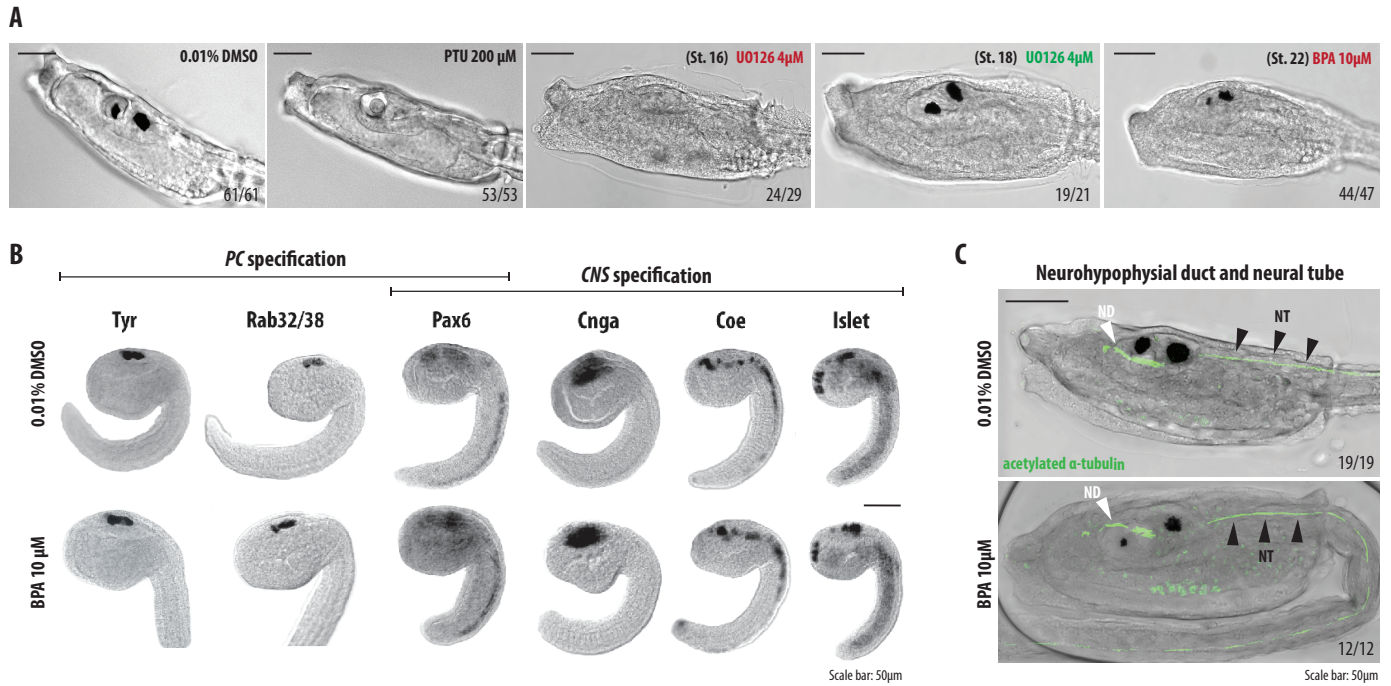


Figure S2. Bisphenol A does not affect *P. mammillata* pigment cell (PC) nor central nervous system (CNS) specification lineages. (A) BPA phenotype is different from PTU and UO126. Embryos were exposed to the tyrosinase enzyme inhibitor phenylthiourea (PTU, 200 μ M) at stage 1, and to the MAPK inhibitor UO126 (4 μ M) at stages 16 and 18 (corresponding to before and after the last MAPK signal induction for PC specification, respectively). PTU and UO126 at stage 16 completely abolished pigmentation, but not when embryos were exposed to UO126 at stage 18. BPA still induced the phenotype (reduced pigmentation and otolith blocked movement) even when incubated at stage 22, but not at stage 24. **(B-C) Spatial expression of PC and CNS specification genes are not affected by BPA.** The mRNA of genes driving/involved in **(B)** PC (*Opsin*, *Pax6*, *Rab32/38*, *Tyr*) and in **(C)** CNS (*Cnga*, *Coe*, *Eya*, *Islet*) specification were assessed by *in situ* hybridization, in both non-exposed (WT) and BPA-exposed (10 μ M) embryos. Expression does not seem to be affected by BPA. **(D) Cilia of the neurohypophysial duct and neural tube are not affected by BPA.** Expression of acetylated tubulin, marker of the ascidian neurohypophysial duct and neural tube cilia, was assessed by antibody staining, in both non-exposed (WT) and BPA-exposed (10 μ M) embryos. Expression does not seem to be affected by BPA. Note: all embryos were exposed to drugs from 1-cell stage, unless specified.

4. GENERAL DISCUSSION & PERSPECTIVES

My PhD project is aimed at deciphering the toxicity of the endocrine-disrupting chemical bisphenol A (BPA) on the embryonic development of the ascidian *Phallusia mammillata*. BPA has been shown to affect multiple targets and may have diverse and pleiotropic modes-of-action. Moreover, embryonic development has been shown as the most critical window of action for EDCs, particularly during neurodevelopment. Studies in vertebrates suggest that BPA can impair brain development and may thus be involved in the etiology of neurodevelopmental disorders in humans. In my study I found that 4 (ERR, PPAR, PXR/VDR α) are expressed within the ascidian brain (also called sensory vesicle, SV), or nearby (TR), corresponding to the human orthologues known to bind BPA. I showed that BPA acts during a precise window of the ascidian embryonic development to induce neurodevelopmental toxicity, by affecting the development of the neurosensory organ. Strikingly, BPA was found to reduce pigmentation of the ocellus/otolith (Oc/Ot), to reduce the distance between them and to diminish the size of the sensory vesicle lumen. In addition, a pharmacological approach revealed that the observed phenotype was specific to bisphenols (BPE, BPF), but not to biphenyls (2,2DPP). Finally, we hypothesize that BPA's effect on ascidian larval brain formation may be mediated by ERR as suggested by a screen of ERR agonists and antagonists and the zygotic expression of ERR in a few cells of the brain vesicle. However, other NRs are expressed in the ascidian brain and may therefore also participate in the phenotype exerted by BPA.

4.1. Bisphenol A affects *P. mammillata* embryo development in a dose-dependent manner

Chemical substances like EDCs induce several different responses among organisms depending on crucial factors such as concentration, time of exposure, route of uptake, sex, amongst others (Wright and Welbourn, 2002). Particularly, EDCs may behave like hormones and act at low and high but not at intermediate concentrations, displaying a U- or inverted U-shape response curve, i.e. a non-monotonic dose-response curve (NMDRC) (Vandenberg et al., 2012), which complicates the task of governmental organizations to define whether a chemical might be considered as an EDC or not (Zoeller et al., 2012).

In my study, I showed that *Phallusia mammillata* embryos exposed to BPA displayed a dose-dependent sensitivity, with a common monotonic dose-response curve (MDRC). BPA induces lethality to half of the embryos at 21 μM (LC50), and at 40 μM BPA is an aneugen, because it prevents formation of normal mitotic spindle and results in numerical chromosome aberrations (Parry et al., 2002). Even though BPA is an EDC and NMDRC are expected with these kind of chemicals (e.g. BPA effect on *Drosophila* (Weiner et al., 2014)), the obtained MDRC is not new, as it has been shown in other invertebrate and vertebrate species (Matsushima et al., 2013; Ozlem and Hatice, 2007; Tse et al., 2013) The LC50 identified in my study is higher than the ones described for other ascidian species *C. robusta* (LC50: 5.2 μM (Messinetti et al., 2018) and 5.4 μM (Matsushima et al., 2013)), and also sea urchin species *Paracentrotus lividus* (3.1 μM (Ozlem and Hatice, 2007)) and *Hemicentrotus pulcherrimus* (1-10 μM (Flint et al., 2012)), but lower than the vertebrate zebrafish (LC50: 73.4 μM (Tse et al., 2013)). BPA aneugenic activity has been previously seen in other invertebrate species like *C. elegans* (Allard and Colaiacovo, 2010) and sea urchin (George et al., 2008), suggesting tubulin as a direct target for BPA genotoxicity (George et al., 2008). In

mouse, not only BPA (Hunt et al., 2003), but also BPS (Horan et al., 2018) showed aneugenic activity, clearly demonstrating that new BPA replacement molecules in the market can be as toxic as BPA.

Regarding lower doses of BPA, I have found that with 11.8 μM BPA, half of the embryos displayed malformed trunks and/or bent tails (EC50). *P. mammillata* was less sensitive than other ascidian species such as *C. robusta* (EC50: 0.7 μM (Matsushima et al., 2013)), but more sensitive than the cnidarian *Hydra vulgaris* (EC50: 30.2 μM) and the crustaceans *Tigriopus japonicus* (EC50: 18.9 μM) and *Daphnia magna* (EC50: 52.5 μM) (Mihaich et al., 2009). This is particularly interesting because *D. magna* is used since the 2000's in OECD chemicals toxicity testing (<https://doi.org/10.1787/9789264069947-en>; (Guilhermino et al., 2000)), suggesting that *P. mammillata* embryos might be a more sensitive model than *D. magna* to test acute toxicity of chemicals (i.e. within 24h). Compared to vertebrates, *P. mammillata* was less sensitive than medaka fish *O. latipes* (0.9 μM (Flint et al., 2012)), but more sensitive than *Xenopus* (20 μM (Flint et al., 2012)) and zebrafish (EC50: 25 μM (Tse et al., 2013)). Overall, we can conclude that BPA induces embryonic malformations in *P. mammillata* in the same micromolar range as other invertebrates and vertebrates. Nevertheless, comparison might be hard to interpret since several factors, such as the choice of endpoints, the exposure time (e.g. ascidians take 24h to reach larvae stage, while zebrafish take 48h) and choice of solvent (DMSO, ethanol, final solvent percentage), may influence the final outcome. Moreover, the use of reference chemicals should be implemented to assess embryo quality (as it is done with ASTM (American Society for Testing and Materials) and OECD protocols).

It is also important to point out that the concentrations used in my study are overall higher than the concentrations reported in the environment (Canesi and Fabbri, 2015; Flint et al., 2012). The presence of BPA in the aquatic system is not negligible and neither is its leaching into the environment, as some authors demonstrated that the presence of nanoplastics can increase BPA bioavailability and cause neurotoxicity in adult zebrafish (Chen et al., 2017). Even if in my study the major BPA concentrations used were higher than those that can be found in the environment, bioaccumulation events have been reported in several organism systems (Corrales et al., 2015; Wright and Welbourn, 2002). Bioaccumulation is the accumulation of chemicals in an organism, and it occurs when the absorption is faster than its catabolism and excretion. Indeed, after collaborating with a chemistry master student, we have found that *P. mammillata* adults are capable of filtering the BPA available in the seawater (20L aquariums) and accumulate BPA in their body tissues (H  lo  se Gendre, unpublished data), suggesting BPA potential toxicity to ascidian progeny. The take-home message is that the low concentrations that are initially non dangerous will result in bio-accumulated BPA in the adult and increased exposure to the gametes of this adult that will become harmful to the reproduction of the organism.

Intriguingly, *P. mammillata* embryos that at first sight did not present any general malformation did in fact display malformations in the brain, detectable due to their two pigmented cells in the sensory vesicle. Neurodevelopmental defects induced by BPA have been found in invertebrates: BPA affected organism behavior in *Drosophila* (Kaur et al., 2015) and *C. elegans* (Mersha et al., 2015); in *Ciona*, exposure to BPA affected pigmented cells within the sensory vesicle (EC50: 0.16 μM (Messinetti et al., 2018)). Moreover, other molecules such as pesticides have been shown to affect the ascidian sensory vesicle (reviewed in Dumollard et al. 2017), yet no mode-of-action has been proposed so far. In vertebrates like

mouse, fish and frog, BPA has been proposed to affect neurogenesis, neuronal migration, gliogenesis and synaptogenesis (reviewed in Inadera 2015; Nesan, Sewell, and Kurrasch 2017). Time-lapse live imaging of the transparent *Phallusia* embryos allowed me to describe in detail the differentiation of ascidian neurosensory organ (Esposito et al., 2015; Racioppi et al., 2014) and how it is affected by BPA.

4.2. Exposure to Bisphenols affects ascidian melanogenesis

In my study, I showed that BPA reduced melanogenesis of the ascidian pigmented sensory organ. Moreover, my data suggests that melanogenesis during pigment cell (PC) differentiation is being targeted by BPA, rather than PC specification.

Several studies described pigmentation problems caused by BPA both in invertebrates and vertebrates. Concerning invertebrates, a similar phenotype was observed in another ascidian species, *C. intestinalis*, where both ocellus and otolith pigment spots were absent in embryos exposed to BPA at concentrations higher than 20 μ M (Matsushima et al., 2013). In zebrafish, more than 50% of the embryos exposed to 25 μ M BPA displayed reduced otolith pigmentation (Gibert et al., 2011).

As I referred in the introduction, vertebrate melanogenesis depends on genes such as MSHR, MITF, Tyr, Pmel and Rab GTPases (Wasmeier et al., 2008). Because BPA is affecting ascidian melanogenesis, one of the main questions would be "Is BPA impairing key melanogenesis genes to reduce pigmentation in ascidians?". First of all, MSHR receptors were not found in *Ciona* genome (Kamesh et al., 2008) nor in cephalochordates, but potentially appeared first in jawed vertebrates (Haitina et al., 2007), suggesting that MSHR is a vertebrate innovation, and thus cannot be a BPA target in ascidian melanogenesis. Secondly, it has been shown that *Ciona* pigment cell (PC) specification is dependent on Mitf, Tyr, Tyrp and Rab32/38 (Abitua et al., 2012; Racioppi et al., 2014). This specification is regulated under several FGF/MAPK signals from stage 13 up to stage 16 (Racioppi et al., 2014). In my study, the time-window of action of BPA (i.e. after stage 22) is after the final FGF-mediated induction of the PC lineage (at stage 16). Additionally, inhibiting PC specification in *P. mammillata* embryos by inhibition of MAPK signaling (using UO126) at stage 16 completely abolishes pigmentation, but not at stage 18, suggesting that specification of PC in *P. mammillata* happens before stage 18, similarly to *C. robusta* (Racioppi et al., 2014). Moreover, several marker genes for PC and CNS specification in ascidians (Tyr, Rab32/38, Pax6, Cnga, Coe and Islet) do not seem overtly affected by BPA exposure (quantitative measurement of expression by qPCR is necessary to confirm ISH data). Finally, total inhibition of tyrosinase enzyme (using PTU) did not phenocopy the BPA phenotype, and personal observations of PTU-exposed embryos showed that PTU abolishes melanin but not melanosome synthesis, while BPA reduces both melanin and melanosome structures, suggesting another mode of action. Together, these observations suggest that PC melanisation but not PC specification is affected and that BPA probably does not directly inhibits the tyrosinase enzyme.

Currently, it is not clear the mechanism by which BPA is interfering with ascidian melanogenesis. My data strongly suggests that BPA is affecting ascidian PC differentiation rather than specification. However, the gene regulatory networks (GRNs) involved in ascidian PC differentiation are not known and more efforts will be needed in the future to uncover this. The evolution of molecular tools, such as single-

cell RNA-seq for example, would allow us to, in a first phase, reveal all the gene populations in the fully developed ascidian sensory vesicle (st. 26) and, in a second phase, compare the GRNs of non-exposed to BPA-exposed embryos to assess whether expression of melanogenic genes is affected by BPA.

4.3. Bisphenols affects ascidian otolith differentiation and expansion of the sensory vesicle lumen

Ascidian otolith development has been quite well characterized in the first (st. 16-20) (Racioppi et al., 2014) and final stages (st. 26) (Dilly, 1964, 1962) of embryogenesis, but not in between. At the beginning, the otolith is the neighboring cell of the other pigment cell, the ocellus, and both are present in the sensory vesicle epithelium (Abitua et al., 2012; Racioppi et al., 2014). When fully developed (st. 26), the otolith is a unicellular structure that protrudes into the lumen of the sensory vesicle (Dilly, 1962). But how does the otolith (initially dorsally positioned and within the sensory vesicle epithelium) become ventrally positioned and alone in the sensory vesicle lumen? In my study, timelapse imaging allowed me to describe in more detail the formation of the otolith. In fact, between stages 22 and 26, the otolith swells from the sensory vesicle epithelium, producing a foot on its basal side and a temporary epithelium invagination. The otolith foot then moves towards the ventral side of the sensory vesicle. Even if this otolith movement has been previously defined as an epithelial-to-mesenchymal (EMT) transition (Stolfi et al., 2015), a characteristic of neural crest cells in vertebrates (Green et al., 2015), this is the first live imaging following such cell behavior. Furthermore, at the same time as the otolith protrudes into the sensory vesicle lumen, this lumen expands and will consequently generate a certain distance between the otolith and the ocellus.

In the present study, I showed that BPA clearly affects the described otolith movements. Consequently, BPA induced a significant reduction of the distance between otolith and ocellus. By imaging transgenic *Phallusia* embryo expressing GFP markers in the PC lineage (*Tyrp*, *βγCristallin* and *Msx*), it was possible to visualize that in BPA-exposed embryo, the otolith tries to protrude from the epithelium, as both epithelium invagination and otolith foot formation are still visible, but its detachment never happens. Indeed, this behavior was not a delay in development as the embryo entered metamorphosis (seen as the notochord invagination in the trunk in supplementary video 2). Additionally, the lower Oc/Ot distance observed in BPA-treated embryos may also be due to a reduced expansion of the sensory vesicle lumen. It has been shown that the ammonium transporter AMT-1a (Marino et al., 2007) and the ion transporter SLC26a (Deng et al., 2013) proteins are involved in sensory vesicle lumen expansion in ascidians. Like *Ciona robusta*, two *AMT1* genes (*Aniseed* gene IDs: *Phmamm.g00000383* and *Phmamm.g00018002*) and the *SLC26α* gene (*Phmamm.g00014562*) are present in *P. mammillata* genome, showing a higher transcriptional activity at later stages (21-26). It would be thus interesting to assess whether the expression of such genes is being targeted by BPA, as well as to quantify the lumen of both non-exposed and BPA-exposed embryos. For this, I would assess *AMT1a* and *SLC26α* expression (at st. 26) in non-exposed and BPA-exposed larvae by qPCR. To measure the volume of the sensory vesicle lumen, I would incubate embryos in a membrane dye for live cell imaging, e.g. *Cell Mask* (McDougall et al., 2015),

during neural tube closure (between stages 16 and 19) and perform high resolution z-scans (two z steps per optical section) over the critical time window, using confocal microscopy to later segment the sensory vesicle lumen using Imaris software.

Furthermore, by actin staining, I showed that exposure of embryos to the BPA analogues BPE and BPF elicited the same phenotype, i.e. all bisphenols inhibited the otolith movement towards the ventral side of the sensory vesicle, as their otolith foots are all dorsally positioned and sensory vesicle lumen seems reduced. My data suggests that the observed phenotype is specific to bisphenols since exposure to the biphenyl 2,2 DPP had no effect on larvae sensory vesicle (neither pigmentation, neither otolith movement nor sensory vesicle lumen). Interestingly, Tohmé and colleagues showed a similar bisphenol-specificity effect in zebrafish otolith development, suggesting that bisphenols may share a similar mode of action in both ascidians and fishes (Tohmé et al., 2014). Because BPA, BPE and BPF can bind human ERR γ and not 2,2 DDP due to the lack of hydroxyl groups on its phenyl rings (Okada et al., 2008; Starovoytov et al., 2014), this suggests that BPA may impair otolith development by affecting ERR activity.

4.4. BPA may target ERR during otolith differentiation in ascidians larvae

Recent evidence demonstrated that BPA is a weak ligand for estrogen receptor (K_d ER α : 1040 nM; K_d ER β : 1320 nM (Takayanagi et al., 2006)) but high affinity ligand for estrogen-related receptor gamma (ERR γ K_d : 5-13 nM) (Liu et al., 2014; Takayanagi et al., 2006). I sought to explore further this possibility in *Phallusia* embryo, as the *Ciona* genome was reported to possess only one gene coding for ERR, and no steroid receptors such as Estrogen or Androgen Receptors (Yagi et al., 2003). Indeed, phylogenetic analysis showed that *Phallusia mammillata* ERR clustered together with other ascidian ERR genes (supplementary figure S1A in Annexes). A pharmacological approach showed that ERR agonists and antagonists partially phenocopied the BPA phenotype, by affecting the distance between ocellus and the otolith. Analysis of ERR expression in *P. mammillata* embryos revealed that ERR is expressed in the sensory vesicle around the ascidian PC, during PC differentiation, suggesting that *Pm*-ERR might, indeed, be the mediator of the effect of BPA on otolith movement.

Firstly, lack of effect of the estrogen estradiol β -benzoate (E2B) is consistent with the fact that no ER-like or GPER-like proteins can be found in the ascidian genome (Kamesh et al., 2008; Yagi et al., 2003).

Secondly, it is known that ERRs do not respond to estradiol despite their significant homology with ERs in the ligand binding domain (LBD) (Greschik et al., 2002). While DES was shown to bind to all the three human ERRs, GSK4716 and 4-OHT only bind to human ERR β and ERR γ (Coward et al., 2001; Zuercher et al., 2005). I observed that both ERR agonist GSK4716 and ERR antagonists DES and 4-OHT significantly reduced the distance between ocellus and otolith. Additionally, they also seem to reduce trunk elongation, and I will discuss this topic later on section 4.5. Because of Oc/Ot distance phenotype induced by ERR agonists and antagonists and the fact that *Pm*-ERR is expressed in the ascidian sensory vesicle around the pigment cells, I hypothesize that *Pm*-ERR might bind BPA to affect ascidian neurodevelopment.

In silico analysis revealed that the aminoacid sequence of *Pm*-ERR is well conserved with human ERR γ , particularly regarding DBD and LBD domains, as well as the presence of sumoylation motifs (PDSM/SM) and nuclear localization signals (NLS) (supplementary figure S1B in Annexes) (Tremblay et al., 2008). Furthermore, aminoacid residues known to be crucial for the binding of BPA, DES and 4-OHT to human ERR γ were mostly conserved between human and *Pm*-ERR (supplementary figure S1C in Annexes). Within 17 aminoacid residues, 10 are conserved (black arrows) and 5 are similar (grey arrows), with only 2 (red arrows) not conserved. Concerning the 2 non-conserved aminoacids, in human ERR γ sequence, one is the asparagine 346 (N346) and the other is the histidine 434 (H434), which are replaced by a tyrosine (Y409) and a phenylalanine (F502) in the *Pm*-ERR, respectively. Nevertheless, Liu and colleagues have shown that mutations of the N346 to tyrosine (N346Y) and of H434 to phenylalanine (H434F) would still allow BPA binding to ERR γ , with a lower affinity though (5 nM in the wild-type against 14 nM for N346Y and 45 nM for H434F) (Liu et al., 2014). Altogether, these data suggests that BPA might bind to the *Pm*-ERR.

However, even if pharmacological and *in silico* data suggests that *Pm*-ERR might bind BPA and be the mediator to explain impaired ascidian neurodevelopment, we cannot be sure of the specificity or effect of human ERR agonists and antagonists in the ascidian organism, and protein-ligand binding is a complex subject that cannot be solved with modest *in silico* analysis. The crucial experiments to perform are **1**) to prove BPA binding to *Pm*-ERR by radioligand binding assay or two-hybrid screening using a ERR-LBD reporter plasmid (Grimaldi et al., 2015); **2**) to prove that ERR is necessary for the BPA phenotype by knock-out (KO) by Crispr/Cas9 (Stolfi et al., 2014); and **3**) to assess if ERR is sufficient for the BPA phenotype by *Pm*-ERR overexpression (ERR::Venus mRNA). I am now performing these experiments to confirm my hypothesis.

4.5. BPA may be targeting other nuclear receptors in the ascidian larvae

Nuclear receptors (NRs) play essential roles in development, metabolism and physiology, consequently their dysfunctions are associated with many diseases. NRs are, therefore, major drug targets (Delfosse et al., 2015b). As I previously discussed, several clues led me to suggest ERR as the potential mediator for a part of the observed BPA phenotype, i.e. the otolith movement within the sensory vesicle. However, it is important to note that BPA is affecting the Oc/Ot distance twice stronger than ERR agonists and antagonists (31% vs ~60%), suggesting that BPA may have a pleiotropic action by targeting other nuclear receptors (figure 10). Pregnane X Receptor/Vitamin D Receptor alpha (PXR/VDR α), Thyroid Receptor (TR) and Peroxisome Proliferator-Activated Receptor (PPAR) are all potential BPA targets for several reasons. First, *P. mammillata* PXR/VDR α , TR and PPAR are expressed in the ascidian brain and during brain development (figures 9 and 10A). Second, human PXR, TRs and PPARs bind BPA (Ahmed and Atlas, 2016; Delfosse et al., 2014; Moriyama et al., 2002; Sui et al., 2012) (figure 10B). Third, while ascidian PXR/VDR α has already been shown to bind BPA (*Ciona robusta*, K $_d$: 5 μ M) and other chemicals (Richter and Fidler, 2015), TR and PPAR reveal good LBD conservation with human orthologues, particularly concerning the key aminoacid residues involved in BPA binding (data not shown).

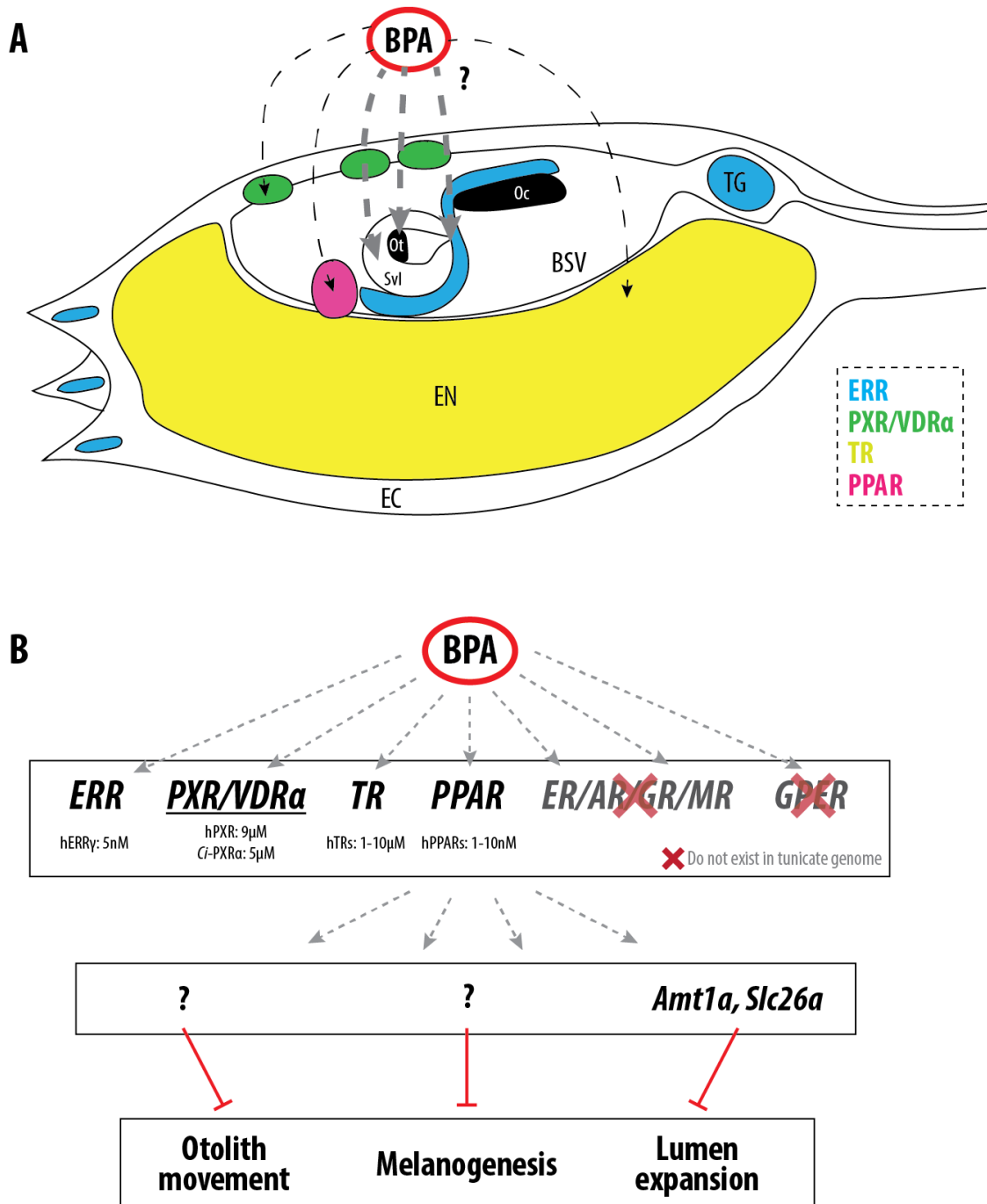


Figure 10. Proposed model to explain the BPA phenotype targets in the ascidian brain. (A) BPA affects pigmentation, otolith movement and sensory vesicle lumen expansion in *P. mammillata* embryos. Besides ERR (blue areas), BPA might also be targeting other *P. mammillata* NRs expressed within the sensory vesicle (PXR/VDR α in green and PPAR in pink) or nearby (TR in yellow), which their vertebrate orthologues are known to bind BPA. (B) Because ascidians do not possess any estrogen/steroid receptors in their genome (red crosses), BPA neurodevelopmental toxicity might be mediated by the previously referred NRs until the final observed phenotype. While mediators of otolith differentiation and melanogenesis pathways are totally unknown, *Amt1a* and *Slc26a* genes have been previously showed to be involved in sensory vesicle lumen expansion. BSV: brain sensory vesicle, SVI: sensory vesicle lumen, Ot: otolith, Oc: ocellus, TG: trunk ganglion, EN: endoderm, EC: ectoderm.

As referred in my introduction, PXR is expressed in a wide range of tissues, particularly in the liver due to its role in detoxification processes. As a consequence, PXR is known to possess a large binding pocket in order to bind a wide range of molecules (Kliewer, 2015; Kliewer et al., 2002; Watkins et al., 2001). We can assume a similar argument for ascidian PXR/VDR α , since *Ciona* PXR/VDR α has been shown to bind both natural occurring molecules such as marine algal toxins, as well as human-made chemicals such as BPA (Fidler et al., 2012; Richter and Fidler, 2015). Moreover, preliminary data of *Pm*-PXR/VDR α exposure to human PXR agonists (SR12813) suggests that this NR might also be involved in the BPA phenotype (Gazo et al., in preparation). This is intriguingly interesting, since cross-regulation between different nuclear receptors might happen but such a research field is today poorly explored, maybe because of the complexity to test it. It would be exciting to perform ERR and PXR/VDR α gene knock-out individually and together to test this hypothesis. It is important to note that because the ascidian genome only possess one copy of each NR, it facilitates the task when compared to other chordates such as fish or human cell lines, revealing the powerful tool that the ascidian larvae can be when assessing NR role in development. It is worth mentioning that PXR is also expressed in the vertebrate brain (Bertrand et al., 2007; Reschly and Krasowski, 2006; Shen, 2017), but its function in this tissue has been largely overlooked. The discovery of PXR expression in the ascidian brain raises the intriguing question about PXR role in the brain. Is it involved in both tunicate and vertebrate neurodevelopment?

Thyroid hormones and receptors in the human brain have several crucial roles (Bernal, 2007; Préau et al., 2014). Among them, TR β and T3 have been shown to be important for ear and auditory system development (Ng et al., 2013), and low levels of thyroid hormone (hypothyroidism) have been associated with the Meniere's disease, a disorder of the inner ear manifesting as vertigo, tinnitus and sensory neural hearing loss (Santosh and Rao, 2016). When Gibert and colleagues found a BPA-induced otolith phenotype in zebrafish, they wondered whether zebrafish TR would be involved in the phenotype (Gibert et al., 2011). The authors suggested that the BPA otolith phenotype was not TR-dependent, since the addition of T3 (1 μ M) did not interfere, either positively or negatively, indicating that the thyroid signaling pathway does not seem to be responsible for the BPA-induced otolith malformations in zebrafish (Gibert et al., 2011). *Pm*-TR is expressed in the ascidian endoderm from late neurula (st. 16) up to larva (st. 26), at the same time as neural tube closure and sensory vesicle development. Thus, can TR be involved in ascidian neurodevelopment and work as a potential mediator of BPA neurodevelopmental toxicity?

The PPAR family of nuclear receptors plays a major regulatory role in energy homeostasis and metabolic function, explaining its main expression in adipose tissues. Besides adipose tissues, the mRNA of all three human PPAR subtypes were found to be expressed in melanocytes (Kang et al., 2004). While two PPAR agonists (WY-14643 and ciglitazone) stimulated melanogenesis via stimulation of the tyrosinase enzyme (Kang et al., 2004), a PPAR antagonist (GW9662) inhibited melanogenesis in murine melanoma cells (Chen et al., 2014), revealing an interesting role of PPAR in vertebrate melanogenesis. This raises the question: can BPA be acting as a PPAR antagonist to reduce melanogenesis in ascidian pigment sensory organ? *P. mammillata* PPAR expression occurs next to the otolith, at the beginning of the

melanogenesis process, thus making this hypothesis very intriguing. Furthermore, the role of PPAR in ascidians is not known today.

Additionally, when performing live timelapse imaging of BPA-exposed embryos, the trunk of the larvae did not seem affected. However, analysis of a high amount of BPA-exposed larvae indicated a significant reduction in the length of the trunk, in a similar way as ERR agonists and antagonists (this study) and as other type of chemicals like pesticides (data not shown), suggesting that affected trunk elongation might be the result of non-specific toxicity. To this date, I cannot propose a potential mediator of trunk toxicity, as the mechanisms involved in the elongation of the ascidian trunk are not currently known.

Besides this potential pleiotropic effect, i.e. the capacity of one molecule to activate several signaling pathways, resulting in a complex phenotype, there is also the fact that in the environment a mixture of chemicals is present, rather than one single molecule. Chemicals may act synergistically to activate NRs and induce a complex phenotype, as recently shown by PXR activation (Delfosse et al., 2015a), reinforcing the awareness that binary chemical mixtures are more dangerous than one molecule by itself and are strongly recommended in laboratory studies.

Altogether, the available studies clearly demonstrate that there is an enormous scarcity of information concerning the roles of these NRs in ascidian development. Importantly, cofactors are present in the ascidian genome, similarly to cephalochordates (Schubert et al., 2008), suggesting that the machinery needed for NR response is present in the ascidian embryo. It would be of greatest interest to decipher NRs roles in ascidian development to shed more light on the roles of NRs during embryogenesis of early chordates.

Conclusions

This is the first study to show the embryonic expression of *Phallusia mammillata* nuclear receptors. While the expression of some NRs was previously reported in another ascidian species (TR, RAR, LXR), expression pattern of PPAR, Rev-erb, ROR, PXR/VDR α , HNF4, COUP, ERR and GCNF were reported for the first time in this study.

Similar to vertebrates, BPA induces genotoxicity at high doses and neurodevelopmental toxicity at medium doses to *Phallusia mammillata*, resulting in a monotonic dose-response curve. Neurodevelopmental toxicity was characterized by the capacity of BPA to impair pigmentation (melanogenesis) of the ascidian pigmented sensory organ, otolith differentiation (by inhibition of the otolith movement towards the ventral side of the sensory vesicle) and sensory vesicle lumen expansion. Additionally, elongation of the trunk was reduced, yet probably due to non-specific toxicity as it was common to other chemicals. Moreover, the need to produce BPA-free products leads the industries to use other types of bisphenols, raising the question of whether these are less dangerous to human health.

We further show that the neurodevelopmental effect of BPA is still visible in the presence of other bisphenols, and that it is specific to bisphenols over biphenyls.

Within the vertebrate NRs known to bind BPA, it has been shown that human ERR γ binds BPA with high affinity, and it has been suspected as a potential mediator in neurodevelopmental diseases as it is expressed in the vertebrate brain. Vertebrate estrogen-related receptor (ERR) agonists and antagonists partially phenocopied BPA phenotype in *P. mammillata* embryos, suggesting an involvement of *Pm*-ERR. With *Pm*-ERR expressed in the larval sensory vesicle close to the ascidian pigmented sensory organ and its aminoacid sequence highly conserved with human ERR γ , *Pm*-ERR can thus be a target of BPA in mediating neurodevelopmental toxicity in ascidians. Finally, the first description of other NRs (COUP, PPAR and PXR/VDR α) in the ascidian larval brain raises the possibility of their involvement in both ascidian development and mediating toxicity, thus they should not be overlooked.

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ANNEXES

Table S1. Gene ID numbers of *Phallusia mammillata* and *Ciona robusta*, available at Aniseed database. In dark red, the genes that were unsuccessfully cloned in this study. The PXR/VDR β NR was not found in *Aniseed*, so the clone number from *P. mammillata* cDNA library is shown. The NR5A was not found either in *Aniseed* or *P. mammillata* cDNA library.

Nuclear receptor	<i>P. mammillata</i> (Phmamm)	<i>C. robusta</i> (Cirobu)
TR	g00013148	g00002460
RAR	g00001201	g00010140
PPAR	g00014986	g00008750
Rev-erv	g00002605	g00003453
ROR	g00011119	g00005327
LXR	g00006094	g00004682
PXR/VDR α	g00002941	g00004909
PXR/VDR β	? (clone 196YC16)	g00001883
HNF4	g00011809	g00006049
RXR	g00000679	g00010482
TR2/4	g00007988	g00009602
COUP	g00007626	g00011734
ERR	g00012306	g00013110
GCNF	g00011487	g00000021
FXR	g00006103	g00004334
NR4A1/2/3	g00003933	g00009986
NR5A	?	g00007614
Co-factors	<i>P. mammillata</i> (Phmamm)	<i>C. robusta</i> (Cirobu)
NCOA	g00007309	g00011562
PGC1a	g00006680	g00007502
CBP/p300	g00003049	g00003884
NCOR	g00004419	g00001791
TRAP220	g00001467	g00002395
Other genes	<i>P. mammillata</i> (Phmamm)	<i>C. robusta</i> (Cirobu)
Tyr	g00005364	g00003025
Rab32/38	g00006935	g00013795
Pax6	g00010911	g00010249
Cnga	g00015101	g00004428
Coe	g00012706	g00012207
Islet	g00000576	g00011396

Figure S1. *In silico* analysis of *P. mammillata* ERR. (A) Phylogenetic tree of *Pm*-ERR. The *P. mammillata* ERR clustered next to other ascidian ERR proteins. Human estrogen receptors (ER) were used as outgroup. The phylogenetic tree was constructed by maximum likelihood method (PhyML), with 1000 bootstrap replicates, using Seaview software. **(B) *Pm*-ERR sequence.** The *P. mammillata* ERR sequence cloned in this study is shown. For comparison, human ERR γ was used, together with *Ciona robusta* and *Halocynthia roretzi* ERR. Nuclear receptor important domains are shown, as well as sumoylation motifs (PDSM/SM) and nuclear localization signals (NLS) (see legend). *Pm*-ERR is highly conserved with human ERR γ , and it displays one PDSM and one SM, and two predicted NLS. **(C) Comparison of human ERR γ ligand binding domain with ascidian ERR.** Within 17 aminoacid residues known to be important for BPA, DES and 4-OHT binding to human ERR γ , 10 are conserved (black area and black arrows) and 5 are similar (grey area and grey arrows), with only 2 not being conserved (red arrows) (for reference, see Liu *et. al*, 2014).

