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Quels tests spécifiques pour identifier les perturbateurs endocriniens ?

François BRION

Unité d'écotoxicologie *in vitro* et *in vivo*

INERIS

maîtriser le risque
pour un développement durable

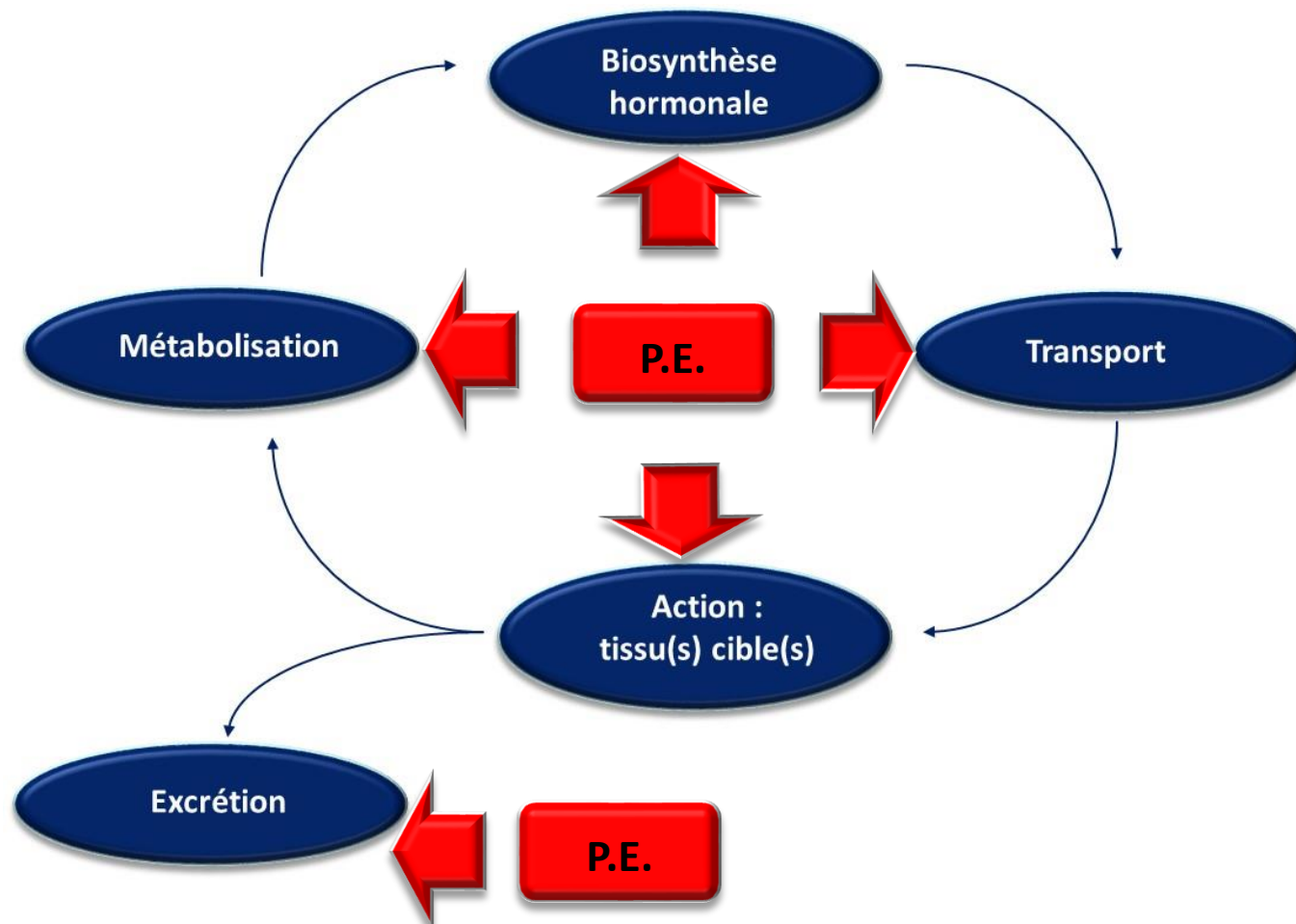
Perturbateurs Endocriniens: définitions

- Substances qui « interfèrent avec les processus de synthèse, de sécrétion, de transport, d'action ou d'élimination des hormones responsables de l'homéostasie, de la reproduction et du comportement » (Kavlock *et al.*, 1996)

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Dynamique hormonale



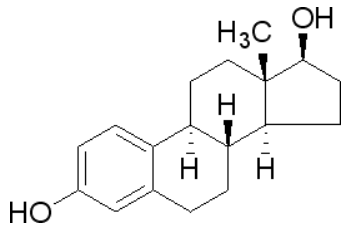
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- « Substances exogènes qui provoquent des effets néfastes sur la **santé** d'un **organisme** ou sa **descendance**, secondairement à des changements de la fonction endocrine » (OCDE, 1997)

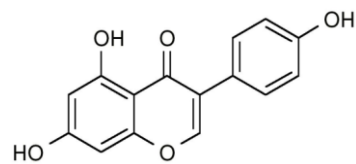
Perturbateurs Endocriniens: définitions

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- « Substances exogènes qui provoquent des effets néfastes sur la **santé** d'un **organisme** ou sa **descendance**, secondairement à des changements de la fonction endocrine » (OCDE, 1997)
- « Substance ou **mélange de substances**, qui altère les fonctions du système endocrinien et de ce fait induit des **effets néfastes** dans un **organisme** intact, chez sa **progéniture** ou au sein de **(sous)-populations** » (OMS, 2002)

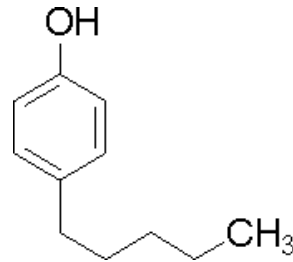
Perturbateurs Endocriniens: une grande diversité de molécules



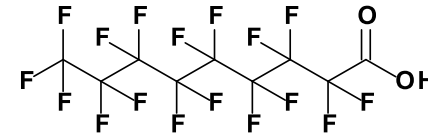
17β-oestradiol



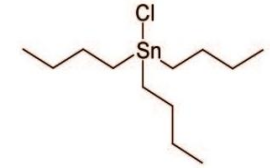
génistéine



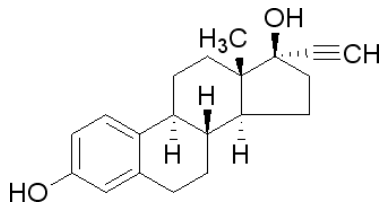
Pentylphenol



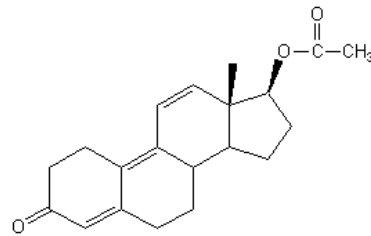
PFOA



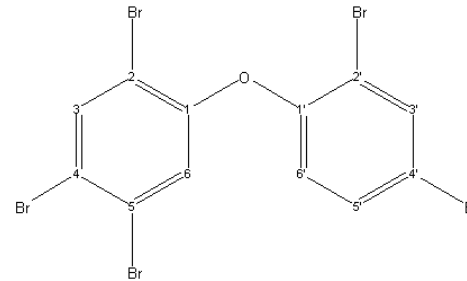
tributyltin



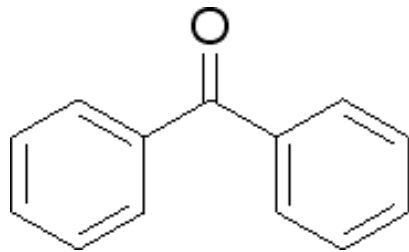
17α-Ethinylloestradiol



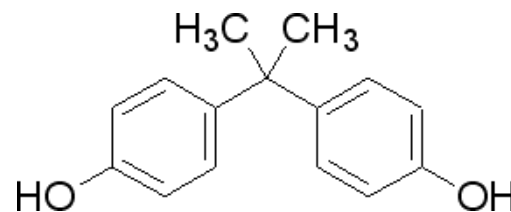
Trenbolone acetate



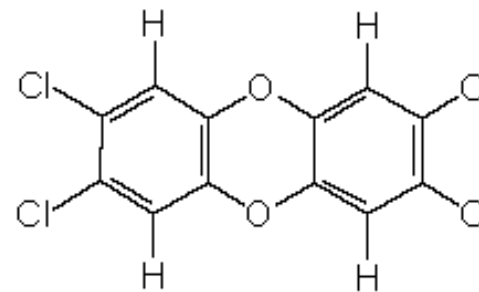
Polybromodiphenyléthers (PBDEs)



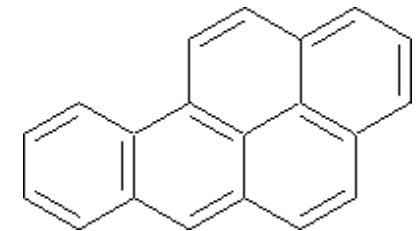
BP 1



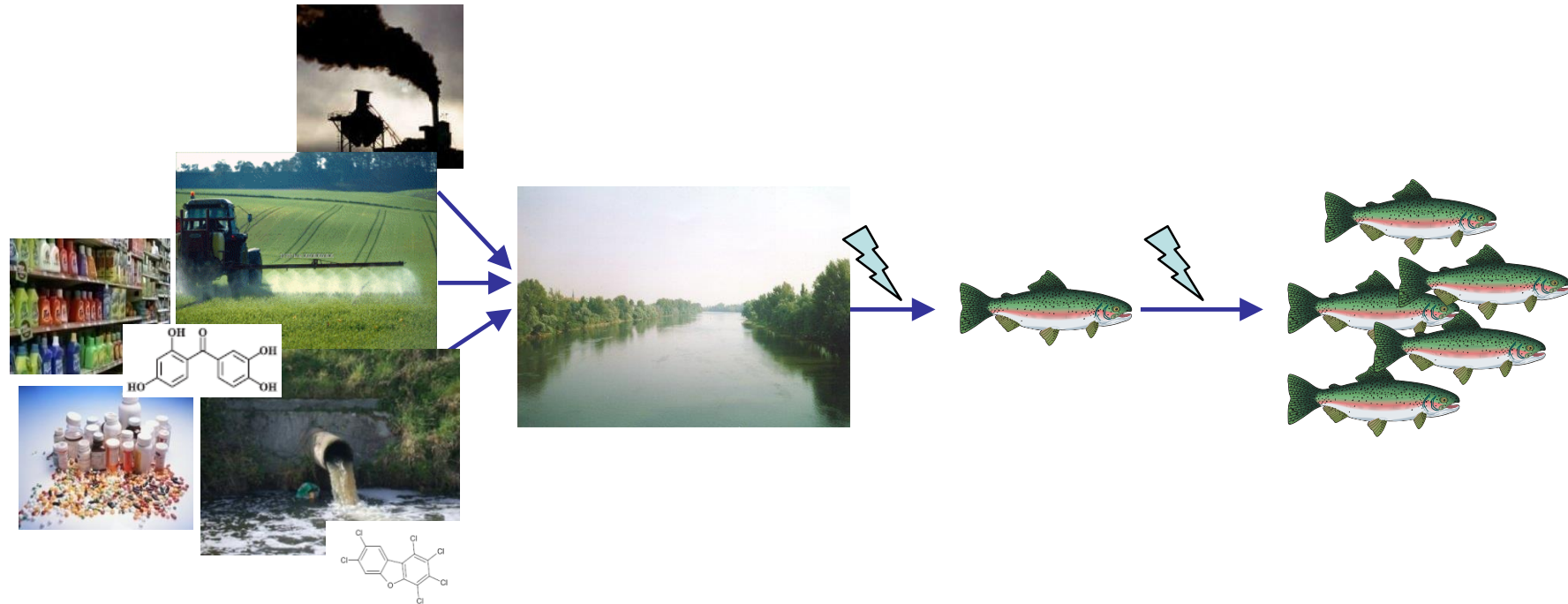
BPA



TCDD



Environmental ED = chronic exposure, individual and population responses



multiple source of contamination
complex mixtures

chronic exposure

low concentration

Effect in individuals

Effect at the population level

ED in the aquatic environment

Endocrine Disrupting Chemicals (EDCs)
in aquatic systems



Disruption of development, sexual
differentiation, reproduction



Risks for aquatic species

Natural & synthetic estrogens
Xeno-estrogens

(Nash et al., 2004, Brion et al., 2004, Kidd et al., 2007)



Environmental estrogens can act at low concentrations on
various biological levels
(from molecular to population level)



Derivation of EQS for EE2, E2, E1
(Watchlist of the Water Framework Directive)

Environmental EDs : How to deal with ?

Endocrine Disrupting Chemicals (EDCs) in
aquatic systems



Disruption of development, sexual
differentiation, reproduction



Risks for aquatic species



Environmental hazard and risk assessment posed by EDs ?

Environmental EDs : How to deal with ?

Endocrine Disrupting Chemicals (EDCs) in
aquatic systems



Disruption of development, sexual
differentiation, reproduction



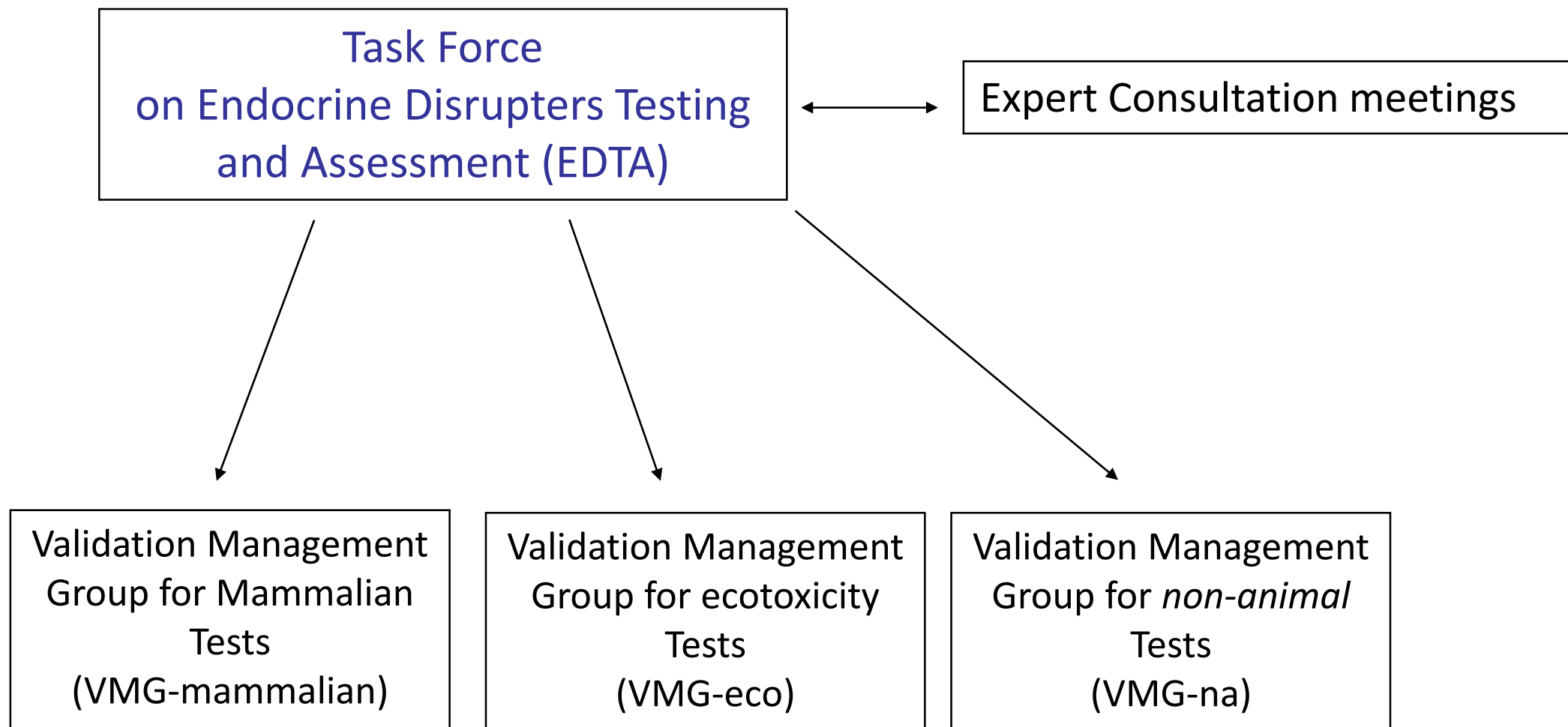
Risks for aquatic species



Hazard and risk assessment posed by EDs ?

Development and validation of specific tests for ED,
Implementation of screening and testing strategies

Development of specific test guidelines at OCDE level



OCDE conceptual framework

- **Level 1** : Existing data and non-test information
- **Level 2**: *In vitro* assays providing data about selected endocrine mechanism(s) / pathway(s)
- **Level 3**: *In vivo* assays providing data about selected endocrine mechanism(s) / pathway(s)
- **Level 4**: *In vivo* assays providing data on adverse effects on endocrine relevant endpoints
- **Level 5**: *In vivo* assays providing more comprehensive data on adverse effects on endocrine relevant endpoints over more extensive parts of the life cycle of the organism

Environmental EDCs : How to deal with ?

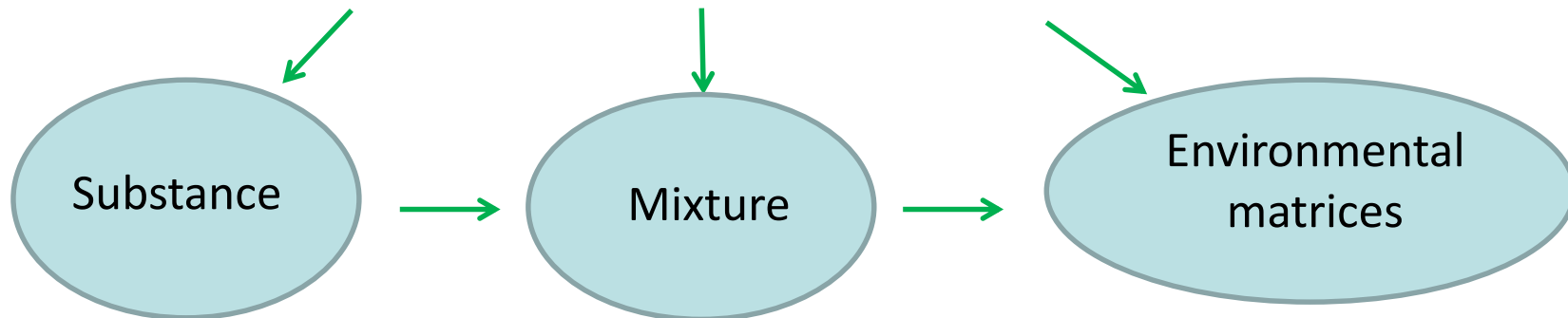
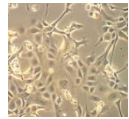
Level 1 Sorting & prioritization based upon existing information	- Physical & chemical properties/fate (MW, reactivity, volatility, persistence and bioaccumulation, pH, Po/w, ...) - exposure information/models (production volume, release and use pattern, human and environmental monitoring data, etc.) - hazard information (e.g. QSAR, human data, available toxicological data)	
Level 2 <i>In vitro</i> assays providing mechanistic data	- ER, AR, TR receptor binding affinity - transcriptional activation assays - aromatase and steroidogenesis <i>in vitro</i> - aryl hydrocarbon receptor recognition/binding - QSARs	- Thyroid function - Fish hepatocyte VTG assay
Level 3 <i>In vivo</i> assays providing data about single endocrine mechanisms	- Uterotrophic assay (estrogenic related) - Hershberger assay (androgenic related) - Non -receptor mediated hormone function	- Fish VTG (vitellogenin) assay (estrogenic related)
Level 4 <i>In vivo</i> assays providing data about multiple endocrine mechanisms	- Enhanced OECD 407 (endpoints based on endocrine mechanisms) - male and female pubertal assays - adult intact male assay	- Fish Sexual Developmental Test - Frog metamorphosis assay
Level 5 <i>In vivo</i> assays providing adverse effects data from endocrine & other mechanisms for RA	- 1-generation assay (TG415 enhanced)¹ - 2-generation assay (TG416 enhanced)¹ - reproductive screening test (TG421 enhanced)¹ - combined 28 day/reproduction screening test (TG422 enhanced)¹ 1 potential enhancements will be considered by VMG mamm.	- Partial and full life cycle assays in fish, birds, amphibians & invertebrates (developmental and reproduction)

- The mechanisms of ED investigated are limited
- Only human cell-based assays as screening assays (Level 2)
- In vivo fish screening assays (Level 3) are difficult to implement for rapid screening purposes
- Limited to detect potential ED acting as estrogen, androgen or aromatase inhibitor on peripheral organs (liver, gonads)
- Transgenerational effects through epigenetism are not take into account
- Mixture effects are not take into account

An integrated approach to investigate the effect of ED in a unique model fish species

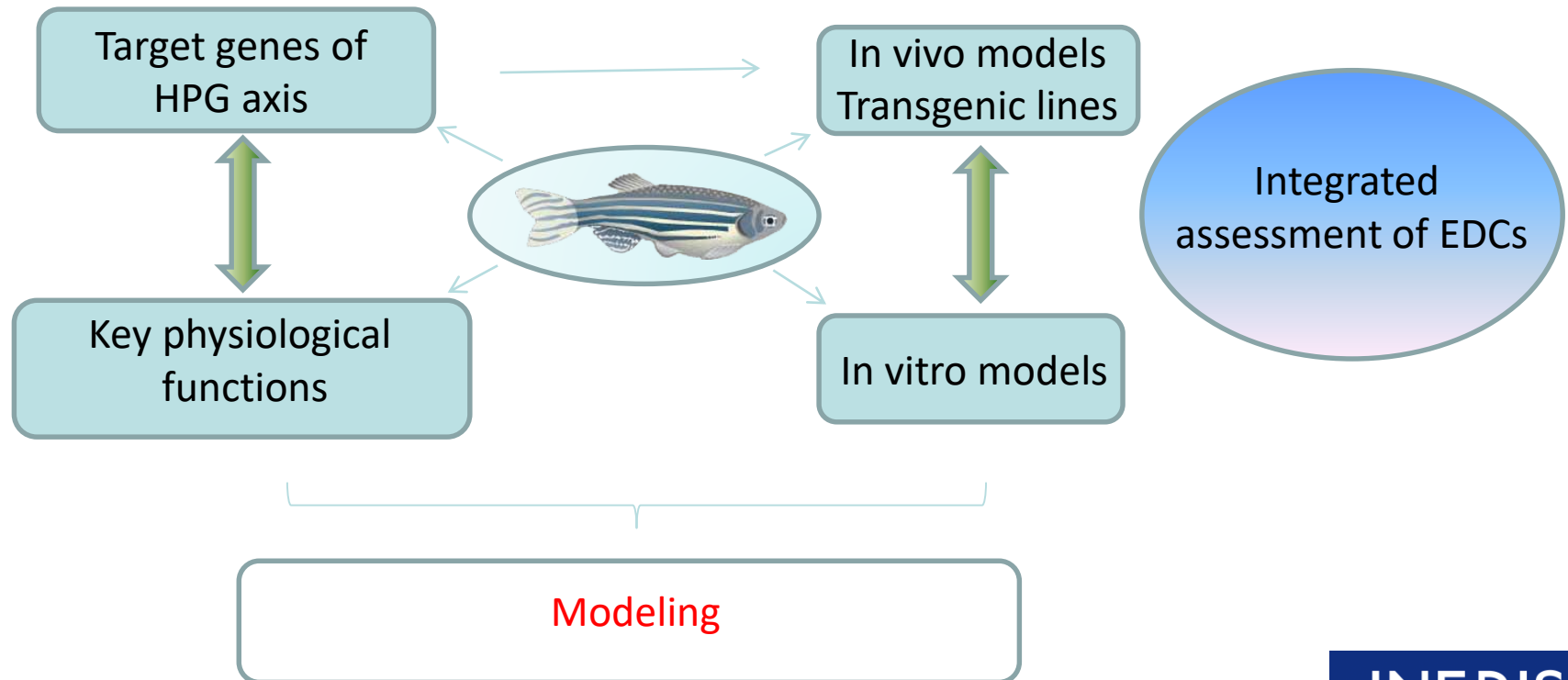
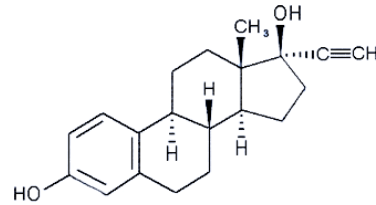
Environmental hazard and risks posed by ED?

in vitro & *in vivo* species-specific mechanism-based bio-assays



The zebrafish as a model organism for investigating endocrine disruption and assessing effects of endocrine disrupting chemicals

Mechanisms and effects ?



Les récepteurs nucléaires (RN)

Différents récepteurs nucléaires, différents ligands endogènes, différentes distribution tissulaires et cellulaires, supportent des fonctions différentes

Steroid Receptors



RXR Heterodimers



Dimeric Orphan Receptors

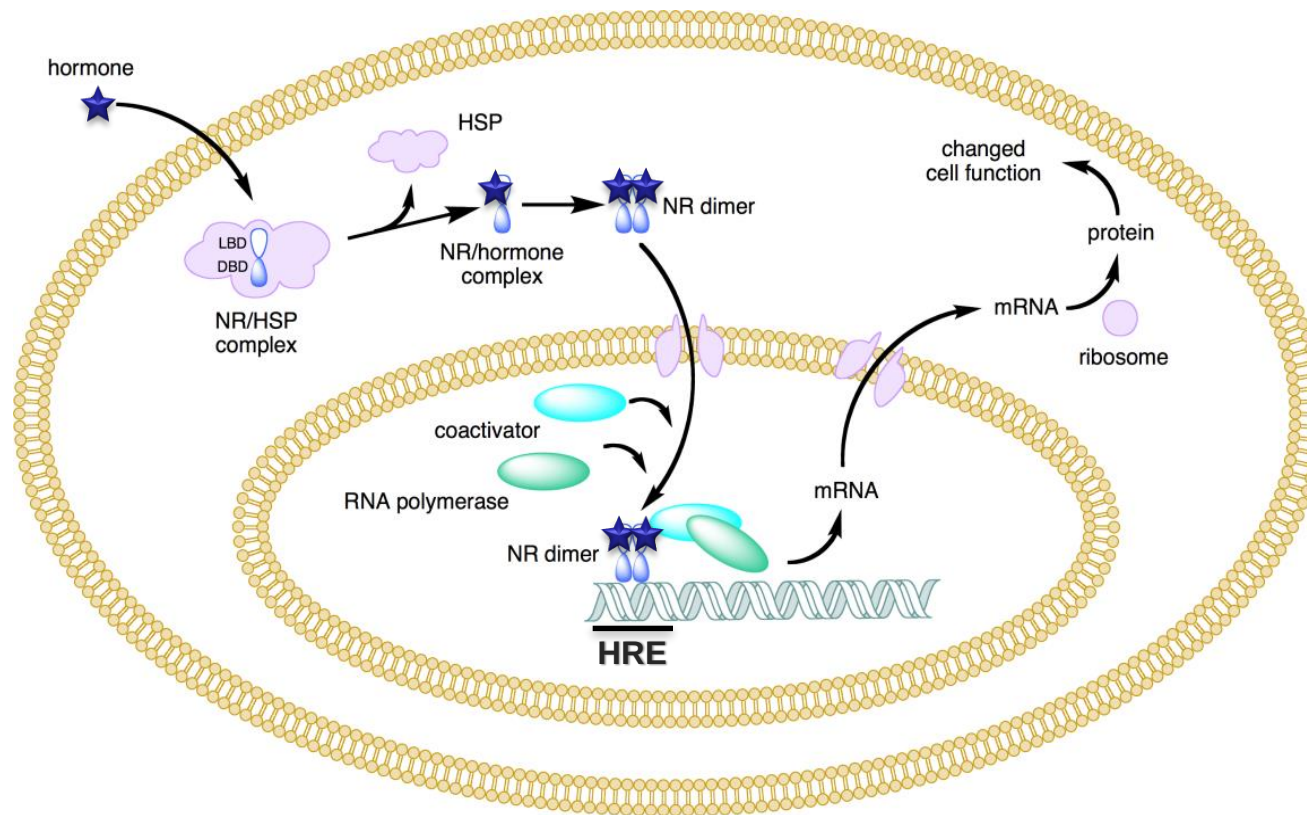


Monomeric / Tethered Orphan Receptors



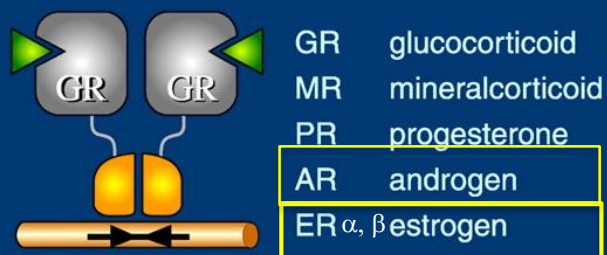
Les Récepteurs Nucléaires – Fonction biologique

Les RN = facteurs de transcription ligand-dépendant: la liaison du ligand induit des changements de conformation et permet le recrutement de co-facteurs et la fixation des RN activés dans la région promotrice.



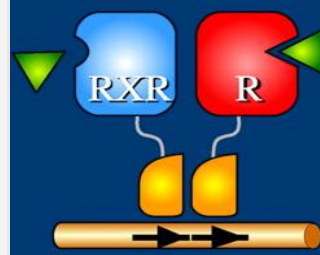
Interactions des PE avec les RN

Steroid Receptors



Bisphenols, alkylphenols,
cosmetics, pesticides

RXR Heterodimers



T ₃ R	thyroid hormone
RAR	all-trans RA
VDR	1,25-(OH) ₂ -VD
PPAR α	fatty acids /
PPAR γ	15d- $\Delta^{12,14}$ -PGJ
EcR	ecdysone
FXR	bile acids
CAR	androstane
LXR	oxysterol
PXR/SXR	xenobiotics

flame
Retardants,
perfluorinated
compounds,
phthalates,
organotins

estrogens, bisphenols, alkylphenols,
pesticides, phthalates, pharmaceuticals

Dimeric Orphan Receptors



organotins

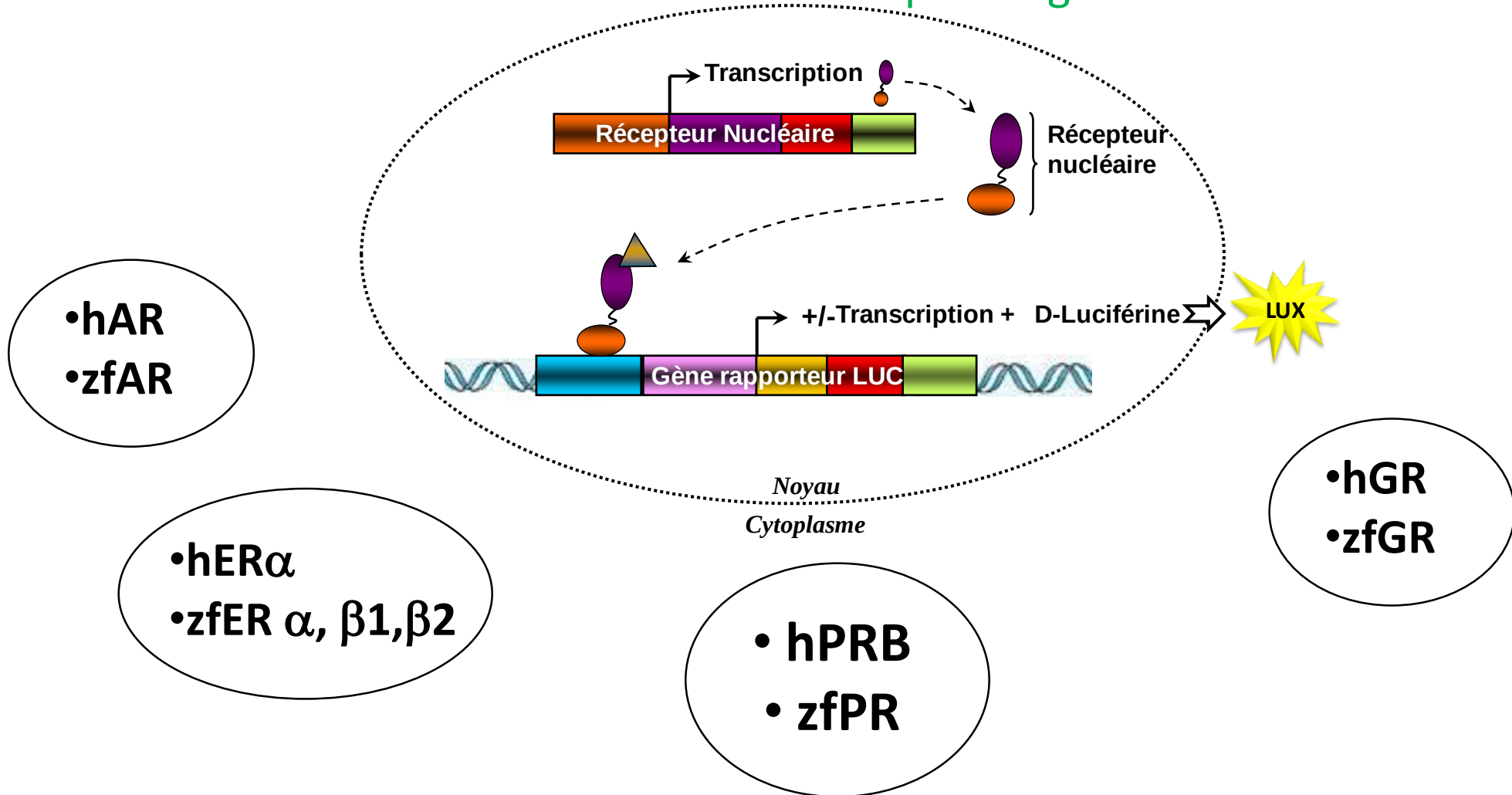
Monomeric / Tethered Orphan Receptors



bisphenols

Interaction of EDs with nuclear steroidal receptors

Human cell lines stably expressing human or zebrafish NRs
Zebrafish cell lines expressing zfNRs



In vitro reporter gene assays for human (h) and zebrafish (Zf) nuclear receptor (NR)

Zebrafish liver cell line (ZFL)

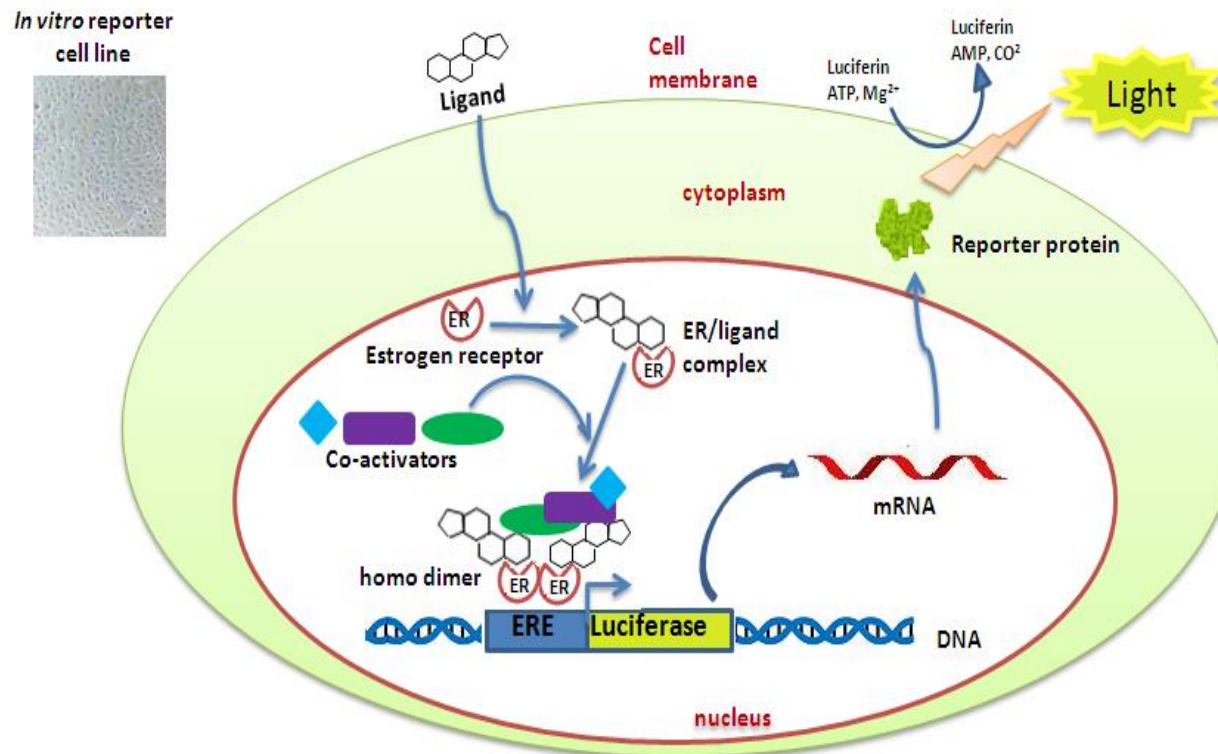
ZELH-zfER α (Cosnefroy *et al.*, 2012)

ZELH-zfER β 2 (Cosnefroy *et al.*, 2012)

ZELH-zfER β 1 (Cosnefroy *et al.*, 2012)

Human mammary cell line (MCF-7)

MELN-hER α (Balaguer *et al.*, 1999)



Zebrafish Liver Cells (ZFL) expressing Zf-ERs

In vitro : selectivity, cross-species differences

Cosnefroy et al., *Tox. Sciences* 2012

Pinto et al., *Tox. Appl. Pharm.* 2014

Chemicals	EC50 (nM)			
	<i>In vitro</i>			
	zfER α	Zebrafish zfER β 1	zfER β 2	Human hER α
17 β -estradiol	0.25*	0.01*	0.03*	0.02*
Estrone	1.8 ^b	0.48 ^b	0.48 ^b	0.69 ^a
Estriol	2.04 ^b	2 ^b	1.24 ^b	0.1 ^a
Ethinylestradiol	0.13*	0.01*	0.01*	0.01*
Hexestrol	0.87 ^b	1.62 ^b	0.89 ^b	0.1 ^a
Diethylstilbestrol	0.06 ^b	0.09 ^b	0.06 ^b	0.18 ^a
Bisphenol A	22667*	22670*	18716*	440*
Bisphenol F	6588*	5337*	5361*	-
4-tert-octylphenol	21725*	2860*	2573*	280*
<i>o,p'</i> -DDT	1359 ^b	w.e. ^b	217 ^b	1700 ^a
α -Zearalanol	68 ^b	127 ^b	265 ^b	0.14 ^a
α -Zearalenol	144 ^b	154 ^b	262 ^b	0.075 ^a
β -Zearalenol	224 ^b	5212 ^b	1893 ^b	0.9 ^a
Zearalenone	3742*	-	6469*	4.9*
Genistein	277 ^b	553 ^b	355 ^b	27 ^a
Benzophenone-1	2195 ^b	w.e. ^b	3895 ^b	9192 ^a
Benzophenone-2	1084 ^b	2216 ^b	1477 ^b	3284 ^a
Benzophenone-3	n.e. ^b	n.e. ^b	w.e. ^b	w.e. ^a

Selectivity towards zfER subtypes

Natural and synthetic steroid

- More affine for zfER β s

Phyto- and myco-oestrogens

- More affine for zfER α

BPA, THB $\rightarrow$ zfER α

op'DDT $\rightarrow$ zfER β 2

- Responses differ from human models
- Zf-SERMs

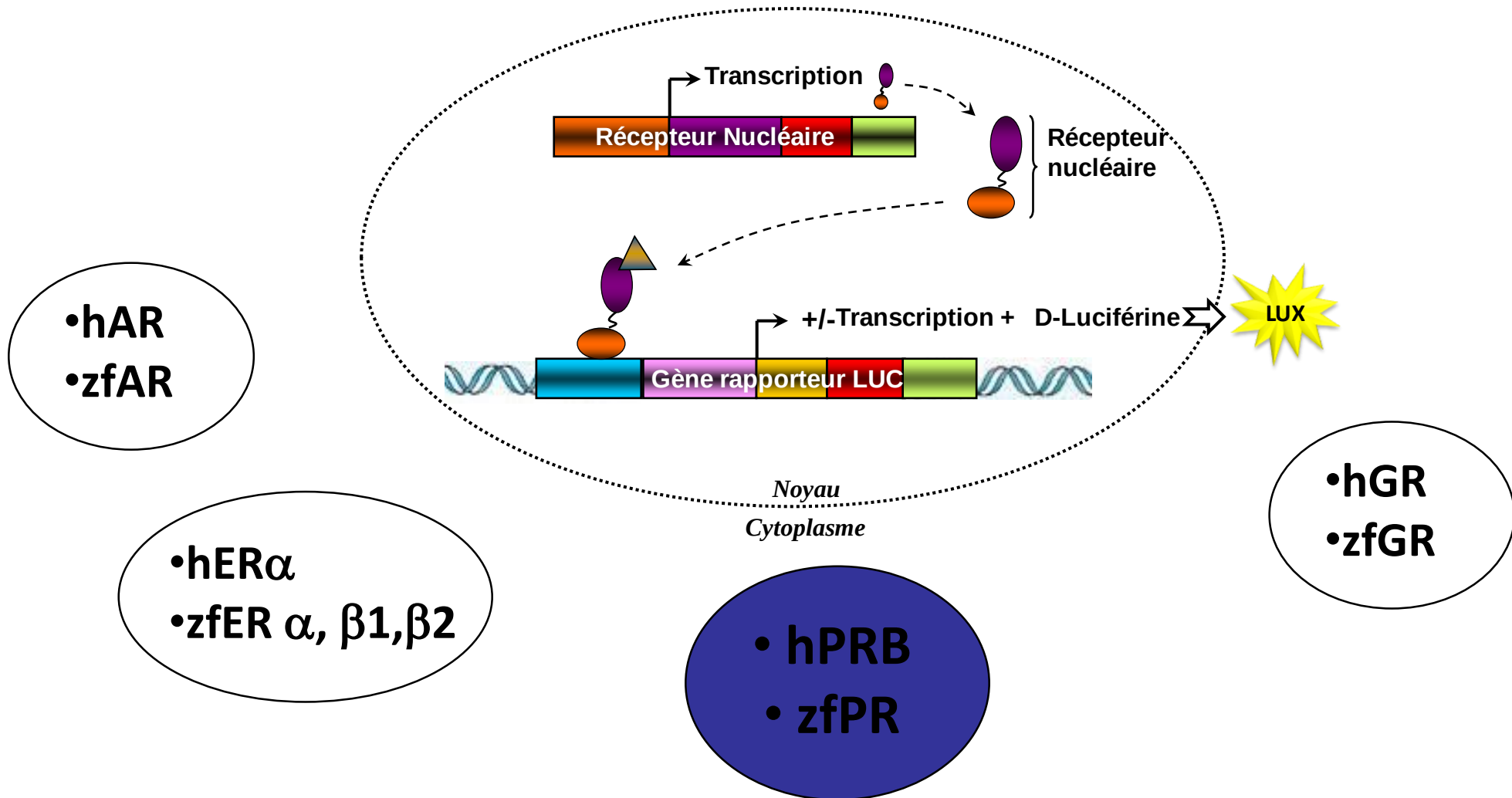
Relevant tools for hazard assessment in aquatic species

In vitro screening of pharmaceuticals progestins

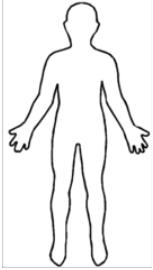
Classification		Compound
Natural estrogen		17 β -estradiol
Natural progestin		Progesterone
Synthetic progestin retroprogesterone		Dydrogesterone
Progestins structurally related to progesterone	Derived from 17 α -hydroxyprogesterone	Medroxyprogesterone Medroxyprogesterone acetate Megestrol acetate Chlormadinone acetate Cyproterone acetate
	Derived from 19-norprogesterone	Promegestone Nestorone Nomegestrol acetate
	Derived from 17 α -hydroxy-19-norprogesterone	Gestonorone
Progestins structurally related to testosterone: derived from 19-nortestosterone	Estranes	Ethisterone Ethinodiol diacetate Lynestrenol Norethindrone acetate Norethindrone Tibolone
	Gonanes	Desogestrel Etonogestrel Gestodene Levonorgestrel Norgestimate Norgestrel
Progestin structurally related to spironolactone		Drospirenone
PR antagonist		Mifepristone

Interaction of pharmaceutical progestins with nuclear steroidal receptors ?

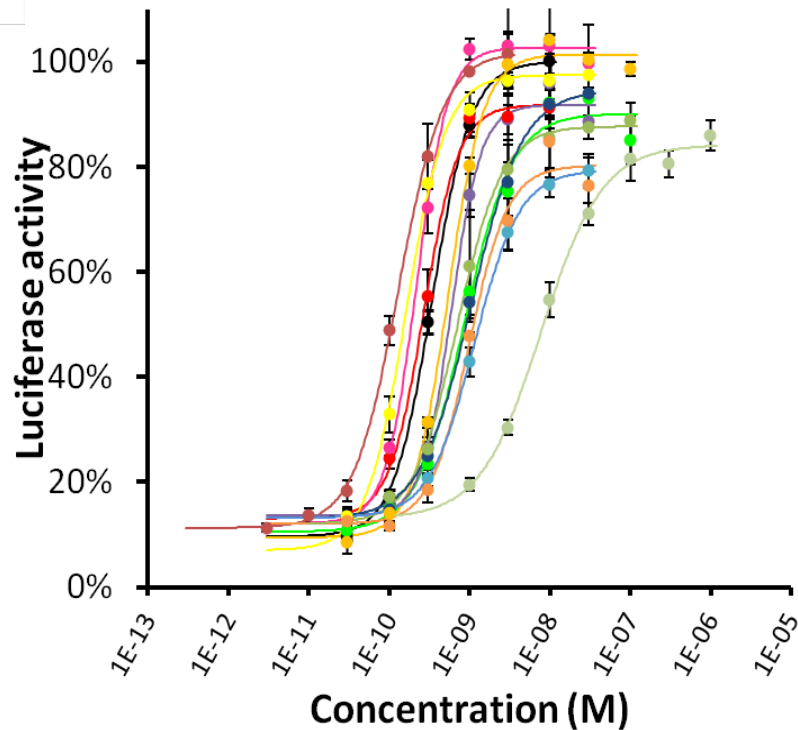
Human cell lines stably expressing human or zebrafish NRs



All the synthetic progestins act as agonist of hPR

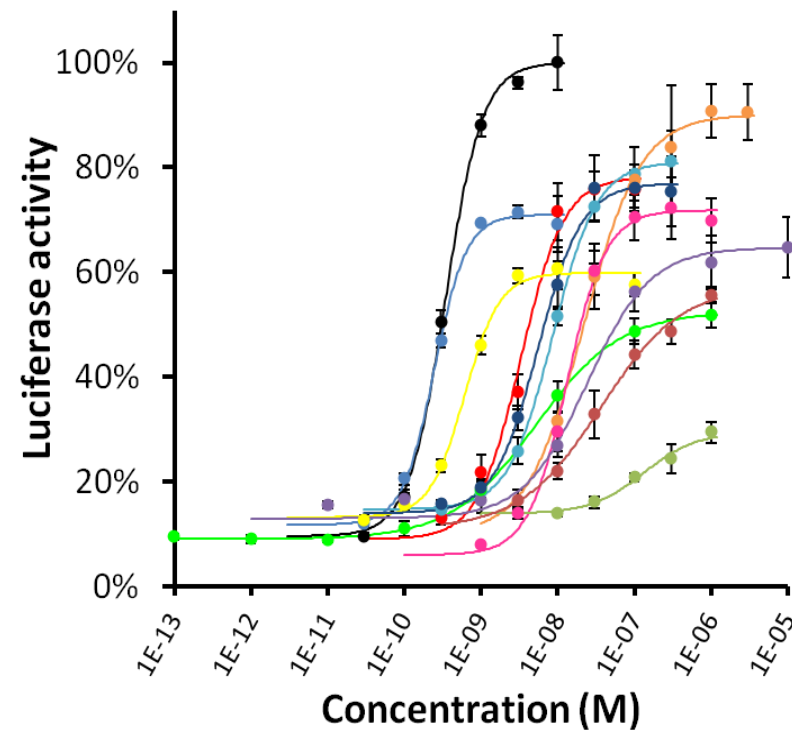


HELN-hPRB full agonists



- EC₅₀ ranging from 0.33 nM (R5020) to 9.97 nM (TIB=Tibolone).

HELN-hPRB partial agonists

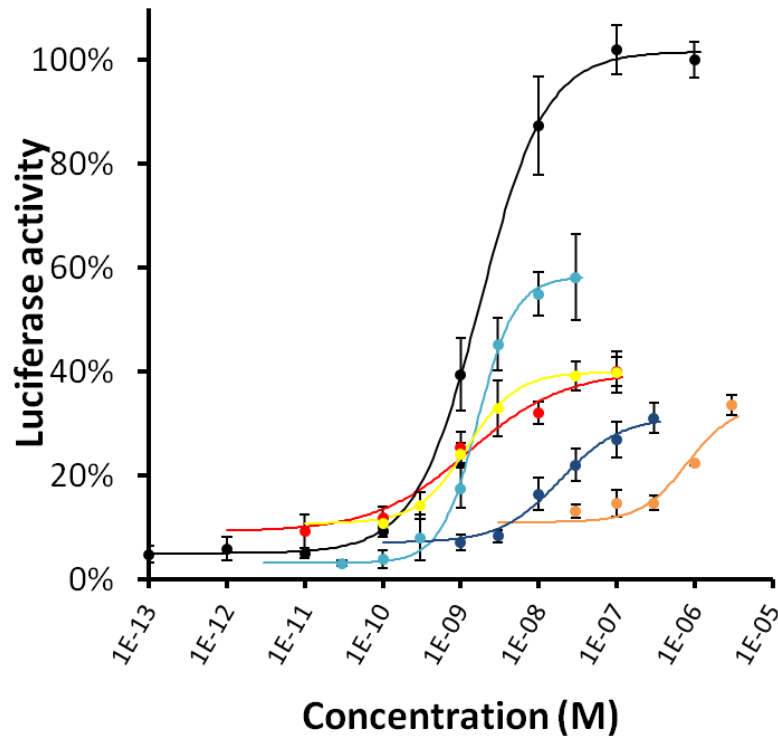


- EC₅₀ ranging from 0.20 nM (medroxyprogesterone acetate) to 249 nM Medroxyprogesterone).

Most of the progestins act as zfPR antagonists



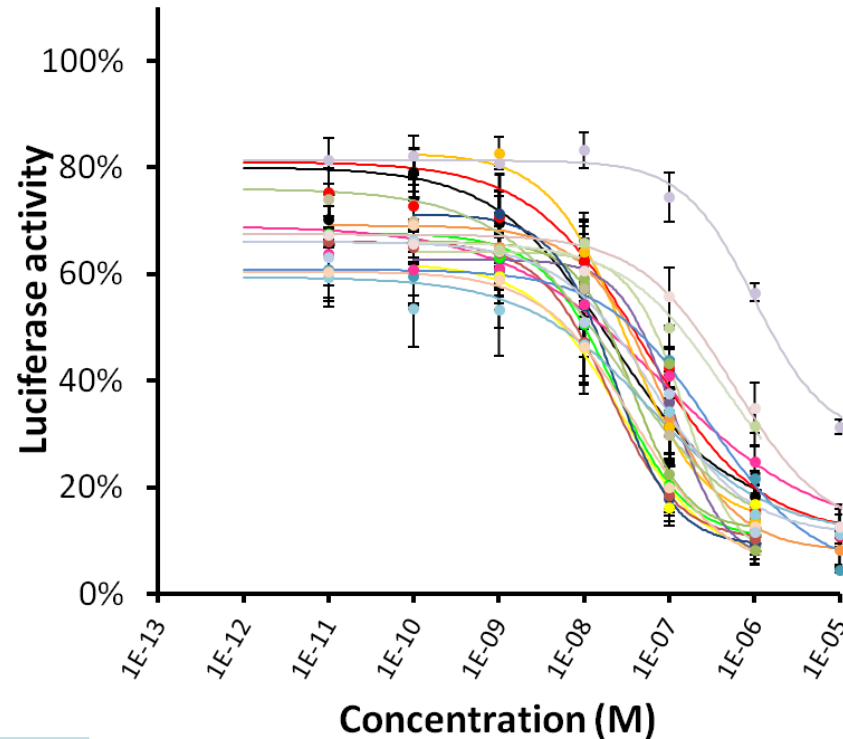
U2OS-zfPR agonists



EC₅₀ ± sd (nM)

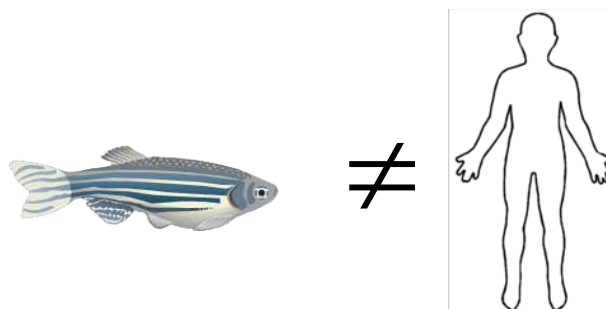
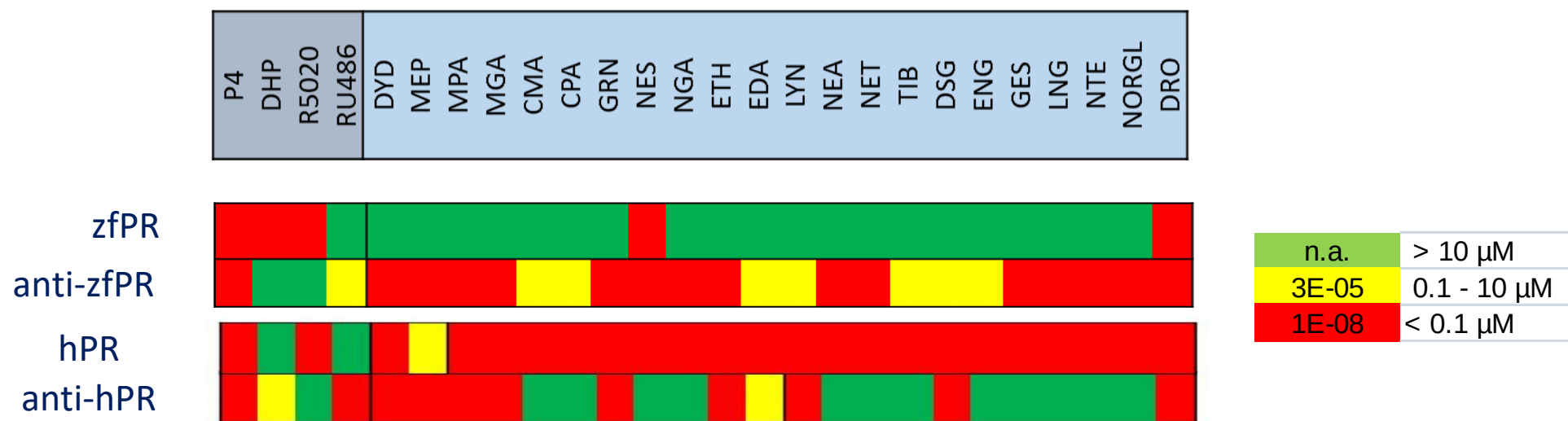
P4	1.97 ± 0.57
DHP	1.87 ± 0.44
R5020	1.93 ± 0.60
Nestorone	1.41 ± 0.41
Drospirone	14.1 ± 4.49
Spironolactone	674 ± 300

U2OS-zfPR antagonists



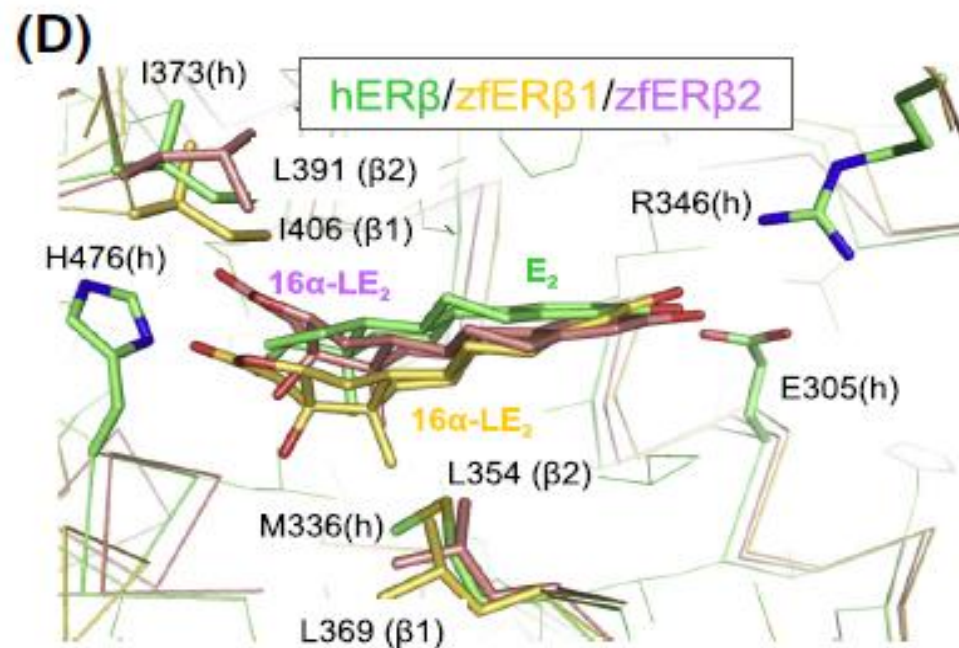
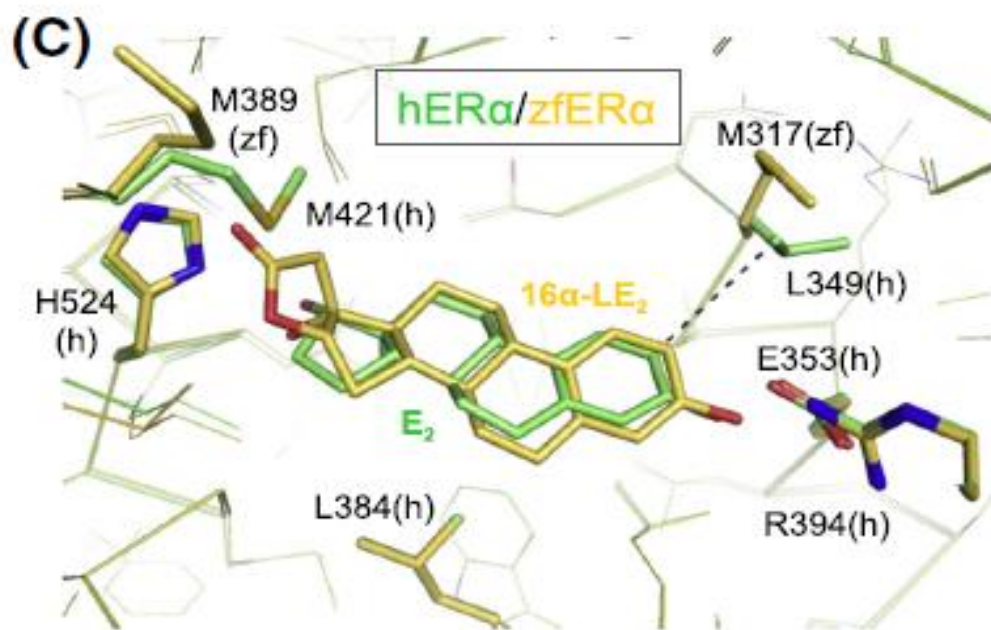
- IC₅₀ ranging from 14 nM (levonorgestrel) to 1000 nM (desogestrel).

Complex toxicological profiles of progestins towards zfNRs



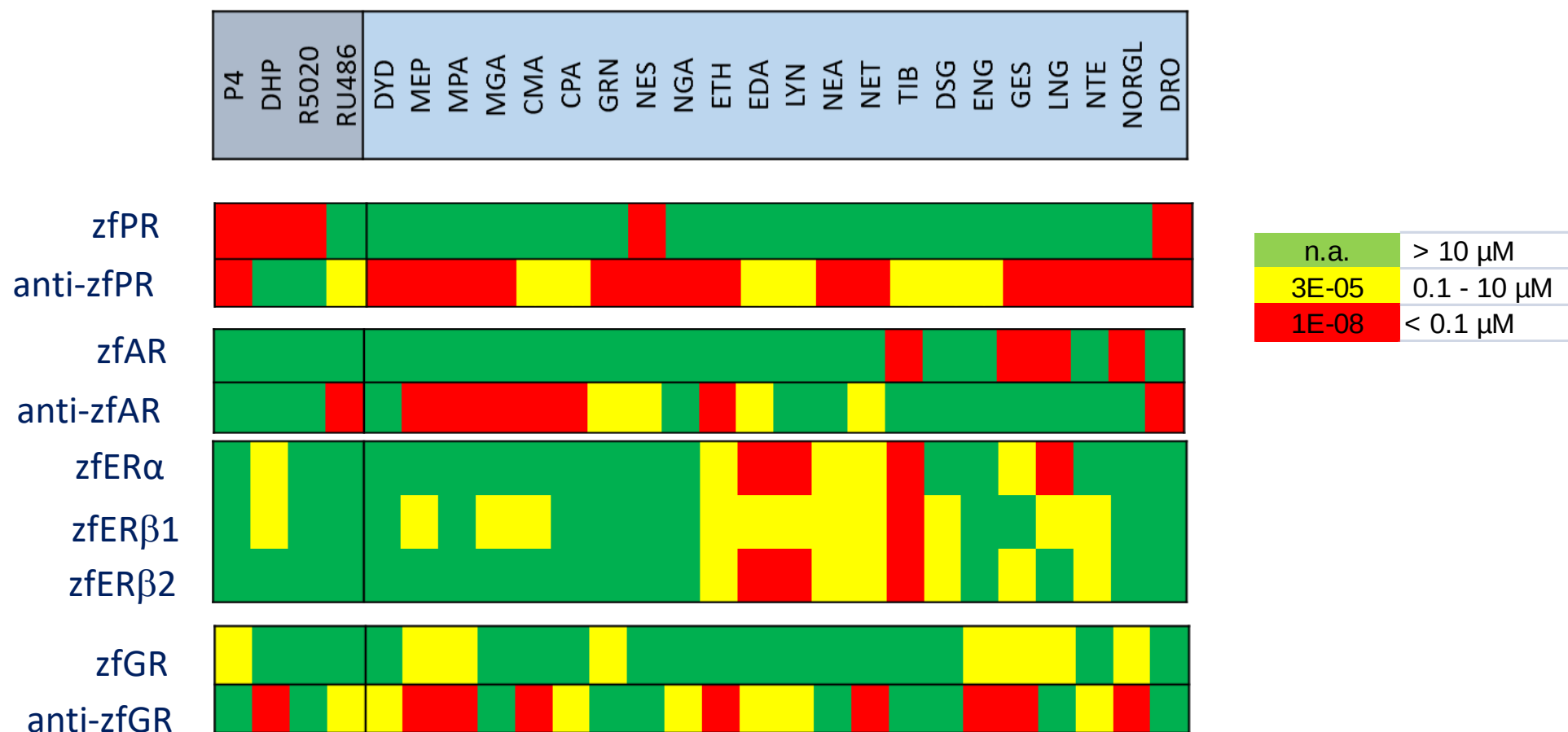
Modeling of interaction of the PR ligand with h and zf PR

- Collaboration with William Bourget (Inserm, Montpellier).



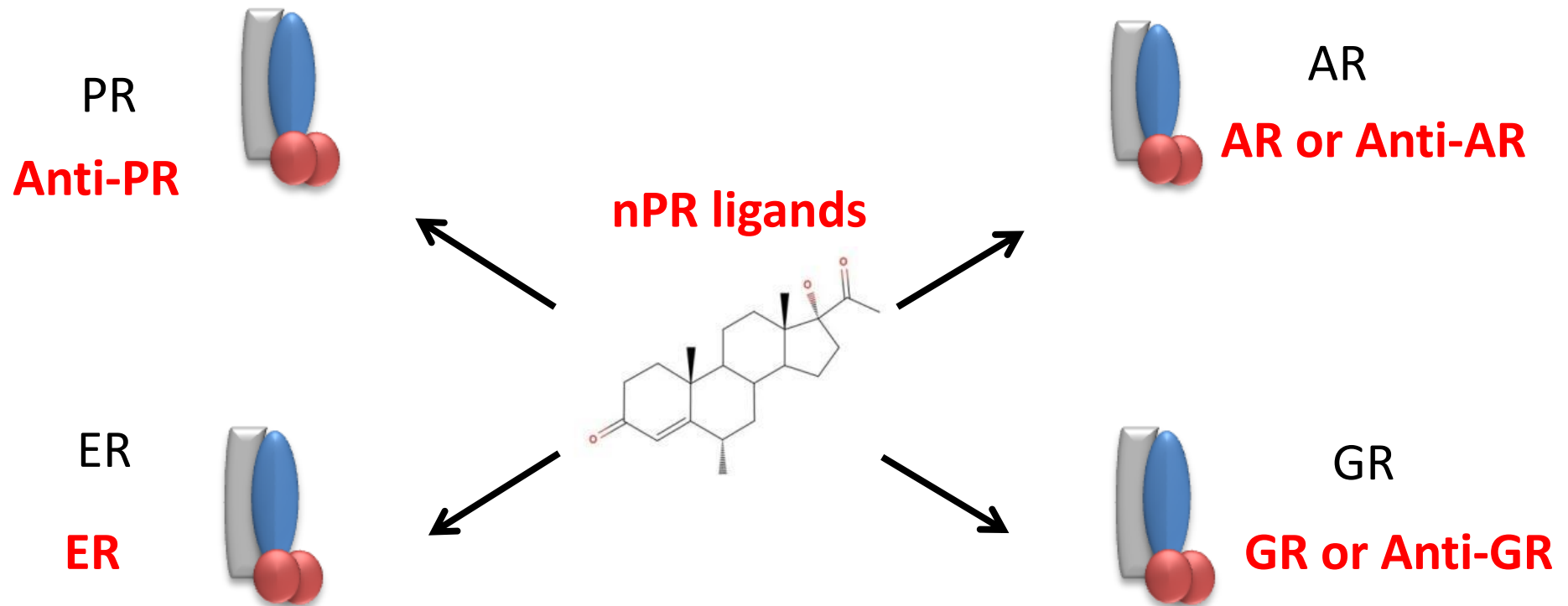
Pinto et al., 2014 Tox. Applied Pharmacol.

Complex toxicological profiles of progestins towards zfNRs

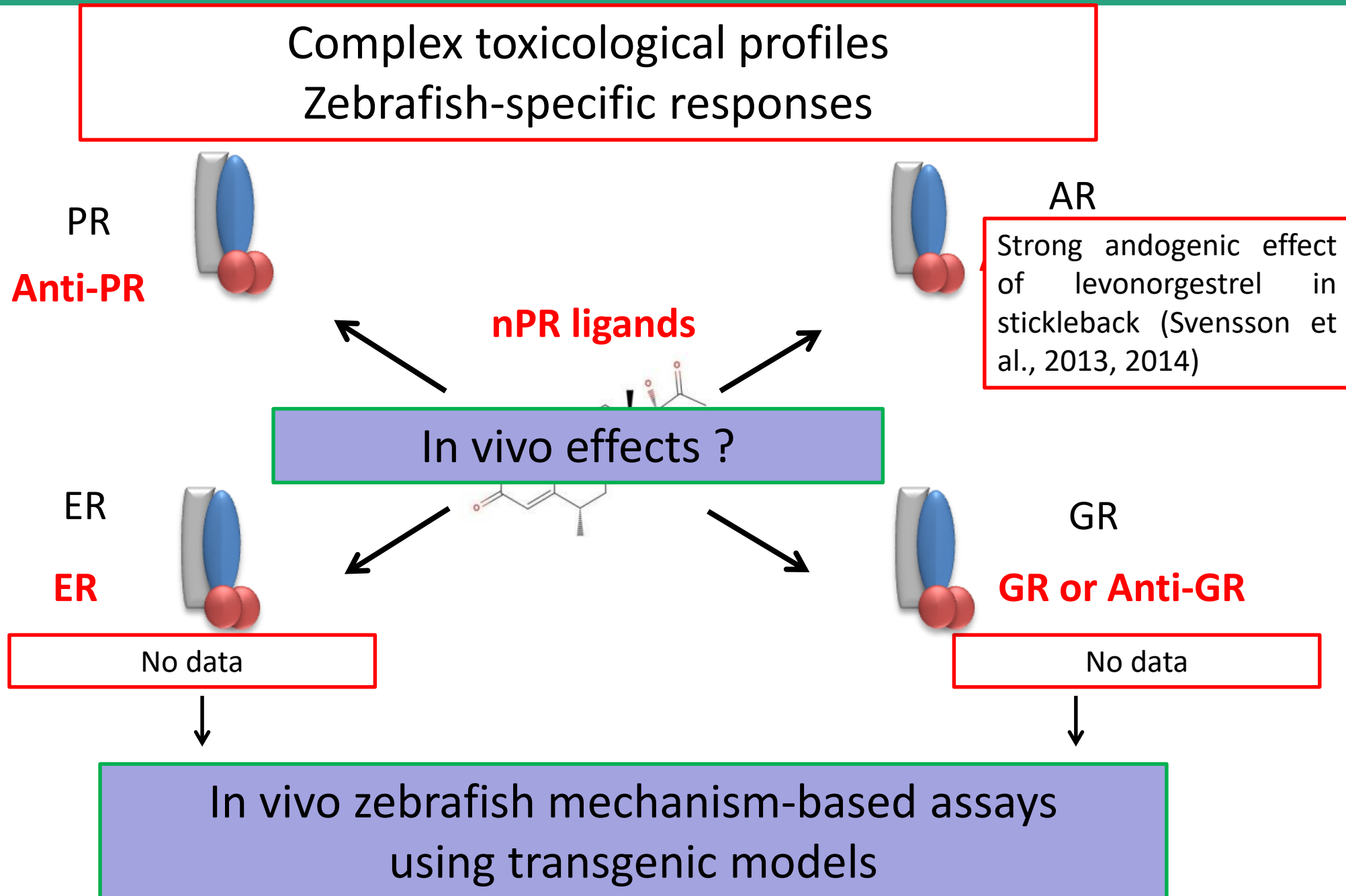


Interactions of progestins towards zf nuclear steroidal receptors

Complex toxicological profiles
Zebrafish-specific responses

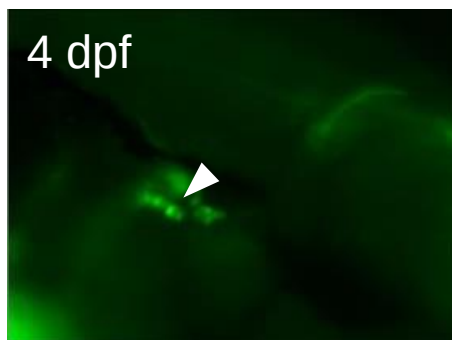
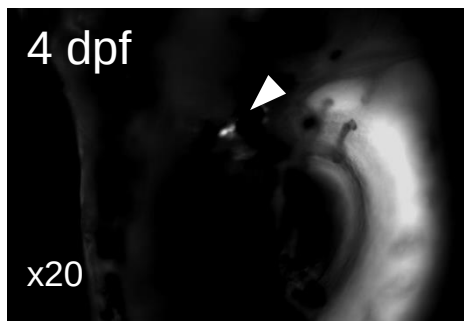


Interactions of progestins towards zf nuclear steroidal receptors



Engineering *in vivo* zebrafish models for EDCs

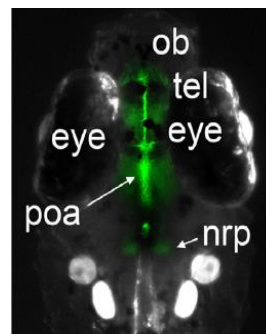
11beta-hydroxylase
Cyp11c1-GFP



Interrenal cells
expressing GFP

Garoche et al., submitted

P450 aromatase B
Cyp19a1b-GFP

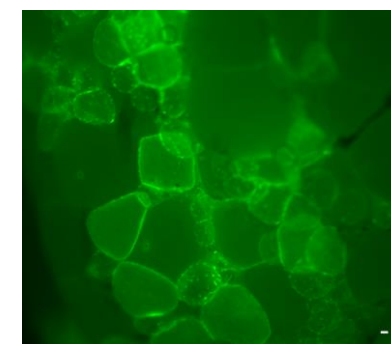
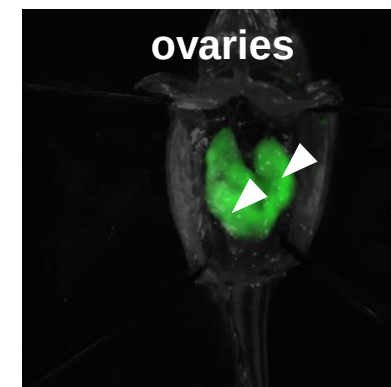


Radial Glial Cells
Brion et al., 2012



**New zebrafish models
for EDCs (Nemo project)**

P450 aromatase A
Cyp19a1a-GFP

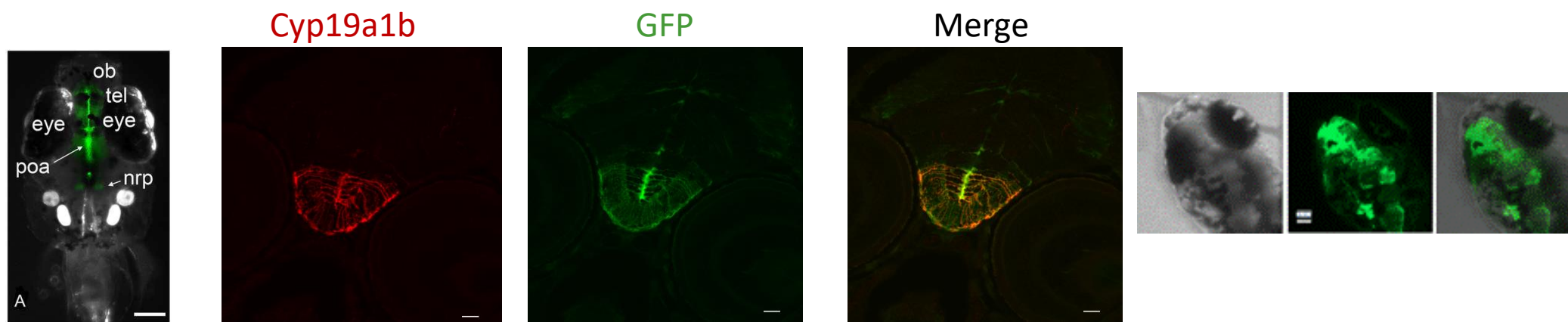


ovarian cells
expressing GFP

Hinfray et al., GCE 2018

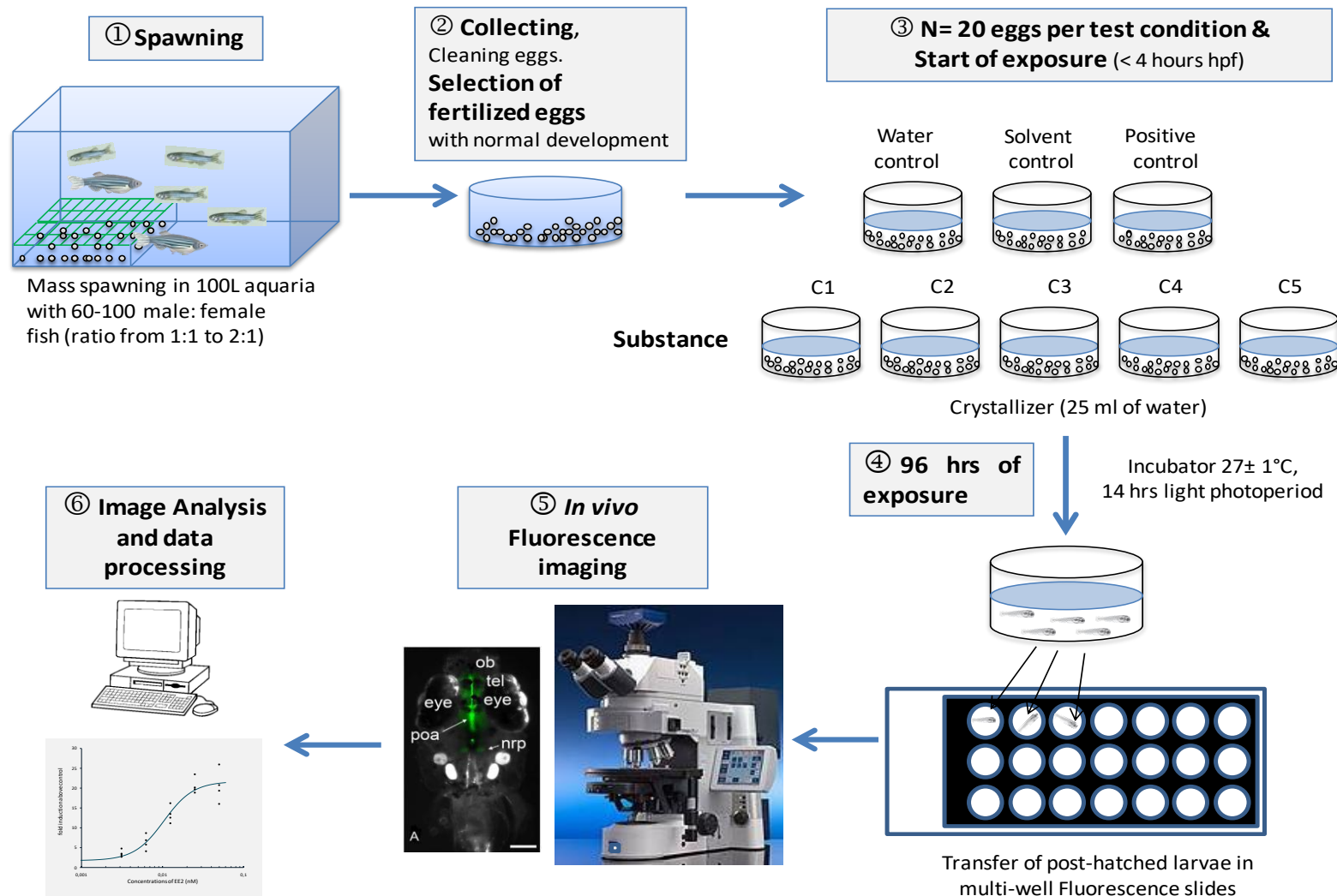
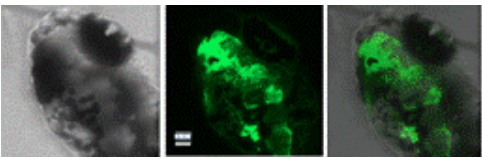
In vivo quantification of estrogenic activities using EASZY

- A zebrafish-specific mechanism-based non invasive *in vivo* assay allowing quantification of the **estrogenic activity of chemicals** in fish early life stages (4-dpf old zebrafish)
- Use of transgenic *cyp19a1b*-GFP zebrafish (Tong *et al.*, *Genesis* 2009)
 - *cyp19a1b* is :
 - a ER-regulated target gene coding for brain aromatase (Menuet *et al.*, *J. Comp. Neurol.* 2005)
 - extremely sensitive to (xeno)-estrogens (Vosges *et al.*, *Aq. Toxicol.* 2010, *Reprod. Toxicol.* 2012)
 - exclusively expressed in Radial Glial Cells (Pellegrini *et al.*, *J. Comp. Neurol.*, 2007)
- EASZY is a usefull model to assess a true-brain specific response of fish exposed to EDCs



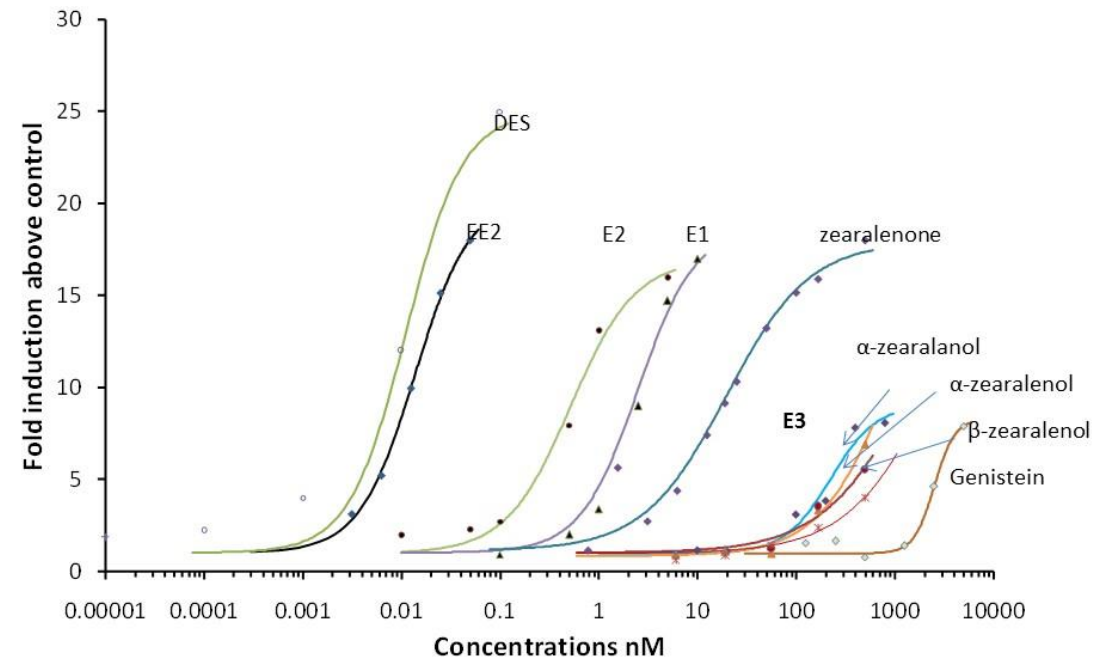
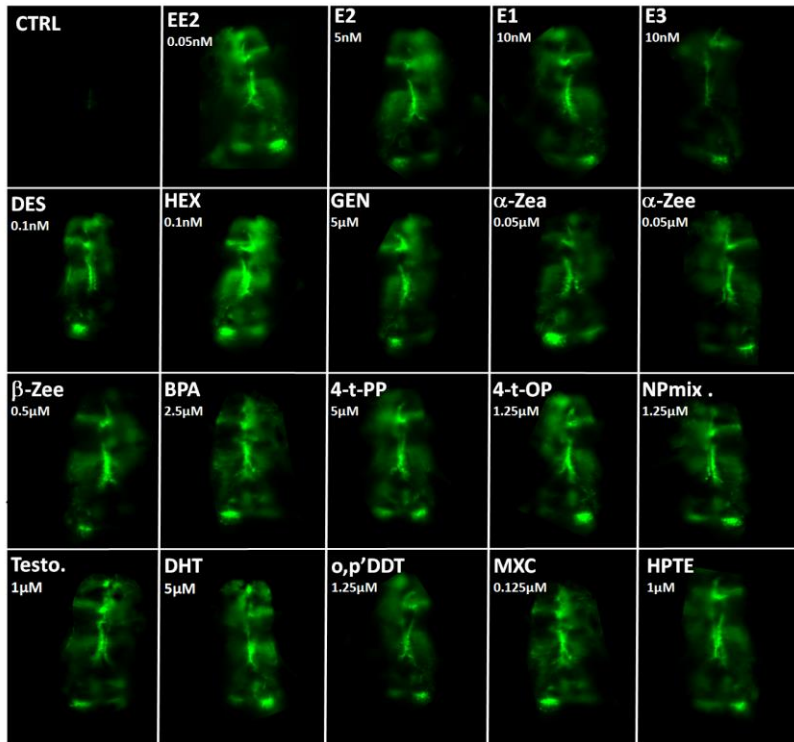
In vivo screening estrogenic activity of EDs

Detection of Endocrine Active Substance, acting through estrogen receptors, using transgenic cyp19a1b-GFP Zebrafish Embryo (EASZY)



Brion et al., 2012
Plos One

Effects of ED on the ER-regulated brain aromatase expression



Brion et al., 2012 Plos One

$$REP = \frac{EC_{50} \text{ E2}}{EC_{50} \text{ test chemical}}$$

EASZY: screening estrogenic activity of substances

OPEN ACCESS Freely available online



Screening Estrogenic Activities of Chemicals or Mixtures In Vivo Using Transgenic (*cyp19a1b*-GFP) Zebrafish Embryos

François Brion¹, Yann Le Page², Benjamin Piccini¹, Olivier Cardoso¹, Sok-Keng Tong³, Bon-chu Chung³, Olivier Kah^{2,*}

Toxicology and Applied Pharmacology 305 (2016) 12–21



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44 substances belonging to diverse chemical families

- natural and synthetic estrogens
- phyto- and mycoestrogens
- industrial chemicals (e.g., Akyphenols, pesticides)
- Steroidal compounds: androgens, progestins, glucocorticoids, mineralocorticoids
- « inactive » compounds

24 pharmaceutical progestins

Several synthetic progestins disrupt the glial cell specific-brain aromatase expression in developing zebra fish



Joel Cano-Nicolau^{a,1}, Clémentine Garoche^{b,1}, Nathalie Hinfrey^b, Elisabeth Pellegrini^a, Nouredine Boujrad^c, Farzad Pakdel^c, Olivier Kah^{a,*}, François Brion^{b,*}

Ecotoxicology and Environmental Safety 142 (2017) 150–156



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Ecotoxicology and Environmental Safety

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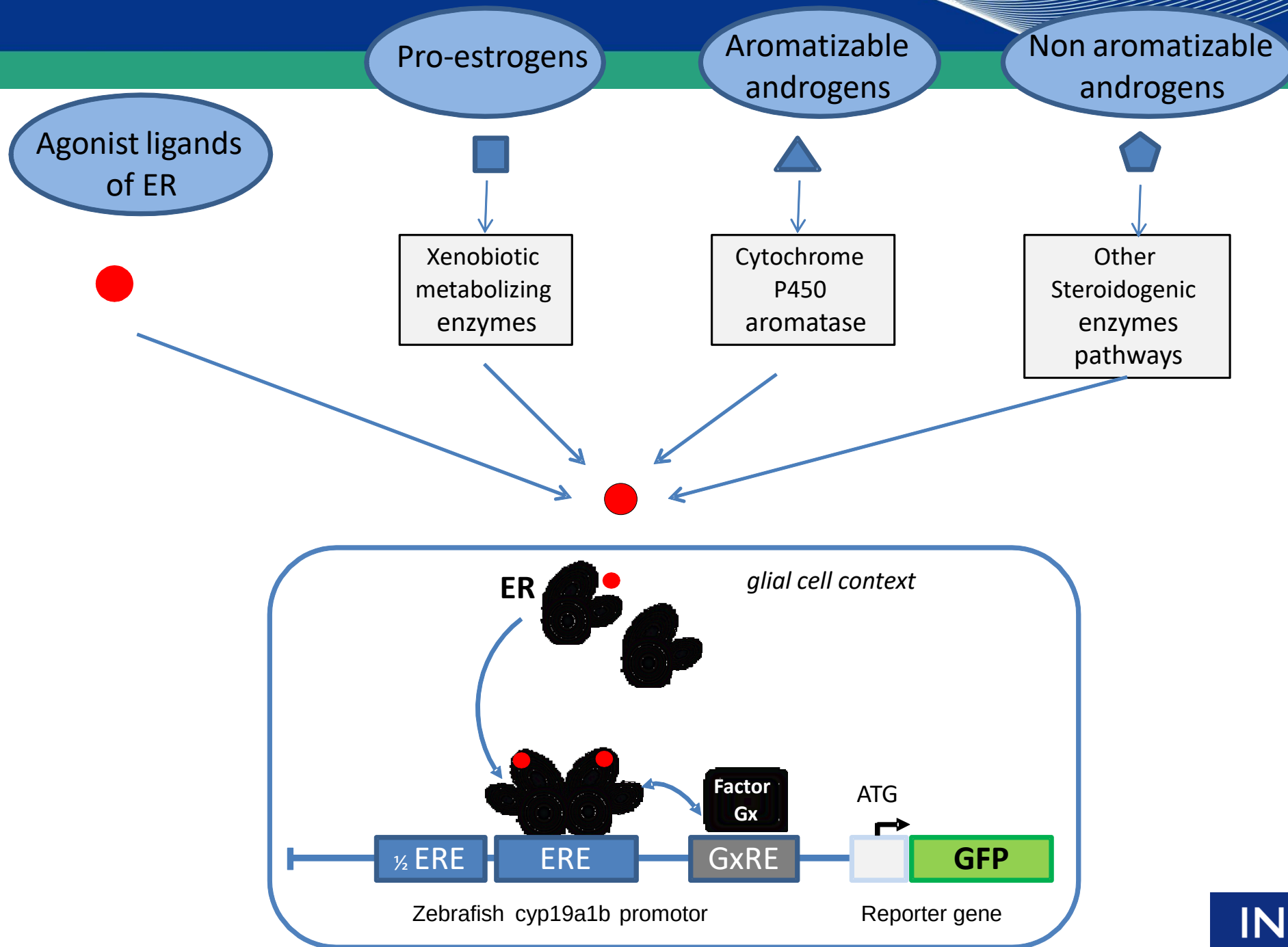
Bisphenol substitutes

In vitro and in vivo estrogenic activity of BPA, BPF and BPS in zebrafish-specific assays



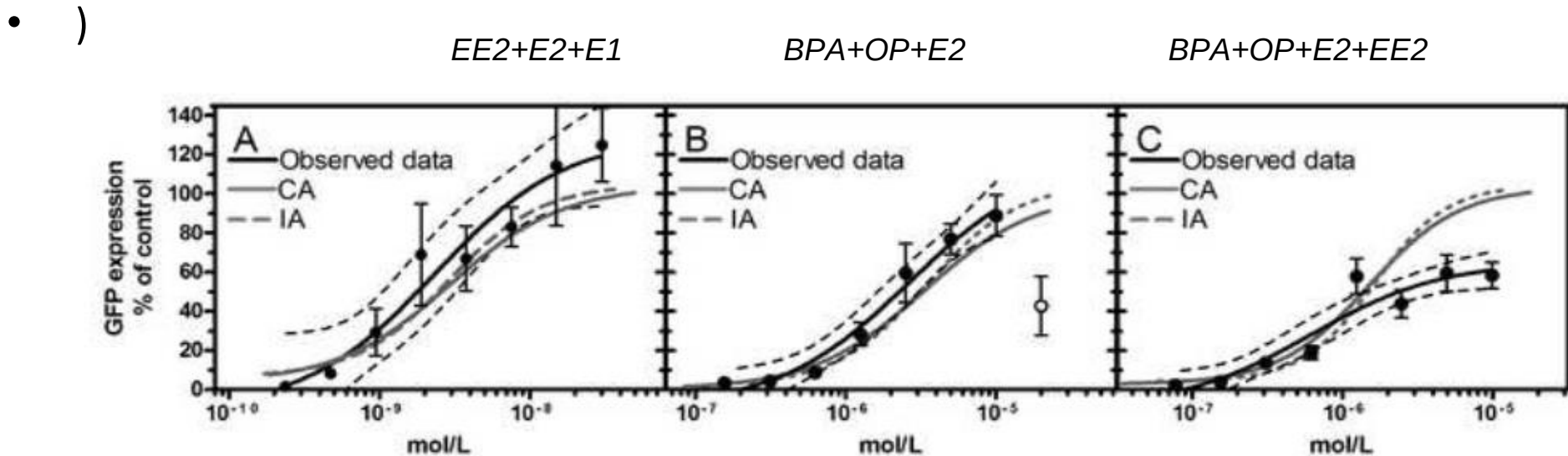
Vincent Le Fol^{a,b,c}, Selim Aït-Aïssa^{a,*}, Manoj Sonavane^a, Jean-Marc Porcher^a, Patrick Balaguer^{d,e,f,g}, Jean-Pierre Cravedi^{b,c}, Daniel Zalko^{b,c}, François Brion^{a,*}

Substances detected by the EASZY assay

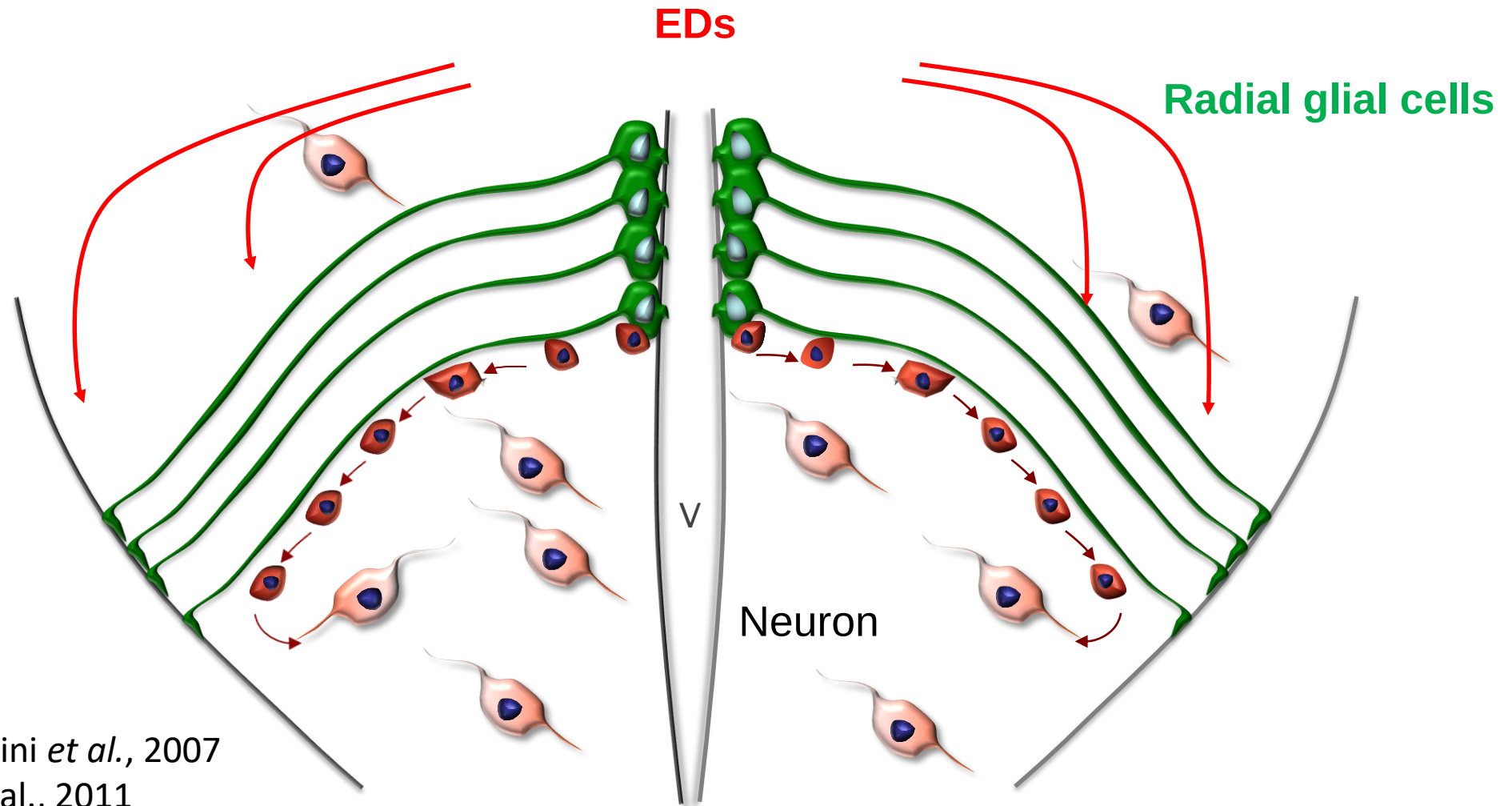


EASZY: screening estrogenic activity of mixtures of EDs

Binary or multi-component mixtures of EDs (Brion et al., Plos One 2012, Petersen et al., Aquat. Toxicol., 2013, Hinfrey et al., 2016 Tox. Appl. Pharmacol)



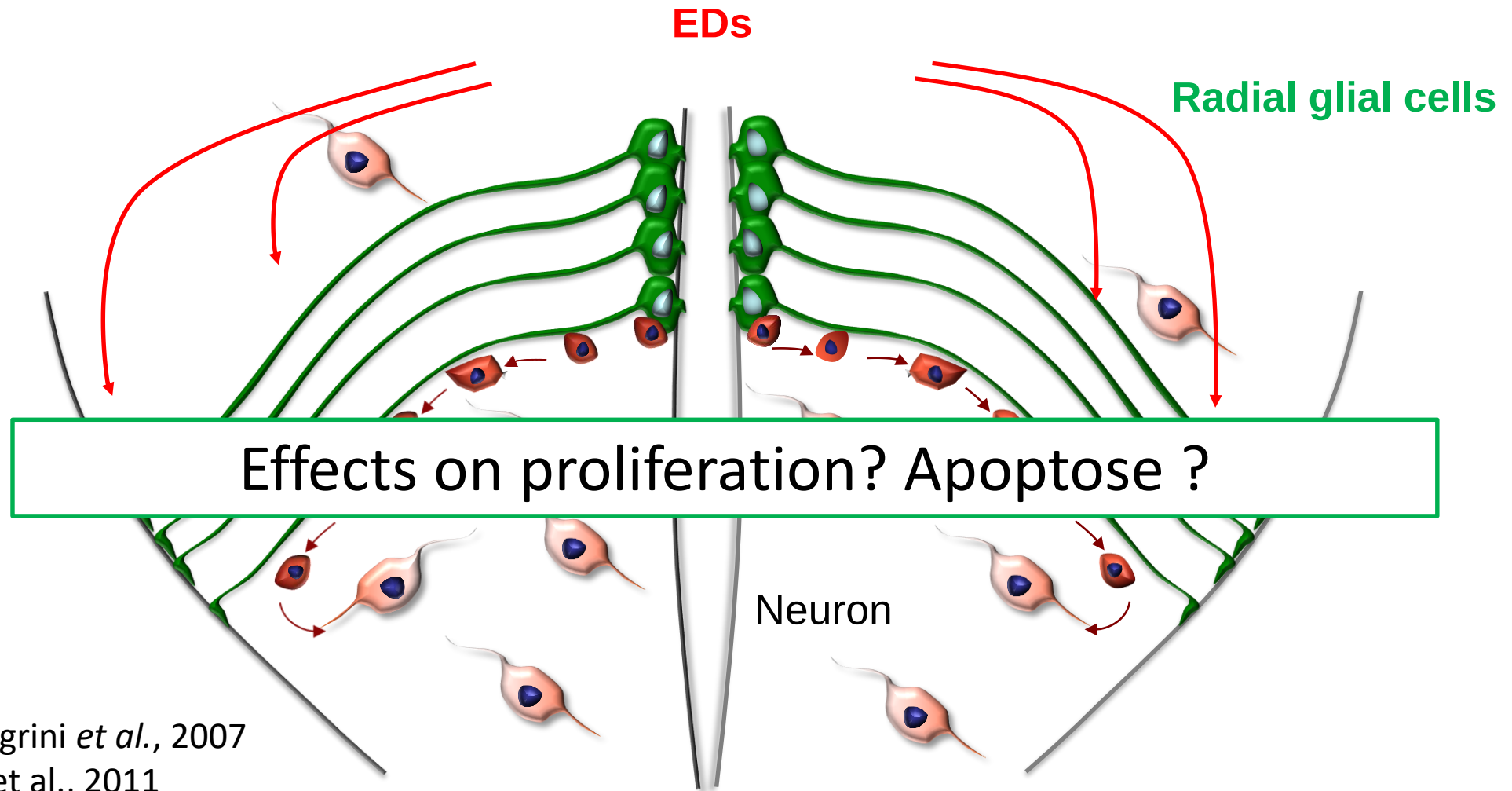
Induction of the glial cell-specific expression of the brain aromatase



From Pellegrini *et al.*, 2007
Kah *et al.*, 2011

Radial glial cells = progenitor cells of neurons

Induction of the glial cell specific expression of the brain aromatase



From Pellegrini *et al.*, 2007
Kah *et al.*, 2011

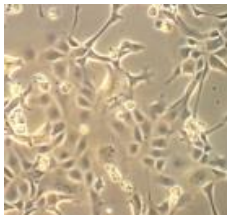
Radial glial cells = progenitor cells of neurons

Effect on apoptosis and cell proliferation within the brain

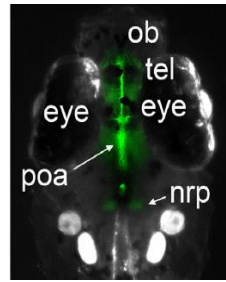
	nM	Poliferation	Apoptosis	
Progesterone	0.1	=	=	
	1	=	=	
	10	=	=	
Norethindrone	0.1	=	=	
	1	=	+	Pro-apoptotic effects seen in different brain regions in zebrafish larvae (Hypothalamus++)
	10	=	+	
17 β -estradiol	0.1	=	=	
	1	-	=	Anti-proliferative effect of E2. Similar to adult zebrafish treated wiht E2 (Diotel et al., 2011)
	10	-	=	

Potential Adverse Outcome Pathways (AOP) for brain aromatase

Molecular initiating event



Altered gene expression and function



brain responses

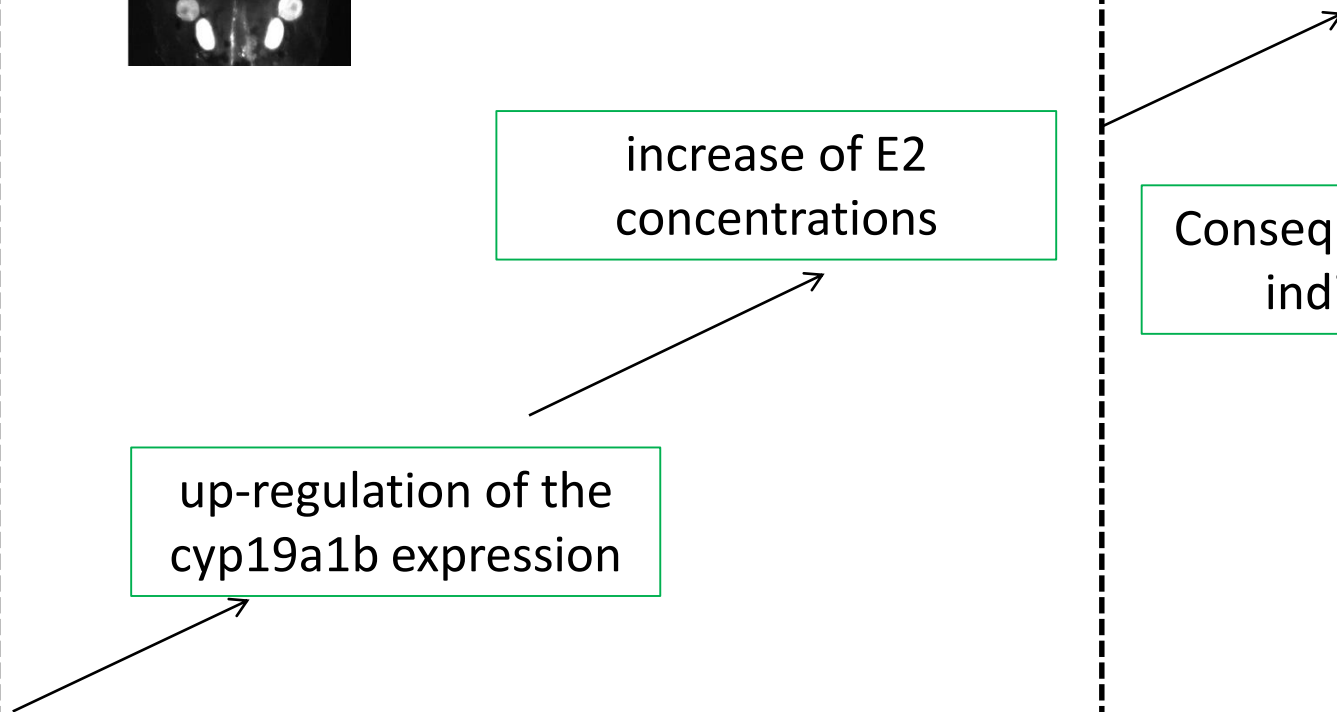
Proliferation,
apoptose

increase of E2
concentrations

Consequence for the
individuals ?

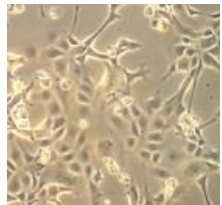
up-regulation of the
cyp19a1b expression

Activation of the zf-
ERs receptor



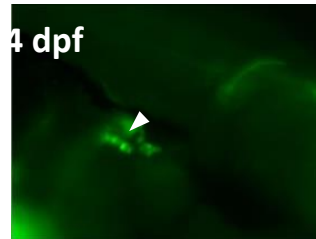
Norethindrone : potential AOP on GR-signaling pathways

Molecular initiating event



Inhibition of the zf-GR receptor

Altered gene expression and function



Down-regulation of the cyp11c1 expression

Decrease of whole – body cortisol concentrations

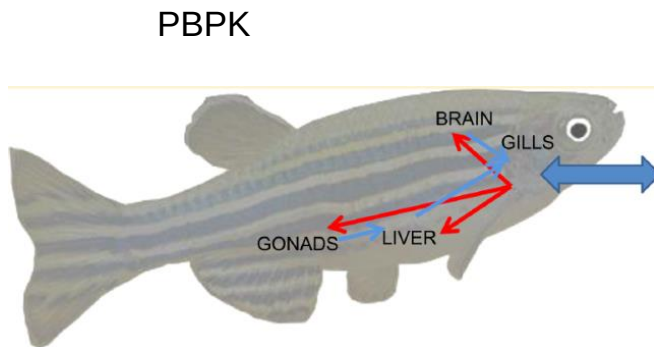
Behaviour responses

Stress responses?

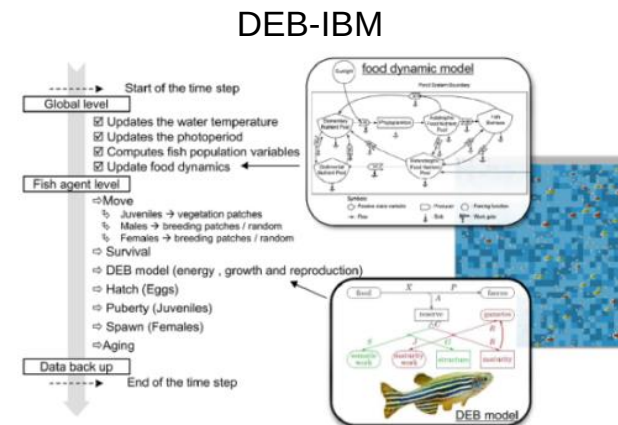
Norethindrone induce a suite of molecular events which supports the disruption of GR signaling pathways in zebrafish larvae

Intergrated approach using zf-based assays: further improvement

- **Zebrafish models (collaboration P. Balaguer, INSERM)**
 - Zebrafish PXR in vitro and in vivo (PNRPE PestR 2017-2020)
- **Establishment/refinement of fish based using newly developed transgenic ZF models**
 - (AIDEZ Project ANSES 2017-2020)
- **In silico models for predictive (ecot)toxicology: collaboration R. Beaudouin and F. Bois, INERIS, Modelling unit**



Pery et al., ES&T
2014



Beaudouin et al., Plos One
2015

Conclusions

1. **Quantification of ED** activities of substances, alone or in mixtures
2. Complementary assays providing information on **mechanism of action and effects at cellular and individual levels**
3. Together with modeling, such integrated approach can **enhance the efficiency of chemical screening** and add value to environmental hazard and risk assessment

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Frédéric Bois

Cléo Tebby



NEMO project
(2009-2014)



ANR NEED (2008-2010)
ANR PROOFS (2013-2017)



MIXEZ & MOZAIC
2010-2014



Olivier Kah, Elisabeth Pellegrini



Frédéric Sohm, Joanne Edouard (transgenesis)

Inserm

Institut national
de la santé et de la recherche médicale

Patrick Balaguer (reporter gene cell lines)

