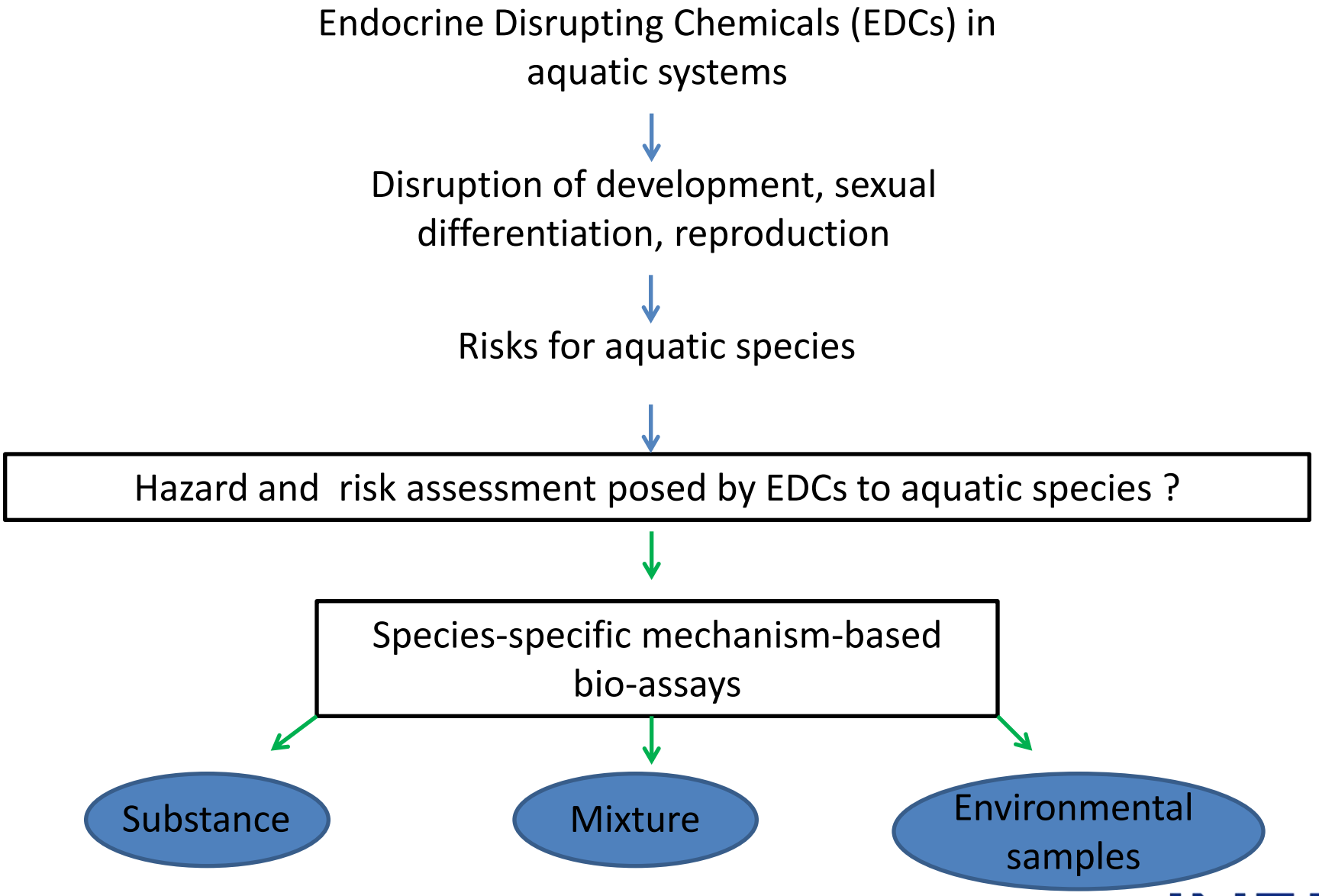
Assessment of the estrogenic potency of chemicals, alone or in mixtures, and environmental samples using a novel transgenic cyp19a1b-GFP zebrafish embryo assay (EASZY assay)

François BRION

Environmental EDCs



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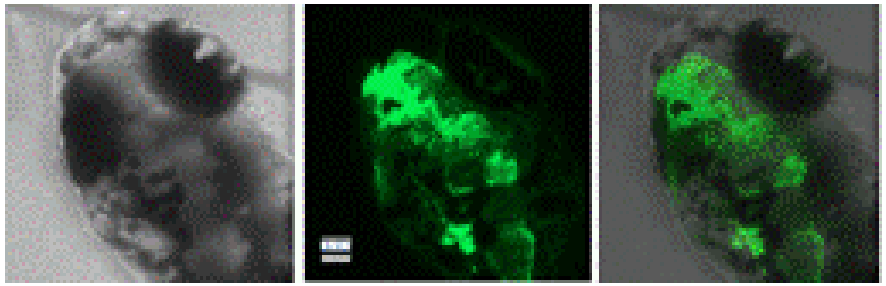
Detection of **E**ndocrine **A**ctive **S**ubstance, acting through estrogen receptors, using transgenic cyp19a1b-GFP **Z**ebrafish Embr**Y**os

(EASZY)



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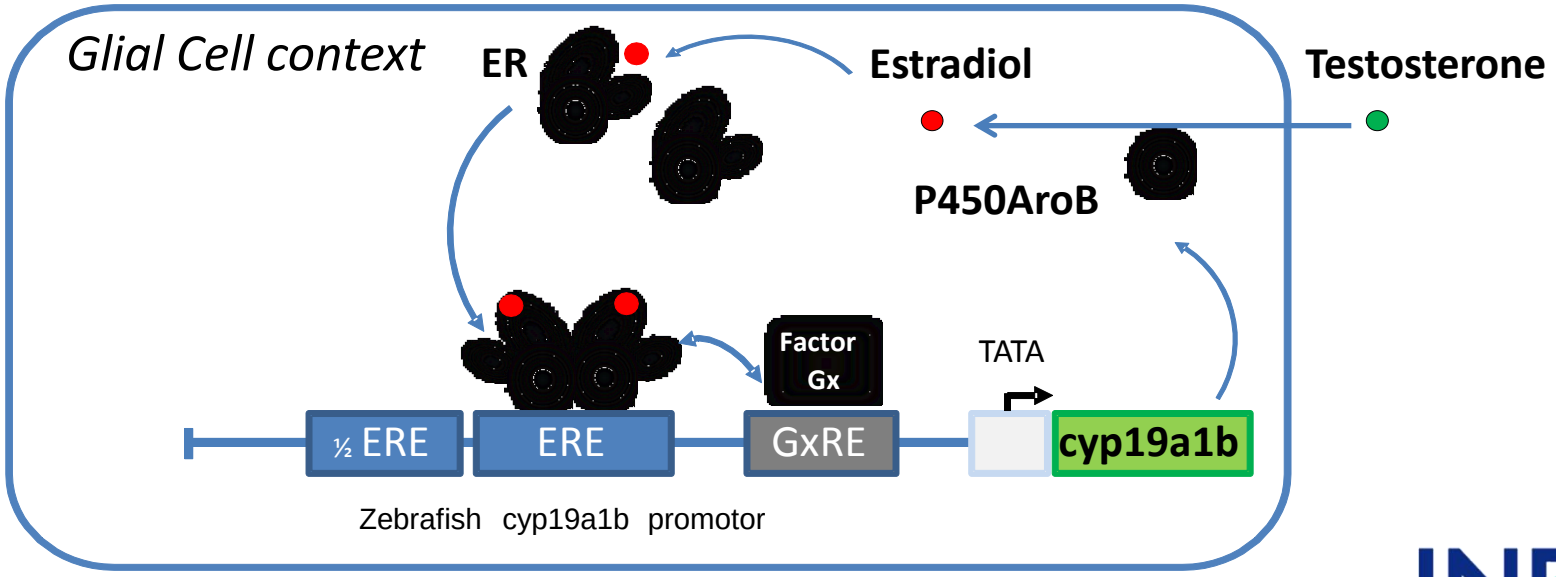
What is EASZY?

- A zebrafish-specific mechanism-based *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 (P450AroB) *(Menuet et al., J. Comp. Neurol. 2005)*



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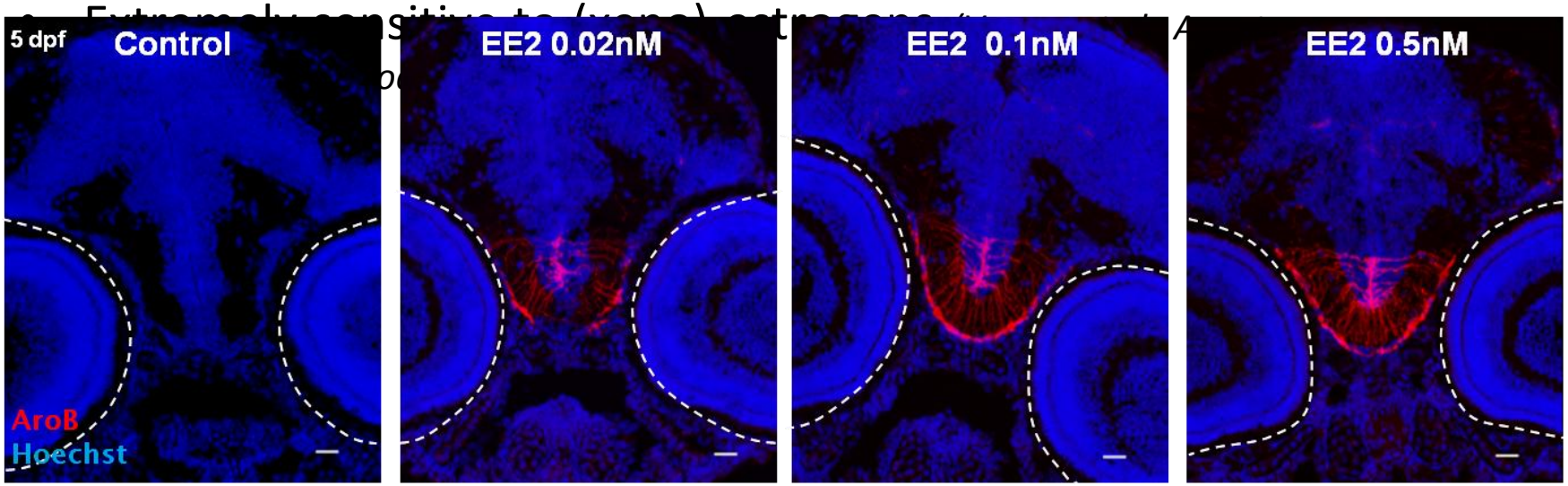
  - Extremely sensitive to (xeno) estrogens *(*

Treatment of zebrafish embryos to EE2 from 0 to 5dpf



Vosges et al., *Aquat. Toxicol.* (2010)

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  - extremely sensitive to (xeno)-estrogens *(Vosges et al., Aq. Toxicol. 2010, Reprod. Toxicol. 2012)*
  - Is exclusively expressed in Radial Glial Cells (*Pellegrini et al., J. Comp. Neurol., 2007*)

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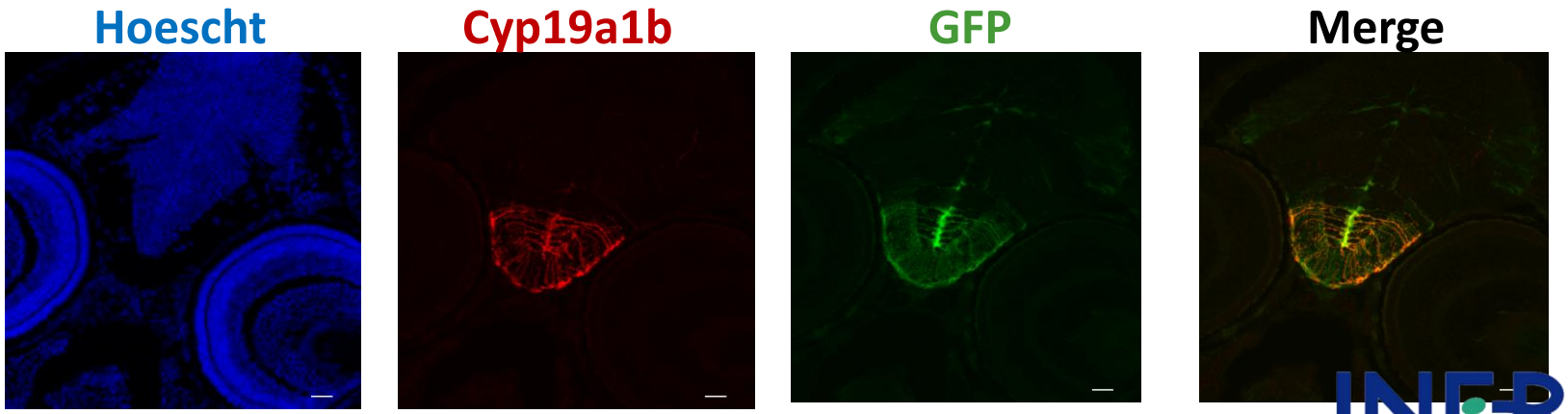
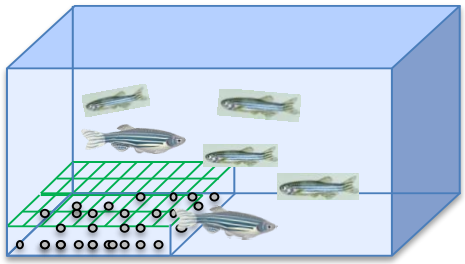
GFP faithfully mimics the endogenous brain Cyp19a1b expression



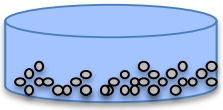
Principle of the EASZY assay

① **Spawning**

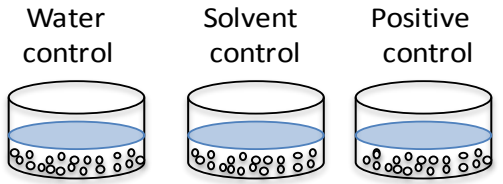


Mass spawning in 100L aquaria with 60-100 male: female fish (ratio from 1:1 to 2:1)

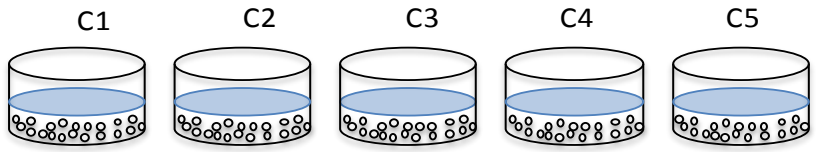
② **Collecting,**
Cleaning eggs.
Selection of fertilized eggs
with normal development



③ **N= 20 eggs per test condition & Start of exposure** (< 4 hours hpf)



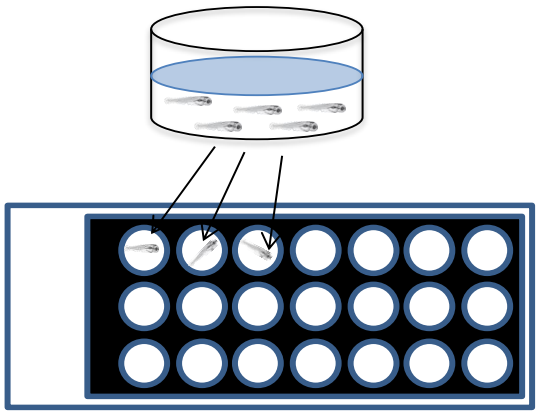
Substance



Crystallizer (25 ml of water)

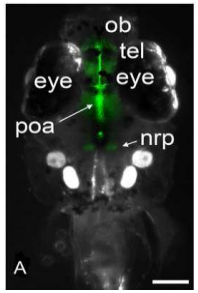
④ **96 hrs of exposure**

Incubator 27± 1°C,
14 hrs light photoperiod

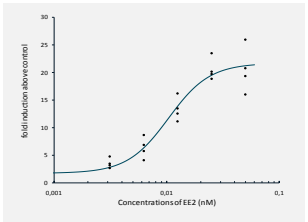


Transfer of post-hatched larvae in multi-well Fluorescence slides

⑤ ***In vivo*** **Fluorescence imaging**



⑥ **Image Analysis and data processing**



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Application to chemical screening (>60 chemicals)

ER ligands

• Hexestrol, Diethylstilbestrol , 17α-Ethynylestradiol , 17β-Estradiol, Estrone, Estriol

• Zearalenone, α-Zearalanol, α-Zearalenol, β-Zearalenol Genistein, 4′,7-Dihydroxy-iso-flavone

•4-n-nonylphenol, 4 nonylphenol (mixture of isomers), 4-*tert*-octylphenol, 4-*tert*-pentylphenol

• Methoxychlor & 1,1,1-Trichloro-2,2-bis(4-hydroxyphenyl)ethane (HPTE), o,p'-DDT, Endosulfan, Chlordecone,

•BPA and substitutes

• Benzophenone and its derivatives

AR ligands

• Testosterone,, 17α Methyl-testosterone,
•11 KT-testosterone
Dihydrotestosterone,
17β-trenbolone

PR ligands

• Progesterone,
• Medroxyprogesterone, levonorgestrel, norethindrone, tibolone, desogestrel

MR ligand

• Spironolactone,

GR ligands

• Corticosterone, dexamethasone

« Negative » compounds

• Acetone, methanol, ethanol, potassium permanganate

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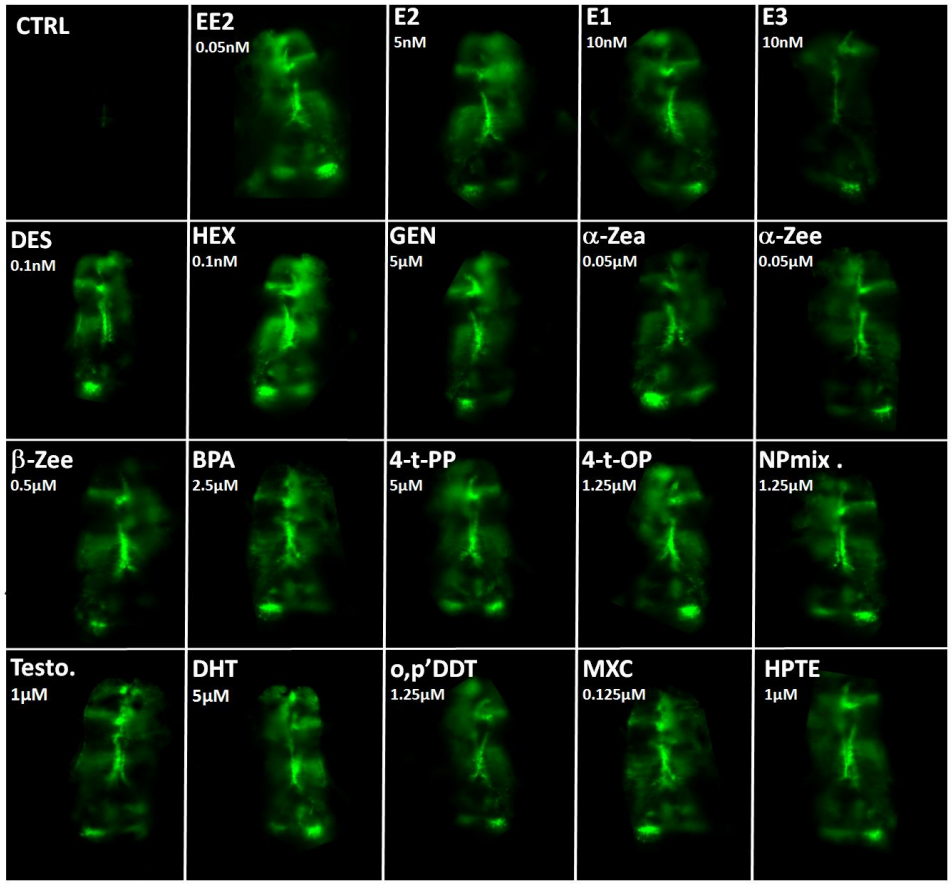
SER-dependent induction of GFP expression by substances

ER ligands



Brion et al., Plos One 2012

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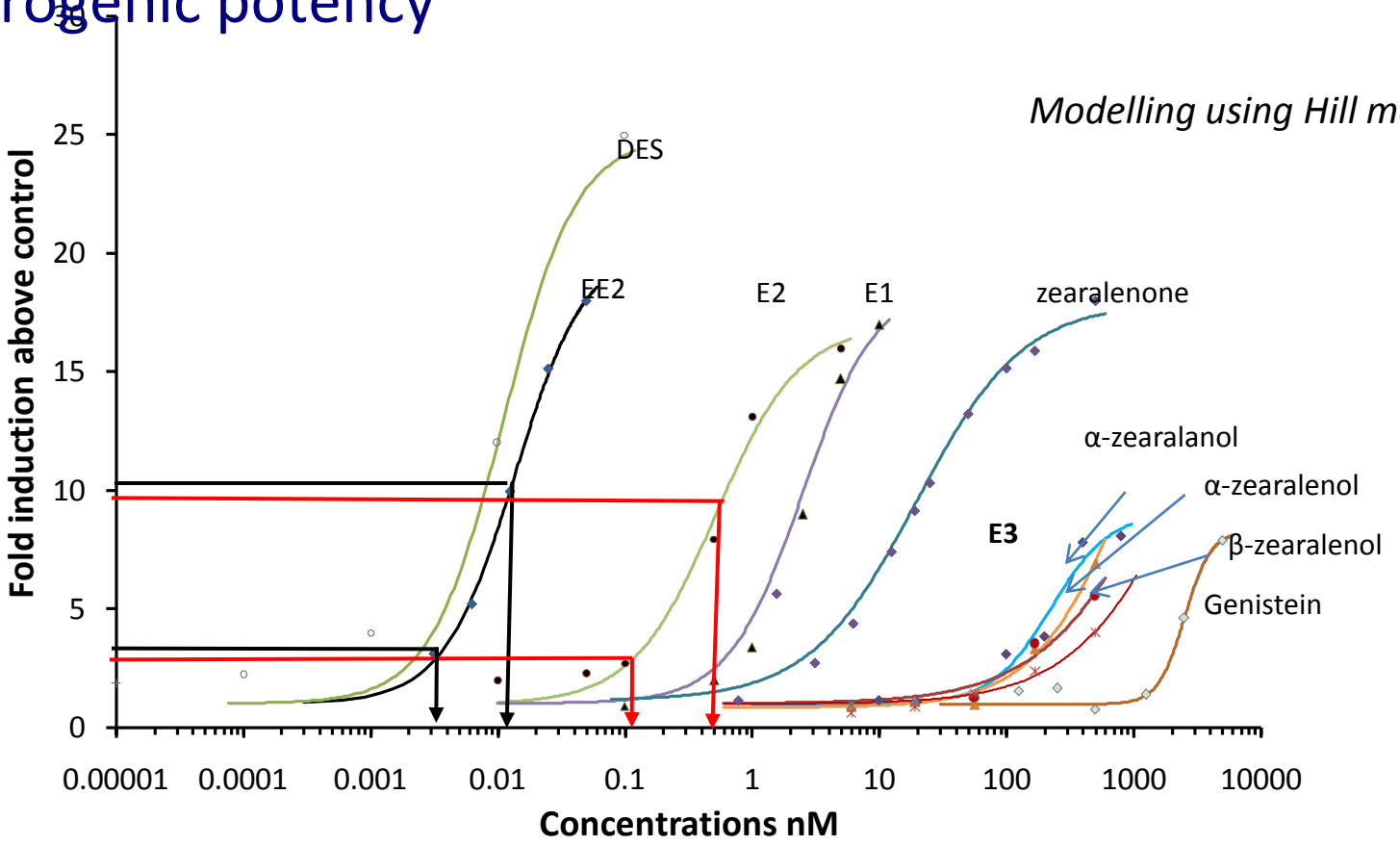
Modelling concentration- responses curves & Quantification of the estrogenic potency



- Effective Concentrations EC_x

  Example of EE2:

  EC_{50} = 13 pM

  EC_{10} = 3 pM

- *In vivo* Relative Estrogenic Potency

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

$$REP_{EE2} = 36$$

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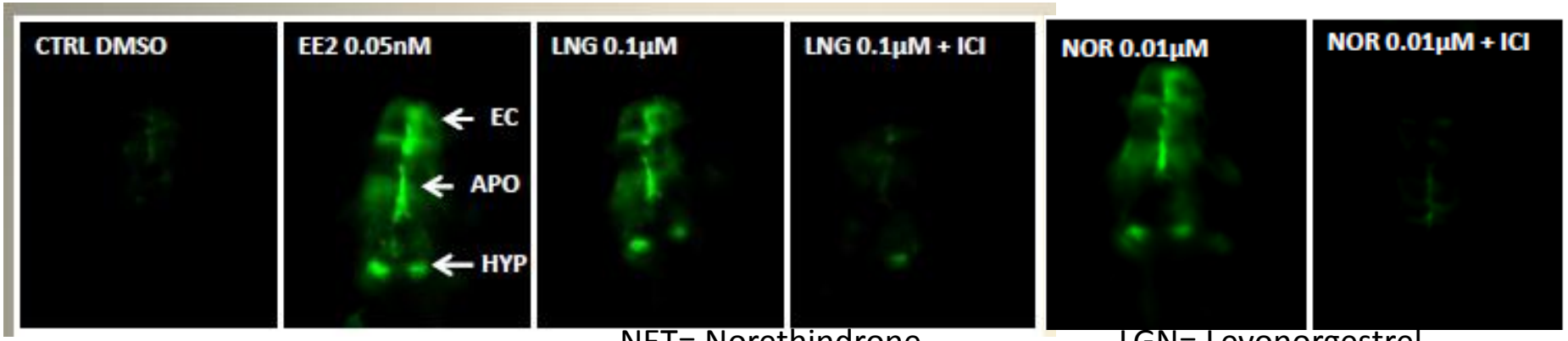
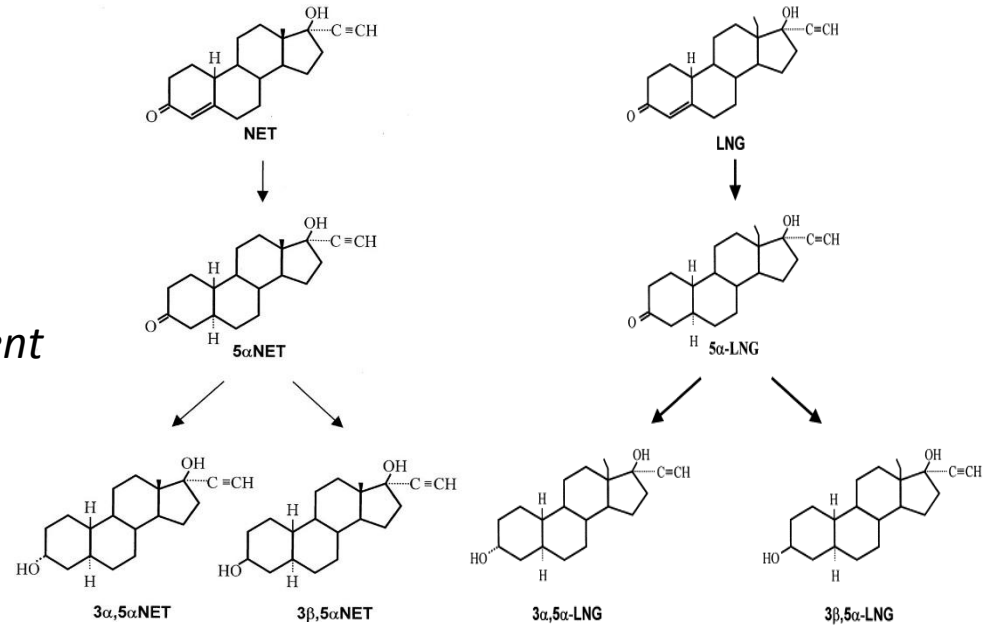
ER-dependent induction of GFP expression by various substances



NET= Norethindrone

LGN= Levonorgestrel

ANR PROOFS 2013-2016
Occurrence and effects of
Progestins in aquatic environment



3α,5α- and 3β,5α-tetrahydro derivated of NET and LNG are estrogenics in mammalian models (Larrera et al., 2001 , Garcia-Becerra et al., 2002)

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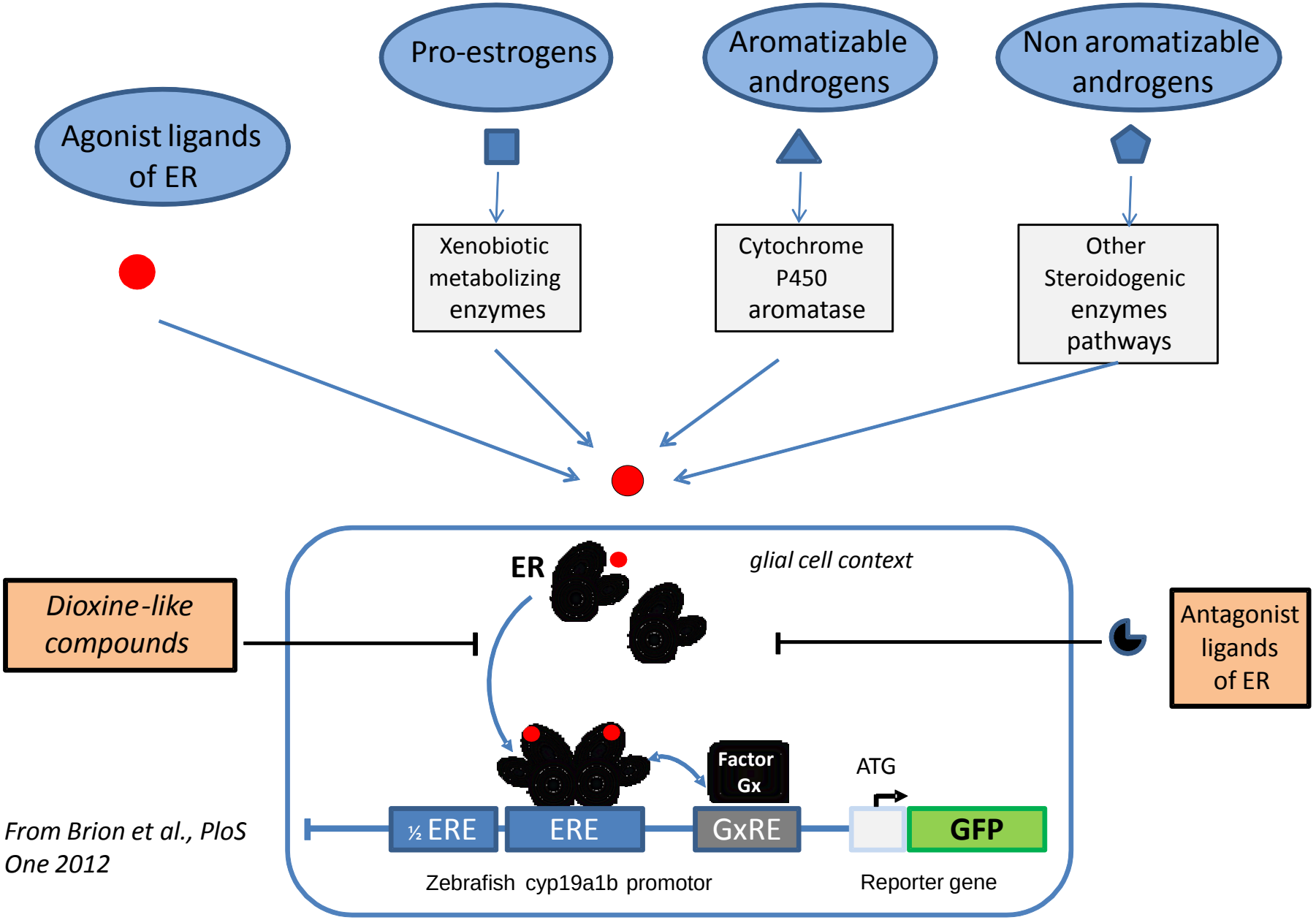
Overview of the substances detected by EASZY

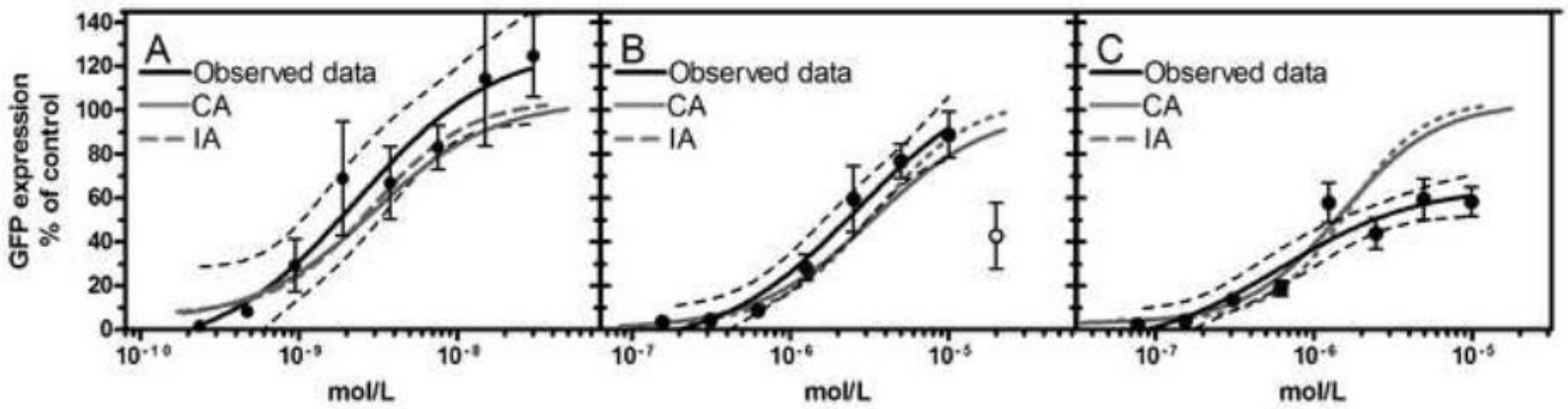


From Brion et al., PloS One 2012

Application to mixture effects

- Binary or multi-compounds mixtures of ER agonists tested at fixed ratio concentration in EASZY (Brion *et al.*, Plos One 2012, Petersen *et al.*, Aquat. Toxicol., 2013)
- CA & IA models can predicted the combined effects of ER ligands

EE2+E2+E1 *BPA+OP+E2* *BPA+OP+E2+EE2*



Collaboration with KE Tollefsen & K Petersen (NIVA); S. Scholz E & Fetter (UFZ)

- Deviations from the CA / IA models
  - high concentrations of *BPA+OP+E2+EE2*
  - antagonism of E2+ Genistein at low concentrations (PNRPE, MIXEZ)

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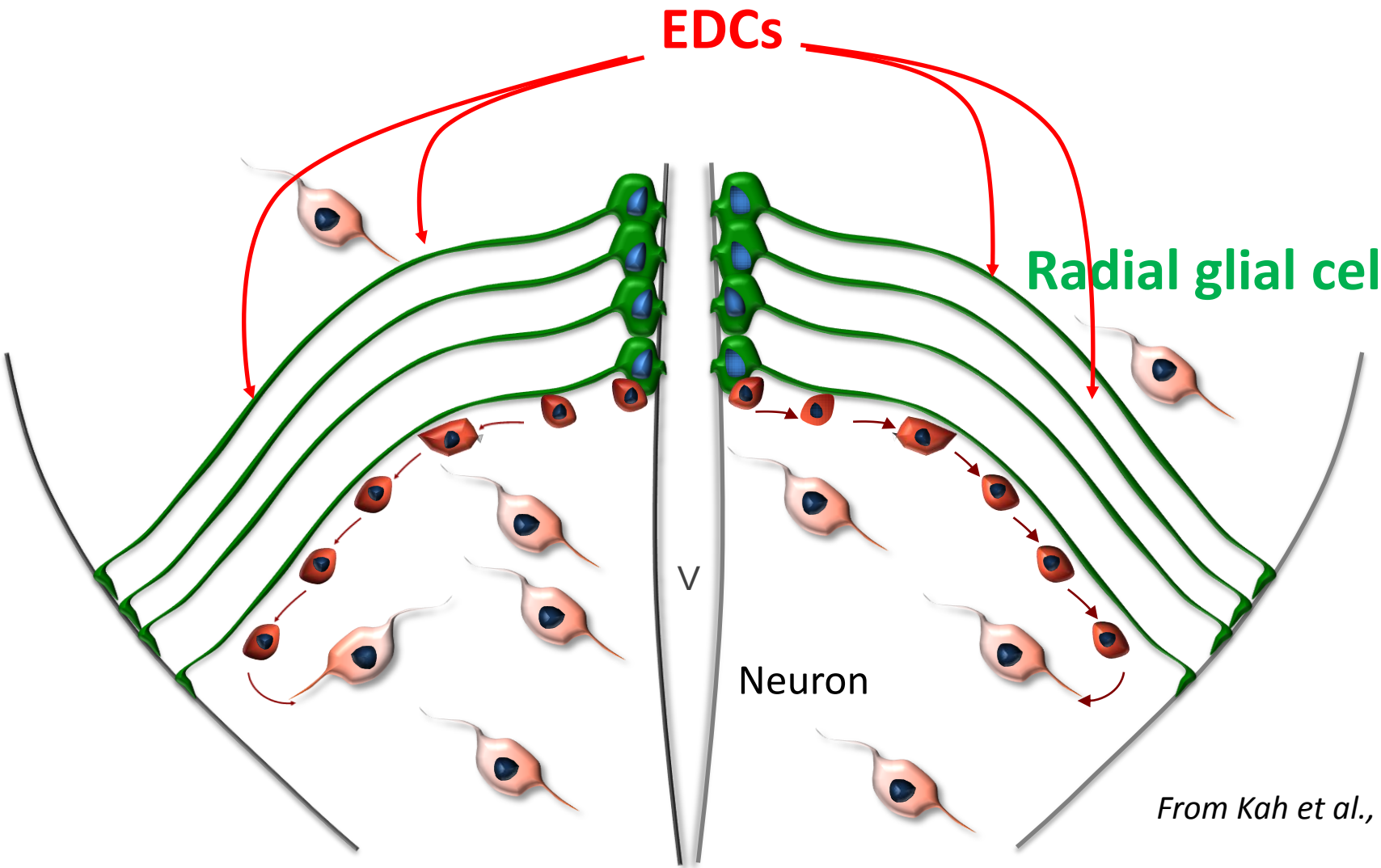
Toxicological relevance of brain aromatase disruption ?

Neurogenesis



From Kah et al., 2011

Radial glial cells = progenitor cells of neurons

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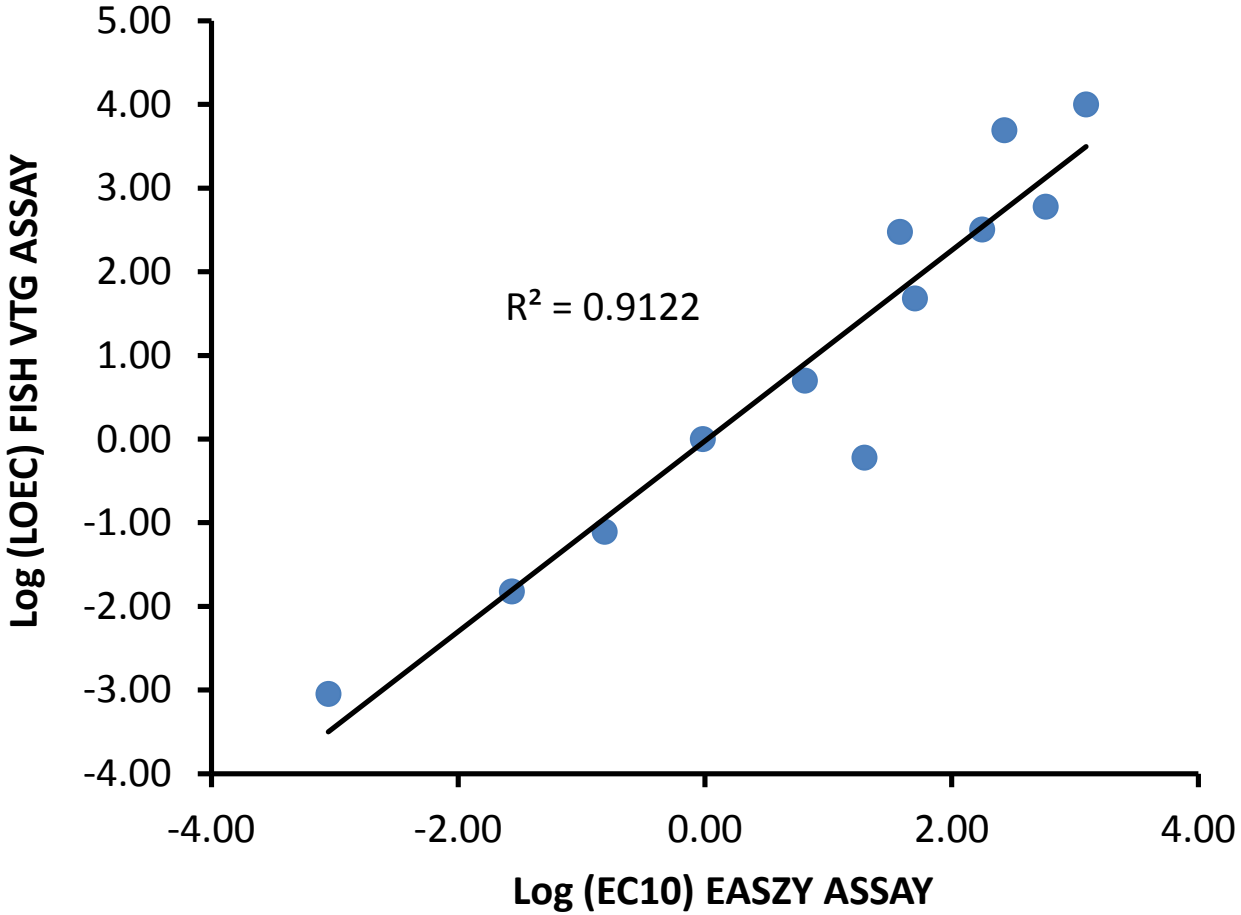
Toxicological relevance of brain aromatase disruption ?

Vitellogenin



Brion, unpublished data

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Conclusions

- Provides **mechanistic information:** activation the ER-signalling pathway *in vivo* while considering the biodisponibility and phamracodynamics of test chemicals.

- Provides **toxicological relevant information** regarding the brain development (neurogenesis) by measuring a true brain-specific response

- Could provide **predictive information** regarding the reproductive effect of substances

Conclusions and future works

- EASZY as an ***in vivo* screening assay** in fish

  - sensitive, specific, rapid, cost-effective
  - low sample volume
  - quantification of *in vivo* estrogenic activity

    ECx, $REP_{in\ vivo}$

- Under validation at the OCDE
  - Acceptance of the EASZY test to the OECD working program (May 2013)
  - Validation program accepted by VMG-eco expert group (October 2013)
  - 1st phase of the validation plan in 2014 (5 laboratories)

Acknowledgements

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Ecotoxicological Unit
Sélim Aït-Aïssa
Nathalie Hinfray
Nicolas Creusot
Benjamin Piccini
Cyril Turies
Jean Marc Porcher

Modelling Unit
Cléo Tebby
Alexandre Péry

中央研究院 分子生物研究所
Institute of Molecular Biology, Academia Sinica

Bon-Chu Chong

irset
Institut de recherche sur la santé l'environnement et le travail

Olivier Kah

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MIXEZ project
2011-2014