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Highlighting the gaps in hazard and risk assessment of unregulated Endocrine Active Substances in surface waters: retinoids as a European case study

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Abstract

Regulatory hazard and risk assessment of endocrine-active substances currently specifies four modes of action: interference with sex hormone (oestrogen, androgen) pathways, steroidogenesis, and thyroid hormone signalling. This does not encompass the full complexity of the endocrine system and its extended interfaces with environmental pollutants that can potentially disrupt the carefully maintained balance. Here we take the retinoid signalling pathway as a European case study for both, under- and unregulated endocrine pathways and outline the different levels of interference, discuss their adversity, and indicate crosstalk to other signalling pathways. Retinoid compounds already exist in drinking water sources, occur naturally in cyanobacterial blooms and/or enter surface waters via wastewater discharge, where they pose a potential hazard to the environment and human health - a situation that can be expected to worsen due to water shortages induced by climate-change and population growth. We briefly review relevant aspects of current endocrine disruptor (ED) testing for regulatory purposes and then expand upon the needs for inclusion of disruption of retinoid signalling in (ED) regulatory safety assessment contributing to adverse health outcomes that include cognitive function and neurological disease. An overview of developmental effects of retinoid signalling disruption across species highlights critical processes and potential crosstalk with other signalling pathways. A focused weight of evidence-based evaluation of the biologically plausible associations between neurological disorders and altered retinoid signalling highlights the evidence gaps. We show that monitoring only a limited number of anthropogenic priority chemicals in water is insufficient to address the environmental risks of retinoid signalling disruption. To comprehensively assess impacts on the endpoints, processes, and pathways of the endocrine system that are most vulnerable to chemical interference we need further investigation of the true mixture composition in environmental matrices. On a weight of evidence-basis this information can then be integrated into a reliable, inclusive, quantitative approach that ultimately accommodates all the critical pathways. By focusing on the retinoid signalling pathway, we intend to improve the scope and relevance of an integrated approach for the risk assessment of endocrine disruptors.

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Introduction

Internationally, chemical substances are currently screened for endocrine activity in regulatory risk assessments (as for example in the European Union's Biocides regulation [1, 2]), utilizing standard test methods that refer to chemical substances as endocrine active when interfering with sex hormone (oestrogen, androgen) receptors, steroidogenesis, or thyroid hormone signalling (EATS; the available tests are introduced in the "infobox" below) [3]. It is recognized that the endocrine system, however, is a complex interplay of different, often evolutionary highly conserved, mechanisms that by far exceed the above-mentioned four modes of action. It includes all hormone signalling pathways, interlinking and regulating an extensive set of functions, including development, growth, reproduction and metabolism [4–8], and this is being actively examined at inter-governmental levels [8–16]. The endocrine system is highly sensitive and circulating hormone levels are in the pM–μM range, making it highly susceptible to interfering compounds [17, 18]. Interference of exogenous chemicals with the tightly regulated endocrine system may result in adverse health effects, that, especially when encountered during development, may have sustained and life-long [12 and references therein, 19] or even transgenerational impacts on individuals or contribute to non-communicable diseases like metabolic disorders and cancer [4, 18, 20–29].

To allow an assessment of risks related to chemicals in the environment, information on the ecological or human health hazard of these chemicals is needed [2, 3] together with information on exposure to these chemicals and/or mixtures, i.e. their levels and fate in the environment, to conclude as to whether there is a risk of adverse outcomes or not [30, 31].

Despite the progress in the development of test methods screening for endocrine disrupting activity, endocrine pathways other than EATS remain under-investigated. Whilst currently there are no specific test methods available with respect to other endocrine mechanisms, these are being actively explored at the European level (https://eurion-cluster.eu/), and internationally, for retinoids [13, reviewed in 14]. Such comprehensive reviews together with identification of relevant assays with reference and test chemicals are needed to address regulatory needs, prior to the development of the test method tools that can be included into legislative mechanisms.

In surface waters, endocrine disruption gained public attention when altered sex ratios, genital malformations, and reproductive impairment were discovered in aquatic vertebrates [6, 33–38]. Most strikingly, feminization of male fish occurred at oestrogen levels below the limit of detection by analytical methods available at that time and also led to the collapse of a fish population in a Canadian experimental lake [39]. The oestrogen levels in the respective water bodies have been frequently attributed to poor treatment of communal wastewaters, containing high levels of human contraceptives [40]. More recently progesterone has been detected in UK shores in molluscs at concentrations equivalent to those used in contraceptives and hormone replacement therapy [41]. The striking impact of compounds interfering with the oestrogen hormone system has expanded the field of environmental endocrine disruption and enabled investigation of other endocrine pathways sensitive to environmental interference [42].

It is intended that this review provides a useful contribution to the discussion of under- and unregulated endocrine pathways, particularly in relation to the gap in hazard and risk assessment approaches to address anthropogenic and naturally occurring toxic retinoid substances for water quality. We focus on two key aspects of environmental chemicals' potential to interfere with retinoid signalling: (1) with respect to the presence of chemicals that elicit retinoid-like activity via retinoid receptors, and (2) the potential of the endogenous retinoid system to be a target for an expanded range of chemicals which could disrupt this system. In addition to retinoid signalling pathway-related developmental and reproductive endpoints [reviewed in 13, 14], here we facilitate the addition of the less well studied endpoints of cognitive function and neurological disease. We thereby intend to contribute to the evidence base needed for the development of the tools and approaches to address endocrine adverse outcomes related to disruption of retinoid signalling pathway.

Infobox: Available OECD standardized test methods for the hazard assessment of Endocrine Active Substances

OECD Guidance document 150 [3] provides detailed information and guidance on standardised test methods for Endocrine Active Substance (EAS) and endocrine disruptor (ED) testing, and the EFSA/ECHA have developed ED guidance for the use of these test methods in relation to current EU regulatory needs [2]. While EAS refers to the endocrine activity of the substance it does not necessarily result in an adverse health outcome. If the observed effect is irreversible then the substance is considered an ED with adverse health outcome [3, 43, 44].

Oestrogen receptor (ER) **binding** can be measured using the ER binding assays in OECD Test Guideline 493 [45], and ER agonist and antagonist activity can be screened using *in vitro* reporter-gene assays [46]. In the OECD Endocrine Disrupter Conceptual Framework for testing, (anti) oestrogenic activity can be confirmed by the *in vivo* uterotrophic bioassay in rodents [47, 48].

In vitro and *in vivo* assays for the assessment of (anti) **androgenic activity** are also standardised and available [49–52]. The *in vitro* assay for androgen receptor (AR) activation [49] uses the same principle of reporter gene assays as the ERα assay. Although not adopted as a Test Guideline, the validated *in vivo* method to test for and/or confirm (anti) androgenic activity in aquatic animals is, for example, the androgenised female stickleback screen (AFSS [50, 53]), where a species-specific biomarker, spiggin, can be measured as an indicator of androgenic activity in the three-spine stickleback (*Gasterosteus aculeatus*) [54, 55]. For mammals, higher-tier testing for (anti)androgenic endocrine disruption relies on the Hershberger bioassay in rats, which is still used since its approval in the 1960s [51].

For both oestrogenic and androgenic activity, there are further whole animal (fish and amphibian) systems currently under validation, under the auspices of the OECD and specifically transgenic models are now on the OECD workplan that are intended to address the gap between *in vitro* and *in vivo* test methods. These include the "Rapid Estrogen ACTivity *In Vivo*" [56, REACTiV; 57, 58], the "Endocrine Active Substance, acting through estrogen receptors, using transgenic cyp19a1bGFP Zebrafish embrYos" [EASZY; 59] and the "Rapid Androgen Disruption Adverse outcome Reporter" [RADAR; 60, 61] assays. Whilst the *in vitro* ER test methods correlate well with *in vivo* models [2, 62], *in vitro* AR assay data do not correlate well with the Hershberger assay [63]. So in terms of refinement, it is expected that the RADAR assay will potentially be a great improvement on the Hershberger method.

Interference of chemicals with the **steroidogenesis** pathway, and therefore also with the biosynthesis of sex hormones, is covered by the Test Guideline 456 [64]. Similar to the ER and AR transactivation assays, this *in vitro* screening test provides mechanistic data on the potential of a substance to interfere with the production of corticosteroids and sex steroids, such as 17β-estradiol or testosterone.
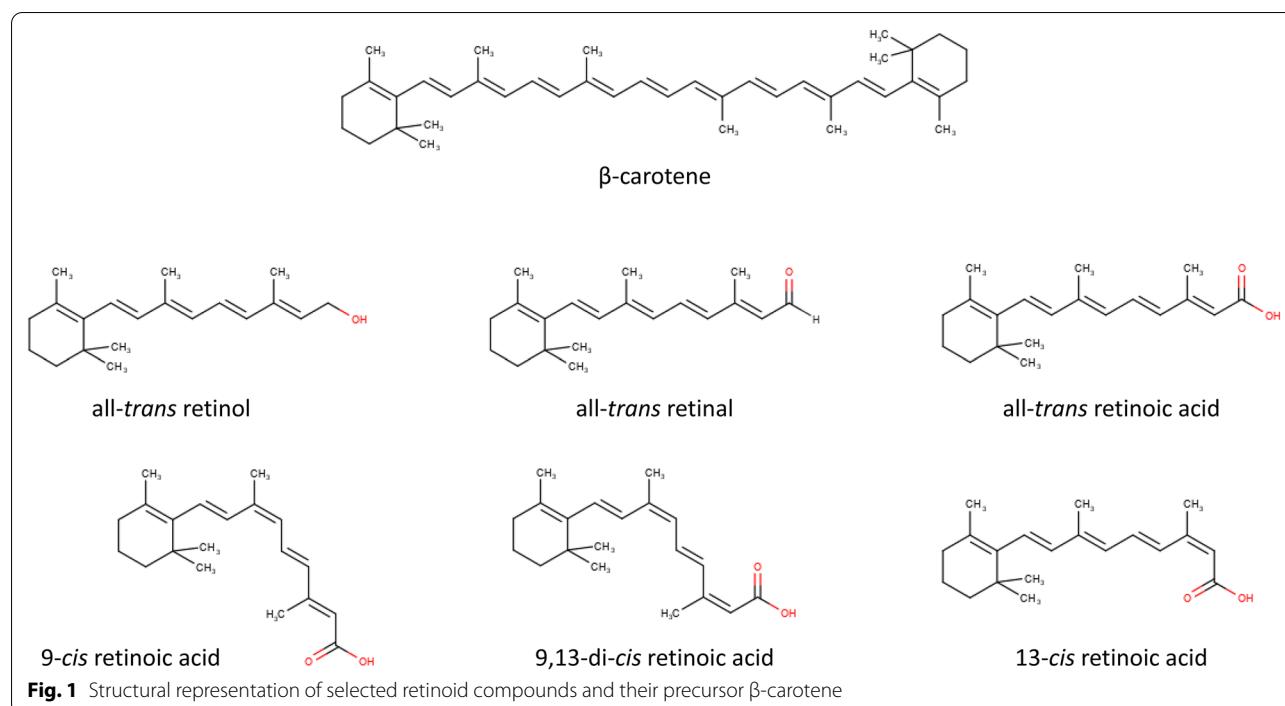
In vitro screening tests for interference with the mammalian **thyroid hormone** system are currently under development [32] and those documented in the OECD thyroid scoping document [10] are being validated by EURL ECVAM [65, 66]. For amphibians, eleutheroembryos of transgenic *Xenopus laevis* can be utilized to obtain qualitative information about interference with thyroid hormone signalling [67]. The recorded response is the expression of green fluorescent protein, that is governed by a thyroid hormone receptor sensitive promoter [67, 68].

Hazard characterization of retinoid substances - from molecular interactions to developmental and neurological outcomes in vivo

Retinoid substances are chemically related to retinol (vitamin A). They are small organic molecules biosynthesized from isoprenoid precursors, mostly by photosynthetic organisms like phytoplankton and plants [69]. Retinoids, generally obtained from the diet [70–72], particularly retinoic acid, play a pivotal role during early development, driving anterior–posterior patterning in developing embryos and development of the vertebrate brain [73–77]. At the same time, retinoic acids are classified as teratogenic, due to the pronounced dependence on the spatio-temporal distribution of retinoids in the tissues of developing organisms [73, 78–81]. The

developmental processes in which retinoids are involved are further discussed in "Phenotypic patterns of interference with retinoid signalling during development" section. Besides the tissue distribution and metabolization of retinoid isomers, also the expression pattern of retinoic acid receptor (RAR) and retinoid X receptor (RXR) isoforms and variants in tissues determines retinoid effects [78, 82].

The endogenous ligand for RAR is all-*trans* retinoic acid (atRA; Fig. 1); for RXR it appears to be either 9,13-di-*cis* retinoic acid or 9-*cis* retinoic acid (9cRA; Fig. 1). Additional retinoids that have been shown to activate retinoid receptors include 9-*cis* didehydro retinoic acid, all-*trans* 5,6 epoxy retinoic acid, all-*trans* 4-oxo retinoic acid, and all-*trans* 4-oxo retinal [83–88]. In humans,

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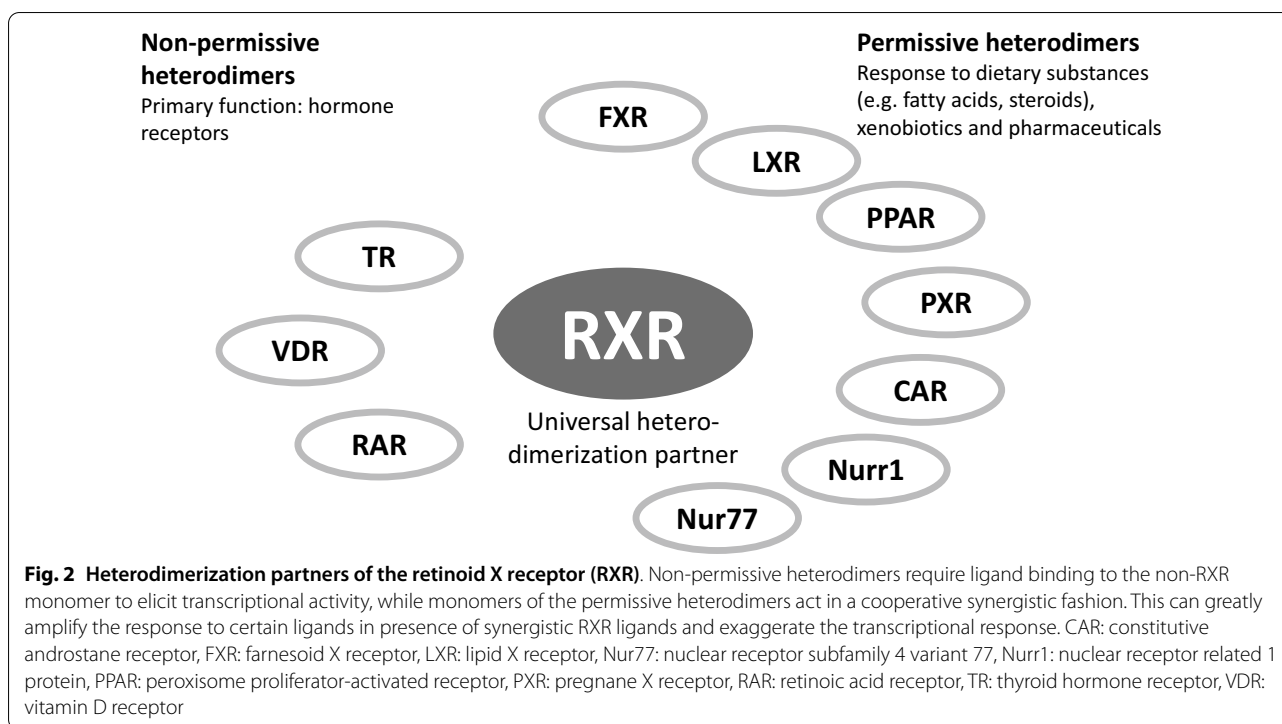**Fig. 1** Structural representation of selected retinoid compounds and their precursor β-carotene

the biologically active and most potent atRA is obtained by sequential oxidation from all-*trans* retinol (vitamin A; Fig. 1) via alcohol dehydrogenases (esp. retinol dehydrogenase 10) [89] and retinal dehydrogenases (RALDHs, mainly RALDH2 in mammals) [90, 91]. Retinol is stored in the liver as retinyl esters [92–94]. atRA cannot be synthetized de novo in vertebrates and requires nutritional sources, which can be in the easily metabolized precursor forms such as β-carotene [80, 94–96]. The Population Reference Intake ranges between 250 μg retinol equivalent/day in infants below the age of 1 year and up to 750 μg retinol equivalent/day in children and adults [97] and is within the same range as the daily vitamin A intake recommended by Public Health England [72].

Binding of retinoids, primarily atRA, to RAR results in their heterodimerization with RXR and subsequent transcriptional activation of retinoic acid-responsive elements (RAREs), which govern a number of crucial cellular processes, including inflammation, proliferation, differentiation and carcinogenesis [28, 98, 99]. RAR–RXR heterodimers furthermore can recruit co-repressor complexes and, depending on the presence of natural or synthetic ligands, modulate or suppress gene expression [100–102].

While RARs show a higher specificity towards retinoid compounds binding and are the main driver in retinoid-mediated patterning and teratogenicity [e.g. recently reviewed by 103], the role of RXRs is broader.

One of the reasons is the molecular promiscuity of RXR. Type II nuclear receptors, characterized by forming heterodimers with RXR, govern the transcription of a large variety of target genes [104]. They are involved in the biological responses to many endogenous ligands, anthropogenic and natural chemicals and therapeutic drugs. The affected functions include lipid metabolism (peroxisome-proliferator activated receptor, PPAR), steroidogenesis, xenobiotic response (pregnane X receptor, PXR; constitutive androstane receptor, CAR), vitamin D receptor (VDR), liver functions (FXR, LXR), orphan nuclear receptors (Nurs), and thyroid hormone signalling (thyroid hormone receptor, TR [67]; Fig. 2) [7, 85, 88, 105–107]. Whilst the receptors TR, VDR, and RAR form non-permissive heterodimers, the others (Fig. 2) form permissive heterodimers with RXR, where the transcriptional activity is regulated by a ligand binding to one of the dimerization partners [85, 104, 106]. Dimerization is achieved via the asymmetrical so-called *identity box* - a small region within the ligand binding domain, which, in the case of RXRα, consists of 40 amino acids [108, 109]. This subdomain shows a very high degree of conservation. Especially, the two amino acids A416 and R421 have been shown crucial for dimerization of RXRα with RAR [108, 109]. The high conservation of the RXR identity box even across animal phyla underlines the evolutionary importance of RXR [110, 111].

Fig. 2 **Heterodimerization partners of the retinoid X receptor (RXR)**. Non-permissive heterodimers require ligand binding to the non-RXR monomer to elicit transcriptional activity, while monomers of the permissive heterodimers act in a cooperative synergistic fashion. This can greatly amplify the response to certain ligands in presence of synergistic RXR ligands and exaggerate the transcriptional response. CAR: constitutive androstane receptor, FXR: farnesoid X receptor, LXR: lipid X receptor, Nur77: nuclear receptor subfamily 4 variant 77, Nurr1: nuclear receptor related 1 protein, PPAR: peroxisome proliferator-activated receptor, PXR: pregnane X receptor, RAR: retinoic acid receptor, TR: thyroid hormone receptor, VDR: vitamin D receptor

Molecular crosstalk in the RXR signalling pathway

The fact that nuclear receptors share the common heterodimerization partner, RXR, indicates the potential for molecular crosstalk between signalling pathways dependent on RXR heterodimerization. The sequestration of ligand-bound RXR from the pool of active RXR monomers with downstream modulating activities is indicated [7], and also direct ligand activation of, e.g. the PPAR family by retinoic acids [112, 113] has been reported in addition to activation of retinoid receptors. Additionally, there is evidence of crosstalk to the thyroid hormone signalling pathway by heterodimers of TR with RXR in vitro [114] and augmentation of thyroid hormone-related effects by RXR activation in vivo [67]. Most often, the ubiquitous RXRα isoform is involved in heterodimerization and it is essential for xenobiotic metabolism [7, 13, 115]. Competitive decrease of effect due to RXRα sequestration by retinoic acid/RAR has been reported for CAR [116], LXR, FXR, PPARα [106], and PPARγ [117] and may be implied in the metabolization and detoxification capacity mediated via activation of, e.g. PPARs. The capacity of RXRs to form heterodimers with several dimerization partners allows integration of signals from simultaneous and independent signalling pathways that can be further modulated by transcription co-factors [13, reviewed in 87]. The importance of allosteric modulators has been also stressed in a recent study on nuclear receptor binding

to DNA target sequences (direct repeats and half-sites), where in vitro binding was predictive of in vivo binding, but not of in vivo function [104].

The importance of co-evolution of nuclear receptors and overlapping *cis*-regulatory elements also becomes apparent at the intersection of RAR/RXR and ERα signalling pathways. RAR/RXR signalling has been demonstrated several times to antagonize ER binding to respective DNA target sequences [118–121]. Besides the therapeutic use of this observation particularly in ER-responsive breast cancer [121], ERs play a critical role in organogenesis and maturation processes that, thus, can be affected by dietary and environmental factors.

Steroidogenesis critically influences the production and subsequently the circulating amount of the prototype sex steroids oestrogen and testosterone [13]. RAR/RXR play a pivotal role at the beginning of the steroidogenesis pathway, but RXR also as the essential dimerization partner for adjacent and subsequent steps interlinked with lipid metabolism (PPARs, LXR) and xenobiotic response (CAR, PXR) [13, 106]. At this interface, delivery of retinoic acid to the various nuclear receptors (RAR, PPAR or VDR) can have different consequences with respect to adiposity, such that, for example VDR activation in fibroblasts induces non-adipogenic gene transcription, whilst PPARγ/RXR heterodimers contribute to adipogenic processes [7, 122].

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Whilst the therapeutic potential of the interdependency amongst many pathways and retinoid signalling via RXR has already been discovered and drugs specific to RXR, so-called "rexinoids" (e.g. bexarotene) are available to treat certain types of cancers [123], the implications of unintentional deregulation of retinoid signalling remain to be elucidated.

Phenotypic patterns of interference with retinoid signalling during development

Interference with retinoic acid signalling has the highest impact on humans during development and was first observed in vitamin A (retinol) deficiency. This has been understood since the early twentieth century from studies that investigated the teratogenic effects of both excess and a lack of retinoid activity [124–130]. To date, vitamin A deficiency is still a concern, especially in developing countries where one-third (33.3%) of pre-school age children and 15.3% of pregnant women have serum retinol levels below 0.7 μM [131], resulting in severe risk of vitamin A-preventable blindness that has a fatality rate in children of 50% within one year [131–133]. Without nutritional supplementation within the first year of life, this can be considered as irreversible retinoid disruption and interpreted as an example of an (irreversible) adverse outcome in humans justifying classification as an endocrine disruption pathway.

Retinoic acid gradients determine the dorso-ventral and anterior–posterior patterning of the embryo in the first trimester [73, 75, 78, 82]. Furthermore, the differential expression and activation of retinoid receptor variants and isoforms, together with the spatio-temporal regulation of RA synthesis and metabolism, drives organogenesis and elongation of the spinal axis [74, 76, 78, 134]. It also determines progenitor cells to the neural lineage, thus initiating the formation of the neural system including the spinal cord and the brain [73, 76, 82]. Notably, retinoid signalling drives the formation and segmentation of the hindbrain and neural network formation even before thyroid receptors are being expressed [73, 135, 136], and is involved in the correct development and positioning of other vital internal organs [137], including the heart [138–140], the lungs [139, 141–143], (male) genitals [13, 144–147], and the kidney [148, 149]. Overall, RA governs a multitude of developmental effects and also influences processes in adults, namely neuronal plasticity in the brain [150–153], peripheral neural regeneration [154–156], immune function [157–159], epithelial function [139, 160], and reproduction [13]. An overview on the processes sensitive to or dependent on retinoic signalling is outlined in Table 1.

The most critical elements for interference in the retinoid signalling pathways, beyond RARs and RXRs, are the metabolizing enzymes of the cytochrome P450 subfamily 26 (CYP26) CYP26A1, CYP26B1, and CYP26C1 [94, 161, 162], the retinol-converting alcohol dehydrogenase (ADH), and retinal-oxidizing dehydrogenases (RALDHs) [90, 94, 163, 164]. Efforts to identify key players and switches in the hierarchical signalling network and sort them into adverse outcome pathways (AOPs) continue to be undertaken with respect to vertebrate axial and neural tube development [14, 165, 166], as well as mammalian reproduction [reviewed in 13].

Besides retinoids themselves, other environmental contaminants, such as pharmaceuticals (e.g. valproic acid), flame retardants (e.g. polybrominated diphenyl ethers), plasticizers (phthalate esters), and pesticides (triazole fungicides) have been reported to alter retinoid signalling biomarkers and induce retinoid-like teratogenic effects [111, 167–170] (see also Table 1). Exposure assessment of pharmaceuticals with the retinoid mode of action may require different regulatory approaches, depending upon the route of exposure, i.e. whether there is oral intake/dermal application, as opposed to their occurrence and potency in (waste)waters.

Contribution of retinoids to chronic neurological disorders
The contribution of endocrine disruptors to neurological disorders is receiving more attention, and increasing resources are being put into funding such research [e.g. 32, 171]. In addition to known adverse teratogenic effects during development, particularly, brain and neurodegenerative conditions such as Alzheimer's and Parkinson's disease, or depression may be linked to altered retinoid signalling [172, 173].

While retinoid compounds are well described as early morphogens of the central nervous system (CNS) during development, their role in postnatal development of the brain is less investigated. Retinoid signalling is implied in neural plasticity, required for the formation of new memories and for learning [151, 174–176], in affective disorders [177], and in ageing - namely in Alzheimer's disease and dementia [178].

To date, neurodegenerative diseases such as dementia, Parkinson's, Alzheimer's, and Huntington's disease, are the 6th leading cause of deaths in adults in the US [179, 180]. Unlike mortality due to heart disease, stroke, or HIV, deaths linked to Alzheimer's disease have more than doubled between 2000 and 2018 [179]. Even more severe is the situation regarding neurological disorders in general, which are the second leading cause of death after heart disease and the leading cause of disability worldwide [181, 182]. To date, there is no cure for dementia and treatment focuses on ameliorating the symptoms of

Table 1 Examples of the impacts of retinoic acid signalling effects on morphology, phenotype, and/or development. Explanations and abbreviations are at the end of this table

Apical Effect/ Key mechanism/ Endpoint	Morphological alteration level	Developmental stage	Test system (species)	Main relevant and related pathways, genes, enzymes	Endpoint/hallmark affected cross-reactions	Substances associated with key mechanism/ adverse outcome	Associated human pathology	Reference(s)
Axial development, Anterior-posterior patterning, osteogenesis	Tissue	Embryo, larvae	*Branchiostoma floridae* (amphioxus), zebrafish (*stocksteif* mutant), chicken, mouse	ADH/ RALDH, Cyp26b1, **Cyp26a1**, RARα, RARβ, RARγ, Wnt, **FGF**, *Hox* (esp. 1&3), BMP	RA acts as an early developmental morphogen along the anterior-posterior axis; it coordinates the position of endoderm-derived organs along the anterior-posterior axis. Cyp26/ RA concentration drives the osteogenesis in the vertebral column (in osteoblasts); posteriorization of gills and mouth in invertebrate chordates. Hyperactive RAR induces higher expression levels of RA-metabolizing Cyp26a1 and acts in a paracrine way	RA, retinol		[76, 148, 313–322]
					Symmetric somite development is mediated by RA			[323–325]
			Human, zebrafish, rat, mouse	Dhrs3, Cyp26(a1)	Axial skeletal and craniofacial defects upon exposure	Triazole fungicides (flusilazole, triadimefon)		[165, 326]
Neural tube formation	Organ/organism	Embryo	Quail	FGF and Wnt gradients/signaling, CYP26A1, RALDH activity	Mesodermal segmentation, somite formation, and neurogenesis in caudal neural tube (future spinal cord) are RA dependent	RA		[73, 315, 327–329]
			Human, zebrafish, rat	Dhrs3, Cyp26(a1)	Neural tube (and axial) defects; proposed Adverse Outcome Pathway: "for neural tube and axial defects mediated by modulation of retinoic acid homeostasis"	Triazole fungicides (flusilazole)		[165]
Neural differentiation and spinal cord formation	Tissue/organ	Embryo, (adult)	Mouse, zebrafish, *Xenopus*	FGF and Wnt, CYP26A1, RALDH, RARβ, RARα, *Hox*	The nervous system develops sequentially along this axis, starting anteriorly (CNS/brain), continuing via hindbrain to spinal cord. Determination of cell fate and differentiation of ventral neuronal cell types in developing spinal cord. Neurite outgrowth in embryos and adults is dependent on RARβ expression; *RARα* knockdown abolishes atRA-mediated dendritic growth	RA (endogenous)		[76, 196, 314, 327, 330–336]

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Table 1 (continued)

Apical Effect/Key mechanism/Endpoint	Morphological alteration level	Developmental stage	Test system (species)	Main relevant and related pathways, genes, enzymes	Endpoint/hallmark affected cross-reactions	Substances associated with key mechanism/adverse outcome	Associated human pathology	Reference(s)
Early neural differentiation	Tissue/organ system	Embryo	Mouse	RAR, TR, MCT8	Organization of the (central) nervous system; RA signalling precedes TRα expression/TR signaling; RA induces MCT8 expression in the developing brain allowing TH transport	RA, TH		[135, 136]
			Zebrafish		RA co-administration (1 nM) prevents adverse effects (behavioural and histological) of ethanol (150 mM) exposure during gastrulation	RA, ethanol		[337]
Hindbrain segmentation, Ear development and hearing recovery	Tissue/organ	Embryo, adult	Mouse, rat, zebrafish, VAD quail model, chicken, *Xenopus*	RARα, RARβ, (CRABP), SHH, Wnt, FGF, *Hox*; CYP26A1, CYP26C1, RALDH activity	RA guides the formation of 8 segments (rhombomeres) that give rise to e.g. otic vesicle, sensory tract. RA determines the forebrain–hindbrain and hindbrain-spinal cord boundary (excess leads to posteriorization); midbrain-hindbrain boundary is unaffected (in mouse and *Xenopus*). RA directly influences the differentiation of branchiomotor neurons (zebrafish)	RA	Congenital hearing loss [338, 339]	[91, 124, 148, 321, 331, 340–359]
			Mouse, zebrafish		Development of the olfactory region requires RA. RA stimulates regeneration of auditory hair cells			[360–363]
							Fetal alcohol syndrome; cerebellar maldevelopment	[364, 365]
Head and forebrain development; Eye development	Tissue/organ	Embryo	Mouse, VAD quail model, pig, rabbit, cattle, sheep, rat, zebrafish	RA	Formation of optic vesicle (retina precursor; invagination of neuroepithelium); micro-/anophthalmia in absence of RA. However: head development in general requires absence of RA [76]	RA		[90, 125, 127, 129, 163, 363, 366, 367]
			Rat, human	RALDH, CYP26, AhR	Craniofacial malformations are linked to RA deregulation	atRA, 13cRA	Cleft palate and lip	[368–372]

Table 1 (continued)

Apical Effect/Key mechanism/Endpoint	Morphological alteration level	Developmental stage	Test system (species)	Main relevant and related pathways, genes, enzymes	Endpoint/hallmark affected cross-reactions	Substances associated with key mechanism/adverse outcome	Associated human pathology	Reference(s)
Telencephalon differentiation	Tissue	Embryo	Mouse		Changed population of ganglia; altered precursor population; RA stimulates production of dopaminergic neurons	RA		[373–377]
Cerebral cortex	Tissue	Embryo, postnatal	Mouse	RAR	Influence on neurogenesis/migration/ differentiation in other brain regions/ at other developmental stages. Sensitivity to RA is retained in the mature cortex	RA (endogenous), 13cRA	Affective liability and behavioural disinhibition upon 13cRA treatment, depression [177]	[73, 374, 378–380]
Hippocampus, neuronal plasticity	Cell/ tissue	Adult	Mouse, rat, zebra finch	RAR/RXR, esp. RARβ, RXRγ	Defects in spatial learning and memory, and recognition working memory (RXRγ) upon vitamin A deprivation; restoration of cognitive impairment with vitamin A supply; cognitive impairment also in excess RA scenario (13cRA). Decreased ability to learn mating song in zebra finches	RA, vitamin A	Learning and memory impairment, depression	[150–153, 177, 192, 381–383]
Hippocampus	Tissue/organ	Adult	Rat, human (Alzheimer's disease patients), mouse	Calbindin D28K, neurogranin, somatostatin, CHAT; RARα, RARβ, RXRγ, amyloid pathway, RBP4, CYP26, RALDH2 (in adjacent meninges)	Regulation of memory and spatial learning; RA acts as a proamnesic molecule Deprivation leads to amyloid-β accumulation, RARα downregulation, CHAT expression loss in forebrain cortical neurons RA regulates proteins linked to protection from Alzheimer's disease	Vitamin A	Alzheimer's disease, ageing	[82, 151, 178, 192, 205, 211, 384–392]
Hippocampus	Organ	Postnatal	Mouse, rat	RARs (esp. RARα), RXRs, GR, somatostatin, RALDH2 (in adjacent meninges)	RARβ-/- and RXRγ-/-: deficiency in spatial learning and memory, like VAD rats (rescue by RA treatment). Degradation of hippocampal function in aging mice via proliferation/ differentiation of hippocampal stem cells VAD increases GR binding capacity and modulates the somatostatinergic and acetylcholinergic hippocampal system	Vitamin A, atRA, 13cRA	Memory/ learning impairment, dementia, Alzheimer's disease, depression	[73, 151–153, 173, 188, 192, 193, 203, 374, 381, 391, 393–398]
			Rat		Hippocampal volume is reduced after 3 weeks of 13cRA treatment			[190]

Table 1 (continued)

Apical Effect/ Key mechanism/ Endpoint	Morphological alteration level	Developmental stage	Test system (species)	Main relevant and related pathways, genes, enzymes	Endpoint/hallmark affected cross-reactions	Substances associated with key mechanism/ adverse outcome	Associated human pathology	Reference(s)
Learning	Organism	Embryo	Rat	RALDH, PC2	Exposure during gestation (day 11–13 in rat) impaired amphetamine-stimulated activity and avoidance learning, but not performance in complex spatial maze or auditory startle response in offspring. A signalling decline is observed in aging and associated with cognitive impairment, decreased acquisition of new memories; reversible by RA administration	atRA, 13cRA, 9cRA	Possibly: affective disorders, neurodegenerative disorders, schizophrenia, autism	[172, 186, 188, 203, 220, 395, 399–401]
		Postnatal	Rat, Mouse	RARβ, RXRγ	Vitamin A deprivation and RARβ-/- mutants show spatial learning and memory impairment			[151–153]
Behavioural changes	Organism	Adult	Mouse, rat		Extended low-dose exposure in mice induced depression-like behaviour. This was partially confirmed in 91 days-old rats, but not in older rats.	13cRA	Depression	[177, 187, 232, 234]
			Human	D2, (D1), Ser1A	13cRA use/treatment is associated with depression and suicidal behaviour with longer onset (~4–8 weeks; long-term effect).		Case studies are reviewed in the reference	[177]
Striatum	Tissue/organ	Adult	Mouse, rat, human	D2, RAR(α)**β**/**RXRβγ**, Nurr1/RXR, RALDH1, RALDH3, neurogranin, GAP43	The striatum shows the highest endogenous RA concentrations in the adult brain. Dopaminergic neurons; autocrine action on neuro-transmission, paracrine action on striatal cells; locomotor impairment in RAR/RXR and Nurr1/RXR mutant mice. RXRγ-/- mutants: increased despair behaviour, anhedonia (reversible by re-expression of RXRγ). Induction of Parkinsonism and catatonia by lesions in basal ganglia	RA (endogenous), disulphiram[a]	Depression, (potentially: Parkinson's and Huntington's disease), mood disorders	[177, 226–231, 374, 395, 402–404]
					Extended 13cRA dose (in rat) increases dopamine and serotonin metabolites			[187]

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Table 1 (continued)

Apical Effect/ Key mechanism/ Endpoint	Morphological alteration level	Developmental stage	Test system (species)	Main relevant and related pathways, genes, enzymes	Endpoint/hallmark affected cross-reactions	Substances associated with key mechanism/ adverse outcome	Associated human pathology	Reference(s)
GABAergic (inter-) neurons	Cell/ tissue	Embryo/foetus	Mouse	Raldh3	Enhanced differentiation; migration to olfactory bulb and cortex. Raldh3 activity is required for efficient differentiation of GABAergic interneurons, while Raldh2 is not	RA (endogenous)		[163, 380]
		Adult			GABAergic interneurons of the olfactory bulb are RA sensitive			[405]
Branchial arches	Tissue/organ	Embryo	Mouse	RAR, Hoxa1, Hoxb1, Pax1/9	3rd–6th arch are RA responsive, give rise to: endodermal pouches, thymus, parathyroid glands, aorta and associated large blood vessels, nerves etc. Linked to rhombomeric (hindbrain) origin of mesenchyme/ neural crest cells	RA	DiGeorge syndrome, CATCH22 syndrome	[343, 358, 406–410]
Heart development	Organ	Embryo	Mouse, chicken, zebrafish	RARs, RXRs (esp. RXRα), Hoxb1, Hoxb5b,Tbx1, RALDH2, STRA6, CYP26A1, FGF8, NR2F5	Congenital heart disease, incl. conotruncal and aortic arch artery malformations (patterning defects); defects in RA synthesis can be, in some cases, partly rescued by maternal RA levels/RA supplementation. STRA6 mutations (vitamin A transport/ cellular uptake) may result in developmental defects in atrial and venous vessels. Later in development, RA is cardiotoxic (in zebrafish)	RA	Rarely observed/ undocumented, maybe due to embryonic death. Matthew-Wood syndrome (STRA6 mutation) [411,412], DiGeorge syndrome [413]	[89, 138, 140, 164, 196, 369, 414–425]
			Zebrafish, rat	RARα, SHH, AhR		Flame retardants (miTP, TPP, PBDE), TCDD		[169, 170, 370, 426]
Lung development and regeneration	Tissue/organ	Embryo, adult	Mouse; embryonic explants, rat	RALDH2, Wnt, TGF-β, FGF10, BMP, SHH	Lack of RA/RAR activity prevents induction and growth of primary lung buds; RA induces regeneration of alveoli in rat and rescues lung functionality in experimental hypoplasia. RA is not required for endodermal lung cell fate, but essential for primordial lung bud formation	RA		[141–143, 427–433]
				VEGF, FGF18	RA regulates angiogenesis and elastin production in the maturing lung			[434]

Table 1 (continued)

Apical Effect/Key mechanism/Endpoint	Morphological alteration level	Developmental stage	Test system (species)	Main relevant and related pathways, genes, enzymes	Endpoint/hallmark affected cross-reactions	Substances associated with key mechanism/adverse outcome	Associated human pathology	Reference(s)
Pancreas formation	Tissue/organ	Embryo	Zebrafish, *Xenopus*, mouse	*Cyp26a1*, *Cdx4*, RALDH	Formation of dorsal pancreatic bud (pancreatic and hepatic endoderm); specification of pancreatic endocrine cell lineages. RA is required for ventral pancreas patterning. In mouse and human pancreas, β-cell differentiation may be influenced by RA	RA		[148, 334, 435–442]
Kidney formation	Organ	Embryo	*Xenopus*, zebrafish, mouse	*RARα, RARβ,* RALDH2, Notch signalling, *mecom*	Inactivation of RARα and RARβ results in renal malformation (mouse); ureteric bud cell signalling depends mainly on RALDH2-generated atRA. Specification of renal progenitor cells depends on RA signaling (*Xenopus*, zebrafish)	RA		[149, 443–448]
					Competition for RALDH2	Ethanol		[449, 450]
Limb (and tail) development and regeneration	Organ	Embryo, postnatal	Amphibians, chicken, mouse, zebrafish	FGF8, SHH, FGF4, RALDH2, Cyp26(b1), *Hoxb8*	RA can induce development (embryonic) or regeneration (postnatal) of supernumerary limbs or digits, when locally applied to the limb bud. In mice, RA exposure on gestational day 12 (33–41 somite pairs) affected rather forelimbs, on gestational day 13 (40–51 somite pairs) rather hindlimbs. Also, tail and tail vertebrae development is impaired by RA exposure	RA, 9cRA		[130, 134, 363, 451–459]
Gene expression repression by unliganded receptors (RARs)	Cellular	Embryo	*Xenopus*, mouse	RAR, NCoR-1/2, SMRT, CYP26. (further: *Fgf, Wnt, Hox* genes)	Head development in *Xenopus* and skeletal development in mice requires gene repression by unliganded RAR (otherwise: malformation of the head, anterior/posterior shift)	RA		[460–463]
							Acute promyelocytic leukemia	[462]

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Table 1 (continued)

Apical Effect/Key mechanism/Endpoint	Morphological alteration level	Developmental stage	Test system (species)	Main relevant and related pathways, genes, enzymes	Endpoint/hallmark affected cross-reactions	Substances associated with key mechanism/adverse outcome	Associated human pathology	Reference(s)
Invertebrate development	Organ/organism		Chordata, Arthropoda, Mollusca, Porifera	RAR, RXR, CYP26, RALDH	Conservation of the retinoid signalling pathway and involvement in invertebrate development; incl.: all body, digestive glands, gonads, limb buds, regeneration of body parts	RA, retinol		Reviewed in [322]
			Crustacea, *Drosophila*	EcR, USP	Ecdysone is the driver of invertebrate molting. EcR dimerizes with USP (RXR-homolog), which increases dimer stability and affinity towards target DNA sequences	Ecdysone, tributyltin		[464–467]
	Organ		Mollusca, Gastropoda	RXR	Imposex (gastropods), shell thickening, reproduction perturbation (*Crassostrea gigas*); RAR/RXR heterodimers have a repressive function (instead of activating)	Tributyltin, HX630 (only linked to imposex in gastropods), 9cRA		[111, 468–470]
Reproductive tract development	Organ		Molluscs	RXR; potential crosstalk with PPARγ	Induction of outgrowth of male reproductive structures (*T. clavigera*), regulation of male/female seasonality (reproductive tract recrudescence; *Ilyanassa obsolete*)	9cRA		[111, 144, 322, 469, 471–473]
Reproductive organ development, testes; male fertility	Organ/organism	Embryo, adult	Mouse	RARα, RARγ, RXRβ, RALDH2, **Cyp26b1**, SHH, BMP4, STRA8	Degeneration of testes after knockout of all RARα isoforms; sterility of male mice after knockout of RARγ; male sterility in RXRβ knockout mice. RA is required for spermatogenesis	RA	Sterility in adult men in absence of RA [124, 160]	[13, 76, 145–147, 474–478]
			Mouse, in vitro (P19 and C3H10T1/2), human	RALDH, Cyp26, *Hoxa1*, **HDAC**-I, AR (via SHH)		Phthalate esters (esp. containing aryl and cyclohexane groups), valproic acid		[168, 479–482]
Peripheral nervous system, regeneration	Organ system	Adult	Human	RARα, RARβ, RBP	Regeneration of spinal cord motor neurons depends on RA-induced RARβ(2) expression; RA peaks 4-7 days after encountering the injury	RA, 9cRA		[82, 154–156, 158, 483]
Nervous system	Organ system	Adult	Human	RARα, RALDH2, RBP(1)	Motor neurons: neurofilament accumulation, astrocytosis, decrease in neuron number, elimination of RARα and reduction of RALDH2 expression, reduction in retinol binding protein levels in spinal cord	Vitamin A, RA	Amyotrophic lateral sclerosis (ALS)	[82, 484–486]
		Embryo	Rat		Gestational exposure (day 11–13) led to difficulty to swallow milk (motor control); delayed righting reflex at 35 days. Decreased locomotor activity, motor coordination, and learning (90 days)		Depression, suicidal behaviour [223]	[177, 186, 395, 487–490]

Table 1 (continued)

Apical Effect/ Key mechanism/ Endpoint	Morphological alteration level	Developmental stage	Test system (species)	Main relevant and related pathways, genes, enzymes	Endpoint/hallmark affected cross-reactions	Substances associated with key mechanism/ adverse outcome	Associated human pathology	Reference(s)
Vision		Pregnancy	Human (pregnant women)			Vitamin A	Night blindness; associated with miscarriage	[131]
		Pre-school age children, adult	Human		Retinal is required in the retina	Vitamin A, RA	Blindness; 50% fatal in children within 1 year if untreated; impaired vision	[131, 133, 491]
Keratinization of epithelia	Tissue	Adult, embryo	Human, rat		Mucous epithelia (as with the trachea/respiratory/gastrointestinal tract) become keratinized in absence of RA; RA is required for continuous renewal of skin epithelia. Treatment of cystic and nodular acne with "Accutane" (13cRA)	RA, 13cRA		[124, 139, 160, 221, 492]
			Human (in vitro)	AhR, RA-signaling	TCDD alters matrix protein (esp. collagen) deposition; atRA shows an additive effect. The increased protein deposition is due to promoter activation and increased mRNA stability	TCDD, atRA		[29]
Immune function	Organ system	Adult	Human, mouse	RARα	Immune function severely compromised in absence of RA. RA is required for (CD4+) T cells in the thymus	RA		[124, 126, 157–160, 493–496]

Bold indicates high level of confidence. Gene and protein nomenclature has been adapted to human, though homologous genes/proteins in other species may have been assessed in the original studies

ADH: alcohol dehydrogenase, AR: androgen receptor, BMP: borne morphogenetic protein, 9cRA: 9-*cis* retinoic acid, 13cRA: 13-*cis* retinoic acid, Cdx4: Homeobox protein transcription factor, CHAT: choline acetyl transferase, CRABP: cellular retinoic acid-binding protein, Dhrs3: short-chain dehydrogenase/reductase 3, EcR: ecdysone receptor (homologous to vertebrate farnesoid X receptor, FXR, though endogenous ligand is the ecdysone steroid), FGF: fibroblast growth factor, GR: glucocorticoid receptor, HDAC-I: class I histone deacetylase, *Hox*: Homeobox gene family, HC630: selective RXR agonist, MCT8: monocarboxylate transporter 8, *mecom: mds1/evi1 complex* transcription factor, miTP: monosubstituted isopropylated triaryl phosphate, NCoR-1/2: nuclear receptor corepressor 1/2, NR2F5: nuclear receptor 2F5 (COUP-transcription factor family), PBDE: polybrominated diphenyl ether, RA: retinoic acid, PPARγ: peroxisome proliferator activator receptor γ, RALDH: retinal dehydrogenase, RAR: retinoic acid receptor, RBP: retinol-binding protein, RXR: retinoid X receptor, Ser1A: serotonin receptor 1A, SHH: sonic hedgehog gene family, SMRT: silencing mediator of RAR and thyroid hormone receptor, TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, TH: thyroid hormone, TPP: triphenylphosphate, TR: thyroid hormone receptor, USP: ultraspiracle (nuclear receptor in *Drosophila*, homologous to vertebrate RXR), VAD: vitamin A deficiency

[a] Disulfiram acts a selective antagonist to RALDH1, hence prevents endogenous RA synthesis. Therefore, effects are often reported as RA-dependent rather than disulfiram-sensitive

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cognitive decline and increasing or maintaining quality of life [179].

While risk factors associated with dementia are mostly age, genetic predisposition (family history of dementia), or life-style related (high BMI, non-healthy diet, lack of physical and cognitive exercise) [179, 182], the contribution of environmental exposure has also been considered [183–185].

Most observations regarding retinoid signalling and adverse health outcomes in adolescents and adults have been derived from human intervention and clinical studies together with animal in vivo modelling. Of particular relevance are studies with pharmacological application of 13cRA for the treatment of acne or cancer [177, 186, 187].

Retinoic acid signalling is necessary for the differentiation and speciation of cell types, particularly in neurons [82]. While the differentiation of neurons is often perceived as restricted to early developmental stages, in fact many postnatal processes, including memory and learning, are dependent on neural differentiation and speciation (also known as neural plasticity) throughout life [174, 188–190].

Cognitive function, memory, learning, and dementia

Postnatal disturbance of RAR/RXR signalling impairs cognitive functions, especially in the forebrain and hippocampus [191]. Indeed the hippocampus region retains high postnatal RAR expression, and so is most susceptible to RA signalling interference [192–194]. One potential role of RA signalling in the adult brain is the modulation of synaptic plasticity, that is required for learning and the formation of memories [173, 174, 188]. In a mouse model, functional expression of retinoid receptors has been shown to be critical for long-term potentiation (RARβ) and long-term depression (RARβ and RXRγ) [151]. The same study linked the decreased synaptic plasticity with a substantial performance loss in spatial learning and memory tasks in mice. While Chiang et al. [151] did not study the role of RARα, Aoto et al. [195] observed a rapid increase in synaptic strength upon treatment of primary rat neurons in vitro with 1 μM atRA or increased endogenous atRA synthesis due to decreased neuronal activity. The homeostatic modulation of synaptic strength was mediated by atRA via dendritic, i.e. non-nuclear, RARα and upregulated postsynaptic glutamate receptor 1 (GluR1) expression in a transcription-independent fashion [195]. Membrane-bound RARα was also shown to be involved in the differentiation of spine neurons from the hippocampus [196], suggesting a non-transcriptional role of RARs and perhaps of RA as a paracrine signalling molecule.

Interestingly, the involvement of RA signalling in learning processes in vertebrates is not limited to mammals and may even play a role in invertebrate learning [197]. It has been shown that RA is critical for learning and song maturation in songbirds (zebra finches) [198, 199]. Unlike vocalization in mice, song maturation involves a learning aspect similar to that in human speech [200]. Convergent signalling mechanisms of forkhead box protein P2 and RA have been hypothesized to play a role in learned vocalization in both, birds and humans [200].

Involvement of RA signalling in these key cognitive functions raises the questions of its role in neurodegenerative diseases. Age-related cognitive decline with impaired spatial learning and memory is associated with decreased RA signalling in elderly vertebrates (human and mice) [201, 202]. In elderly mice with impaired cognitive function, the administration of 13cRA re-established RA signalling and hippocampal RARβ and RXRβ/γ expression and rescued the cognitive impairment [153, 201, 203].

Therapeutic applications

As a specific form of dementia, Alzheimer's disease (AD) is characterized by formation of amyloid-β plaques in the CNS, which leads to inflammation and subsequent neurodegeneration [179, 204]. Due to a reported decrease in RA signalling in AD patients' brains, particularly in the hippocampus region responsible for the formation of memories [201, 202], retinoids, especially 13cRA, are proposed for AD treatment as neuroprotectants [123, 205–209]. Furthermore, specific synthetic agonists of RARα/β, such as tamibarotene, that are in use for cancer treatment, have been explored for AD treatment [209], but the clinical trials seem not to have progressed since [210]. For AD, disruption of RA signalling was linked to increased amyloid-β deposition in rats [211], and RAR-agonism was effective to act both preventively and therapeutically to decrease amyloid-β-induced damage to human cell cultures in vitro and to mice in vivo [212–218]. Besides RARs, also RXRs are being explored for their pharmacological potential in neurodegenerative and inflammatory disease treatment, though the results are not conclusive so far [123, 219].

In contrast to the recovery of learning and memory abilities in elderly mice, longer-term (6 week) administration of 13cRA at a therapeutic dose (1 mg/kg/day, i.p.) during young adulthood decreased cell proliferation in the murine hippocampus and was associated with impaired learning and memory formation [192]. The authors consider this result to be due to an insufficient growth factor supply to maintain a large differentiating neuron population, leading to premature neuronal death and longer-term decreased performance in RA-sensitive

tasks. However, the impaired cognitive functions could also be linked to affective depression disorders, as reported in humans after extended periods of RA treatment [177].

Affective disorders - altered mood, depression, and suicide
RA was first linked to altered behaviour in rats in 1986 [220]. Later a link between affective disorders and RA in humans was proposed after 13cRA was approved for medical use as a treatment of severe cystic and recalcitrant acne in 1982, leading to the inclusion of a warning on the label [221, 222]. A systematic review conducted by Marqueling and Zane [223] and an almost parallel review of studies by Strahan and Raimer [224] concluded that the current data available neither confirm nor disprove the association. The latter however noted that changes in mood can be accounted to 13cRA [224]. Further case studies and reports on the involvement of retinoid exposure in affective disorders have been summarized by Bremner et al. [225].

Here, we briefly introduce mechanistic data generated from animal models, with respect to the involvement of RA signalling in the dopaminergic system, as this is of particular interest with respect to the development and manifestation of affective disorders, as well as schizophrenia.

In the late 1990s, mutation and knockout of RARβ, RXRβ, and RXRγ in mice were observed to be linked to impaired locomotion and decreased signalling via dopamine receptors 1 and 2 (D1R and D2R) [226, 227]. Also, the involvement of the orphan nuclear retinoid receptor 1 (Nurr1) in the differentiation and/or maturation of dopaminergic neurons was hypothesized [226, 228]. Consecutively, the involvement of RA signalling in the development of the dopaminergic system, particularly in the expression of D2R, was confirmed in mice [229] and rats [227, 230, 231]. Of interest are also studies of chronic 13cRA administration in mice that better reflect an extended exposure to retinoids. Administration of therapeutic doses (1 mg/kg/day) to young adult mice over 6 weeks did not alter general locomotor activity, but increased depression-like behaviour in the forced swim test and tail suspension test [232]. A follow-up literature review to this study proposed alterations to the serotonin neurotransmitter system rather than dopamine [233]. This is in line with an extended 13cRA exposure study in rats, which affected the serotonin rather than the dopamine neurotransmitter system [187]. Interestingly, a parallel study with chronic exposure to 13cRA or atRA in rats did not confirm the observed behavioural despair (forced swim test) observed by O'Reilly

et al. [232], indicating species differences in sensitivity to RA [234].

Another parallel between depression in (elderly) human and in mice was drawn rather recently, when Qi et al. [235] observed *post-mortem* a decrease in mRNA levels of brain-derived neurotropic factor (BDNF) and RA signalling pathway elements in the brain of depressed patients and were able to confirm this observation in mice (BDNF is a biomarker also considered for inclusion in AOPs for developmental neurotoxicity and learning impairment [236–238]). Additionally, they identified a RA-responsive element in the tropomyosin receptor kinase B (TrkB; receptor for BDNF) promoter region specifically targeted by RARα and thereby confirmed crosstalk between the RA and BDNF signalling pathways [235].

Schizophrenia
While the multifactorial aetiology of schizophrenia includes genetic and environmental risk factors, critical areas are early neurodevelopment, social behaviour and cognitive ability [239, 240]. In fact, genetic predisposition by itself is not necessary nor sufficient for the development of schizophrenia and the developmental cascade leading to the disease should include interactions with the environment [241]. Whilst no discrete substance has been proven to cause schizophrenia, it has been hypothesized that the neurochemical processes affected by some recreational drugs play a role in the development of schizophrenia and psychoses [summarized in 241]. These processes are signal transduction via dopaminergic [242] and glutamergic synapses [243], the endocannabinoid system [244], and (neuro-)inflammation [245].

Twenty years ago retinoid signalling was postulated to be involved in the development of schizophrenia [246], and whilst further mechanistic evidence is present, it is not sufficient for confirmation. The link has been established based on a predisposition for schizophrenia in children with congenital anomalies similar to RA signalling disturbance, convergent gene loci of schizophrenia risk factors and the RA signalling cascade (esp. CYP26B1) [240, 247], and the already mentioned sensitivity of the dopamine neurotransmitter system, particularly D2R, to RA interference (see "Affective disorders - altered mood, depression, and suicide" section) [outlined in 246]. Besides the dopamine system, also γ-amino butyric acid (GABA)-ergic interneurons in the prefrontal cortex, whose aberrant development is associated with neurological disorders including schizophrenia, have been shown to be sensitive to RA [248].

Although a considerable number of studies address the biologically plausible link between retinoid signalling and

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neurological diseases, on balance, the evidence supporting the link is currently insufficient to attribute causation (see Table 2).

Whilst the causal link between aberrant retinoid signalling and neurological disease is currently weak, the biological plausibility of the association is high: RA is a morphogen during early development and is strongly involved in shaping the CNS, including differentiation and maturation of neurons. Despite the significant role during development, the role of RA signalling in the adult or postnatal brain is less clear. Still, the conserved mechanisms of RA signalling are most likely to act also in the adult brain, though the effects may be less evident due to the multitude of parallel processes and potential influencing factors. Also, it is difficult to simulate and assess the many hues of neurological disease in animal models that are distinctly different from humans and it is not possible to assess behavioural changes in in vitro systems.

The "Cognitive function, memory, learning, and dementia"–"Schizophrenia" sections introduced several neurological conditions that share common affected personality traits due to changed connections in the CNS and altered neural plasticity. While modified RA signalling is not the single cause of adverse psychological and neurological outcomes, it is a strong candidate for connecting environmental exposure to neurological and/or neurodegenerative disease by modulating neurotransmitter systems (i.e. the dopaminergic system) and altering the base-line population of (non-)differentiated cells in the CNS. The role of the retinoid signalling pathways is especially pronounced, because interference of environmental chemicals does not have to be mediated via the molecular initiating events of the nuclear receptors (RARs and RXRs) directly, but could interfere with the endogenous retinoid homeostasis, e.g. by altering RA degradation (via CYP26 enzymes) or its biosynthesis (via ALDH and/or RALDH).

Sources of retinoids in surface water - exposure

Whilst retinoids are an intrinsic part of the diet for terrestrial animals and humans, aquatic animals in particular may be susceptible to involuntary exposure to excess retinoids at critically sensitive early-life stages [79] due to the prevalence of retinoid sources, both natural - cyanobacteria (blue-green algae) in eutrophic (fresh)water ecosystems [249–252] - and/or anthropogenic - wastewater discharge [253].

Retinoid-like activities in environmental matrices mediated via RAR or RXR can be measured by in vitro receptor transactivation assays, similar to ER and AR (see "Infobox"). In fact, ligand binding to specific nuclear receptors that leads to the transcription of target genes which, in the case of reporter assays, govern the expression of an easily detectable (e.g. luciferase) product, has become the method-of-choice for recent screening programmes targeted at uncovering endocrine activities of chemicals in a high-throughput manner [254–256]. The signal reflecting the extent of receptor transactivation can be quantified relative to the reference ligand (atRA for RAR, 9cRA for RXR) [257–259]. Detected retinoid-like activity for the different types of samples is expressed as equivalent concentration of the reference ligand that would cause the same response. These retinoic acid equivalent concentrations integrate the potential of a given mixture to activate the transcriptional response of the receptor and are more informative than targeted analyses for a limited set of compounds.

Cyanobacteria

An important source of retinoids to surface waters is cyanobacterial blooms in eutrophic freshwater ecosystems. Anthropogenic eutrophication of water bodies is driven by agricultural activities and insufficient removal of nutrients (mainly nitrogen and phosphorous) from communal wastewaters [260, 261]. The ability of cyanobacteria to fix dissolved carbon dioxide (HCO_3^-) by photosynthesis makes their occurrence independent of bioavailable carbon [262]. Together with global climate change, these are the biggest factors enhancing cyanobacterial blooms in (fresh-)water environments [260, 263]. As a result, the main limiting nutrients are bioavailable inorganic nitrogen (nitrate, NO_3^-) and phosphorous (phosphate, PO_4^{3-}) [261]. These nutrients are further concentrated in long, dry warm periods in summer, that are increasing with, and exacerbated by, global climate change. Evaporation and increased abstraction from surface water bodies leads to increasing water temperatures especially in shallow surface waters, further fuelling the development of cyanobacterial blooms [264–266]. Greater abstraction will also be expected with the growth in human population. In addition, climate change increases the frequency and size of flooding events which, in turn, (a) increase sediment loss to surface water (which is a key mechanism via which phosphorus enters water [267]) and (b) promote resuspension of nutrient-laden benthic sediment, both of which further exacerbate cyanobacterial blooms [268]. Besides being an integral part of the aquatic ecosystem, cyanobacteria produce a large variety of secondary metabolites, many of which show bioactive or even toxic properties [269, reviewed in 270]. Amongst others, cyanobacterial bloom biomass and affected waters were shown to contain retinoids, elicit retinoid-like activity in vitro, and to cause in vivo

teratogenic effects in *Xenopus laevis* tadpoles and *Danio rerio* embryos, which implies relevance towards wildlife populations [79, 249, 251, 252, 271–275]. Although algae contain retinoids at comparable levels in their biomass to cyanobacteria [251, 271, 274], it is the latter that are major contributors to retinoids in surface waters due to their proliferation. While the occurrence of cyanobacteria themselves is natural, their hazardous massive blooming events are strongly driven by human actions making it an "anthropo-natural" phenomenon.

European and Asian environmental case examples

The chemical assessment of environmentally occurring retinoids or a quantification of retinoid-like activity is a monitoring data gap. However, the few studies systematically analysing water samples reveal highly concerning levels of retinoids or their activity.

Measured in Czech lake waters, retinoid-like activities reached up to 263 ng atRA equivalent (REQ) × L^{-1} [249]. While this concentration does not exceed the nominal EC20 of atRA in zebrafish embryos, total bloom biomass extracts did cause teratogenic effects at these concentrations [272]. This indicates that environmental retinoids extend beyond atRA and 9cRA. Indeed, a broad spectrum of retinoids has been detected in field samples of cyanobacterial blooms and their surrounding water, as well as in laboratory cultures and their exudates [84, 249, 251–253, 271, 275, 276]. Among the retinoids detected are retinoic acids (atRA, 9cRA, 11cRA, 13cRA), RA derivatives (5,6-epoxy atRA, 7-hydroxy atRA, 4-oxo atRA, 4-oxo 9cRA, 4-oxo 13cRA), retinal and its derivatives (all-*trans* retinal, all-*trans* 4-oxo retinal) [249, 251–253, 271, 272, 276]. However, the chemical analysis of retinoids could not entirely explain the retinoid-like bioactivity observed in the biological assays, hence it underestimates the endocrine active potential arising from these waters.

Besides occurring in cyanobacterial blooms, retinoids also enter the environment via wastewater effluents [reviewed in 253]. Humans, as well as animals, excrete retinoids most often as 4-oxo derivatives [277–279]. Even though retinoids are sensitive to oxidation and isomerization processes that significantly alter their bioactivity, in municipal wastewater treatment plants the treatment efficiency may not be sufficient for their complete removal and, consequently, retinoids can be released to the receiving water bodies at concentrations of up to 11.5 ng REQ × L^{-1} [253, 271, 280–283]. Amongst the detected isomers, oxidized (i.e. 4-oxo-) derivatives of retinoic acids dominate over the parent compounds. Besides dietary excreted retinoids, pharmaceutical retinoids (tretinoin (atRA), alitretinoin (9cRA), isotretinoin

(13cRA), bexarotene, and others; pharmaceutical use: cancer, acne (≥ 0.5 mg isotretinoin/kg/days), eczema treatment [222, 284, 285]) and cosmetically used retinoids (retinol, retinyl palmitate, retinyl acetate; cosmetic use: body lotion ($\leq 0.05\%$ retinol equivalent), hand/face cream or rinse-off products ($\leq 0.3\%$ retinol equivalent) [279, 286]) can be excreted into wastewaters or washed-off after topical application. The use of retinoic acid in cosmetics is restricted within the EU and Norway [70, 286, 287], recommendations are provided by the European Medical Agency, with respect to oral use of the retinoid containing medicinal products in pregnancy, and also for those suffering from neuropsychiatric disorders [222]. The potential contribution routes to retinoid compounds in surface waters are also summarized in Fig. 3.

Anthropogenic and natural endocrine-active substances

The presence of unregulated endocrine-active substances (EAS), like retinoids, in waters raises the questions: to what extent do they contribute to mixture effects? Do we need to update the assessment of water safety for human consumption? To date, EAS in the environment are managed by regulating their commercial release: the manufacturer is responsible for correct labelling of the product and has to assure an acceptable risk of the active ingredient, or the preparation, to the environment. While this management scheme covers anthropogenic releases of chemicals, it cannot capture natural sources that contribute to the cumulative effects observed in the environment in situ. Hence, it does not reflect the need to tackle mixture effects in the environment directly or just before release of complex mixtures like waste water effluents into the environment [288–291].

The European Union's visionary concept of water legislation, encompassed in the Water Framework Directive (WFD, [291]), the Urban Waste Water Treatment Directive [288, 292], the Nitrates Directive [293], and the Drinking Water Directive [DWD, 294], aims to secure safe drinking water now and for future generations. However, the demand for water, for nutritional, recreational and agricultural purposes, increasingly challenges water supply managers and may require switching to less-favourable water sources to provide the consumers with the desired supply [295, 296].

To meet the high expectations for surface and drinking water safety, a holistic assessment at the point of abstraction for drinking water, including sub-acute and/or longer-term effects of EAS and putative endocrine disruptors, rather than managing only anthropogenic inputs of active substances (e.g. pesticides, fertilizers, pharmaceuticals) to the environment would be beneficial for risk assessment and more reflective of the true burden of EAS exposure via drinking water [44, 290, 297, 298].

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Table 2 Summary weight-of-evidence matrix for retinoid signalling perturbation by environmental contaminants

Associated condition	Line of evidence, uncertainties, and limitations	Biological relevance (B), strength (S) of the study, and correlation of data (Corr.)	Reliability of the source and "test system" with respect to human health	Conclusion: incl. identification of data gaps
Developmental effects (teratogenicity, malformations)	Various (see Table 1); strongest associations are supported by in vivo loss-of-function and developmental toxicity/teratogenicity studies in, amongst others, mammals, fish and amphibians (also recently reviewed in [103]	B: ++, S: ++	Very high	
Affective disorders	RAS contributes to the development of: mood changes, aggressive behaviour, depression, and/or suicidal thoughts [177, 222, 223, 232, 234]	B: +, S: +	Very high (confirmed independently multiple times in human and rodent studies)	
	Mood changes, aggressive behaviour, depression, and/or suicidal thoughts [222] (Basis for decision to include it on the label is not provided)	B: -, S: ++, Corr.: ?/↑	Very high (official note for isotretinoin treatment); human	Though a comment on the association of 13cRA with affective disorders is included on medication labelling, the causal link is not definitively established yet
	Depression and suicidal behaviour [223] Systematic review; many of the sources are reported to be of limited use to the review, esp. with respect to suicidal thoughts	B: +, S: +, Corr.: 0/↓	High (systematic review); human	A trend, but no statistically significant correlation between depression/suicidal thoughts and 13cRA treatment, but epidemiologic data are insufficient to draw associations esp. with respect to suicidal thoughts
	Depression [187, 234] Very thorough and broad studies, covering longer-term (28 days) and chronic (> 100 days) exposure in adult/elderly rats; doses of 13cRA and atRA reflect serum levels in human during 13cRA treatment. No histopathological examination was conducted	B: +, S: ++, Corr.: 0	Moderate (original research); rat	The data do not substantiate the hypothesis of 13cRA inducing depression. No effects were observed on behaviour, nor on monoamine levels in the brain. Observations differ from other studies perhaps due to interspecies variation
Autism, schizophrenia, ADHD, depression	RA modulates the correct distribution of GABAergic interneurons in the prefrontal cortex via corticothalamic interaction [248] Focus on CYP26B1 (co-)detection with parvalbumin-positive GABAergic interneurons in the prefrontal cortex in mice. The study concludes on the role of thalamic signalling (controlling CYP26B1 expression) during interneuron maturation, not on RA signalling itself	B: 0, S: +, Corr.: ↑	Weak/moderate (original research); mouse	The study satisfactory links altered corticothalamic influence on CYP26B1 expression in the prefrontal cortex, together with subsequently altered RA signalling, however, the study only indirectly assessed RA signalling

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Table 2 (continued)

Associated condition	Line of evidence, uncertainties, and limitations	Biological relevance (B), strength (S) of the study, and correlation of data (Corr.)	Reliability of the source and "test system" with respect to human health	Conclusion: incl. identification of data gaps
Schizophrenia	CYP26B1 mutation in humans is a risk factor for schizophrenia [240, 247] Genome-wide association study (GWAS) including 36,989 cases of schizophrenia and 113,075 controls, and a multi-laboratory combined cohort of 153 patients vs. 153 controls examined for gene expression changes. While both studies identified CYP26B1 as a potential schizophrenia risk factor, other enzymes in the RAS cascade/pathway (e.g. RALDH, RARs, RXRs) were not identified	B: +, S: ++, Corr: 0/↑	High (genome-wide association study, large cohort); human	Identification of 108 conservatively defined loci of genome-wide significance towards schizophrenia, including CYP26B1 (rank 74). While GWAS is a powerful tool to uncover rare mutations and genetic risk factors, the causal link needs to be confirmed in mechanistic studies
	RAS is disrupted in schizophrenia [497] GWAS on Australian Schizophrenia Research Bank cohort (425 schizophrenia cases and 251 controls included in study). The polygenic risk score of 22 retinoid genes was significantly associated with the disorder. In addition, a rare variation in the gene encoding RARβ was associated with severe cognitive deficits	B: +, S: ++, Corr: ↑	High (genome-wide association study, large cohort); human	The study strengthens the link between neurological disease, particularly schizophrenia with cognitive deficits, and disturbed (decreased) retinoid signalling This study paves the way for preventive or therapeutic use of retinoids in schizophrenia, though responsiveness of cognitive symptoms needs to be investigated individually first
Learning disabilities	Prolonged post-natal vitamin A deficiency decreases RAS and RAR expression in the brain [153] Vitamin A deficiency simulated age-related decline of RAS in mice (C57BL6 Jico). Unlike in age-related decline in function, RA treatment (150 μg/kg) could not rescue the effects to control levels The results need to be taken with caution; the trends observed in treated and control mice are very similar	B: +, S: 0, Corr: ↑	Moderate (original research); mouse	Vitamin A deprivation during adolescence is associated with a sustained decrease in learning/memory function and, which cannot be rescued by RA treatment These results need further investigation and independent reproduction

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Table 2 (continued)

Associated condition	Line of evidence, uncertainties, and limitations	Biological relevance (B), strength (S) of the study, and correlation of data (Corr.)	Reliability of the source and "test system" with respect to human health	Conclusion: incl. identification of data gaps
	Decreased retinoid signaling impairs locomotion [226] Open field behaviour of morphologically and histologically normal mutant mice (RXRβ–RXRγ double null or single mutant and WT) was observed. Double null mutants showed impaired locomotion. Additionally, double mutants (but not single-mutants) showed a decreased striatal expression of dopamine receptors, which are involved in voluntary movement Though the authors are addressing mechanistic endpoints (retinoid and dopamine receptors), the mechanistic investigation is insufficient	B: +, S: +, Corr.: ↑	Moderate/high (original research); mouse	This study is amongst the first to link retinoid signalling and cognitive function mechanistically. Follow-up studies based on these initial findings have been conducted, though the link is still not understood entirely
	RARα modulates synaptic plasticity and affects hippocampal learning [174] In mice (homozygous RARα floxed mice, C57BL/6 background. 10 days environmental enhancement in young adults before assessment at PND 60-70), deletion of RARα in hippocampal circuits blocks homeostatic synaptic plasticity and enhances long-term potentiation via/together with mTOR. The Morris water maze was used to assess memory/learning capacity (RARα KO mice performed better than WT) and for reversal learning/ memory modification testing (RARα KO mice performed worse than WT) The results are the first to look into stable (i.e. not chronically suppressed) synaptic plasticity, which makes comparisons to other studies difficult	B: ++, S: +, Corr.: ↑	High (original research); mouse	Hippocampal RARα deletion enhances spatial learning but decreases learning flexibility. In this study, RARα alters AMPA receptor expression indirectly by an intermediate pathway rather than having direct transcriptional/ translations effects The conflicting observation between initial learning and memory modification, and its implications for cognitive performance has to be investigated further This publication may contribute to teasing out the reasons for apparently conflicting findings regarding retinoid signalling in cognitive functions

Table 2 (continued)

Associated condition	Line of evidence, uncertainties, and limitations	Biological relevance (B), strength (S) of the study, and correlation of data (Corr.)	Reliability of the source and "test system" with respect to human health	Conclusion: incl. identification of data gaps
Dementia/neurodegenerative diseases	Modulation of RAS contributes to Parkinson's disease (PD) by modulating the dopaminergic system. [498] No statistically significant correlation between vitamin A/carotene blood levels and PD was identified in the pooled dataset; significant associations detected only in case-studies. Out of 362 potentially relevant papers, the sample size included in the meta-analysis was low ($n = 8$ studies)	B: ++, S: -, Corr.: 0	Very high (23-y. epidemiology-based meta-analysis)	Epidemiological data on the association of PD with vitamin A/carotenoid blood levels are insufficient to draw conclusions; recommended reporting schemes for epidemiological data need to be followed
	Age-related memory deficit in mice (C57BL6 Jico) due to decreased RAS associated with decreased RAR expression [153, 203] Observations are clearly linked to RAS and RAR activity due to the experimental design	B: +, S: ++, Corr.: ↑	Moderate (original research); mouse	Age-related, but not postnatal vitamin A deficiency-induced, decline in RAS and learning/memory could be restored to normal adult levels by subcutaneous injection of 150 μg RA/kg bw. These results need independent reproduction

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, KO: knock out, mTORC1: mammalian target of rapamycin, PD: Parkinson's disease, PND: post-natal day, RA: retinoic acid, RAR: retinoic acid receptor, RAS: Retinoic acid signalling/retinoid signalling, RXR: retinoid X receptor, WT: wild type. +: relevant/strong/reliable line of evidence, 0 neutral, - not relevant/strong/reliable line of evidence. "Biological relevance" is interpreted as: "the working hypothesis to be tested is scientifically sound", "strength" is interpreted as: "the tools to investigate the hypothesis are fit-for purpose". Arrows indicate if the results of the study support the hypothesis (positive correlation, ↑), contradict the hypothesis (negative correlation, ↓), are neutral (no correlation/effect, 0), a trend was observed but no statistical significance (0/↓; 0/↑), or are still under development (?)

The impact of substances such as pesticides, fertilizers, and pharmaceuticals upon the growth of cyanobacterial blooms causing increased "anthropo-natural" retinoid production is also a critical factor to include in the hazard and risk assessment process.

Discussion - implications for risk assessment

We have presented evidence here indicating that altered endogenous retinoid signalling is plausibly implicated in a variety of major public health areas of concern. In particular these include brain and neuro-degenerative conditions such as Parkinson's disease, dementia, schizophrenia, and depression (see sections "Contribution of retinoids to chronic neurological disorders"–"Schizophrenia", Tables 1 and 2), as well as developmental effects. However, the available evidence causally linking these diseases with aberrant retinoid signalling is currently weak - particularly with respect to environmental exposure.

Retinoids in the form of vitamin A and its precursors are essential nutritional requirements, and it is well established that too little or too much can lead to adverse health outcomes. Whilst vitamin A dietary reference values are clearly specified [70, 72, 97], these are advisory and on the whole are not controlled by regulatory bodies, with the exception of fortified functional/novel foods, where fortification can impact upon vulnerable populations, as for example, with infant formula [71]. Retinoid-based orally administered pharmaceuticals can also contribute to the daily exposure, while not being accounted for by nutritional reference values [222]. Critical limitations in attributing altered retinoid signalling to environmental exposure are the lack of monitoring of retinoid compounds and virtual absence of effect-based screenings for retinoid-like activity, even though monitoring reports from the Czech Republic and Asia indicate significant (anthropo-)natural sources of retinoids [249–252, 271]. This data gap is aggravated by the retinoid signalling pathway not (yet) being included as a contributory pathway that can be adequately assessed by standardized test methods, as part of endocrine disruption hazard assessment and by the unknown hazards related to environmental mixtures of anthropogenic contaminants and (anthropo-)natural compounds adversely interfering with this pathway. Retinoid signalling is directly involved (via RAR) in a multitude of developmental, neurological and repair processes as well as indirectly via the universal heterodimerization partner RXR. This contributes to the non-linear intercommunication web of cause–effect relationships that are observed upon disturbed retinoid signalling and additionally allows pleiotropic effects through crosstalk with other pathways such as TR [114], or PPARβ/δ signalling [113] and steroidogenesis [7]. In addition, the substantial evidence for the teratogenicity of retinoids is usually addressed under developmental toxicity hazard assessment, rather than endocrine disruption per se (see Table 1), whilst the proposed adverse outcomes in relation to spermatogenesis and male reproduction [summarized in 13], fall under reproductive toxicity, and the biologically plausible hypothesis of involvement in the development and manifestation of neurological disease, under (developmental) neurotoxicity (see Table 2). Furthermore, the elucidation of the link between neurological disease and altered RA signalling also requires more basic investigation into the role of retinoid signalling in the brain (using experimental models and clinical investigations) together with population-based studies - as has been done, e.g. for polychlorinated biphenyls [299, 300]. This would need a characterization of the exposure to compounds with potential retinoid signalling disruptive effects - as depicted in Table 2.

In addition to hazard assessment, the characterization and quantification of environmental levels of retinoid compounds and retinoid-like activity is key for the exposure evidence base needed to assess whether they are likely to pose a risk to the environment and human health. This needs to include a consideration of exceedance of vitamin A nutritional requirements and dietary sources of exposure. (Anthropo-)natural sources of EAS such as cyanobacterial blooms often exhibit pronounced cyclic recurrent (i.e. seasonal, non-continuous) patterns [270, 301]. Consequently, derived exposure limits should consider intermediate longer-term values in addition to lifetime-daily exposure, such as seasonal, monthly, or weekly exposure. This has been recently proposed and conducted by the WHO for a few selected cyanobacterial toxins [270]. In surface waters, wastewater treatment effluents are a constant contributor of anthropogenic bioactive compounds including retinoids [253]. To address transformation of pharmaceutically and cosmetically used retinoids, it is suggested that work being initiated at the OECD to develop a test guideline on anaerobic transformation of veterinary pharmaceuticals, organic compounds and biocides in liquid farmyard manure [302] could include retinoid-like monitoring endpoints. This would contribute to filling the assessment data gap of retinoid-like activity originating from farmyard manure and its fate in the environment. This could also be a tool of potential value in addressing subsequent monitoring of retinoid removal in urban wastewater effluents.

Besides recognizing the occurrence of endocrine disruptors in the environment, it is also critical to develop more accurate tools to assess their potential impact and hence any associated risk. In the case of interference with retinoid signalling, this means mainly to direct research efforts into the augmentation of already existing test

Fig. 3 Exposure scenario to environmentally occurring retinoids, including challenges for water catchment management. Retinoid-like activity in the environment is not only associated with anthropogenic contaminants, but also from human-driven naturally occurring ("anthropo-natural") cyanobacterial blooms. N & P: nitrogen and phosphorous
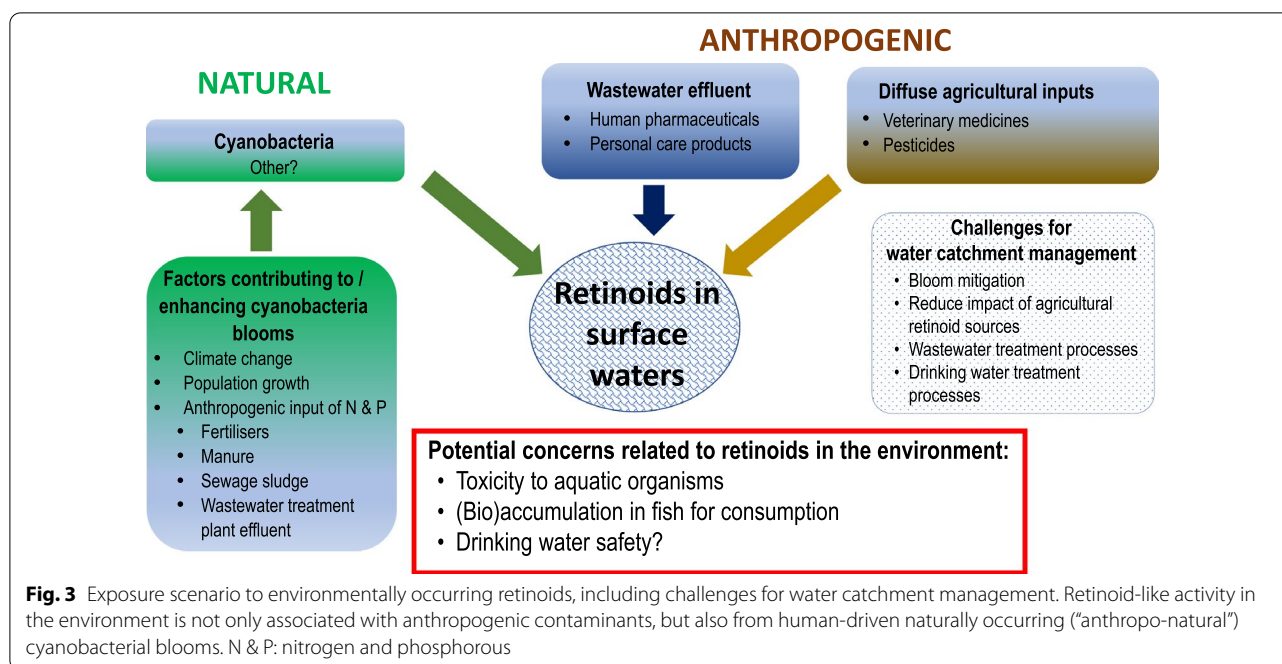
guidelines and the validation of (non-animal alternative) methods for regulatory testing of retinoid signalling pathway disruption, which is already initiated at international intergovernmental levels (see "Introduction" section).

In addition to distinct test methods, AOPs are being developed with the intention of regulatory applications, to convey biologically plausible hierarchical structures of causes, effects, and outcomes from basic research to regulatory actions. It is important to refine and strengthen AOPs under development for retinoid signalling disturbance [13, 303]. Besides only linking the sequential "event-train", recent efforts to define tipping points for transition between key events could make AOPs become quantitative, thus more useful for computational predictive approaches [21, 303, 304]. An analogous approach to AOPs has also been taken in exposure science with aggregate exposure pathways (AEPs). They aim to summarize exposure from different sources, and integrate target site exposure, e.g. at a receptor in the tissue [305]. AEPs take into account potential environmental or metabolic transformation of a substance or cumulative effects of structurally similar substances in mixtures and are inclusive to substances of natural origin that may contribute to target site effects [305, 306]. The integration of exposure and effect assessment is also called for by European partnerships to achieve the ambitious goals laid out in the WFD [307].

Substances of emerging concern often show endocrine activity and/or are candidate endocrine disruptors [308, 309]. Although not intended, these substances often find their way into the environment, and, most importantly their environmental occurrence is augmented by human actions [309]. For the sustainable development of society, we need to recognize our environmental impact and try to retain or re-establish the delicate balance of maintaining and protecting landscapes and ecosystems (see Fig. 3). Only then will we be able to achieve the visionary milestones identified and articulated, e.g. by the United Nations Organization as the "Sustainable Development Goals" [310, Goal 6: "Clean water and sanitation"], by the European Commission in water-related directives (e.g. WFD [291], DWD [294]) and, most recently, by the European Green Deal, which aims at ensuring a "toxic-free environment", including a zero pollution approach and the development of an action plan regarding endocrine disruptors in the environment and circular economies [311]. With respect to mixtures in the environment, it was recently proposed to combine all EU chemical-related legislation, independent of the use scenario, in order to allow an inclusive mixture impact assessment [312]. A further proposal is to formulate "human health protection goals", similar to the protection goals defined in the WFD for aquatic environments, with respect to involuntary and cumulative exposure to chemicals [312].

Conclusion

Here, we have presented the (anthropo-)natural occurrence of retinoids in freshwater environments as a case study example to highlight the importance of regulatory recognition of non-EATS endocrine disruption pathways,

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specifically the retinoid signalling pathway. Elaborating on diffuse and especially (anthropo-)natural sources of these teratogenic EAS, we highlight the necessity of including exposure to mixtures from different environmental media and evaluating environmental and human health impacts of compounds, irrespective of and independent to their initial use, e.g. biocide/plant protection product; environmental matrices like water or soil are indifferent to the use-case of a product.

The (anthropo-)natural occurrence and production of retinoids in water bodies in addition to anthropogenic sources suggests a human health hazard. However, due to insufficient data on environmental levels of retinoids, especially spatio-temporal screening data, an adequate risk assessment cannot be conducted to date. Future monitoring studies need to take into account both point sources such as wastewater treatment plants and diffuse (anthropo-)natural sources of EAS that include retinoids.

The retinoid signalling pathway is conserved at least across vertebrates and plays a pivotal role during prenatal development, such that its disturbance can cause teratogenic effects that range from mild malformations to lethality. Phenotypically similar developmental defects were observed in aquatic vertebrates exposed to environmental cyanobacterial bloom extracts with retinoid-like activity.

Postnatal roles of retinoids include epithelial integrity and spermatogenesis, and retinoid signalling disruption may play a role in the epidemic of neurological and neurodegenerative disease. A preliminary weight-of-evidence matrix for the association of disturbed retinoid signalling with neurological disease was presented to flag uncertainties in the experimental design or the biological link, however despite biological plausibility, the weight of evidence to date is insufficient to support the causality of retinoid signalling disturbance in neurological diseases.

Also, agonistic/antagonistic and additive actions that are not covered by the current assessment methods may occur due to the high degree of molecular crosstalk between different endocrine signalling pathways, as depicted for example for RXR.

To strengthen the retinoid relevant AOPs for regulatory applications, future toxicological studies need to further address and elucidate the toxicological tipping points from one key event to the next. Understanding the adaptive stress response in a concentration and time-dependent manner is crucial to derive not only acute and chronic (i.e. life-time daily) exposure limits, but also more realistic prolonged-short time exposure limits that, for example, reflect seasonal variations in exposure scenarios as recently conducted by WHO [270], although retinoids are not currently included in this proposed approach. It may also lead to a better understanding of life-stage and gender differences in toxic effects. The development of high-throughput methods and an increasing number of validated non-animal methods will enable more rapid and efficient understanding of these differences that could ultimately contribute to safer waters in the future - for humans and ecosystems.

Abbreviations
9cRA: 9-*cis* Retinoic acid, alitretinoin; 13cRA: 13-*cis* Retinoic acid, isotretinoin; AD: Alzheimer's disease; AEP: Aggregate exposure pathway; AOP: Adverse outcome pathway; AR: Androgen receptor; atRA: All-*trans* retinoic acid, tretinoin; BDNF: Brain-derived neurotropic factor; CAR: Constitutive androstane receptor; CNS: Central nervous system; CYP26: Cytochrome P450 monooxygenase subfamily 26 (detoxifying enzyme); D1R, D2R: Dopamine receptors 1 and 2; DWD: Drinking Water Directive; EAS: Endocrine-active substances; EATS: Sex hormone (oestrogen, androgen) receptors, steroidogenesis, and thyroid hormone signalling; EC: European Commission; EC20: Effective concentration affecting 20% of the tested population; ER: Oestrogen receptor; GABA: γ-Amino butyric acid; OECD: Organisation for Economic Co-operation and Development; PPAR: Peroxisome-proliferator activated receptor; PXR: Pregnane X receptor; RA: Retinoic acid; RALDH: Retinal dehydrogenase; RAR: Retinoic acid receptor; RARE: Retinoic acid-responsive element; REQ: atRA equivalent; RXR: Retinoid X receptor; TG: Test guideline; TH: Thyroid hormone; TR: Thyroid hormone receptor; VAD: Vitamin A (retinol) deficiency; VDR: Vitamin D receptor; WFD: Water Framework Directive; WHO: World Health Organization.

Availability of data and materials
Not applicable.

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Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no conflict of interest. MNJ is on the Scientific Advisory Board of ERGO.

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