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Retinoids and Developmental Neurotoxicity: utilizing toxicogenomics to enhance adverse outcome pathways and testing strategies

Hao Chen[1], **Megan Chidboy**[1], **Joshua F. Robinson**[1,*]

[1]Center for Reproductive Sciences and Department of Obstetrics, Gynecology, and Reproductive Sciences, University of California, San Francisco (UCSF), San Francisco, CA, USA

Abstract

The use of genomic approaches in toxicological studies has greatly increased our ability to define the molecular profiles of environmental chemicals associated with developmental neurotoxicity (DNT). Integration of these approaches with adverse outcome pathways (AOPs), a framework that translates environmental exposures to adverse developmental phenotypes, can potentially inform DNT testing strategies. Here, using retinoic acid (RA) as a case example, we demonstrate that the integration of toxicogenomic profiles into the AOP framework can be used to establish a paradigm for chemical testing. RA is a critical regulatory signaling molecule involved in multiple aspects of mammalian central nervous system (CNS) development, including hindbrain formation/patterning and neuronal differentiation, and imbalances in RA signaling pathways are linked with DNT. While the mechanisms remain unresolved, environmental chemicals can cause DNT by disrupting the RA signaling pathway. First, we reviewed literature evidence of RA and other retinoid exposures and DNT to define a provisional AOP related to imbalances in RA embryonic bioavailability and hindbrain development. Next, by integrating toxicogenomic datasets, we defined a relevant transcriptomic signature associated with RA-induced developmental neurotoxicity (RA-DNT) in human and rodent models that was tested against zebrafish model data, demonstrating potential for integration into an AOP framework. Finally, we demonstrated how these approaches may be systematically utilized to identify chemical hazards by testing the RA-DNT signature against azoles, a proposed class of compounds

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*Corresponding author: Joshua F. Robinson, Ph.D., Center for Reproductive Sciences, Department of Obstetrics, Gynecology, and Reproductive Sciences, University of California, San Francisco (UCSF), 513 Parnassus Ave, Rm. 1621, San Francisco, CA 94143-0665, Phone: (415) 476-0549, Joshua.Robinson@ucsf.edu.

Author contributions

JR managed the project and conducted the statistical analyses; HC, JR drafted figures and interpreted the data, and HC, MC, and JR wrote the manuscript.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Competing Interests

The authors have no conflicts to declare.

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that alters RA-signaling. The provisional AOP from this study can be expanded in the future to better define DNT biomarkers relevant to RA signaling and toxicity.

Keywords

retinoids; retinoic acid; human; embryonic stem cells; whole embryo culture; zebrafish; neurogenesis; neurotoxicity; transcriptome; alternative model; *in vitro* ; azoles; adverse outcome pathway; transcriptomics

Introduction

Retinoic acid (RA), also known as *all-trans*-retinoic acid, is required for mammalian development. Mammals are unable to synthesize RA *de novo*, deriving the compound through dietary intake of vitamin A or other retinoid precursors [1]. RA, a small lipophilic molecule, is synthesized in specific cells, released, and taken up by surrounding cells [2, 3]. Upon cellular entry via diffusion, RA transfers to the nucleus and acts as a transcriptional activating ligand by binding to nuclear receptors known as retinoic acid receptors (RARs) [4–6] that form heterodimer complexes with retinoid X receptors (RXRs) [7]. RA levels and ligand-activity is tightly regulated by the regional-and temporal-dependent expression of a combination of molecules including RAR/RXRs, co-factors, binding proteins, and metabolizing enzymes.

RA signaling is important for several aspects of central nervous system (CNS) development including 1) regional patterning and the 2) differentiation and maintenance of progenitors into neuronal populations [8–10]. Subsequently, disruption of RA signaling due to imbalances in RA bioavailability, in deficiency or in excess, can lead to developmental neurotoxicity (DNT) [11, 12]. While the effects of RA may be dependent on model and exposure paradigms utilized [13], CNS (and related axial) defects associated with perturbations in RA signaling include: anterior (exencephaly or anencephaly) and posterior (spina bifida) NTDs, hindbrain abnormalities, microcephaly, irregular somitogenesis, delayed or reduced caudal elongation, vascular damage, notochord defects, irregularities in the neural folds and other variants [14, 15]. Morphological alterations due to disrupted RA signaling are linked with abnormal changes on the cellular and molecular level (*e.g.,* neuronal proliferation, differentiation, viability (apoptosis), and migration) [16, 17].

Classically, chemicals are evaluated for neurotoxicity [18, 19] and DNT [20–22] with standardized *in vivo* tests based on guidelines developed in the US and Europe (*e.g.,* OECD TG 426). While informative and predictive in identifying potential developmental neurotoxicants [22], traditional *in vivo* assessments are costly, time-consuming, and suboptimal to rapidly screen the thousands of compounds being released in the environment [23]. In line with strategies to integrate the 3R principles (replacement, reduction, refinement) into toxicological testing and to appropriately model the complexities of the developing CNS, incorporating alternative methodologies as part of a DNT battery can increase screening-throughput, hasten validation efforts, and reduce reliance on traditional *in vivo* studies [24, 25]. Recent technological advancements in evaluating mRNA expression have enhanced our ability to determine global molecular perturbations that correlate with

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or precede toxicity in relation to chemical exposures (reviewed, [26]). The identification of molecular signatures linked with normal/abnormal neurodevelopmental processes [27] and/or toxic chemical exposures [28] may provide benchmarks that can be applied to determine common effects of developmental neurotoxicants across models. While toxicogenomic changes are highly dependent on several toxicological factors (*e.g.,* dose, time, model, sex), the integration of like-datasets may lead to the discovery of common and consistent features that can be applied in hazard identification and screening approaches. As RA signaling is highly conserved among vertebrates and regulates various developmental processes in addition to CNS formation (*e.g.,* skeletal [29], lung [30], heart [31], germ cell [32]), genomic approaches can define susceptible molecular players associated with toxicological interference, thereby facilitating and enhancing DNT hazard identification.

Adverse outcome pathways (AOPs) provide a framework to link environmental exposures to perturbations on molecular, cellular and functional levels that contribute to changes to DNT and disease [33]. AOPs can be implemented with *in vitro*, *in silico,* and *in vivo* toxicological testing strategies aimed at determining potential hazards. Recent work by Tonk et al. [34] constructed an AOP connecting perturbations in RA signaling and neural tube/axial patterning defects, with this framework also serving as a case study for a developmental toxicity ontology [35]. RA's critical role across a diversity of developmental processes underscores the need to define key molecular interactions mediating organismal defects, as chemicals that directly or indirectly disrupt components of RA signaling—such as retinoids [14], azoles [36], or ethanol [37]–confer (neuro)developmental toxicity by disrupting RA homeostasis. Key examples of enzymes involved in RA metabolism were provided by Tonk et al. and their diminished function were linked with imbalances in RA and defects in the neural tube, axial patterning, and other malformations such as craniofacial and cardiac defects. As a proof-of-principle, the AOP framework was validated for hazard prediction utilizing pre-existing experimental data of flusilazole— an antifungal agent and proposed RA-signaling disruptor— in rat whole embryo culture (WEC), zebrafish (Zf) embryo, and embryonic stem cell (ESC) models.

Building on this approach, we constructed a provisional AOP framework connecting RA signaling and DNT, with a focus on CNS patterning and neuronal differentiation. In the following sections, we summarize existing literature linking RA signaling to CNS development and propose models and endpoints relevant for DNT assessment. Next, we integrated toxicogenomic datasets across mammalian model systems and performed a meta-analysis to identify common gene expression changes from excess RA exposure during early neurodevelopment, informing the design of our AOP framework. The resulting RA-DNT gene list was compared to a Zf embryo dataset to illustrate the applicability of these proposed gene targets in other model systems. Lastly, as a case study, we utilized the AOP and RA-DNT gene set to provide supporting evidence that azoles, a class of antifungals, disrupt RA signaling and cause DNT.

Molecules regulating RA signaling during early CNS development

Vitamin A is transformed into RA in two steps (Figure 1). First, retinol conversion to retinaldehyde is mediated by two classes of oxidizing enzymes (alcohol dehydrogenases

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and retinol dehydrogenases). In mouse, retinol dehydrogenase-10 (*Rdh10*) is necessary for conversion of retinol to retinaldehyde during early embryogenesis [38, 39]. Enzymes such as Short-chain Dehydrogenase/Reductase 3 (*Dhrs3*) facilitate the reverse conversion of retinaldehyde to retinol [40, 41]. In the second step, retinaldehyde is irreversibly oxidized to form RA by aldehyde dehydrogenases (ALDHs). Specifically, ALDH1A2, also known as retinaldehyde dehydrogenase 2 (*Raldh2*), is critical for RA synthesis during early CNS development [42]. The cytochrome p450 26 subfamily enzymes (*Cyp26a1/Cyp26b1/Cyp26c1*) are also necessary in regulating RA levels in the embryo by catalysing reactions converting RA to less active RA metabolites, thereby reducing bioavailability [43]. Although less defined, other enzymes, including other CYPs (*Cyp1b1*) [44, 45], may play secondary roles in RA metabolism during early CNS development.

Cellular binding-proteins such as CRABP1 and CRABP2 facilitate RA transfer to the nucleus, though these proteins also play roles in RA cellular sequestration, metabolism, and function [46]. In the nucleus, retinoic acid receptors RARs and RXRs are activated by different forms of RA. The primary endogenous ligand for RARs is RA, while 9-cis-RA binds to both RARs and RXRs *in vitro* [47, 48]. After ligand binding, RARs form heterodimer complexes with RXRs and bind to motifs known as RA response elements (RAREs) to mediate gene transcription. Though the co-factors specific to RA-mediated CNS development remain undefined, transcriptional activation is influenced by binding of RAR/RXR heterodimer complexes, leading to the release of factors (*e.g.,* SMRT [49], histone deacetylases) that restrict chromatin access and the recruitment of co-activators (*e.g.,* CBP/p300 [50]) and co-repressors (*e.g.,* LCoR [51, 52]). A suite of molecules that include RAR/RXRs, co-factors, binding proteins, and metabolizing enzymes are critical for controlling RA bioavailability and ligand-activity in the developing CNS.

RA signaling and CNS regional patterning

Neural induction is initiated by the release of signaling factors from notochord mesoderm to neighboring ectoderm cells overlying the notochord, giving rise to the neuroectoderm (as reviewed [53]). Along the dorsal region of the embryo, the neuroectoderm transforms to give rise to the neural plate. Thickening of neural plate tissue and elevation at the lateral edges of the neural tube results in neural folds which eventually merge at the dorsal midline and establish the neural tube, forming the backbone of the CNS.

Early in development, RA contributes to the antero-posterior and dorso-ventral patterning of the neural plate and neural tube via a tightly-regulated spatiotemporal concentration gradient [2, 54]. The specification of distinct regions in the CNS—forebrain, midbrain, hindbrain, and spinal cord— arises from the regulation of gene expression in each of these domains [55]. RA signaling influences transcription in these domains via “sources” and sinks,” represented by RALDHs in the posterior and CYP26 enzymes in the anterior ends of the embryo respectively. The bioavailability of RA is tightly controlled by the coordinated expression of these, and numerous other metabolic enzymes, throughout development [56]. During the presomite stages, *Raldh2* and *Rdh10* expression in the paraxial mesoderm produces RA in posterior trunk regions [57, 58]. The anterior neuroepithelium restricts diffusion of RA from these posterior regions via *Cyp26a1* expression [43]. Meanwhile RA

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in the posterior regions activates FGF and WNT signaling to repress expansion of *Cyp26a1* at the posterior regions [59]. Patterning of the CNS is regulated by this RA gradient.

The critical influence of RA on hindbrain patterning is well-established. During hindbrain formation, transient metameric units called rhombomeres develop (Figure 2). The hindbrain is composed of 7 rhombomeres and each segment generates a specific repertoire of genes that confers its distinct regional identity. RA regulates the formation of rhombomeres through interactions with RAREs on *Hox* genes. The function of *Hox* genes and their targets in establishing rhombomere identity has been thoroughly reviewed [60–62]. Briefly, RA regulates expression of *Hox* genes 1–4 [63] during hindbrain segmentation, with the highest concentrations of RA beginning at the caudal end and shifting throughout development [64]. In general, *Hox* expression varies within one or two-segment rhombomere boundary increments. *Hoxa1* and *Hoxb1* are induced early at the presomite stage and are critical for r4 patterning [65, 66]. *Krox20* (Egr2), a zinc finger transcription factor, is subsequently activated and regulates the expression of *Hoxa2* and *Hoxb2* in r3 and r5 [67, 68]. *Hoxa2* expression is required to maintain r2 identity [69], while the interactions of *Hoxa2* and *Hoxb2,* in combination with *Hoxa1* and *Krox20*, are necessary for r3 [70, 71]. *Kreisler (Mafb)* induces expression of *Hoxa3* and *Hoxb3, Hox* genes important for the r5 and r6 segments [72, 73]. While no direct interaction between RA and *Kreisler* is known to us, indirect interactions could affect its expression. *Krox20* and *Kreisler* synergize to regulate *Hoxb3* expression [74], and *Cdx1,* which prevents hindbrain patterning in the spinal cord region [75], is activated by RA and represses *Kreisler* [76]. *Hoxa4/Hoxb4/Hoxd4* maintain the r6/r7 boundaries respectively and all contain RAREs [77].

RA signaling and neuronal differentiation

RA can differentiate cells towards various identities, including serotonergic, GABAergic, glutamatergic, and dopaminergic neurons. Transcriptional regulation of these different differentiation processes is context-dependent, based on factors such as RA levels [78] and positional cues [79]. One such example is the differentiation of cells from the ventral p3 hindbrain domain, which can become glutamatergic V3 spinal interneurons or serotonergic hindbrain neurons despite originating from the same progenitor pool. RA signaling influences the fate of these progenitors through dose-dependent attenuation of *ASCL1* levels via the Notch pathway, with areas of low RA/high *ASCL1* being permissive of serotoninergic differentiation and RA repression of *ASCL1* inducing a glutamatergic V3 identity [80].

RA is critical for differentiation of neurons in the nigrostriatal system and cortical neurogenesis [81]. In a regional- and temporal-dependent manner, *Raldh* enzymes regulate RA bioavailability and, subsequently, differentiation. *Raldh1* is expressed in neurons of the ventral midbrain early [82] and becomes restricted to the dorsal retina and dopaminergic neurons in the substantia nigra and ventral tegmentum [83] later in development. *Raldh1* expression in these regions is transcriptionally regulated by *Pitx3,* as evidenced by loss of the enzyme in the meso-diencephalic dopaminergic neurons of *Pitx3* knockout models and restoration with RA [84]. RA transcriptionally regulates dopaminergic differentiation via activation of the dopamine autoreceptor D2 at a RARE in its promoter region [85].

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In contrast, *Raldh3* expression shifts to the lateral ganglionic eminence (LGE) during development and extends to the piriform cortex and septum [86]. The LGE serves as a local site of RA during striatal neurogenesis, with production originating from these *Raldh3* expressing cells [86, 87] and glia [88]. LGE cells and neuronal subpopulations migrating through the LGE require RA signaling for GABAergic differentiation [89]. RA production in the LGE is mediated by *Gsh2* [90], with *Gsh1* maintaining proliferation of LGE progenitors [91]. *Gad67* upregulation is also a demonstrated effect of RA, though the mechanism is unclear as no RARE has been identified in the promoter region [92]. Maintaining the critical balance of RA during development extends into CNS maturation, as early regions of RA synthesis generally maintain their spatial distribution of RA later [93]. For example, in the postnatal rodent brain the highest levels of endogenous RA possess similar regional distinctions seen early in development (*e.g,* striatum) when measured with mass spectrometry [94, 95]. Thus altering RA homeostasis in sensitive regions or cellular populations may have lasting impact as RA is involved in the initial differentiation, and continued maintenance, of these neurons [96].

Genetic defects in RA signaling and adverse CNS outcomes

Due to the importance of RA bioavailability during hindbrain formation and anteroposterior patterning, proper RA signaling is necessary for development in these CNS regions. Genetic modulation of RA metabolic enzymes, receptors, and downstream signaling targets has provided substantial insight into the role of RA pathway members in hindbrain and AP development. Perturbations of various components of the RA signaling pathway has been linked to impaired patterning, neural tube closure, and CNS morphology (Supplemental Table 3). For example, *Rdh10(−/−)* embryos carrying a RARE-lacZ RA-reporter transgene display a loss of RARE-lacZ expression in the eye/forebrain and hindbrain regions, with defects in patterning and development in those areas [39]. Meanwhile, *Raldh2(−/−)* embryos generated insufficient levels of RA to activate a RAREhsplacZ transgene, subsequently resulting in hindbrain/axial defects [97]. *Cyp26a1(−/−)* embryos exhibit increased hindbrain defects, NTDs (exencephaly and spina bifida), and caudal truncation [43, 98]). *Cyp26a1(−/−)* models are sensitive to excessive concentrations of RA [99] and rescued by eliminating embryonic expression of *Rarg* [100].

Altered expression of *RAR/RXR*, transducers of RA-signaling, are similarly linked with abnormalities of the CNS. Observed phenotypes are related to the spatiotemporal patterns of the target molecule. For instance, *Rarg* is expressed prior to closure of the neural tube, while *Rarb* and *Rara* are expressed as the neural tube folds adjoin [101–103]. *Rara(−/−):Rarg(−/−)* double null mouse mutants [104] have significantly higher levels of exencephaly, while *Rarg(−/−)* are less susceptible to abnormalities in the neural tube [105, 106]. The genetic loss in expression of downstream target genes of RA, including *Hoxa1* [107, 108], *Hoxb1* [109, 110], *Krox-20 (Egr2)* [111] and *Kreissler (Mafb)* [112, 113] are also associated with abnormalities in hindbrain development.

Evidence of RA level imbalances in experimental models and developmental neurotoxicity

Various experimental models have a demonstrated concentration-dependent effect of RA on CNS development, with higher concentrations causing more overt impairments and

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teratogenicity (examples provided in Table 1). In animal models, including mouse [114], rat [115, 116], hamster [117], rabbit [118], and monkey [119], prenatal imbalances in retinoid levels —in excess or deficiency—results in DNT. In general, the most sensitive windows to retinoid-induced DNT appear when imbalances in RA signaling occurs during neurulation and early organogenesis [120–123], while later periods in development may be more vulnerable to deficiencies in brain weight and potentially postnatal survival [124]. Numerous studies in rodent models have exogenously manipulated RA signaling through diet or injection, leading to description of specific CNS malformations and structural variations. Sprague Dawley rats exposed on embryonic day 10 to RA (50–70 mg RA/kg maternal body weight, oral gavage) were significantly associated with multiple CNS deficiencies, including myelomeningocele, lumbosacral defects, exencephaly, and caudal regression syndrome [125]. Meanwhile, embryos of Vitamin A-deficient (VAD) pregnant rats (Sprague Dawley) display loss/disorganization of cranial nerves, loss of caudal hindbrain segmentation, expansion of rhombomeres R3–4, and otic vesicle abnormalities [126].

Alternative models, including rat WEC, ESCs, and Zf embryo have also been utilized to evaluate retinoids in the context of DNT. The WEC model is a proposed tool to study the influence of both environmental and genetic factors on early embryonic development and has proven to be a valuable model to evaluate the teratogenic potency of retinoids. Morris and Steele demonstrated the use of the WEC model to profile the teratogenic effects of excess retinol and RA exposures during early organogenesis, validating *in vivo* observations that elevated exposures to retinoids during neurulation results in defects of the neural tube as well as malformations of the palate and limb [127]. Recent studies examining RA effects in WEC have incorporated transcriptomic approaches to identify global changes in gene expression which may underlie and precede morphological alterations. Luijten et al. utilized transcriptomic profiling to identify early responses to teratogenic levels of RA (0.5 μg/mL) in single cultured rat embryos initially exposed at 2–4 somites [16]. RA affected 260 genes after a 4h exposure duration, including genes involved in embryonic patterning and CNS development (*e.g., Gbx2* and *Otx2)* as well as genes linked with RA metabolism (*Cyp26a1, Cyp26b1, Dhrs3*) and RA gene activation (*Rarb*). In a study by Robinson et al., RA effects on morphological and transcriptomic levels were compared between the rat postimplantation WEC model (0.5 μg/mL) and embryos *in vivo* undergoing neurulation (50 mg/kg) [128]. Consistent with Luitjen et al., perturbations were found in 845 genes and enriched for processes involved in CNS and embryonic development as well as neuronal differentiation; more than 50% of identified differentially expressed genes in either model overlapped. Thus, the WEC may be considered a useful alternative system to test retinoids and other environmental compounds in their effects on RA signaling in the context of early CNS development.

While the predictive value of ESCs for DNT screening has yet to be fully defined [24], early investigations suggest that these model systems can be used to study a wide-range of risk factors on multiple aspects of neurogenesis [24, 129, 130], including retinoids and other environmental chemicals that cause neurotoxicity by perturbing RA-signaling pathways. Retinoids (RA or Vitamin A) are commonly added in ESC cultures to induce neural differentiation [15, 131] and can regulate patterning genes in a similar manner to *in vivo* [132, 133]. Utilizing a transcriptomic approach at four time points during

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mESC-embryoid body differentiation, Akanuma et al. (2012; [134]) demonstrated the stage-dependent sensitivity to RA at two concentrations (0.01 or 0.1μM). These analyses provided information regarding gene networks influenced by RA exposure at 0, 2, 8, and 36 days of mESC differentiation in parallel with cell morphological changes (increased growth and neuronal differentiation), and identified particular targets (*e.g.,* Gfap, Gbx2) that may amplify RA-signaling and neuronal development.

The effects of RA have also been examined in various hESC models in relation to neurodevelopment and DNT *in vitro*. For example in a study using a hESC-based neural rosette model—a proposed screen to identify toxicants that disrupt early CNS morphogenesis and apical neuroepithelial organization of the neural tube—the concentration-dependent effects on cell morphology, function and global mRNA expression were evaluated [135]. Exposures to RA caused increased cytotoxicity (2μM), disorganized neural rosette morphology (0.2μM), and perturbed expression of molecules critical for CNS patterning (HOXA1, HOXA3, HOXB1, HOXB4) and neural differentiation (FOXA2, FOXC1, OTX2, PAX7)— suspected target genes of RA *in vivo* [136, 137]—at concentrations as low as 0.02μM. As a model of early CNS development, ESC models may be used to assess cellular and molecular changes relevant to RA-induced DNT.

The zebrafish (Zf) embryo model has also been proposed as a predictive alternative screening system for developmental toxicology [138–141]. The relevance of RA regulation in Zf CNS development has been demonstrated through RARE visualization [142] and genetic knockdowns of metabolic enzymes which promote biosynthesis (*rdh10* [143]; *raldh2* [144]) or breakdown (*cyp26a1* [145], *dhrs3* [146]) of RA. Several investigations have examined the relationship between excess or deficiencies in RA and DNT using Zf. In a concentration-dependent manner, Holder and Hill demonstrated RA (0.001–1μM) to cause CNS and tail malformations [147]. Concentrations of 0.1–1μM RA produced loss of the mid-hindbrain border, rhombomere abnormalities and reduced neurons within the anterior lateral line and ganglia. Global transcriptomic analyses of Zf during early stages of embryogenesis demonstrate genes disrupted by RA (0.1μM) overlap with mammals, including *cyp26a/b1, dhrs3a/b, aldh1a2 (raldh2),* and *hox* genes [148]. In the same study, utilizing translational-blocking morpholinos, the authors demonstrated that the expression levels of a subset of genes (~27%; 85 genes) altered by RA are dependent on *rar* expression. These studies illustrate the ability of the Zf embryo model to be used to evaluate RA-induced DNT on morphological and molecular levels.

Material and Methods

Processing of transcriptomic datasets and identification of a RA-induced developmental neurotoxicity signature in mammalian models

To define a transcriptomic signature associated with RA and DNT, we searched for datasets in public online repositories maintained by the National Center for Biotechnology Information (Gene Expression Omnibus) and European Molecular Biology Laboratory-European Bioinformatics Institute (ArrayExpress) using search terms [“retinoic” or “retinoid”] and [“toxicity” or “neurotoxicity”]. Additional searches were completed *ad hoc*. In total, we identified twelve transcriptomic studies evaluating RA exposures in

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vertebrate model systems (Supplemental Table 1). First, we determined a common signature related to RA and DNT in mammalian models. We selected three datasets from two studies [E-MEXP-3577 and GSE33195] for meta-analysis. These studies examined the: 1) toxicological-response of RA in human embryonic stem cells (hESCs) differentiating towards a neural cell fate; or 2) time-dependent responses of a developmentally toxic concentration of RA in cultured embryos (WEC) or in embryos *in vivo*. As previously described [27], datasets were independently processed, *i.e.,* log2 transformed, normalized via the Robust Multi-array Average (RMA) algorithm, and annotated. To identify genes commonly dysregulated by RA in association with DNT, a fixed effects linear model (ANOVA) was independently applied in each study to determine significance of RA across concentration or time. In the case of multiple probes per gene, the one with the lowest p-value, *i.e.*, most significant changes with RA, was used for comparison purposes. Next, we determined genes commonly altered in all three studies by applying a cutoff of $p<0.005$, and incorporated a secondary filter that required genes to trend in the same direction in all 3 datasets with RA effects as determined by Pearson's Correlation (across dose; E-MEXP-3577) or the computed average fold change in expression across time (GSE33195). This subset was termed the "RA-DNT" gene set (Supplemental Table 2). Datasets were merged using the Official Gene Symbol and R statistical package. Hierarchical clustering of fold change (FC) values for differentially expressed genes was determined using average linkage and Euclidean distance [149]. We performed enrichment analysis of RA-DNT genes utilizing Gene Set Enrichment Analysis [150] and a cutoff of $q<1*10^{-10}$.

We examined the conservation of patterns of expression of RA-DNT genes, identified in mammalian model systems, in Zf embryos exposed to RA using the dataset GSE43755 (Supplemental Table 1). This transcriptomic study included profiles of RA-exposed (100nM at the 2 cell embryo stage; ~45 minutes post-fertilization) and control embryos at 3, 5, and 8h post exposure. In Zf embryos, exposures of 100nM RA have been shown to induce significant teratogenic effects, including malformations of the CNS. We processed, annotated, and merged the dataset with the RA-DNT gene set based on the OGS identifier. Genes identified as significantly altered due to RA exposure were defined with a $p<0.05$ (ANOVA). We performed a secondary filter to verify homology (60% similarity in protein sequence) between mammals and Zf via the National Center for Biotechnology Information Basic Local Alignment Search Tool (BLAST) [49].

We demonstrated the applicability of the RA-DNT gene set in two independent toxicogenomic assessments of azoles in cultured rat whole embryos (WEC) generated at the National Institute of Public Health and the Environment (GSE102082 [151] and [152]; Supplemental Table 1). The dataset GSE102082 [151] includes whole rat embryos exposed to a single concentration of either: difenoconazole, fenarimol, flusilazole, ketoconazole, miconazole, propiconazole, prothioconazole, tebuconazole, triadimefon, B595, B599 or B600, estimated to significantly induce developmental toxicity (reduce total morphological score (TMS) by 10%) after 4h exposure. The other dataset [152] utilized whole rat embryos exposed to either one of four concentrations of flusilazole or one of two concentrations of cyproconazole or triadimefon after 4h exposure. As described above, these datasets were normalized via Robust Multichip Average (RMA) and annotated. We identified RA-DNT genes significantly dysregulated by each compound (ANOVA; $p<0.05$). Within these

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gene subsets, we determined the number of differentially expressed RA-DNT genes that displayed similar directional changes, up or downregulated, as excessive RA exposure in the evaluated mammalian systems. Fold enrichment (observed vs. expected) and the significance of identifying the number of differentially expressed RA-DNT genes for each compound (Chi-Squared test with Yates' continuity correction; https://www.graphpad.com/) was also calculated. In the case of multiple probes per gene, the one with the lowest p-value corresponding with all azoles tested in each study was used for comparison purposes.

An Adverse Outcome Pathway of RA-induced developmental neurotoxicity and the integration of a toxicogenomic signature of RA-DNT

We constructed a provisional RA-DNT AOP framework (as reviewed [153]) utilizing diverse literature sources (detailed above in the introduction). First, we assembled evidence supporting the role of RA in hindbrain formation/patterning and neuronal differentiation on the molecular, cellular, tissue, and organism level. Next, we connected a series of key events (KEs) to predict adverse outcome(s), with the first triggering KE being the molecular initiating event(s) (MIEs) that describe primary chemical-molecule interactions [33]. As the levels of RA and the activation of RA-dependent genes may be dependent on many factors—including spatiotemporal and concentration-dependent restrictions—multiple MIEs plausibly exist. Thus, we developed the RA-DNT AOP in a general manner to capture potential chemical-molecular interactions that contribute to the dysregulation of RA and RA-mediated transcription. Subsequent secondary KEs were categorized based on: 1) genes regulated by RA-RAR/RXR activation with evident roles in patterning and differentiation; 2) cellular regional identity and neuronal differentiation; 3) hindbrain (tissue) development; and 4) brain development and maturation on an organism level. Our defined transcriptomic signature associated with RA and DNT was incorporated into the AOP as a potential broad indicator of RA-dysregulation.

Results

Integration of toxicogenomic datasets to define a signature of RA-induced developmental neurotoxicity

We integrated genomic datasets related to RA exposures to identify a transcriptomic signature applicable across mammalian model systems, with the aim of defining predictive biomarkers of RA response for DNT assessments. We identified three datasets from two relevant studies: E-MEXP-3577 and GSE33195, examining the: 1) concentration-response of RA in hESCs differentiating towards neural rosettes or 2) time-dependent response of a developmentally toxic concentration of RA in cultured embryos (WEC) or *in utero* during neurulation/early organogenesis. Differentially expressed genes due to RA were identified via generalized linear models (ANOVA), and datasets were compared to identify common genes dysregulated across all three datasets. In total, we observed 131 differentially expressed genes ($p<0.005$; Figure 3A). Within this subset, 95 genes displayed common trends in regulation, of which 74 were upregulated, and 21 were downregulated. In general, the magnitude in RA-induced effects on expression were concentration- or time-dependent (Figure 3B). We performed enrichment analysis of the "RA-DNT" genes utilizing Gene Set Enrichment Analysis [150]. RA-DNT genes were significantly enriched for processes

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related to regulation of cell differentiation, embryo development, embryonic morphogenesis and regulation of gene expression ($q<1*10^{-10}$; Figure 3C). Gene targets identified through this analysis (Figure 3D; Supplemental Table 2) included molecules involved in: RA metabolism (*CYP26A1, CYP26B1, DHRS3*), RA signaling (*RARA, RARB*), RA binding (*CRABP2*), patterning (*HOXA1, HOXB2, MEIS1, MEIS2*) and differentiation (*MAFB, PBX1, SIX, SKIL*). We propose that this gene set may be utilized to: 1) identify potential compounds that cause DNT via RA-mediated pathways, and 2) compare across mammalian and non-mammalian model systems for common/unique mechanisms of RA-induced DNT.

Zebrafish case study

As a proof of principle, we examined for conservation of patterns of RA-DNT gene expression in the Zf embryo using the dataset GSE43755 [154], which included profiles of RA-exposed (100nM) and control embryos at 3, 5, and 8h post exposure, initially exposed at the 2 cell embryo stage. The concentration of RA (100nM) used in this study is significantly teratogenic (*e.g.,* brain malformations) in the Zf [155, 156]. We identified homologues for 62 genes of the RA-DNT gene set. We observed an enrichment of differentially expressed RA-DNT genes ($p<0.00001$). In total, 16 genes were found to be altered with RA in the Zf embryo model, 15 of which were upregulated, 1 downregulated; and 100% of differentially expressed genes followed similar trends, *i.e.,* up or downregulated, with observed responses in mammalian models (Figure 3E). The dysregulated genes included: *crabp2a, cyp26a1, cyp26b1, dhrs3a, hoxb2a, lhx5* (downregulated), *meis1b, meis2b, mmp11b, nrip1, raraa, rarab, skib, spsb4a, tiparp,* and *tshz1*. Thus, our analysis led to the identification of relevant genes linked with mammalian RA-DNT that may be evaluated as biomarkers of RA exposure in Zf. These data support previous evidence suggesting significant conservation of members involved in the RA signaling pathway across vertebrates.

Integrating toxicogenomic signatures into the AOP framework

Based on available data in the literature and the RA-DNT gene set, we outlined a preliminary AOP of RA-induced DNT that describes the mechanistic links between excessive or deficiency in RA bioavailability and defects in hindbrain development (Figure 4).

The initial development and segmentation of the hindbrain occurs within the period of neurulation and early organogenesis, approximately between the 3rd and 4th weeks of human pregnancy, GD7.5–10 in mouse, and GD9.5–12 in rat. RA bioavailability is regionally controlled by a suite of enzymes. *Rdh10* and *Raldh2*, expressed in the paraxial mesoderm, promote biosynthesis of RA and diffusion to proximal tissues. *Cyp26a1* is highly expressed in the anterior region and strictly limits RA bioavailability in this region. Nuclear transfer of RA and activation of *RAR/RXR* leads to the recruitment of co-activators and co-repressors which mediate specificity of transcription. In a tightly regulated sequence, segments of the hindbrain are formed due to RA bioavailability and subsequent RA-induced gene expression. RA regulates the regional expression of several genes and signaling molecules that underlie hindbrain segmentation and regional identity, including *Krox20(Egr2), MafB,* and *Hox* family members [157] as well as target genes involved in the differentiation of cells within each region.

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Alterations in the bioavailability of RA, in excess or deficiency, can result in severe malformations and/or variants in hindbrain development and posterior segmentation. These changes can occur on the molecular, cellular, and tissue level in the CNS, and depend on the timing/duration of RA exposure, dose, and factors such as genetic variants in RA metabolic enzymes. Excess RA results in hyper activation of *RAR/RXR*-mediated gene expression, whereas deficiency in RA, leads to lower activation of *RAR/RXR*-mediated gene expression. Subsequently, potential MIEs can occur through various means, such as 1) altering the availability of RA via enzymes that control the molecule's spatiotemporal gradient or 2) interaction with RAR/RXR and/or its cofactors to alter RA-mediated gene expression. Regulation of RA-mediated target genes represents a key event (KE), as regional cellular identity (*e.g., Hox* family members, *Krox20, MafB, Gbx2, Ncam1)* and neuronal differentiation (*e.g., Ngn2, Dbx1, Pax6*) can be altered by aberrant expression of these genes. Perturbations in regional cell identity and differentiation underlies disruption of hindbrain expansion and disorganization on the tissue level. Postnatally, morphological defects may appear as irregularities in CNS morphology (abnormal segmentation), NTDs, and/or changes in brain patterning and size. Disruptions in the development of these regions can potentially manifest in, or increase the risk of, adverse neurobehavioral outcomes such as deficiencies in motor control or sleep on the organismal level. In the preliminary framework, we propose that alterations in either direction in specific gene/gene families may be biomarkers of changes in RA status.

Azole case study

Direct or indirect chemical-molecular interactions may lead to the disruption of RA levels and activation of RA-dependent gene expression, critical MIEs, which may result in subsequent perturbations on the molecular, cellular, tissue, and organism level. Here, utilizing the provisional AOP and the RA-DNT predictive signature, we classify evidence supporting the ability of azoles to cause DNT during early brain formation by altering RA signaling.

Azoles represent a diverse class of compounds (*e.g.,* triazoles, imidazoles) which contain a five-membered ring comprised of at least one nitrogen atom and at least one other non-carbon atom. These compounds are widely-used as antifungal agents due to their ability to impair ergosterol formation, a necessary component of fungal cell-wall integrity, via inhibition of lanosterol 14α-demethylase (CYP51A1). In vertebrates, azoles are also suspected to cause DNT by altering RA signaling pathways by inhibiting CYP26 [36]. Metabolic disruption by select azoles leads to altered effects on the molecular, cellular, and organism level similar to observations as RA (at excess levels), including hindbrain segmentation and patterning defects as well as craniofacial and axial malformations [158, 159].

Evidence of azoles disrupting RA signaling has also been previously proposed on a global expression level [151, 152, 160]. Utilizing two transcriptomic datasets examining azoletoxicity in rat WEC (Supplemental Table 1), we interrogated the relationship between azole exposures and the expression of RA-DNT genes. In total, we examined the response of 13 independent azoles; two compounds (flusilazole, triadimefon) were evaluated in

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both datasets under different exposure designs. We identified enrichment of differentially expressed RA-DNT genes with 10 of the 13 azoles (p<0.01, Chi-Square). In both datasets, transcriptomic profiles of flusilazole or triadimefon exposed embryos were significantly enriched for differentially expressed RA-DNT genes (p<0.0001; fold enrichment ≈ 4X), and trends in dysregulation paralleled responses observed with excess RA exposure (96% agreement). Genes significantly altered by 5 compounds (p<0.05) included: *DHRS3, CYP26A1, RARB, PRICKLE1, FNDC5, ZFP36L2, RARA, MAFB, RPS6KA2, WFIKKN1, HOXA1, MEIS1, GSE1, TSHZ1, RAB38, DHX32, SERPING1, DEPTOR, CRABP2,* and *MGAT4A*. These analyses utilizing the independently derived RA-DNT gene set support the ability of azoles to alter expression of genes involved in RA-response and DNT, specific to hindbrain development and neural differentiation.

Discussion

In this study, we utilized existing literature and integrated relevant toxicogenomic datasets to construct a provisional RA-DNT AOP framework. Despite the central role of RA in embryonic growth and development, imbalances can be a human health risk; RA disruption during pregnancy leads to fetal CNS malformations and adverse neurodevelopmental outcomes [161]. Concentration-dependent effects of RA on various experimental models have been observed (Table 1), and as numerous xenobiotics classes may perturb RA signaling—including fungicides [36], styrenes [162], metals [163], and pesticides [164]—constructing an AOP framework could facilitate DNT evaluation of chemicals that disrupt the RA pathway. The use of alternative assays is a core tenet of AOP development and because RA signaling is conserved in vertebrates, we leveraged genomic approaches to create a RA-responsive gene set during CNS development, *i.e.* biomarkers of RA-DNT, that could refine and harmonize screening efforts. In the context of RA signaling, a multitude of enzymes regulate RA levels and their receptors. Accordingly, there potentially exists multiple MIEs, *i.e.,* molecular interactions, that trigger global or local changes in RA bioavailability and signaling cascades. There is a vast literature detailing the components of the RA pathway and identifying the essential molecules for DNT assessment will be crucial. Our provisional AOP notes some of these potential key targets, informed by our review, and incorporates some examples from our toxicogenomic analysis that could inform comparisons across models.

We identified the RA-DNT gene set consisting of significantly responsive targets with shared directionality across hESC and rat embryos (*ex vivo* and *in vivo*) models. Proposed target genes included key enzymes involved in RA metabolism (*CYP26A1*, *CYP26B1*, *DHRS3A*), *RARA* and the transcriptional coregulator *NRIP1,* and the RA binding protein, *CRABP2.* Other identified RA-DNT genes control downstream KEs, including molecules critical in hindbrain patterning (*HOXA1, HOXB2, TSHZ1, MEIS1, MEIS2*) [165], cell migration (MMP11) [166], and neuron differentiation (*LHX5*) [167]. Uncharacterized RA-responsive genes, such as *SPSB4* [168], *SKIL*, and *TIPARP* were also found in our analysis. Disruption of RA-target genes during this vulnerable time period in CNS development, such as those in the RA-DNT gene set, can lead to perturbations in regional cell identity and neuron differentiation during development, impacting hindbrain formation and expansion. Alterations in RA-sensitive brain structures can manifest as overt malformations, structural

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variations, and/or potentially, neurobehavioral deviations [122], such as respiration [169, 170] or motor activity [171]. By integrating like-transcriptomic datasets and identifying common dysregulated genes in mammalian models, we propose targets that can be introduced into the AOP framework to examine RA-DNT.

In an analysis of transcriptomic profiles of RA-exposed Zf embryos, we observed an enrichment of differentially expressed RA-DNT genes (Figure 3E), demonstrating the utility of the molecular signature in non-mammalian model systems. Common targets included well-described conserved elements of the RA-signaling pathway in vertebrates (*e.g., cyp26a1, cyp26b1, crabp2a, dhrs3a, hoxb2a, nrip1, raraa, rarab*). Divergence in RA responsive signatures between mammalian and Zf datasets may be due to several factors, such as differing RA exposure and developmental time-window, and additional comparisons with other Zf datasets is warranted to determine conservation of the RA-DNT signature. In addition, while many aspects of Zf development are comparable to mammals, the Zf may diverge in certain processes such as neural tube formation [288], and the need of RA for body axis extension [289]. The inclusion of hESCs in creating the RA-DNT gene set is also relevant. Though hESCs express and are capable of activating patterning genes, future protocols that emphasize optimizing culture cytoarchitecture will likely improve comparisons between the WEC and Zf embryo datasets [172]. These caveats also underscore the contextual nature of RA signaling, wherein effects are influenced by variables such as RA concentration and developmental time. Evaluating the responsiveness of multiple genes in the RA-DNT will be important, as the relative contribution of any single target can also vary by model system. For example CRABP2, while significantly responsive in our comparisons and essential for Zf hindbrain patterning [173], is dispensable in the mouse [174].

We applied the RA-DNT gene list to the case study of azoles to reinforce the value of toxicogenomic comparisons, as focusing on identifying common biomarker signatures within a biological pathway could hasten the identification of mechanisms of action by a chemical class. Our results, similar to reported findings which focused on specific RA and cholesterol metabolism genes [175, 176], revealed specific azoles, *e.g.,* propiconazole, triadimefon, flusilazole, cyproconazole, to be more disruptive to RA-signaling at developmentally toxic levels (Table 2). By utilizing the RA-DNT gene set, we quantitatively described potency in terms of disruption of RA-signaling. Alterations in RA-DNT genes in either direction could be used as biomarkers and potentially, trends in expression may indicate excess vs. deficiency in RA levels. For example, in azoles that significantly disrupted the RA-DNT gene set, trends in dysregulation generally matched transcriptomic profiles associated with excess RA.

The RA-DNT gene set and provisional AOP framework is not intended to be comprehensive or conclusive. There are significant challenges associated with developing AOPs informative for DNT, including the complexity and diversity of the brain, the multiple mechanisms that govern its development, and the potential of neurotoxicants to affect numerous molecular targets [177]. The RA signaling pathway is similarly complex and diverse, regulating the development of numerous organs [178]. Over 500 genes are associated with the RA signaling pathway [137] and deciphering transcriptional mechanisms and essential players

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is ongoing. While we have framed our AOP in the context of hindbrain development, the RA-DNT gene set and provisional AOP framework presented here provides only a general framework that necessitates additional, rigorous experimental input to be further developed. Within the AOP framework, alterations in the RA-DNT gene set potentially represents an early KE that signals general DNT risk. Although the cellular-, tissue-, and organismal-responses detailed in the provisional framework are DNT outcomes associated with disruptions in RA signaling, establishing the relative contributions of genomic alterations to these responses would significantly strengthen KE-relationships (KERs). Other MIEs outside of the presented AOP are also not accounted for; for instance, RA signaling can influence synaptic plasticity through non-genomic mechanisms [179], suggesting that RA and/or analogues can influence determinants of neural connectivity through other KEs. As knowledge of RA's function expands, validation and incorporation of endpoints from nongenomic assays—*e.g.,* cellular, functional, behavioral—that define sensitive target molecules or tissues would better elucidate these additional KEs and KERs. Alternative model systems, including those referenced above, will be relevant as they are adaptable to several DNT test methods used to evaluate KEs within the provisional RA-DNT framework. As AOPs are purposed to be deliberate simplifications of biological pathways, incorporating advances in mechanistic understanding will be necessary to both better evaluate chemicals in their ability to disrupt RA signaling pathways and to refine subsequent AOPs for RA-DNT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The Organisation for Economic Co-operation and Development (OECD) Test Guidelines Programme Detailed Review Paper 178 (DRP178) provided an overview of *in vitro* and *in vivo* screening and testing methods and endpoints for evaluating endocrine disruptors. Due to the known interaction between RA signaling and endocrine pathways, within this review, molecular effect biomarkers of the retinoid system for tiered toxicity screening and monitoring studies were also summarized. As an extension of this project, a review of the Retinoid system was initiated (project 4.97b) within the OECD Endocrine Disruptors Testing and Assessment Advisory Group (EDTA AG). As a member of this working group, we reviewed literature *ad hoc* and summarized information related to retinoids and links to DNT to construct a review paper. In-part, language and content were modified from this report (written by Dr. Robinson and colleagues) to develop this study. The authors would like to thank Patience Browne (OECD), Thomas Knudsen (USEPA), and members of the OECD DRP Working Group for their tremendous feedback regarding content and structure of the OECD DRP Neurotoxicity Report, which provided the key elements of this manuscript.

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Abbreviations:

AOP	Adverse outcome pathway
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
CNS	Central nervous system

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DNT	Developmental neurotoxicity
ESC	Embryonic stem cell
FC	Fold change
hESC	Human embryonic stem cell
NTD	Neural tube defect
KE	Key event
MIE	Molecular initiating event
OECD	Organisation for Economic Co-operation and Development
OGS	Official gene symbol
RALDH	Retinaldehyde dehydrogenase
RA	All-trans retinoic acid or retinoic acid
RA-DNT	Retinoic acid-induced developmental neurotoxicity
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RXR	Retinoid X receptor
RDH	Retinol dehydrogenase
RMA	Robust Multichip Average
VAD	Vitamin A deficiency
WEC	Whole embryo culture
Zf	Zebrafish

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Highlights

- Review of retinoic acid (RA) signaling and developmental neurotoxicity (DNT)
- Defined an adverse outcome pathway (AOP) related to imbalances in RA and DNT
- By integrating diverse datasets, identified genes associated with RA exposure and DNT
- This gene set, when applied to the zebrafish model, yielded shared targets of RA
- Demonstrated application of RA-signature in AOP framework and chemical screening

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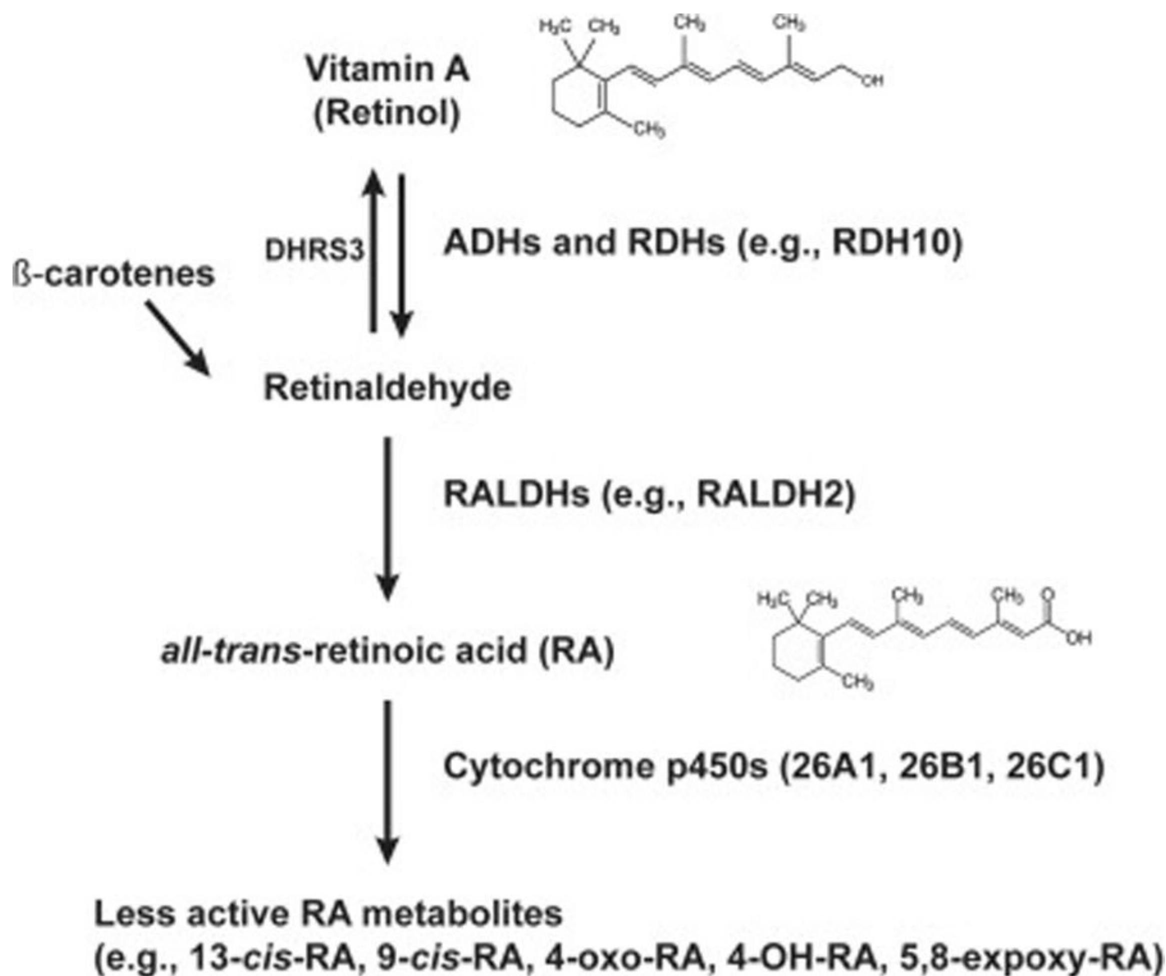

Figure 1. Retinoic acid metabolism.

The availability of RA is tightly controlled in the mammalian CNS. Mammals are unable to synthesize RA *de novo* and require intake of vitamin A or other precursors (β-carotenes) from food sources. Vitamin A is converted to retinaldehyde by alcohol dehydrogenases (ADHs) and retinol dehydrogenases (RDHs). In mouse, RDH10 is necessary for conversion of retinol to retinaldehyde in the developing embryo [38]. Enzymes such as Short-chain Dehydrogenase/Reductase 3 (DHRS3) facilitates the reverse transformation of retinaldehyde to retinol [40, 68]. Retinaldehyde is further oxidized to form RA by aldehyde dehydrogenases (ALDHs or RALDHs) in an irreversible step. RALDH2 is critical for RA synthesis during early CNS development [42]. Cytochrome p450 26 subfamily enzymes regulate RA levels in the embryo and catalyze reactions to reduce RA bioavailability by converting RA to 4-OH-RA, 4-oxo RA, and other oxidized, less active metabolites [43]. These metabolites undergo glucuronidation which promote elimination pathways [180].

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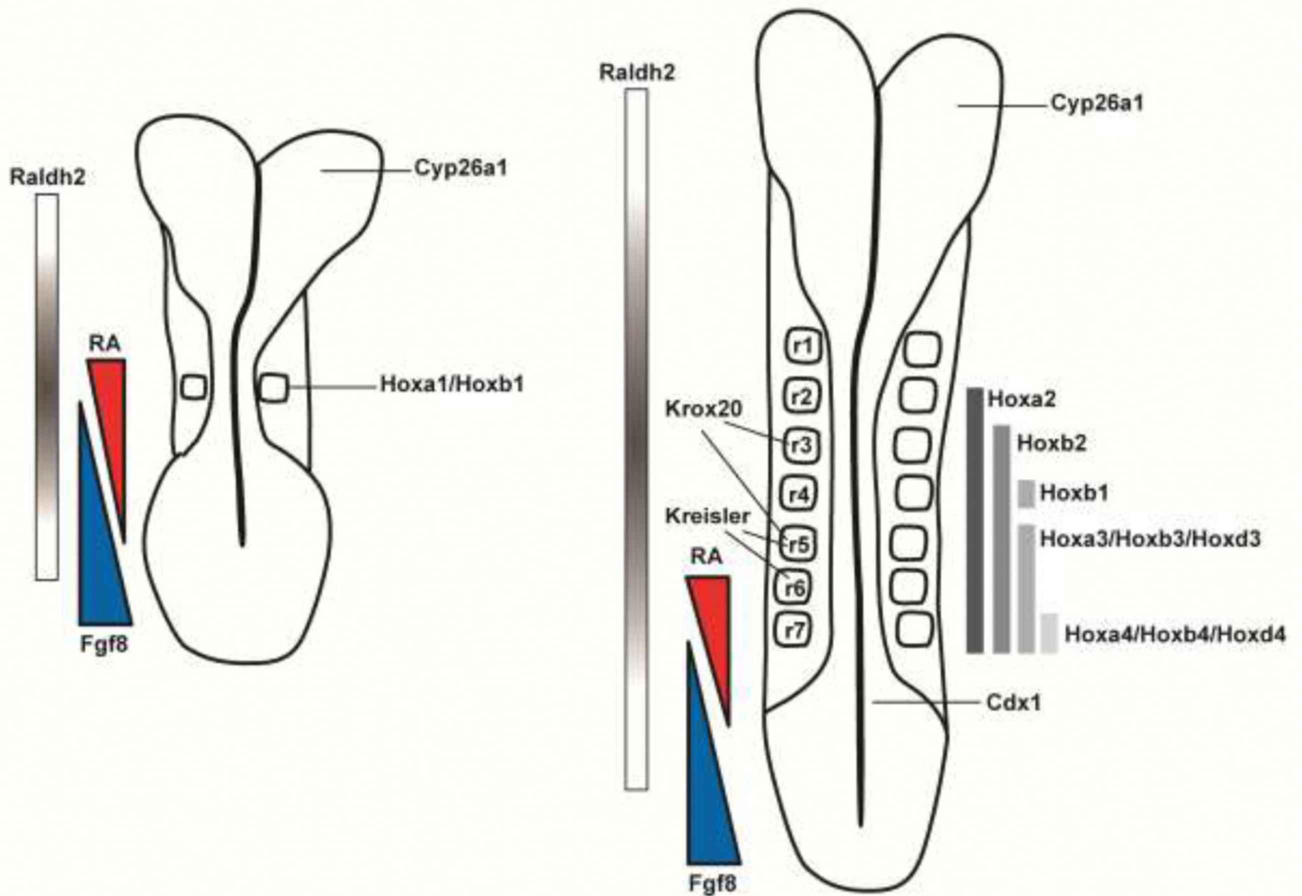

Figure 2. Retinoic acid and anterior-posterior axis formation.
During the formative stages of CNS development, retinoic acid (RA) is regionally restricted and induces posterior embryonic growth. CYP26A1 is exclusively expressed in anterior tissues leading to the breakdown of RA and inactivation of RAR/RXR-signaling. Growth factors (Fgf, Wnt) which promote a posterior phenotype inhibit CYP26A1 and promote RALDH2 biosynthesis of RA, contributing to a gradient of RA availability in the intermediate zone, enabling RAR/RXR activation of HOX genes and other molecules which promote expansion of the posterior domain. Modified from [10, 181].

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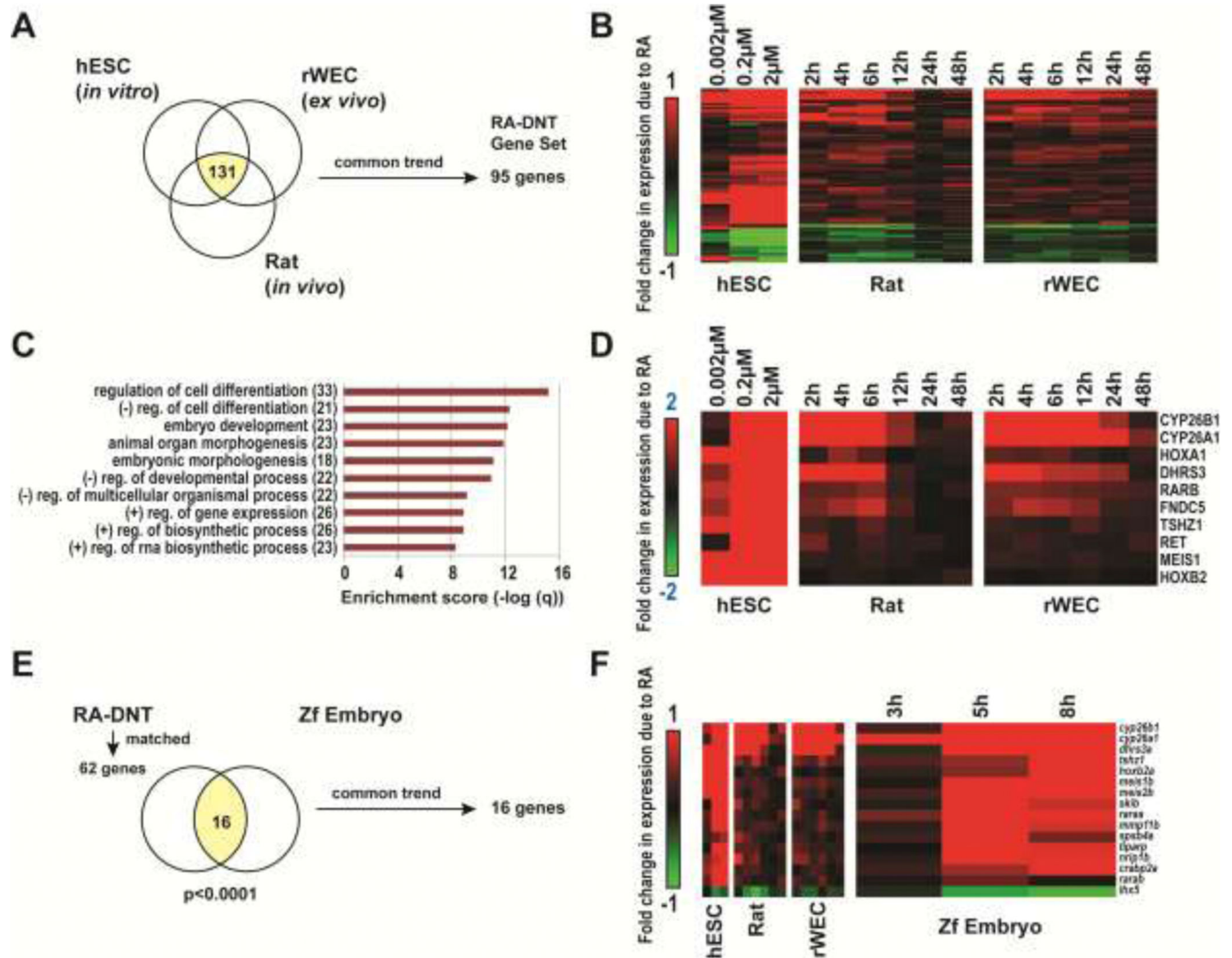

Figure 3. Characterization of common genes identified to be differentially expressed with RA exposure and associated with developmental neurotoxicity in mammalian models.

(**A**) We identified 131 genes to be commonly differentially expressed in association with DNT across *in vitro*, *ex vivo* and *in vitro* models (p<0.005, ANOVA). (**B**) Hierarchical clustering of the subset of 95 genes that displayed common trends in regulation, of which 74 were upregulated and 21 were downregulated with RA exposure in a concentration or time-dependent manner. This subset of genes was termed the RA-DNT gene set. (**C**) Identified enriched GO Biological Processes within the RA-DNT gene set ($q<1*10^{-10}$). (**D**) RA-DNT genes with absolute average fold change 2 (log 2 scale) comparing RA vs. control per study. (**E**) We identified homologues for 64% (61 total genes) of the RA-DNT gene set. Within this subset, we identified 16 genes to be altered with RA in the Zf embryo model. All 16 genes trended in similar fashion to RA-exposed mammalian model systems. Referenced datasets: E-MEXP-3577 [135], GSE33195 [128], and GSE43755 [154].

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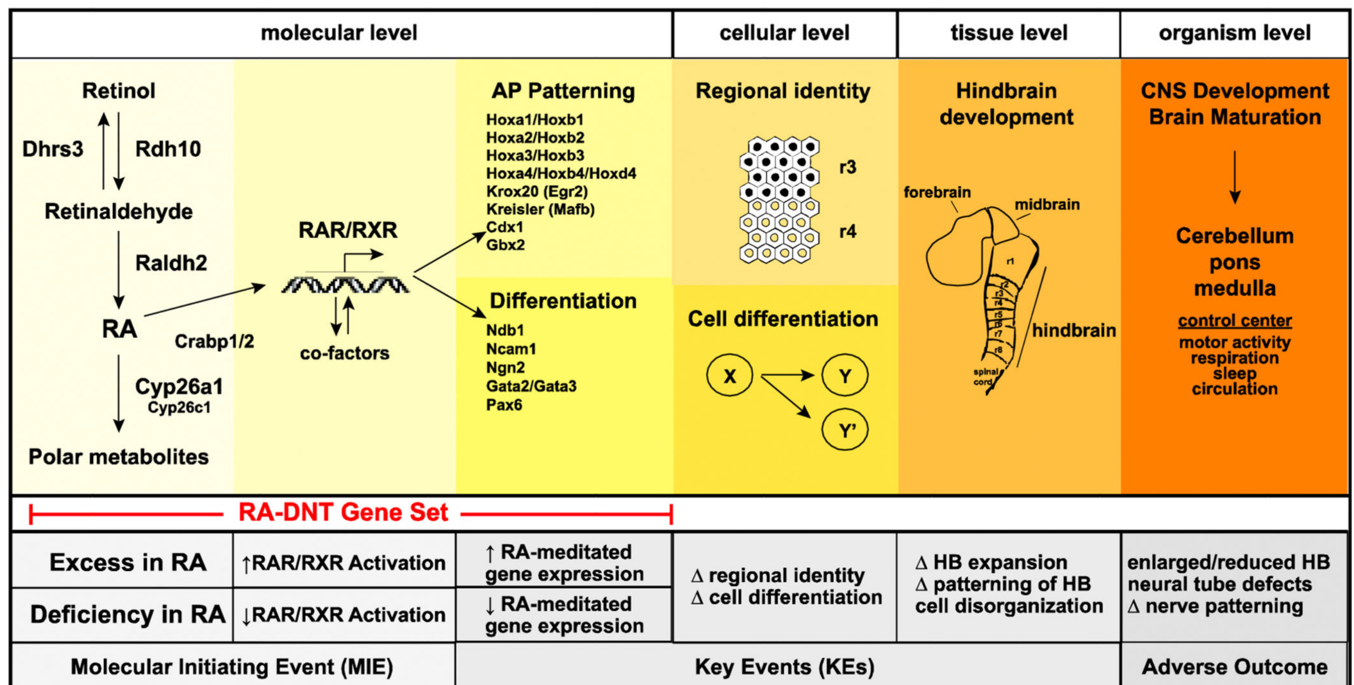

Figure 4. A provisional adverse outcome pathway for RA and hindbrain development.

Under normal conditions during the initial stages of CNS development, RA is regionally restricted by enzymes that promote biosynthesis (*Rdh10, Raldh2*) or elimination (*Cyp26a1, Cyp26c1*) of RA. Binding proteins (e.g., *Crabp1/2*) facilitate RA transfer to the nucleus. RA binding of RAR/RXRs leads to recruitment of co-activators and co-repressors which mediate specificity of transcription. RA mediates expression of multiple gene and gene families involved in patterning and differentiation. On a localized level, changes in expression lead to cell specification (patterning) and cell differentiation, and underlie expansion of the hindbrain and maturation of the CNS. The hindbrain serves as the basis for the cerebellum, pons, and medulla. A prospective MIE that alters RA availability and/or activation of RAR/RXR can lead to KEs that change the expression of genes responsible for regulating cell regional identify and differentiation. These altered pathways underlie perturbations in hindbrain expansion and cell organization, which manifest as adverse outcomes brain development and structure.

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Table 1:

Examples of concentration-dependent effects of RA in diverse models of CNS development and neurotoxicity.

We summarized a selection of studies detailing RA exposures in various models of DNT and their corresponding phenotypes based on increasing concentration.

Model	Conc.	Effect	Reference
human ESC neural rosette	0.002μM	dysregulated expression of RA-dependent signaling pathway members (*e.g.,* Hox genes)	[135]
mouse ESC	0.01μM	increased neural outgrowth and differentiation	[134]
human ESC neural rosette	0.02μM	increased neural cell proliferation and differentiation; dysregulated expression of RA signaling pathways	[135]
zebrafish embryo	0.33μM	dysregulated expression of RA metabolism enzymes, RA receptors, and downstream signaling molecules	[41]
rat maternal plasma (**Cmax; single dose RA, oral)	1.2μM	low teratogencity	[116]
rat whole embryo culture	1.7μM	moderate teratogenicity; dysregulated expression of RA metabolism enzymes and downstream signaling molecules	[128]
human ESC neural rosette	2μM	inhibition of neural differentiation; dysregulated expression of RA signaling pathways	[135]
rat maternal plasma (**Cmax; single dose RA, ip)	4.6μM	low-moderately teratogenic	[118]
rat maternal plasma (**Cmax; single dose RA, ip)	6.3μM	moderately teratogenic	[118]
rat maternal plasma (**Cmax; single dose RA, ip)	9.9μM	highly teratatogenic	[118]

Abbreviations: concentration (Conc.); embryonic stem cells (ESC); concentration maximum (Cmax); intraperitoneal (ip).

** = estimate from figure in manuscript.

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Table 2:

Differentially expressed RA-DNT genes in cultured rat embryos exposed to triazole compounds.

We utilized two datasets (Study A: [152] and Study B: GSE102082 [151]) to assess the transcriptomic effects of triazole compounds in rat whole embryo culture (WEC) at concentration(s) associated with altered morphology, *i.e.*, decline in total morphological score (TMS). Datasets were processed and analyzed for differentially expressed genes using a cutoff of $p<0.05$ (uncorrected). Within this subset, we determined the number of differentially expressed RA-DNT genes, the associated fold enrichment (FE) (*i.e.*, ratio of observed vs. expected), statistical significance (Chi-Squared test with Yates' continuity correction) in identifying with differentially expressed RA-DNT genes, and the agreement in trend with RA response in mammalian models.

Study	Compound (# concentrations)	DE Genes	DE RA-DNT	FE	p-value	Trend w/RA
A	Flusilazole (4)	728	21	4.4	<0.0001	100%
	Cyproconazole (2)	828	22	4.0	<0.0001	95%
	Triadimefon (2)	869	25	4.0	<0.0001	100%
B	Propiconazole (1)	920	31	5.1	<0.0001	100%
	Triadimefon (1)	1056	31	4.4	<0.0001	100%
	Flusilazole (1)	1110	28	3.8	<0.0001	96%
	Ketoconazole (1)	1372	27	3.0	<0.0001	89%
	B599 (1)	1214	20	2.5	<0.0001	95%
	Difenoconazole (1)	747	14	2.8	<0.0001	86%
	B600 (1)	961	16	2.5	0.0002	100%
	Fenarimol (1)	1471	20	2.1	0.0009	95%
	B595 (1)	944	16	2.6	0.0054	100%
	Tebuconazole (1)	969	11	1.7	0.0928	100%
	Miconazole (1)	834	4	0.7	0.7254	100%
	Prothioconazole (1)	1029	8	1.2	0.7806	63%