Ecotoxicology (2011) 20:580–594
DOI 10.1007/s10646-011-0623-3

Conserved toxic responses across divergent phylogenetic lineages: a meta-analysis of the neurotoxic effects of RDX among multiple species using toxicogenomics

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Accepted: 14 February 2011 / Published online: 29 March 2011

Abstract At military training sites, a variety of pollutants such as hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), may contaminate the area originating from used munitions. Studies investigating the mechanism of toxicity of RDX have shown that it affects the central nervous system causing seizures in humans and animals. Environmental pollutants such as RDX have the potential to affect many different species, therefore it is important to establish how phylogenetically distant species may respond to these types of emerging pollutants. In this paper, we have used a transcriptional network approach to compare and contrast the neurotoxic effects of RDX among five phylogenetically disparate species: rat (Sprague-Dawley), Northern bobwhite quail (*Colinus virginianus*), fathead minnow (*Pimephales promelas*), earthworm (*Eisenia fetida*), and coral (*Acropora formosa*). Pathway enrichment analysis indicated a conservation of RDX impacts on pathways related to neuronal function in rat, Northern bobwhite quail, fathead minnows and earthworm, but not in coral. As evolutionary distance increased common responses decreased with impacts on energy and metabolism dominating effects in coral. A neurotransmission related transcriptional network based on whole rat brain responses to RDX exposure was used to identify functionally related modules of genes, components of which were conserved across species depending upon evolutionary distance. Overall, the meta-analysis using genomic data of the effects of RDX on several species suggested a common and conserved mode of action of the chemical throughout phylogenetically remote organisms.

Keywords Genomics · Meta-analysis · Species comparison · $GABA_A$ receptor · RDX

Electronic supplementary material The online version of this article (doi:10.1007/s10646-011-0623-3) contains supplementary material, which is available to authorized users.

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Introduction

The environment is constantly being challenged with an increasing number of stressors that can have serious adverse effects on wildlife. For instance, the release of chemical compounds into various environmental matrices represents a complex risk not only to wildlife, but also to human health. 1,3,5-Trinitro-1,3,5-triazacyclohexane (RDX) has been observed as an environmental contamination primarily at munitions manufacturing plants, load and park operations, firing ranges, and demilitarization areas (Jenkins et al. 2001). Once it enters the environment, RDX has the potential to affect many species. Impacts on the nervous system have been observed in a variety of species exposed to RDX including humans, dogs, rats,

birds and earthworms (Goldberg et al. 1992; Talmage et al. 1999; Gong et al. 2008; Gust et al. 2009). Williams et al. (2011) recently demonstrated that the molecular initiating event for neurotoxicity is binding of RDX to the $GABA_A$ (γ-aminobutyric acid) receptor ($GABA_AR$) where it blocks membrane repolarization.

Chemicals released into the environment can affect very specific targets, such as the endocrine or nervous system. Drug and specific chemical receptors such as the $GABA_AR$ are evolutionarily conserved; therefore the chemicals may potentially affect many different species, even phylogenetically distant organisms depending on sequence conservation of binding sites (Gunnarsson et al. 2008). For example, mechanisms for cell-to-cell communication in pre-metazoan cells also serve as the molecular foundation for the most complex communications network in multicellular organisms, the nervous system (Tsang et al. 2007). Given the known impacts of RDX exposure on nervous system processes, investigation of the molecular functions affected by RDX exposure, including GABAergic signaling (Williams et al. 2011), constitute a key focus for understanding the impacts of RDX across disparate phylogenetic lineages.

GABA receptors have been described in many different phyla such as social amoeba (*Dictyostelium discoideum),* cnidarians, mollusks, annelids, arthropods, nematodes, and chordates (Kass-Simon and Pierobon 2007; Tsang et al. 2007; Kehoe et al. 2009; Fountain 2010). The $GABA_AR$ is an ionotropic receptor and ligand-gated ion channel. When activated, $GABA_AR$ conducts Cl^- through its pore causing a hyperpolarization of the neuronal membrane thereby inhibiting neurotransmission. Several major insecticides, such as α-endosulfane, lindane, fipronil or dieldrin, are non-competitive antagonists for the $GABA_A$ receptor targeting its $\beta 3$ subunit (Ratra et al. 2001). Non-competitive antagonists of the $GABA_A$ receptor can have a very wide structural diversity such as pesticides, picrotoxin, a plant convulsant; or thujone, the active component of absinthe, a popular emerald-green liqueur in the 19th and early 20th centuries (Olsen 2006). All of these non-competitive antagonists are proposed to fit a single binding site in the chloride channel lumen (Fig. 1; Chen et al. 2006). While RDX has a different chemical structure, recent findings via radio-ligand receptor-competition binding assays show that it also acts as non-competitive antagonists through binding to the picrotoxin binding site in the chloride channel of the $GABA_A$ receptor (Williams et al. 2011).

Many receptors and biological systems are highly conserved and operate similarly across different species and conditions. Although still very challenging, cross species analysis of microarray data can lead to insights that cannot be obtained from the analysis of a single species (reviewed in Lu et al. 2009). For example, Ueda et al. (2004)

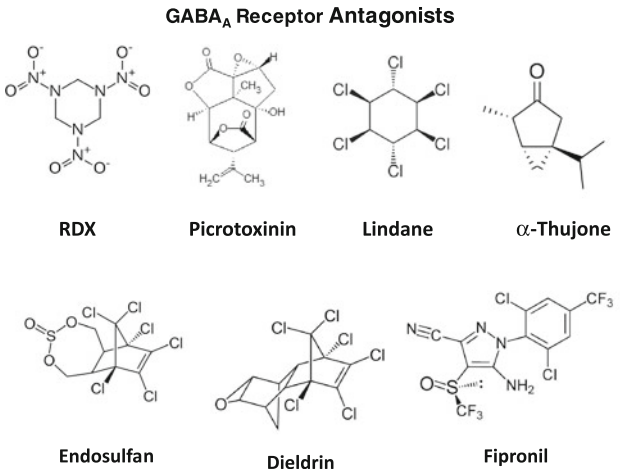

Fig. 1 Chemical structures of several compounds that are noncompetitive agonists against the chloride channel of the $GABA_A$ receptor

identified dynamic expression patterns that are highly conserved across species ranging from bacteria to human. Lu et al. (2007) identified a core set of genes involved in cell cycle across yeast, human, and plants. When comparing microarray datasets across multiple species, researchers face both technological and contextual challenges in comparing expression of divergent genomes. However, successful meta-analysis across species can be used to leverage information in one species with that of less studied species, as well as to find common expression patterns that would reveal core gene functions.

Analysis across species can be divided into two types (reviewed in Lu et al. 2009): expression and co-expression meta-analyses. Expression meta-analysis studies the similarity between expression profiles of homologous genes in different species, while co-expression meta-analysis searches for conserved co-expressed gene clusters across species. One of the advantages of co-expression meta-analysis is that it allows the use of different conditions for the different species studied. Direct cross-species comparison of differentially expressed genes is particularly challenging when trying to compare distant species. Instead, it can be useful to look at enriched GO (Gene Ontology) terms that are conserved across species (Lu et al. 2009). Subramanian et al. (2005) proposed a more sophisticated approach called gene set enrichment analysis (GSEA) to extract biological insight from expression data. GSEA focuses on groups of genes that share common biological function, chromosomal location, or regulation. This analysis can be extended to gene network enrichment analysis (GNEA), based upon the idea that the cell is associated with a protein–protein interaction network and each protein belongs to one or more gene sets associated with biological processes or molecular functions. When the cell is perturbed, some subset of the interaction network becomes affected;

therefore certain functional sub-networks may show significantly altered activity (Liu et al. 2007). Other researchers have used network analysis to identify conserved genetic modules (Stuart et al. 2003) and conserved patterns of protein interactions (Sharan et al. 2005).

In this study, we performed a meta-analysis of previously conducted experiments to understand the neurotoxic effects of RDX across five phylogenetically disparate species: rat (*Rattus norvegicus* Sprague-Dawley), Northern bobwhite quail (*Colinus virginianus*), fathead minnow (*Pimephales promelas*), earthworm (*Eisenia fetida*), and coral (*Acropora formosa*) using toxicogenomics. The rat was used as a model species to examine clinical toxicology of chemicals, because of the sequenced genome, and wealth of information at the physiological level. Other species are environmentally relevant and belong to very different ecosystems that could potentially be exposed to RDX. Northern Bobwhite quail is used as a model wildlife bird species, the earthworm *E. fetida* is a standard toxicity test invertebrate for soils, fathead minnow is a standard toxicity test fresh water fish, and coral belongs to a marine environment where potential exposures could occur. We applied pathway enrichment, GSEA and network analysis to determine the degree to which the impacts of RDX exposure are conserved among these distantly related phylogenetic relatives.

Materials and methods

Exposures

Several datasets contributed to the meta-analysis (Supplementary Table 1). The following provides a brief summary of each of the studies and accompanying datasets that contributed to our current investigation comparing and contrasting the impacts of RDX exposure across species. A more detailed description of methods and materials for the rat, fathead minnow, and coral exposures and microarray measurements of gene expression can be found in Supplementary File 4.

Rat

The effects of RDX on rat brain gene expression were assessed by single gavage exposure of female Sprague-Dawley rats (Habib et al. 2011). Briefly, five separate exposures were conducted consisting of five adult female rats were randomly assigned to an RDX dose administered by oral gavage. Doses consisted of control (5% v/v DMSO in corn oil), and RDX doses of 1.2, 12, 24, and 47 mg/kg in 5% DMSO in corn oil emulsion. Animals were euthanized at 24 h, 48 h, 7 days, 14 days, 28 days, and 90 days post gavage using CO_2. Right and left hemispheres of brain were flash frozen in liquid nitrogen. The left hemisphere was reserved for analytical chemistry analysis and the right hemisphere for RNA isolation. Concentration of RDX in brain tissue was determined by HPLC as in Johnson et al. (2007). Total RNA was extracted from homogenized right hemisphere samples, labeled using an Array900 detection kit (Genisphere, Hatfield, PA) and hybridized to 8 k Sigma/Compugen rat 70-mer oligonucleotide libraries arrayed on glass slides [http://www.cag.icph.or/]. Three biological replicates at each dose including control were examined using a two-color, interwoven loop design. Raw intensity data was normalized using the R package LIMMA (Smyth and Speed 2003) and loess normalization was applied for each group. Channels were separated for each time point and tested for differentially expressed genes by running one-way ANOVA, followed by multiple pair-wise comparisons between the control and the four dose treatments using LIMMA and MultiExperiment Viewer (Saeed et al. 2006). The dataset is available at NCBI GEO (http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE27042 to be inserted.

Northern Bobwhite quail

The details of Northern Bobwhite quail exposures and microarray analysis have been previously described in Gust et al. (2009). This study included two sub-acute (14 days) RDX-exposure bioassays including a high-dose range in which all RDX doses elicited seizures and a low-dose range where RDX induced seizures in a dose–response manner (Johnson et al. 2007; Quinn et al. 2009; Gust et al. 2009). Briefly, whole brain tissue was collected from RDX exposed quail and homogenized. Concentrations of RDX were experimentally determined in brain tissue homogenate as in Johnson et al. (2007). RNA was extracted from brain homogenate. Gust et al. (2009) created a custom 4,119 cDNA spotted microarray based on Northern bobwhite quail brain-tissue RNA. This custom quail array was used with two-color hybridizations and an interwoven loop experimental design to assess RDX impacts on brain tissue gene expression. Statistical analysis of the microarray data was performed using Bayesian Analysis of Gene Expression Levels software version 3.62 with default settings (Townsend and Hartl 2002). Genes were considered differentially expressed if the expression level was different from controls using a 97.5% confidence level measure. The dataset is available at NCBI GEO (http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE27653 to be inserted.

Fathead minnow

Three-day-old fathead minnow (*Pimephales promelas*) fry were exposed to either control water or one of six

concentrations of RDX: 0.9, 1.8, 3.5, 7.0, 13.8, or 27.7 mg/l, in 96-h exposures based on the U.S. EPA (2002) acute testing method 2000.0 for fathead minnow (Warner et al. 2011). The experimental design included four exposure replicates per treatment with ten fish per replicate. Concentration of RDX in exposure water was experimentally determined by HPLC essentially as in Johnson et al. (2007). Fathead minnow samples examined for gene expression effects included solvent control, 0.9, 1.8, 7.0 and 13.8 mg/l RDX treatments. Five individual fathead minnow were pooled together per exposure replicate for RNA extraction. Total RNA was extracted, labeled using the Agilent One-Color Quick Amp Labeling Kit and hybridized to custom 8 × 15 K oligonucleotide microarrays (Agilent Technologies, Santa Clara, CA; NCBI GEO Platform GPL9248) using an Agilent gene expression hybridization kit (Agilent Technologies) following manufacturer's recommendations. RNA was hybridized to arrays using a randomized block design. Microarray data was analyzed using GeneSpring version GX 11.0.2 (Agilent Technologies) to normalize data by 75th percentile shift and conduct baseline transformation to the median of all samples. Statistical analysis was performed using GeneSpring utilizing data from all microarray probes and consisted of one-way ANOVA with Benjamini–Hochberg multiple-testing corrections and a 1.5 fold change cutoff to test for significant expression. The dataset is available at NCBI GEO (http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE27067 to be inserted.

Earthworm

Earthworm (*Eisinia fetida*) exposures to RDX and gene expression analysis have been described in detail by Li et al. (2010) and Gong et al. (2010). Briefly, earthworm exposures were conducted in a field collected silty loam soil in accordance with the American Society for Testing And Materials guideline (ASTM 1997). Earthworms were exposed to soil spiked with RDX (0, 8, 16, 32, 64, or 128 mg/kg) for 0 (control only), 4 or 14 days. RNA from individual replicate worms was hybridized to custom 15 K 60mer oligonucleotide custom microarray (Agilent Technologies, NCBI GEO platform accession number GPL9420) using the Agilent One-Color Microarray Hybridization protocol as recommended by the manufacturer. The dataset is available at NCBI GEO (http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE18495. Differentially expressed genes were identified using the Class Comparison Between Groups of Arrays Tool in BRB-ArrayTools v.3.8 software package (Simon et al. 2007).

Coral

The effects of RDX on coral gene expression were assessed by exposure of the branched coral *Acropora formosa* in aquaria (Gust et al. 2011). Briefly, five replicate coral fragments of *A. formosa* (Oceans, Reefs and Aquaria, Fort Pierce, FL) were exposed to RDX concentrations of 0.49, 1.77, and 7.18 mg/l (measured concentrations) in 10-gallon aquaria for 5 days. RNA samples extracted from the exposures were used to construct a normalized cDNA library, sequenced, and used to develop a 15 K 60mer oligonucleotide custom microarray (Agilent Technologies, Design ID# 020980). Gene expression was measured using Agilent One-Color Microarray Hybridization protocol as recommended by the manufacturer. Microarray data were normalized within array to the 50th percentile and then to the median signal for each gene among arrays using GeneSpring version 7.3 (Agilent Technologies, Sta. Clara, CA). Differentially expressed genes were identified using one-way ANOVA with Benjamini and Hochberg multiple testing corrections (Benjamini and Hochberg 1995) followed by a post-hoc test (volcano plot) including a parametric *t*-test ($p = 0.05$) and fold change cutoff of $\geq$1.5. The dataset is available at NCBI GEO (http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE27624.

Phylogenetic analysis

Sequences for different $GABA_A$ receptor subunits from several species were obtained from NCBI databases (http://www.ncbi.nlm.nih.gov) and their amino acid sequences were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/; Chenna et al. 2003). When the amino acid sequence was not available, the nucleotide sequence was translated using the ExPASy Proteomics server (http://expasy.org/tools/dna.html). ClustalW2 was used to calculate the phylogenetic tree for each subunit using the neighbor-joining method of Saitou and Nei (1987). Distances were calculated first by calculating pairwise scores as the number of identities in the best alignment divided by the number of residues compared (gap positions are excluded). Both of these scores were initially calculated as percent identity scores and were converted to distances by dividing by 100 and subtracting from 1.0 to give number of differences per site.

Pathway analysis

All gene probes from microarrays were annotated with gene accession numbers and mapped to gene symbols of Human homologs and Zebrafish (*Danio rerio*) homologs

using DAVID (Huang et al. 2009). Statistically significant enriched pathways in differentially expressed gene data sets were identified using SubpathwayMiner, an R-based package (Li et al. 2009). *p*-values were calculated using a hypergeometric distribution. The enriched pathways obtained for each species are shown in Supplementary File 1.

Gene set enrichment analysis

Gene Set Enrichment Analysis (GSEA; Subramanian et al. 2005) was run on gene lists from each differentially expressed gene data set representing a subset of genes mapping to gene symbol homologs and corresponding *p*-values with 100,000 permutations. A label permutation was implemented to determine the significance of the enrichment score accounting for differences in set size while correcting for the number of gene sets tested. The canonical pathways were obtained from three different databases: BioCarta gene sets (BIOCARTA; http://www.biocarta.com), Kyoto Encyclopedia of Genes and Genomesgene sets (KEGG; http://www.genome.jp/kegg/), and Reactome gene sets (REACTOME; http://www.reactome.org/). GO terms were obtained from three different sets: Biological Processes (BP), Cellular Components (CC), and Molecular Function (MF). The MOTIF output contains microRNA targets (MIR) and transcription factor targets (TFT).

Network analysis

The information theory based algorithm Context Likelihood of Relatedness (Faith et al. 2007) was used to infer a transcriptional network from normalized expression data of differentially expressed gene data set from rat brain after exposure to RDX using all the genes present in the array. The resulting network was filtered to create a neurotransmission subnetwork (Suppl. Fig. 1) using a list of genes (Suppl. Table 2) related to neurotransmission from KEGG pathways and a few additional genes reported by other studies related to RDX exposure (obtained using *WhichGenes*, Glez-Peña et al. 2009). The subnetwork was filtered from the source network using the list of genes from neurological functions as the seed nodes and their first connected neighbors. Clusters or highly interconnected regions in the subnetwork were identified using MCODE, which measures the "cliqueness" of a vertex (Bader and Hogue 2003), as implemented in Cytoscape (Cline et al. 2007). Genes from the six most highly connected clusters were analyzed for GO and Pathway enrichment using DAVID (Huang et al. 2009) (Suppl. File 3).

Results

Physiological effects

The physiological effects observed in the different exposures are summarized in Table 1. All species showed bioaccumulation of the chemical. Many different physiological effects were observed on rat, such as seizures, lethargy, decreased weight gain, anemic effects, splenic hemosiderosis, and alterations in white blood cells, lymphocytes, and total granulocytes. Acute exposures in Northern bobwhite quail also produced seizures and anemic effects, as well as altered blood chemistry. Liver edema was observed in both quail and rat exposures (Meyer et al. 2005; Johnson et al. 2007; Quinn et al. 2009) while swelling/edema and constriction/autotomy was observed in high dose exposures of earthworms (Gong et al. 2008). Neurotoxicity symptoms observed in earthworms exposed to RDX include rigidity/shrinking (chlonic seizure) and significantly reduced impulse conduction in medial (MGF) and lateral (LGF) giant nerve fiber pathways (Gong et al. 2008). Quail and rat exposed to RDX presented chlonic and tonic seizures (Johnson et al. 2007; Meyer et al. 2005). No neurological effects were observed in exposed fathead minnows and could not be measured in coral. The effects of RDX exposure in coral included an increase in mucocytes and altered bacterial community composition inhabiting the coral surface microlayer (Gust, personal observation). Observed impacts in fathead larvae included spinal deformities and death whereas mortality was the only affect observed in sub-adult exposures (Warner, personal observation).

Relatedness of $GABA_AR$ subunits

The evolutionary relatedness of different $GABA_AR$ subunits—α1 (GABRA1), α6 (GABRA6), β1 (GABRB1), β2 (GABRB2), β3 (GABRB3), and δ (GABRD)—were examined to determine the similarity of different subunits across species (Fig. 2). The phylogenetic analysis shows that sequences from more closely related species, e.g. mammals and avian species, tend to cluster together, while species such as arthropods, annelids or cnidarians, are more distant in the phylogenetic tree.

Pathway analysis

The differentially expressed gene data set for each organism was examined for biological pathways that might be significantly impacted by RDX (Supplementary File 1). The total number of enriched pathways for each species was 148 for rat, 23 for quail, 74 for fathead minnows, 21 for earthworm, and 26 for coral. Only two general

Table 1 Physiological effects of RDX across species

Effects	Rat	Quail	Fathead minnow	Earthworm	Coral
Impacted neuronal function	Chlonic tonic seizures[a]	Chlonic tonic seizures[b]	ND[c]	Chlonic seizures[d]	NA
Edema	Liver[e]	Liver[b]	ND[c]	Whole body[d]	NA
Mucocyte increase	NA	NA	NA	NA	√[f]
Altered blood chemistry	√[e]	√[d]	NT	NT	NA
Spinal deformities	NT	NT	Larvae[h]	NA	NA
Toxic dose	100–187 mg/kg LC50[e]	8 mg/kg LC25[b]	10 mg/l 10 days exposure LC50 Adult[c]; 3.5–13.8 mg/l 96 h larvae LC50[h]	>10,000 mg/kg Soil[g]	>8 mg/l 5 days, no effect seen[f]
Lowest RDX concentration with effect on central nervous system (seizures etc.)	25 mg/kg[a]	8 mg/kg[b]	<10 mg/l showed no behavioural impact reported[c]	20 mg/cm^2 filter paper[d]	NA

[a] Burdette et al. (1988)

[b] Quinn et al. (2009)

[c] Gust et al. (2011)

[d] Gong et al. (2008)

[e] Meyer et al. (2005)

[f] Lotufo (2011)

[g] Simini et al. 2003

[h] Warner et al. (2011)

NA Not applicable, *ND* not detected, *NT* not tested

pathways were common to all species: Metabolic Pathways and Glycolysis/Gluconeogenesis. Pathways related to neurodegenerative diseases such as Alzheimer's, Huntington's, and Parkinson's disease, were significantly enriched in all species except coral. These pathways were also present in coral but were not significantly enriched (p-value > 0.2). Glutathione metabolism was enriched in all species except for quail. The gonadotropin-releasing hormone (GnRH) signaling pathway was enriched in coral as well as in quail and rat. No overlap was seen in homolog gene names between all species and very few between any given species.

Several of the exposures revealed an impact on pathways related to energy and metabolism function. Rat brain transcriptional expression was enriched in metabolic pathways related to Insulin signaling pathway, starch and sucrose metabolism, ascorbate and aldarate metabolism, PPAR signaling pathway, glycolysis/gluconeogenesis, type I diabetes mellitus, tyrosine metabolism, arginine and proline metabolism, tryptophan metabolism, galactose metabolism, fructose and mannose metabolism, fatty acid metabolism, and glycerolipid metabolism. Quail brain gene expression was enriched in gastric acid secretion, metabolic pathways, ribosomes, glycolysis/gluconeogenesis, GnRH signaling pathway, and the insulin signaling pathway. Fathead minnow was enriched several metabolic pathways including amino sugar and nucleotide sugar metabolism, purine metabolism, protein digestion and absorption, galactose metabolism, glutathione metabolism, fatty acid metabolism, PPAR signaling, glycine, serine and threonine metabolism, carbohydrate digestion and absorption, glyoxylate and dicarboxylate metabolism, type II diabetes mellitus, and glycolysis/gluconeogenesis. Earthworm differentially expressed genes were enriched in metabolic pathways including tryptophan metabolism, pyruvate metabolism, glycolysis/gluconeogenesis, ascorbate and aldarate metabolism, glycine, serine and threonine metabolism, and propanoate metabolism. Coral gene expression was enriched in metabolic pathways related to the citrate cycle (TCA cycle), glycolysis/gluconeogenesis, glyoxylate and dicarboxylate metabolism, and one carbon pool by folate.

Gene set enrichment analysis

Enriched canonical pathways

Six Canonical Pathways were found enriched in the rat dataset using GSEA (Supplemental File 2). The GnRH signaling pathway was most highly enriched followed by focal adhesion and integrin pathways. In quail, nine pathways were significantly enriched related to glucose

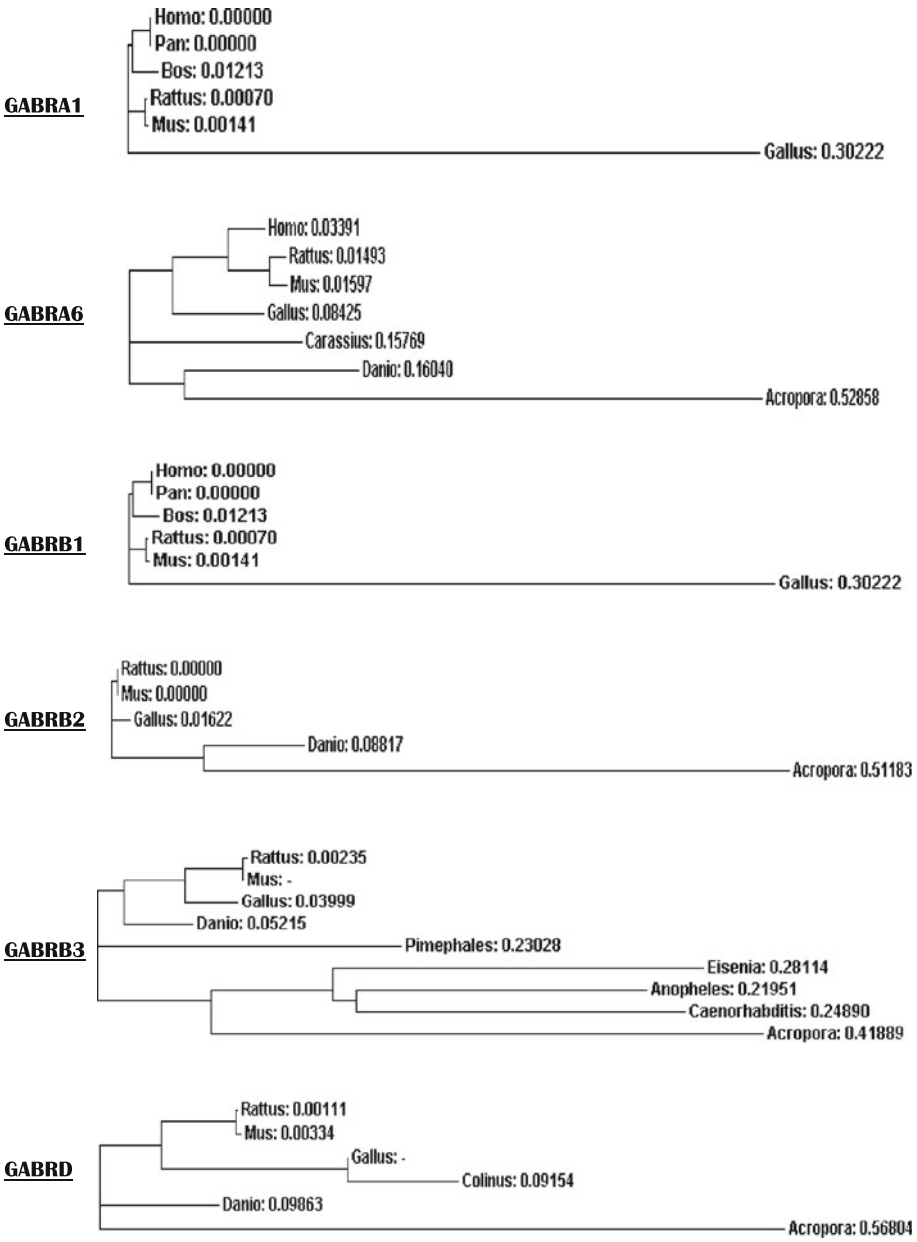

Fig. 2 Phylogenetic trees of different $GABA_A$ receptor subunits using sequences of several species: *Homo sapiens* (GABRA1: NM_000806; GABRA6: NM_000811; GABRB1: NP_000803); *Pan troglodytes* (GABRA1: XM_001144938; GABRB1: XP_001154620.1); *Bos Taurus* (GABRA1: DAA27203; GABRB1: NP_776969); *Rattus norvegicus* (GABRA1: NM_183326; GABRA6: NM_021841; GABRB1: NP_037088; GABRB2: NM_012957; GABRB3: NM_017065; GABRD: NM_017289); *Mus musculus* (GABRA1: NP_034380; GABRA6: AAI45159; GABRB1: NP_032095; GABRB2: NP_032096; GABRB3: NP_032097; GABRD: NP_032098); *Gallus gallus* (GABRA6: NM_205058; GABRB2: XP_414492; GABRB3: NM_205346; GABRD: XM_001234040); *Carassius auratus* (GABRA6: X94342); *Danio rerio* (GABRA6: NM_200731; GABRB2: NM_001024387; GABRB3: XP_695300; GABRD: XP_700099); *Acropora millepora* (GABRA6: EZ037104; GABRB2: EZ006261; GABRB3: EZ023821; GABRD: EZ002403); *Pimephales promelas* (GARBR3: S26846347); *Eisenia fetida* (GABRB3: SMContig_972); *Anopheles gambiae* (GABRB3: XP_311123); *Caenorhabditis elegans* (GABRB3: NP_499661); and *Colinus virginianus* (GABRD: Contig7366). The numbers associated with genes are the genetic distance from a common ancestor scores. Genetic distances were initially calculated as percent identity scores and were converted to distances by dividing by 100 and subtracting from 1.0 to give the number of differences per site

metabolism and regulation of insulin secretion with a tenth related to Alzheimer's disease (Supplemental File 2). Thirty-two pathways were significantly enriched in fathead minnow, many of which are related to G-protein signaling in addition to regulation of insulin secretion. Ten Canonical Pathways were significantly enriched in earthworm including regulation of insulin secretion, Huntington's and Alzheimer's disease. Five Canonical Pathways are

enriched in coral including tight junction pathways, three pathways associated with injury repair (Adenosine diphosphate signaling through the P2Y Purinoreceptor 1, Platelet activation triggers, and Signal amplification for platelets), and Opioid signaling. The GSEA and pathway analysis used different enrichment algorithms and many pathways were identified by both methods, e.g., glycolysis, Alzheimer's disease, and Huntington's disease.

Enriched gene ontology categories

In rat, we found 23 GO categories significantly enriched, some of which were immune system process, cation channel activity, and G protein coupled receptor activity. There were 32 enriched GO terms in the fathead minnow, such as metabolic process, apoptosis, and neurological system process. The most enriched GO term in the earthworm was nervous system development, followed by enzyme regulator activity and system development. No significantly enriched GO terms were found in quail or coral. The only GO terms found in common between organisms were between fathead and earthworm data sets (Anatomical Structure Development, System Development, and Multicellular Organismal Development).

Transcriptional network analysis

We created a reference or 'source' network based on rat gene expression responses to RDX in order to provide functional context for genes impacted by RDX across all species. The network was created using all the genes present in the rat array with all the different values across all conditions. We then filtered the source network to create a 'neurotransmission subnetwork' focusing on a list of genes related to neurotransmission (Suppl. Fig. 1). The neurotransmission subnetwork was filtered from the source network by using the list of genes related to neurotransmission (Suppl. Table 2) as the seed nodes and their first connected neighbors. Approaches using overlapping gene annotation or pathways revealed little overlap between species, perhaps due to several factors including incomplete coverage of transcriptomes, inadequate annotation, and poor overlap of microarray probes. Using the rat neurotransmission network as a reference network, we mapped differentially expressed gene lists from each organism onto the rat network. By using this approach, we associated genes to a common function/transcriptional context (neurotransmission) even with genes whose annotation does not overlap with neurotransmission pathways or directly match genes impacted by RDX in other species. Each organism had significant percentages of differentially expressed genes that mapped onto the rat neurotransmission pathway. Quail shared 50.7% of its RDX affected genes with the rat network. Fathead minnow shared 15.2% of its affected genes with the rat network. Earthworm shared 13.7% of its affected genes with the rat network. Finally, coral shared 20% of its affected genes with the rat network. The significant overlaps of RDX affected genes in each species suggests a common impact of RDX on components related to neurotransmission in all species (Table 2).

The rat neurotransmission sub-network contained six highly connected and enriched clusters (Fig. 3). Cluster 1 was enriched in genes related to oxidative phosphorylation, Parkinson's disease, Alzheimer's disease, Huntington's disease, and Glucolysis/Gluconeogenesis. Cluster 2 was enriched with genes related to proteasome, endocytosis, and insulin signaling pathways. Cluster 3 was enriched with genes related to TGF beta signaling pathway, cytokine-cytokine receptor interaction and Jak-STAT signaling pathways. Cluster 4 was enriched in neuroactive ligand-receptor interaction. Cluster 5 was enriched in genes related to oxidative phosphorylation, Parkinson's disease and Huntington's disease. Finally, cluster 6 was enriched in genes related to Parkinson's, Alzheimer's and Huntington's disease, as well as oxidative phosphorylation.

An investigation of GABA-related genes relative to the six highly connected and enriched clusters showed varying degrees of network connectivity (Fig. 4). The results demonstrated that the first and second neighbors connected to GABA-related genes were highly concentrated in clusters 1 and 5, were connected to a limited degree in cluster 2, and were minimally/not connected to all other clusters.

Table 2 Number of differentially expressed genes for all species, differentially expressed genes present in the sub-network, and differentially expressed genes present in clusters 1 and 5 for all species

Species	DEGs	# DEGs in sub-network	% Genes in sub-network	Genes in cluster 1 & 5	% Genes in cluster 1 & 5	% Subnetwork genes in cluster 1 & 5
Rat	1992	963	48.3	430	22	45
Quail	71	36	50.7	12	17	33
Fathead minnow	2095	419	20	84	4	20
Earthworm	356	49	13.76	13	4	27
Coral	243	37	15.23	6	2	16

DEG Differentially expressed genes ($p < 0.05$)

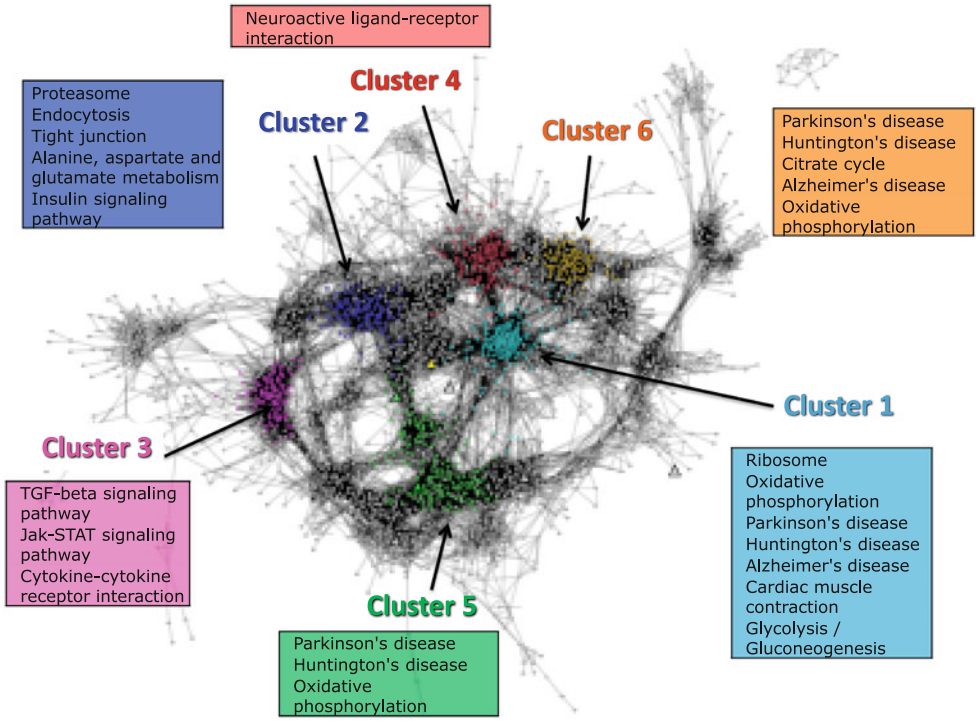

Fig. 3 Neurotransmission subnetwork created filtering the source network using a list of genes related to neurotransmission and their first connected neighbors. Clusters or highly connected regions were identified using MCODE. Genes from the six most highly connected clusters (Clusters 1–6 in the figure) were analyzed for GO and Pathway enrichment using DAVID. Each cluster is highlighted with a different color to differentiate from the other clusters

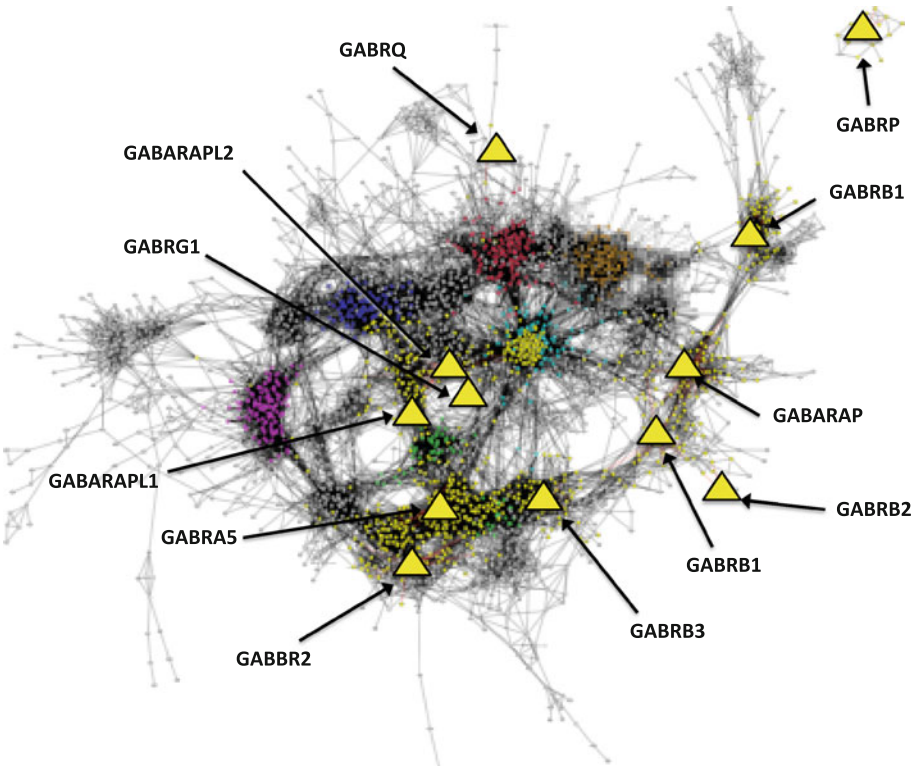

Fig. 4 All GABA-related genes present in the neurotransmission sub-network are marked with a *yellow triangle*. Their first and second neighbors (connected genes) are highlighted in *yellow*

Most notably GABRB3, the $GABA_AR$ subunit specifying the binding site for RDX lies within cluster 5.

Since both GO term enrichment and $GABA_AR$ component linkages indicated that genes in clusters 1 and 5 are associated with neurotransmission and $GABA_AR$ expression, we explored whether genes affected by RDX in other species would map to these regions. Rat homologs from each differentially expressed gene data set for each species were mapped onto genes present in clusters 1 and 5 (Fig. 5a, b) and 2, 3, 4 and 6 (supplemental Fig. 3). A large number of genes present in the sub-network were also differentially expressed in the other species, especially genes included in clusters 1 and 5 with comparatively few included in clusters 2, 3, 4 and 6 (Table 2).

Discussion

Through a combination of phylogenetic comparison of the molecular initiating event for RDX toxicity ($GABA_AR$,), bioinformatic analysis of RDX impacts on pathways and functions in brain and tissue in five different species, and transcriptional network analysis of RDX impacts across species, we have demonstrated that responses to RDX exposure are highly conserved within closely related species. As evolutionary distance increased, effects related to the molecular initiating events, $GABA_AR$ interaction decreased and more general effects on metabolic function increased.

Conserved effects

Prior studies have shown RDX to cause similar toxicological effects on a wide range of species from humans (Davies et al. 2007), rat (Burdette et al. 1988), Northern bobwhite quail (Johnson et al. 2007), lizards (McFarland et al. 2009), to earthworms (Gong, personal communication). While each experimental condition had differences in exposure route, dosages and delivery, comparison of rat, Northern bobwhite quail, fathead minnow, earthworm and coral indicate that RDX has some degree of toxicological effect on all species tested (Table 1). Species with $GABA_AR$ more phylogenetically related to rat tended to have greater similarity in toxicological effects. For example, at high concentrations of RDX liver edema were observed in rat and quail and general edema in earthworms. Seizures were reported as a sub-lethal effect in rat, quail, and earthworms. While quail and rat presented both chlonic and tonic seizures, earthworm only presented chlonic seizures, suggesting that while there are some phylogenetically conserved toxic effects, the evolutionary distance can also be responsible for differences in toxicity. Additionally, 10-d fathead minnow exposures did not elicit seizure like effects although changes in brain gene expression were observed (Gust et al. 2011). However, Mukhi et al. (2005) reported behavioral effects such as whirling movement and lethargic behavior in 96 h larval acute toxicity tests of zebrafish. Overt toxicity due to RDX exposure varied widely across species with earthworms displaying reversible neurological effects at low levels of RDX but no lethal effects to concentrations of 1,000 mg/Kg RDX (Simini et al. 2003).

With the exception of fathead minnow, each of these organisms has been observed to display similar acute effects with high doses of RDX. Impacts on fathead minnow may not have been observed as the maximal dosing examined was 10 mg/l. Sheepshead minnow bioaccumulate RDX by a factor of 0.7 (Lotufo 2011), suggesting tissue concentrations of RDX did not reach concentrations high enough to cause seizures or behavioral effects. In other vertebrates, doses of 8–25 mg/kg body weight are required to elicit seizures. Coral is the most evolutionarily divergent species and has the least amount of similarity in terms of functions related to cellular signaling. This helps demonstrate that the more distant the species, the less conserved the effect if a chemical receptor binding interaction is involved.

Functional analysis

While few differentially expressed genes were common among the experiments examined, a number of pathways and functional groups were found in common between species. We used two different approaches to conduct the functional analyses: GSEA and SubPathway Miner. Although the two approaches utilize unique algorithms, both approaches found glycolysis/gluconeogenesis, GnRH signaling pathway, Alzheimer's disease, Parkinson's disease, Huntington's disease or opioid signaling as highly significant for most species. Results unique to each approach include the observation of enrichment glutathione metabolism (SubPathway Miner) and Guanine nucleotide binding protein (G-protein) related pathways (GSEA).

In addition to neurological effects, there was a common effect observed on significantly enriched Pathways related to energy and metabolism function. Whether this is due to general stress effects or a more direct effect of RDX remains to be determined. General/oxidative/other stress may be an indirect effect or due to oxidative stress. This impact could be a larger component in overall responses once the $GABA_AR$ becomes too divergent to bind RDX and cause an effect.

Network analysis

A transcriptional subnetwork focusing on neurotransmission pathways was created from the rat dataset to compliment the functional analysis describing the most common

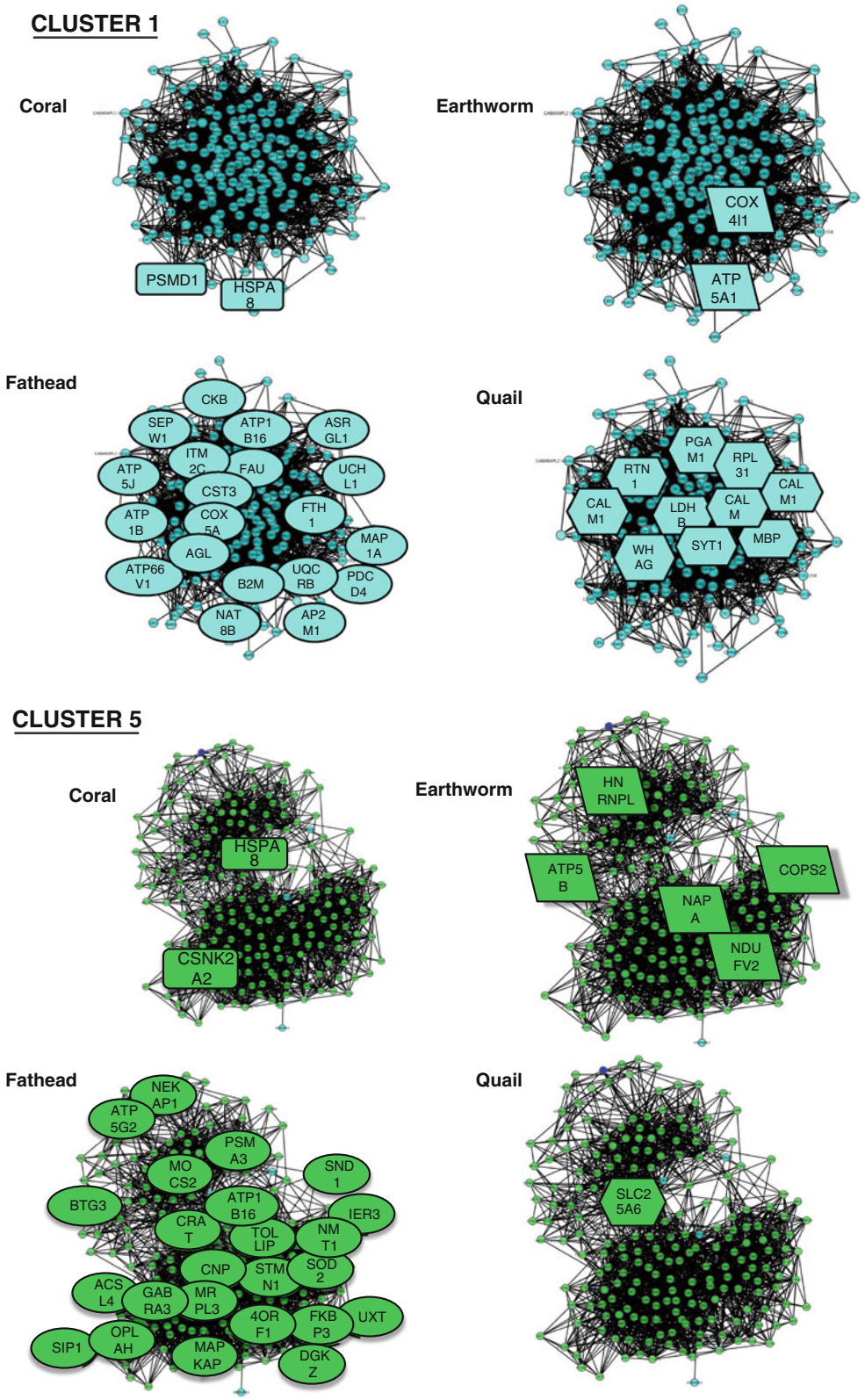

Fig. 5 Clusters 1 (a) and 5 (b) from the neurotransmission sub-network. The differentially expressed genes are indicated with a different shape for each species

effects of RDX exposure among species (Fig. 3). This was used for among-species comparisons to group differentially expressed genes on to common functionally enriched clusters on the rat transcriptional network. Six more highly connected clusters of genes in the rat neurotransmission network were enriched in pathways related to Parkinson's, Alzheimer's, and Huntington's disease; oxidative phosphorylation or glycolysis/gluconeogenesis, and others, in concordance with the results from the functional analysis (Fig. 3). A significant number of differentially expressed genes for each species were mapped to the subnetwork (50.7% for quail; 20% for fathead minnow; 13.8% for earthworm; and 15.2% for coral). The relationship of evolutionary distance of an organism to mapping on the rat neurotransmission network is made difficult by the differences in microarrays in terms of common gene coverage, however many functional responses to RDX exposure are conserved from rat to coral.

The $GABA_AR$ is a known binding target for RDX where it binds to the GABRB3 subunit in the chloride channel (Williams et al. 2011). Therefore, we examined the involvement of genes related to GABA receptors in the rat neurotransmission subnetwork (Fig. 4). $GABA_AR$ related genes are distributed throughout the transcriptional network often in or near dense gene clusters. Genes whose transcriptional responses appear to be connected to the $GABA_AR$ related genes (first and second neighbors) were highly enriched within Clusters 1 and 5 indicating these groups are related to $GABA_AR$ and GABRB3 function. A significant number of differentially expressed genes from each species mapped to these clusters indicating conservation of RDX effects (Fig. 5a and b, Table 2). Given that the network was inferred from rat data, all genes were affected in the rat exposures. Many of these genes are related to ATPases and ATP synthesis, crucial for energy release and ion exchange. Another important gene present in these clusters is calmodulin, which has been linked to the regulation of neuronal excitability (Gust et al. 2009; Marsden et al. 2010). Some of the genes present in the sub-network belong to the heat-shock protein family (271 Da, 70 kDa, and 90 kDa). Heat-shock proteins play a neuroprotective role against harmful insults in the central nervous system (Kiang and Tsokos 1998; Akbar et al. 2003). The network approach is suggesting new connections and targets that could be affected by RDX exposure.

Evolution meets mechanisms of action

Neuronal action potentials are essential for nervous system signaling and depend on a continuous supply of glucose to support overall neuronal physiology (Sokoloff 1977). Endogenous phosphorylation is required for maintaining the $GABA_A$ currents. Laschet et al. (2004) identified GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as the kinase responsible for this endogenous phosphorylation. GAPDH has a dual role: as a dehydrogenase in the glycolysis cascade contributing to ATP production, and as a kinase phosphorylating the $GABA_A$ receptor. The ATP produced locally is consumed for the phosphorylation. Laschet et al. (2004) also demonstrated that $GABA_A$ responses are maintained by a glycolysis-dependent phosphorylation, providing a molecular mechanism for the direct involvement of glycolysis in neurotransmission. Furthermore, dysfunction of $GABA_A$ receptor glycolysis-dependent modulation has been linked to epileptic seizures (Laschet et al. 2007).

These observations, in conjunction with the results of our network analyses identifying cross-species impacts on glycolysis/gluconeogenesis confirms the important role that glycolysis may play in RDX toxicity in all species investigated. Additionally implicated in both the functional and network analyses, Parkinson's, Huntington's and Alzheimer's disease have also been linked to $GABA_A$ receptor and GABAergic neurons (Cepeda et al. 2004; Rissman et al. 2007). Also related to GABA signaling, GnRH (gonadotropin releasing hormone) signaling pathway was also significantly impacted in RDX exposures. GnRH is synthesized and released from neurons within the hypothalamus, and GABA and $GABA_A$ receptor have been shown to be involved in the release of GnRH and subsequent gonadotropin hormones activation and release (Han et al. 2002). Glutathione metabolism was found significantly enriched in most species by the functional approach. Glutathione plays a crucial role in many metabolic and biochemical reactions such as DNA synthesis and repair, amino acid transport or enzyme activation and is recognized as major mechanism for mitigating oxidative stress (Schulz et al. 2000; Fernández-Checa et al. 1998). RDX exposure has been implicated as a potential source of oxidative stress in the brain tissue of fathead minnow (Gust et al. 2011). Further, glutathione metabolism has been linked to anomalies in GABAergic neurons, Parkinson's disease, and chronic seizures (Cabungcal et al. 2006; Zeevalk et al. 2007; Lin et al. 2010). The GSEA approach also highlighted the importance of G-protein related pathways in most species. G-proteins (Guanine nucleotide binding protein) are membrane-associated heterotrimeric proteins that together with their receptors (GPCRs) form one of the most prevalent signaling systems. G-protein signaling pathway has been linked to regulation of seizures (Mazarati et al. 2006). Our results suggest that both methods are very useful in detecting important pathways, and they are also complementary.

Methodology caveats

The brain is composed of many different specialized subregions each with potentially different responses to a stimulant, chemical or drug. The experiments with rats and quail were done prior to understanding where the site of action of the chemical is. Whole brains were examined to capture effects on the brain as a whole and to develop working hypothesis for further investigation. Investigations into impacts on genes involved in neuronal function in the brain were performed using brain homogenate to remove any spatial heterogeneity in expression. Under these conditions we have essentially pooled the different sections of the brain to examine all potential changes in gene expression. By doing so we have been able to detect changes in many specific genes consistent with a chemicals mode of action. The network is essentially constructed based upon gene expression going up or down. The edges are a correlation not a direct interaction, therefore one gene can influence another genes expression indirectly by via signaling and other mechanisms even if they are in different cell types. Nevertheless, we believe that a more specific exposure would provide more accurate information.

The principle goal of the analysis was to use this network approach to build hypothesis about effects across widely different species. The papers analyzed used samples ranging from whole brain to whole animal. Therefore we have made an endeavor to examine on common pathways and components present in the system with a focus on neurotransmission.

Our analysis is an exploratory analysis in applying network analysis to gain a greater understanding of potential function through clustering of expressed genes. Genes of related function have been observed to form coherent clusters in transcriptional networks, much as proteins with common functions are observed to form modules in protein:protein interaction networks. Indeed, we find that the networks does have functional enrichment of genes in the modules 1–6 described in the paper in areas specifically relating to functions that RDX may impact. These modules are statistically enriched for these functions. While admittedly speculative, we have used a "guilt by association" hypothesis building approach that genes mapping to these functionally enriched modules have a significant likelihood of being involved in this function. Additional support is given by the connectivity of modules 1 and 5 to $GABA_AR$ genes. However promising that appears we realize that the network construction is likely imperfect with many false positive edges. Nevertheless, we hope that this approach will be useful when new experiments are planned as well as help with the development of the methodology.

Conclusions

Although there were complicating factors in integrating diverse experimental datasets such as dosing, exposures, species, and array platform, toxicogenomics and network analyses were able to detect common mechanisms underlying toxic effects among these five phylogenetically distant species. Each species has effects consistent with a common molecular initiating event, binding to the $GABA_AR$ or $GABA_A$-like receptor, even in species with no organized neuronal system. Furthermore, our analysis also suggested potential impacts on GnRH hormone signaling and glycolysis/gluconeogenesis that might represent underlying mechanisms for indirect effects. In addition to neurological effects, there is a common effect on significantly enriched Pathways related to energy and metabolism function. Whether this is due to general stress effects or a more direct effect of RDX remains to be determined. Our results indicate that toxicogenomics and network analysis are very useful and complementary tools in comparative genomics studies that examine the extrapolation potential of chemical toxicity across phylogenetically diverse and distant species.

Acknowledgments The authors would like to thank Lynn Escalon, Natalie Barker, Xin Guan, and Robby Body who were responsible for generating the data presented here. This work was funded by the US Army Environmental Quality Research Program. Permission for publishing this information has been granted by the Chief of Engineers.

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