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Effects of Diazepam on Gene Expression and Link to Physiological Effects in Different Life Stages in Zebrafish *Danio rerio*

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We applied zebrafish whole genome microarrays to identify molecular effects of diazepam, a neuropharmaceutical encountered in wastewater-contaminated environments, and to elucidate its neurotoxic mode of action. Behavioral studies were performed to analyze for correlations between altered gene expression with effects on the organism level. Male zebrafish and zebrafish eleuthero-embryos were exposed for 14 d or up to 3 d after hatching, respectively, to nominal levels of 273 ng/L and 273 μg/L (determined water concentrations in the adult experiment 235 ng/L and 291 μg/L). Among the 51 and 103 altered transcripts at both concentrations, respectively, the expression of genes involved in the circadian rhythm in adult zebrafish and eleuthero-embryos were of particular significance, as revealed both by microarrays and quantitative PCR. The swimming behavior of eleuthero-embryos was significantly altered at 273 μg/L. The study leads to the conclusion that diazepam-induced alterations of genes involved in circadian rhythm are paralleled by effects in neurobehavior at high, but not at low diazepam concentrations that may occur in polluted environments.

Introduction

In the past decade, steadily increasing levels of aquatic environmental residues of human and veterinary pharmaceuticals have continuously alerted the scientific community. As a result, multiple research efforts have been initiated to scrutinize possible input pathways and fate of these newly emerging pollutants. Additionally, effects on nontarget species have been studied to protect prospectively the environmental health and to minimize exposure risks (*1–3*). Pharmaceuticals active in the brain and nervous system functions, including antidepressants, antipsychotics, and anesthetics are of direct environmental relevance because of their potential effects on nontarget species. Based on evolutionarily conserved pharmacological targets (receptors, and cellular biomolecules) environmental pharmaceuticals have been shown to provoke adverse effects such as hormonal dysfunction and neurotransmission disruption (*4–6*).

A prominent representative pharmaceutical of this group is the environmental contaminant and highly prescribed neuropharmaceutical diazepam. Diazepam is the active ingredient of valium and belongs to the benzodiazepine group, which has widespread application in human medicine (*7*). Concentrations of up to 0.66 μg/L (*8*) have been measured in effluents of sewage treatment plants. In surface water, concentrations have been found ranging from 0.04 μg/L in Germany (*9*) to 0.66 μg/L in Belgium (*10*). This substantial environmental exposure may lead to negative effects on nontarget species.

Benzodiazepines act through binding to the GABA receptor resulting in central nervous system (CNS) depression. In addition, diazepam has also anxiolytic, sedative, muscle-relaxant, anticonvulsive, and antiepileptic properties (*11*). As in mammals, three subtypes of GABA target receptors ($GABA_A$, $GABA_B$, and $GABA_C$) occur in fish, and the binding site for benzodiazepines in the fish brain is suggested to be highly conserved between fish and mammals (*12*). In nontarget species there is acute diazepam toxicity in the high and chronic toxicity in the low mg/L range (*13*). The measured "no observed effect concentration" (NOEC) of diazepam in early life stage tests with *D. rerio* was found to be 273 μg/L, and the "lowest observed effect concentration" (LOEC) was found to be 2.57 mg/L (*13*). Exposure of fish to diazepam may impair locomotion (*14, 15*), affect vision, (*16*) and can produce anxiolytic effects at concentrations between 1.25 to 5 mg/L, doses that do not cause sedation (*17*). Nevertheless, there is a lack of chronic environmental toxicity studies concentrating on the modes of action of this residual pharmaceutical (*1*). Furthermore, proper ecological risk assessments of diazepam at environmentally realistic concentrations in aquatic organisms have to be investigated.

Classical ecotoxicological tests, e.g. OECD guideline 204 (prolonged 14 d fish test), are important for risk assessment but are often not sensitive enough to identify the subtle effects of pharmaceuticals. Adverse effects of pharmaceuticals may be more accurately determined by focusing on the mode of action (*1, 18, 19*). Based on these considerations, the environmental consequences of diazepam deserve further attention, in particular as effects of diazepam on the global gene expression profile in aquatic organisms are unknown.

In our present study, we determine the neurotoxic mode of action of diazepam in zebrafish to clarify potential molecular effects of this drug by analyzing the global gene expression after exposure for 14 d. This toxicogenomic approach allows the identification of several thousand genes and the corresponding expression profiles upon drug exposure which will assist in the elucidation of both the molecular effects and the compound's modes of action (*20*).

The aim of our study was to focus on the modes of action in the brain and in eleuthero-embryos at low diazepam concentrations using a whole genome oligonucleotide microarray for zebrafish. By analyzing diazepam at the NOEC of zebrafish eleuthero-embryos (273 μg/L) we compared the effects on the transcriptional level with known effects in the early life stages test (*12*). Our hypothesis is that the response of zebrafish to diazepam exposure is similar to the human response, as the GABA receptors are evolutionary highly conserved. On this basis, we searched for alteration of gene expression patterns associated with anxiolytic or sedative

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effects. Thereby, we hypothesize that alterations in gene expression - because of multiple end points and mechanistic information - are more sensitive than physiological or morphological parameters (although they are perhaps less ecologically relevant). Moreover, we compared the global gene expression pattern in the brain to the behavior of zebrafish to analyze, whether the observed molecular effects may correlate with and propagate to higher levels of biological organization. In addition, gene expression analysis may also reveal unknown regulatory mechanisms in fish not directly related to the modes of action of diazepam in humans.

Materials and Methods

Chemicals. Diazepam (≥99%, MW: 284.74, $\log K_{OW}$: 2.82−2.99) was kindly supplied by F. Hoffmann-La Roche Ltd. (Basel Switzerland). Acetonitrile was purchased from Brunschwig (Basel,Switzerland), and methanol and isopropyl alcohol were purchased from Stehelin (Basel, Switzerland). NaH_2PO_4, Na_2HPO_4, HCl, chloroform, ammonium hydroxide, and phosphoric acid were obtained from Sigma Aldrich (Buchs, Switzerland). Paraffin tissuewax, xylol, UltraClear, and hematoxylin were purchased from Medite (Nunningen, Switzerland), and eosin was purchased from Carl Zeiss AG (Feldbach, Switzerland).

Maintenance of Zebrafish. Juvenile zebrafish (*Danio rerio*) were obtained from Harlan Laboratories Ldt. (Itingen, Switzerland). They were transferred to culture tanks (300 L) and raised for 4 months in our laboratory. Fish were held in reconstituted tap water with a total hardness of 125 mg/L as $CaCO_3$ and a conductivity of 270 μS/cm. The water temperature was held constant at 27 ± 1 °C with the photoperiod set at 16:8 h light/dark. Fish were fed twice daily with TetraMin pellets (Tetra GmbH, Melle, Germany) *ad libitum*, once a day with brine shrimp (*Artemia salina*), and twice a week with *Daphnia magna*.

Exposure Experiment. *Exposure of Adult Males.* The experimental setup consisted of four replicates of water controls and two diazepam doses. Twenty male zebrafish per replicate were held in 20-L stainless steel tanks in well-aerated exposure water. Fish were exposed for 14 d to the nominal concentrations of 273 ng/L and 273 μg/L diazepam, respectively. The lower concentration (273 ng/L) was assumed to represent the worst case environmental concentration, and 273 μg/L refers to the NOEC in the early life stages test (*12*). During the experiment fish survival, appearance and behavior were determined.

A 48 h semi static-renewal procedure was used to minimize handling stress for the fish. Thereby after 24 h, food remains and feces were removed by siphoning 1/3 of the water and replacement by new exposure water containing the appropriate diazepam concentrations. After 48 h, the water renewal procedure was repeated, but this time by replacing total tank water (20 L). The quality of the exposure water was continuously monitored by the oxygen concentration determination (>70%), the pH value (6.7−7.2), and the temperature (27 ± 1 °C).

Mortality and abnormal behavior were recorded daily. At the end of the experiment (day 14), all fish were anaesthetized in a clove oil solution (Fluka AG, Buchs, Switzerland). Individual fish length and weight were measured in order to assess the condition factor (CF = weight (g)/length (mm) × 100). The brain of each fish was dissected after 14 d and stored in RNAlater. A total of 15 brains were pooled for microarray analysis and for quantitative real-time polymerase chain reaction (qRT-PCR) confirmation.

Eleuthero-Embryo Exposure. The semi static exposure setup consisted of six replicates of water control and two diazepam doses. A total of 15 fertilized eggs per replicate were exposed up to 3 d after hatching to nominal concentrations of 273 ng/L and 273 μg/L diazepam, respectively. Every 24 h, lethal and sublethal effects were evaluated, and dead eleuthero-embryos were removed. The quality of the exposure water was continuously monitored by oxygen concentration determination (>70%), the pH value (6.7−7.2), and the temperature (27 ± 1 °C). At the end of exposure, eleuthero-embryos were anaesthetized in a clove oil solution. A total of 15 eleuthero-embryos per replicate were pooled in RNAlater for qRT-PCR. RNA was extracted using the RNeasy Mini Kit (Qiagen, Basel, Switzerland).

Chemical Analysis. To determine actual exposure concentrations, 250-mL aliquots of exposure water was taken during the experiment for diazepam concentration analysis. Water samples of each treatment group were taken at the beginning (0 h), before siphoning (24 h), and prior to full water renewal (48 h). This was done on days 1 to 3, 7 to 9, and 11 to 13, respectively, from different randomly selected replicate tanks. The water samples were stored at −20 °C until analysis by HPLC. Extraction of water samples was performed according to ref *21* and chemical analysis according to ref *22*. The chemical analysis was performed separately for the microarray experiment and the behavior experiment. Due to limited amounts of exposure water, the concentration in the exposure water of the eleuthero-embryos experiment could not be measured.

RNA Isolation, Array Hybridization, and Sample Selection. Total RNA was extracted from zebrafish brain pools using the RNeasy Mini Kit (Qiagen, Basel, Switzerland). RNA concentrations were measured spectrophotometrically using a NanoDrop ND-1000 UV−vis spectrophotometer at 260 nm. The integrity of each RNA sample was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Basel, Switzerland). Only samples containing a 260/280 nm ratio between 1.8−2.1, a 28S/18S ratio between 1.5−2, and an RNA integrity number (RIN) > 8 were processed further. A total of 12 arrays (Agilent 4 × 44 K Zebrafish microarray) were used, including four for the control group, four for the 273 ng/L, and four for the 273 μg/L diazepam dose group. Total RNA samples (600 ng) were reverse-transcribed into double-strand cDNA in the presence of RNA poly-A controls with the Agilent One-Color RNA Spike-In Kit. Cy3 labeling and hybridization were performed according to the manufacturer's manual. A short description can be found in the Supporting Information.

RT-qPCR Analysis. Eight differentially expressed genes of interest were selected for microarray result confirmation using qRT-PCR. Gene-specific primers were designed based on published zebrafish sequences (Supporting Information Table S1). Total RNA from a pool of 15 zebrafish brains and a pool of 15 eleuthero-embryos was isolated as described above ($n = 5$ replicates for adults and $n = 6$ replicates for eleuthero-embryos).

One μg of total RNA template was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Promega Biosciences Inc., Wallisellen, Switzerland) in the presence of random hexamers (Roche Diagnostics, Basel, Switzerland) and deoxynucleoside triphosphate (Sigma-Aldrich, Buchs, Switzerland). The reaction mixture was incubated for 5 min at 70 °C and then for 1 h at 37 °C. The reaction was stopped by heating at 95 °C for 5 min.

The cDNA was used to perform SYBR-PCR based on SYBR-Green Fluorescence (FastStart Universal SYBR Green Master, Roche Diagnostics, Basel, Switzerland). The following PCR reaction profile was used: one cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and 61 °C for 60 s followed by a melting curve analysis post run.

The delta CT value was derived by subtracting the threshold cycle (CT) value for the housekeeping gene (*RpL13*α), which served as an internal control, from the CT value of the target gene, respectively. All reactions were run in duplicate using the Biorad CFX96 RealTime PCR Detection System (Biorad, Reinach, Switzerland). The mRNA expression

level of the different genes was expressed as fold-increase according to the formula

$$2^{\Delta CT(\text{untreated sample}) - \Delta CT(\text{treated sample})}$$

PCR efficiencies for each primer were determined from a standard curve using dilutions of pooled brain cDNA ($r^2 >$ 0.96 for all primers). Due to the high efficiency no corrections in the calculation of the delta CT were necessary.

Measurement of Locomotor Activity. *Multispecies Freshwater Biomonitor.* Diazepam effects on locomotor activity were measured in both male zebrafish and zebrafish eleuthero-embryos. Fish activity was observed using a flow-through test chamber with quadrupole impedance conversion as a measuring device, connected to a measuring unit and personal computer with data analysis software (*23*).

Measurement chambers, made of an acryl glass cylinder sealable on both ends, with a size of 15 cm in length and a diameter of 5 cm for the adult fish, and of 4 cm in length and a diameter of 2 cm for the eleuthero-embryos, allowed free movement during measurement. To measure locomotor activity, 12 male zebrafish were exposed to nominal concentrations of 273 ng/L (measured 330 ng/L) or 273 μg/L (measured 260 μg/L) diazepam in addition to a water control for 14 d in stainless steel aquaria as described above. After 3 and 14 d of diazepam exposure the animals were placed into the measurement chambers placed in a stainless steel aquaria filled with 15 L uncontaminated water. The chambers themselves were laid horizontally on the bottom of the test tanks. Following an acclimation time of 2 h measurements were started, and behavior of 12 male zebrafish per treatment was continuously recorded at intervals of 10 min for a 24 h duration. Behavioral recording during these intervals lasted for 4 min each.

For eleuthero-embryos locomotor activity measurements, fertilized eggs were exposed to 273 ng/L or 273 μg/L diazepam as described above. Locomotor activities of hatched eleuthero-embryos were assessed 3 d after hatching for 20 eleuthero-embryos per diazepam dose and the water control, respectively, as described above. Following an acclimation phase of 10 min measurements were started and behavior was monitored with a recording duration lasting 4 min for a period of 2 h with intervals of 10 min each. For locomotor activity measurements, means of locomotor activities (% time spent on locomotion) for each fish were calculated for 2 h during the 2-h or 24-h time period.

Data Analysis and Statistics. Raw microarray data were analyzed using the GeneSpring GX 10 software (Agilent Technologies). In a first step, the Agilent Feature Extraction software output was filtered on the basis of feature saturation, nonuniformity, pixel population consistency, and signal strength relative to background level (Agilent Feature Extraction Manual). Only positively marked entities, in which at least 50% of the values for any out of the three conditions, were accepted for further evaluation. All data were quantile normalized. In a second step, several quality control steps (e.g., correlation plots and correlation coefficients, quality metric plots and PCA) using the quality control tool of GeneSpring were performed to ensure that the data were of good quality.

Differentially expressed genes from the microarray were determined using a Benjamini-Hochberg multiple correction-ANOVA test ($p < 0.05$), followed by a TukeyHSD posthoc test. The genes were considered differentially expressed when $p < 0.05$ and the fold change (FC) ≥ 2. To determine gene ontology (GO) categories of differentially expressed genes, the GO analysis tool in GeneSpring GX 10 was used. Only those categories where $p < 0.05$ were considered differentially altered. MetaCore TM (GeneGo, San Diego, CA) was used to identify and visualize the involvement of the differentially expressed genes in specific pathways (FDR< 0.05). The microarray data used in this analysis have been submitted to NCBI GEO database under accession number GSE23157 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=nfudjckukeakytk&acc=GSE23157).

Data from qRT-PCR and locomotor activity were illustrated graphically with GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Data distribution for normality was assessed with the Kolmogorov−Smirnov test and the variance homogeneity with the Bartlett test. Differences between treatments were assessed by analysis of variance followed by a Tukey test (Bartelett test $p < 0.05$) to compare treatment means with respective controls. Results are given as mean ± standard error of mean. Differences were considered significant at $p < 0.05$.

Results and Discussion

Alteration of Gene Expression by Diazepam. Diazepam concentrations in the exposure water were measured at 0, 24, and 48 h in order to determine the actual exposure concentrations (Table S2). At nominal 273 ng/L, diazepam concentration was 243 ± 16 ng/L ($n = 3$) at 0 h and decreased to 225 ± 30 ng/L ($n = 3$) at 48 h, but this was not significant. At 273 μg/L, diazepam decreased from 315 ± 16 μg/L ($n = 3$) to 272 ± 6 μg/L ($n = 3$). The geometric mean of actual exposure concentrations were 235 ng/L and 291 μg/L. In none of the treatment groups, mortalities occurred. No significant differences on condition factors (CF) were noted (Table S3, Figure S1).

Gene expression profiles derived from microarray results of control and diazepam-exposed adult zebrafish brains (control, $n = 3$; diazepam, $n = 4$) are based on 15 pooled individuals. To test the data quality normalized data were then subjected to a PCA. As shown in Figure S2, the replicates of the 235 ng/L diazepam treatment group differ less from the control than the replicates from the 292 μg/L diazepam treatment group. The similarity to the control group is also reflected in the fact that fewer genes were significantly regulated in the lower concentration (Table S4).

As listed in Table S4, 51 and 103 genes were differentially expressed in the brain of male zebrafish ($\log_2 > 2$, $p < 0.05$) after exposure to 235 ng/L and 291 μg/L diazepam, respectively. At 235 ng/L diazepam, 31 (61%) genes were down-regulated, and 20 (39%) were up-regulated. Of the 103 genes differentially expressed at 291 μg/L diazepam 59 (57%) genes were down-regulated, and 44 (43%) were up-regulated. All 51 genes differentially expressed at the lower dose of 235 ng/L were also altered at 291 μg/L, and all of them were regulated in the same direction. Comparing our results with those obtained in goldfish (*24*), it seems that different compounds acting as GABA agonists alter similar gene families. Similar to our study alterations in the aldehyde dehydrogenase family, GABA receptor subunits, hydroxysteroid dehydrogenase family, solute carrier, rev erb family, DnaJ-like genes, and in the per3 gene have been observed. Diazepam is an allosteric modulator of the $GABA_A$ receptor, which is an inhibitory channel and can decrease the neuronal activity in its activated form (*25*). The $GABA_A$ receptor effects chloride permeability and voltage dependent transmission of neural signals. Hence, interference with this receptor will primarily affect neuronal activity but not gene expression or cellular signal transduction pathways that may lead to changes in gene expression. Therefore, effects of diazepam on gene expression may be regarded as a secondary mode of action and a side effect not directly related to the neurotoxic mode of action. On the other hand, the data may indicate hitherto unknown links and pathways related to the action of diazepam on the brain. In the case of neuropharmaceuticals other than GABA agonists such as mianserin (*26*)

alteration of other gene transcripts occur, as they act in a different manner.

Gene Ontology (GO) analysis was performed to identify functional groups of genes of interest. GO defines terms representing gene product properties and covers three domains namely cellular components, molecular functions, and biological processes. At 235 ng/L diazepam, functionally identified genes fell into 57 different categories, and at 291 μg/L diazepam they fell into 40 categories. The following 8 categories shown in Figures S3A and S3B contain the highest percentage of differentially expressed genes: DNA photolyase activity, photoperiodism, IMP dehydrogenase activity, rhythmic process, circadian rhythm, GMP metabolic process, GMP biosynthetic process, and response to temperature stimulus. In all of these 8 functional groups, a higher percentage of differentially expressed genes occurred at 291 μg/L diazepam as compared to 235 ng/L. Among those categories, alteration of genes involved in rhythmic processes, especially in the circadian rhythm, is of particular significance. Through GABAeric influence on the hypothalamic suprachiastic nuclei (SCN), diazepam has been shown to affect the circadian rhythm in mammals (*27*). The activation of the $GABA_A$ receptor can mediate phase-shifting in the mammalian circadian clock (*28*). In addition, $GABA_A$ receptor activation can lead to gene expression changes of circadian rhythm genes in mammals (*29, 30*). However, the mechanism of this gene expression alteration is not fully understood. It is hypothesized that different stimuli - in our present study $GABA_A$ receptor activation by diazepam - can alter the phase of the circadian clock by increasing and decreasing the expression of different clock genes (*31, 32*). GABA receptors are highly conserved and occur also in fish (*12*). Due to their high homology of up to 90% with the human GABA receptors, a similar mode of action in fish is expected (*33*). Indeed, diazepam showed a dose-dependent effect on the regulation of genes involved in the circadian rhythm (Table 1), including the down-regulation of period genes, which is in agreement with data in mice (*30*). Further experiments are needed to unravel the role of circadian rhythm gene expression alteration as a result of diazepam exposure. Gene knockdowns of circadian rhythm genes could help to elucidate these observed gene expression changes. Additionally to the GO analysis, we performed a pathway analysis with MetaCore. The circadian rhythm is the most significantly altered pathway (Table S5).

In order to confirm microarray results, qRT-PCR was performed for selected genes. Five genes belonging to the circadian rhythm, *arntl2*, *cry2b*, *cry5*, *nr1d1*, and *per1*, were selected. Additionally, the differential expression patterns of *arr3*, *gabrr1*, and *hsd17b3* were verified as shown in Figure 1 and Table 2. The expression of a*rntl2* and *cry2b* showed a dose-dependent induction in adult fish, and a dose-dependent down-regulation in the case of *cry5*, *nr1d1, per1, arr3, gabrr1*, and *hsd17b3* (Figure 1, Table 2). The differential expression of these genes upon exposure of adult male zebrafish was confirmed in zebrafish eleuthero-embryos exposed to the same nominal concentrations of 273 ng/L and 273 μg/L diazepam (15 eleuthero-embryos pooled for each of the six replicates). The same genes were up- and down-regulated, respectively. These changes in transcripts were particularly found in genes involved in the circadian rhythm. It is known that the circadian clock becomes functional already on the first day of development in zebrafish embryos (*34*). Although the same trends in the expression pattern occurred in the microarray and qRT-PCR data of adult fish and eleuthero-embryos for most of the genes, statistical significance and absolute fold-change differed between the methods and developmental stages. Exceptions were g*abrr1* and *hsd17b3*, which presented an up-regulation in eleuthero-embryos but a down-regulation in adults (both microarray and qRT-PCR data). The alteration of a nearly identical set of genes at both concentrations in adults and eleuthero-embryos demonstrates that the effect of diazepam is consistent in both life stages, showing effects on gene expression already at nominal concentrations of 273 ng/L. The almost identical pattern of gene expression profiles also indicates that diazepam acts through a neuropharmacological mode of action in fish on a molecular level, similar to that in mammals. This supports the notion that pharmaceuticals should be assessed for potential environmental risks through their mode of action.

TABLE 1. Fold Changes of Selected Genes Involved in the Circadian Rhythm, Whose Expression Was Significantly Altered in Male Zebrafish Brain after 14 d Exposure at 235 ng/L and 291 μg/L[a]

	fold change (log_2)	
gene name	235 ng/L diazepam (measured concn)	291 μg/L diazepam (measured concn)
aryl hydrocarbon receptor nuclear translocator-like 1a (*arntl1a*)	2.2	2.7
aryl hydrocarbon receptor nuclear translocator-like 1b (*arntl1b*)	2.4	3.2
aryl hydrocarbon receptor nuclear translocator-like 2 (*arntl2*)	2.7	5.6
basic helix−loop−helix domain containing, class B, 2 (*bhlhb2*)	-	−3.1
basic helix−loop−helix domain containing, class B, 3 like (*bhlhb3l*)	−2.3	−3.5
cryptochrome 1b (*cry1b*)	−2.5	−3.2
cryptochrome 2b (*cry2b*)	1.7	3.3
cryptochrome 3 (*cry3*)	−2.1	−2.8
cryptochrome 4 (*cry4*)	1.5	2.1
cryptochrome 5 (*cry5*)	−3.6	−4.3
cryptochrome DASH (*cry-dash*)	−3.2	−3.9
nuclear factor, interleukin 3 regulated (*nfil3*)	2.1	2.5
nuclear receptor subfamily 1, group d, member 1 (*nr1d1*)	−6.3	−3.1
Rev-erbgamma A (*nr1d4a*)	1.6	4.4
Rev-erbgamma B (*nr1d4b*)	2.4	10.5
period homologue 1 (*per1*)	−5.8	−4.5
period homologue 3 (*per3*)	−3.3	−7.9
period homologue 4 (*per4*)	−3.8	−6.5
rar related orphan receptor C (*rorcb*)	4.8	7.4

[a] Fold change (log_2) $\geq$ 2; $p < 0.05$.

Effects of Diazepam on the Circadian Rhythm and Locomotor Activity. In mammals, the circadian rhythm controls a diversity of behavioral and physiological rhythmic processes such as regulation of sleep wakefulness, secretion of hormones, and locomotor activity (*35*). Therefore, a second set of experiments was performed to evaluate whether changes found in the gene expression pattern of circadian genes in the brain were paralleled by changes in the locomotor activity of adult zebrafish and eleuthero-embryos. The behavioral experiments revealed that adult males did not show statistically significant differences to controls in their locomotor activity, both after 3 and 14 d of exposure to diazepam (Figure S4). We chose two time-points (3 and 14 d) to test for possible tolerance to diazepam, as found in *Gambusia holbrooki* (*14*). The lack of significant changes in

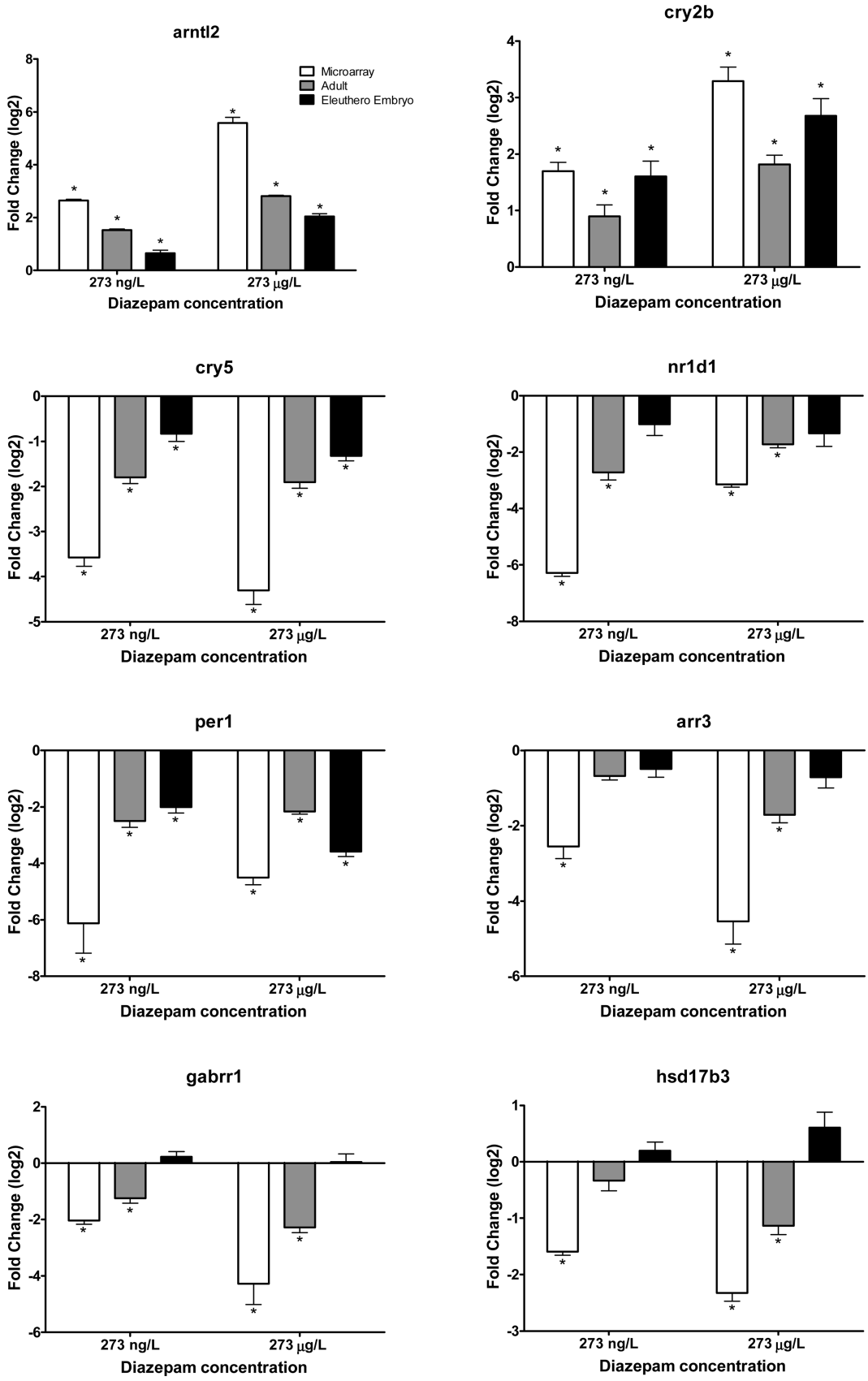

FIGURE 1. Comparison of gene expression in zebrafish brain determined by microarray ($n = 4$ replicates, 15 male fish pooled, white bars), qRT-PCR ($n = 5$, 15 male fish pooled, gray bars) determined in zebrafish brain, and qRT-PCR measured in zebrafish eleuthero-embryos ($n = 6$, 15 eleuthero-embryos pooled, black bars) after exposure to nominal 273 ng/L and 273 μg/L diazepam, respectively. Values are expressed as average fold change (log_2) with standard error compared to control animals as indicated for selected genes. Asterisks (*), statistically significant difference to control ($p < 0.05$).

TABLE 2. Fold Changes of Selected Genes Differentially Regulated in Zebrafish Brain Determined by Microarray and qRT-PCR in Adult Zebrafish Brain As Well As qRT-PCR Determination in Zebrafish Eleuthero-Embryos after Exposure to Low Concentration (235 ng/L) and High Concentration (291 μg/L) of Diazepam (Adults) and Nominal 273 ng/L and 273 μg/L (Eleuthero-Embryos), Respectively[a]

	fold change (log_2)					
	low concentration diazepam			high concentration diazepam		
gene name	array (adult)	qPCR (adult)	qPCR (eleuthero-embryos)	array (adult)	qPCR (adult)	qPCR (eleuthero-embryos)
aryl hydrocarbon receptor nuclear translocator-like 2 (*arntl2*)	2.7	1.5*	0.8*	5.6	2.8*	2.0*
cryptochrome 2b (*cry2b*)	1.7	0.9*	1.6*	3.3	1.8*	2.7*
cryptochrome 5 (*cry5*)	−3.6	−1.8*	−0.8*	−4.3	−1.9*	−1.3*
nuclear receptor subfamily 1, group d, member 1 (*nr1d1*)	−6.3	−2.7*	−1.0	−3.1	−1.7*	−1.3
period homologue 1 (*per1*)	−5.8	−2.5*	−2.0*	−4.5	−2.2*	−3.6*
arrestin 3, retinal (X-arrestin) (*arr3*)	−3.8	−0.7	−0.5	−4.4	−1.7*	−0.7
gamma-aminobutyric acid (GABA) receptor, rho 1 (*gabrr1*)	−2	−1.2*	0.2	−4.1	−2.3*	0.04
hydroxysteroid (17-beta) dehydrogenase 3 (*hsd17b3*)	−1.6	−0.3	0.2	−2.3	−1.1*	0.6

[a] Values are expressed as average fold change (log_2).

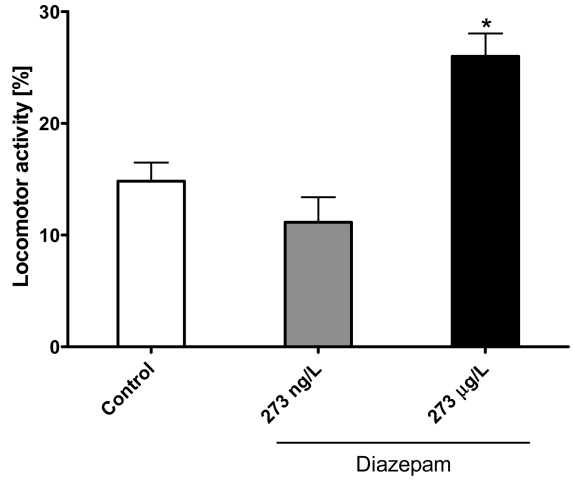

FIGURE 2. Comparison of the locomotor activity (percent of total time spent in locomotion during 2 h) of *D. rerio* eleuthero-embryos exposed for up to 3 d after hatching to nominal concentration of 273 ng/L and 273 μg/L or water control. Asterisks (*), significant differences between treatments ($p < 0.05$).

locomotion in adult fish may be interpreted in a way that the gene alterations were not propagated to significant behavioral changes, or alternatively as rapid tolerance against diazepam exposure. However, there is a dose-dependent trend to a more locally restricted swimming behavior after the 14-d exposure, which is interpreted as an indication of the known sedative effect of diazepam (Figure S4).

In eleuthero-embryos the locomotor activity at nominal concentration of 273 μg/L diazepam was distinctly different from that of control eleuthero-embryos. The diazepam-exposed eleuthero-embryos featured higher activity with less pauses. Movement patterns of controls (Figure S5A) differed from that of diazepam-exposed eleuthero-embryos (Figure S5B). The locomotor activity was significantly increased at 273 μg/L diazepam compared to eleuthero-embryos exposed to 273 ng/L and the controls, which did not differ from each other (Figure 2). This indicates that eleuthero-embryos seem to react more sensitively in their behavior than adults and that diazepam provoked a "paradoxical reaction", e.g. restlessness instead of sedation. This observation was also made in children, who became hyperactive after administration of diazepam (*36*). However, it was observed that zebrafish eleuthero-embryos locomotor activity was reduced when treated with 1 μM (284.7 μg/L) diazepam (*15*). The discrepancy to our observation may be explained that the locomotor activity was determined in the dark, whereas we measured it in the light.

In our study we show that the response of zebrafish to diazepam is similar to the human response. Significant changes in gene expression of circadian rhythm, which is also altered in mammals, occurred at low concentrations of 235 ng/L diazepam. This demonstrates that alterations in gene expression may be more sensitive than behavioral or other toxicological effects (*13*), although no mechanistic support for the potential role of the circadian rhythm genes in mediating neurotoxic effects is provided or yet known. The lack of behavioral and physiological changes at the low concentration, however, raises the question when gene expression changes propagate to toxicological relevant measures such as reduction of the ability to find food, to reproduce, or to escape from predators. In contrast a positive correlation between alteration in gene expression and behavior occurred at the higher diazepam concentration; therefore, the gene expression pattern is interpreted as indicative for effects at the organism level.

In conclusion the present study confirms that the toxicogenomic approach provides important data to identify and characterize molecular effects and to elucidate potential modes of action of a pharmaceutical found in the environment. This allows for establishing new biomarkers, not regularly assessed in routine ecotoxicological studies. However, molecular studies should be paralleled with ecotoxicological investigations of known ecological relevance.

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Supporting Information Available

Condition factor of male zebrafish (S1), PCA analysis (S2), gene ontology categories of differentially expressed genes (S3), locomotor activity of male zebrafish (S4), and typical examples of spontaneous locomotor movement patterns in zebrafish eleuthero-embryos (S5), a list of qRT-PCR primer sequences (Table S1), measured diazepam concentrations (Table S2), body weight, length, and condition factor (Table S3), a complete list of differentially expressed genes (Table S4), and the results of the pathway analysis (Table S5). This material is available free of charge via the Internet at http://pubs.acs.org.

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