Brain Transcriptomic Response of Threespine Sticklebacks to Cues of a Predator

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Abstract

Predation pressure represents a strong selective force that influences the development and evolution of living organisms. An increasing number of studies have shown that both environmental and social factors, including exposure to predators, substantially shape the structure and function of the brain. However, our knowledge about the molecular mechanisms underlying the response of the brain to environmental stimuli is limited. In this study, we used whole-genome comparative oligonucleotide microarrays to investigate the brain transcriptomic response to cues of a predator in the threespine stickleback, *Gasterosteus aculeatus*. We found that repeated exposure to olfactory, visual and tactile cues of a predator (rainbow trout, *Oncorhynchus mykiss*) for 6 days resulted in subtle but significant transcriptomic changes in the brain of sticklebacks. Gene functional analysis and gene ontology enrichment revealed that the majority of the transcripts differentially expressed between the fish exposed to cues of a predator and the control group were related to antigen processing and presentation involving the major histocompatibility complex, transmission of synaptic signals, brain metabolic processes, gene regulation and visual perception. The top four identified pathways were synaptic long-term depression, RAN signaling, relaxin signaling and phototransduction. Our study demonstrates that exposure of sticklebacks to cues of a predator results in the activation of a wide range of biological and molecular processes and lays the foundation for future investigations on the molecular factors that modulate the function and evolution of the brain in response to stressors.

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Introduction

Physiological and behavioral reactions to predators are conserved across organisms, and predation pressure has played an important role in the evolution of brain structures that mediate responses to environmental cues [Cantor, 2009]. Thus, studying how the brain responds to the threat of predation could provide insights into the

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mechanisms underlying neural plasticity and how they have evolved. However, our understanding of the molecular and biological processes underlying the response to predators is in its infancy. Important recent studies have shown that exposure to a predator results in the activation of particular neural circuitry in the brain [e.g. Nanda et al., 2008] and that specific genes, including immediate early genes [e.g. Comoli et al., 2003; Blanchard et al., 2005; Motta et al., 2009], are upregulated in the brain in response to the threat of predation. For example, corticotropin-releasing factor genes were upregulated in the amygdala of rats 3 h after a 10-min exposure to a ferret predator [Roseboom et al., 2007].

We know less about changes in the brain that might occur in response to repeated exposure to predation risk over longer periods of time. Most studies have measured gene expression within minutes after a single exposure to predation risk [Wang et al., 2003; Nanda et al., 2008; Leder et al., 2009; Mori et al., 2009]. However, animals are often recurrently exposed to cues of predators, and other studies suggest that the response of the hypothalamic-pituitary-adrenal axis to acute versus chronic stress is different [McEwen, 2007]. Recent gene expression studies have supported this hypothesis by showing that an acute stressor activates different genes, and a different number of genes, compared to a chronic stressor. For example, different sets of genes are involved in the transcriptomic response to acute versus chronic stress in the brain [Krasnov et al., 2005; Cairns et al., 2008]. Other studies have shown that the number of differentially expressed genes in response to a stressor depends on the duration of the stressor. For example, there were more differentially expressed genes in proximal tubule cells after 1 day of exposure to cadmium compared to 13 days of exposure [Garrett et al., 2011]. On the other hand, another study found that there were more differentially expressed genes in the liver in response to confinement stress in sea bream that had been confined for 20 h compared to fish that had been confined for 120 h [Calduch-Giner et al., 2010]. We also know that exposure to predation risk can have long-term effects on antipredator behavior [Tulley and Huntingford, 1987; Magurran, 1990; Kelley and Magurran, 2003], which suggests that there might be changes in brain gene expression in response to predation risk that are relatively enduring.

The power of genomics has only recently been applied to understand the coordinated action of the entire transcriptome in response to predation [but see Wang et al., 2003; Roseboom et al., 2007]. Whole-genome surveys are an attractive strategy because it is likely that the response to threat of predation involves multiple interacting genes and pathways. In addition, transcriptomic profiling can reveal which genes display similar expression patterns and opens the possibility of more integrated analyses of whole-genome dynamics via techniques such as pathway, network and gene ontology (GO) analyses. Indeed, new insights have emerged from studies measuring the whole transcriptomic response to other stressors, e.g. pollution [Craig et al., 2009], disease [Ching et al., 2010] and confinement [Kennerly et al., 2008].

Threepined sticklebacks *(Gasterosteus aculeatus)* are small teleost fish whose evolution has been strongly influenced by predation pressure. Sticklebacks are subject to a wide range of predators including piscivorous fishes, birds, odonate larvae and snakes [Reimchen, 1994]. Much of the extensive phenotypic variation among freshwater stickleback populations, including morphology [Reimchen, 1980], behavior [Huntingford et al., 1994], physiology [Bell et al., 2010] and life history traits [Moodie, 1972; Baker et al., 1998] can be attributed to variation in predator pressure. For example, sticklebacks from populations that are subject to high levels of predation pressure have more exterior armor (lateral plates) [Reimchen, 1994], show stronger antipredator behavioral responses to the immediate risk of predation [Huntingford et al., 1994] and are more physiologically responsive to the threat of predation as assessed by respiration rate [Bell et al., 2010]. A recent study of the time course of the acute response to predation risk [Bell et al., 2007] showed that acute exposure to predation risk stimulated a glucocorticoid stress response within minutes, with levels of cortisol increasing up to 1 h after exposure to predation risk. While recent studies have successfully pinpointed the genes underlying the evolution of morphological defenses against predators in sticklebacks [Peichel et al., 2001; Cresko et al., 2004; Shapiro et al., 2004; Colosimo et al., 2005; Chan et al., 2010], we know less about the molecular basis of plastic behavioral and physiological reactions to predators within an individual's lifetime. Growing genomic resources for sticklebacks [Kingsley and Peichel, 2007], including a full genome sequence, make this organism a suitable model for investigating the molecular mechanisms that have evolved in response to predation pressure.

Therefore, we investigated the brain's response to cues of a predator using whole-genome microarrays in sticklebacks. We hypothesized that sticklebacks exposed to predation risk would exhibit differential transcriptomic responses compared to a control group.

Materials and Methods

Sticklebacks

We studied laboratory-reared F1 female sticklebacks derived from a wild population in Putah Creek, Calif., USA. We elected to study females in this experiment because males undergo dramatic changes in behavior over the reproductive period, some of which could influence an individual's response to predation risk [Wootton, 1976]. The fish were maintained in our laboratory in 104-liter tanks under 8:16-hour light/dark photoperiod. An average of 10% of the water in the tanks was exchanged each day. The adult fish were fed ad libitum with a mixture of bloodworms, brine shrimp and mysis shrimp.

Adult fish used in the study were sampled from a tank containing representatives of 33 different families. Fish were acclimated together in one tank for 2 weeks prior to the experiment. Fish were placed in six different 26-liter tanks with three fish per tank in a partially recirculating flow-through system. The water was filtered through particulate, UV, biological and charcoal filters, which remove olfactory cues. Half of the tanks were assigned to the control group and the other half to the experimental group. The position of the control and experimental tanks was randomized in the room and the tanks were isolated from external disturbances during the entire duration of the experiment by opaque covers on the back and front of the tanks. The fish were allowed to acclimate for 3 days during which the sides of the tanks were left open to allow the fish to visually interact with fish in neighboring tanks. Dividers were then inserted between tanks for the remainder of the experiment.

Applying Predator Cues

We designed a procedure to mimic the situation of sticklebacks that are repeatedly exposed to a high level of predation pressure in the field. Specifically, over 6 days, sticklebacks were exposed to olfactory, visual and tactile cues of rainbow trout, a natural predator, in order to simulate real predation risk. This procedure elicited enduring changes in stickleback growth and behavior [Dingemanse et al., 2009; Bell et al., 2011], and other studies using this population have shown that they exhibit strong antipredator responses to rainbow trout [Bell and Sih, 2007]. Nanda et al. [2008] used a similar procedure to measure the immediate brain transcriptomic response of rats to olfactory, visual and acoustic cues of predators. Sticklebacks were exposed to two types of olfactory cues. First, olfactory cues of rainbow trout were applied to simulate the presence of trout in the environment. Olfactory cues of trout were prepared by soaking 1 cm² trout skin in 1 liter of distilled water for 2 h. Second, cues of dead stickleback were applied to simulate predation on nearby conspecifics. The dead-stickleback cues were prepared by soaking one sacrificed fish in 1 liter of distilled water for 2 h. Incisions were made in the skin on the sides of the fish with a clean razor to release cues. This procedure releases alarm pheromones in the skin of sticklebacks [Brown and Godin, 1997].

The infusions were frozen in trays and the ice cubes were thawed in a beaker for 1 h prior to use. Once a day for 6 days, at a random time of day, we turned the water off in the tank and added 50 ml of trout infusion and 50 ml of dead stickleback infusion. The olfactory cues were placed within 30 cm of the majority of sticklebacks in the tank. On days 2 and 5 of the treatment period, a trout model was added to the tank at the site of application of the olfactory cues, held still for 30 s, and then quickly moved toward the corner of the tank that contained the majority of the sticklebacks, then to the side and back again. We used a model of a rainbow trout constructed of clay and painted realistically (20.5 cm standard length). The model was thoroughly rinsed in distilled water before being reused. On day 6, the sticklebacks were chased by the model trout for an entire minute. Fish in the control tanks were treated exactly the same except that 100 ml clean water was added instead of olfactory cues, and they were not exposed to the model predator. To control for disruption caused by adding the predator, we splashed the surface of the water in the control tanks once within 30 cm of the majority of the sticklebacks on days 2 and 5 and on day 6, the water was splashed several times for 1 min. The water in the tank was turned on 3 h after the treatment was applied. On the 7th day, all the fish (n = 18) were quickly netted and sacrificed by decapitation using sharp scissors. All methods were approved by IACUC protocol (No. 06178) of the University of Illinois at Urbana-Champaign.

RNA Extraction and cDNA Synthesis

Whole fish brains were individually dissected and placed immediately on ice in 500 µl of Trizol® reagent (Invitrogen, Carlsbad, Calif., USA). Total RNA was isolated immediately from individual brains (n = 18) using Trizol reagent according to the manufacturer's recommendation and subsequently purified on columns with RNeasy kit (Qiagen GmbH, Hilden, Germany). RNA was eluted in a total volume of 60 µl in RNase-free water. In addition, to remove contaminating genomic DNA, all RNA samples were treated with DNase using the Turbo DNA free kit (Ambion, Austin, Tex., USA) following the manufacturer's instructions. The quantity of RNA was estimated using a NanoDrop 1000 (Thermo Fisher Scientific, Inc., Waltham, Mass., USA) and the quality was assessed using the Agilent Bioanalyzer 2100 (Agilent, Santa Clara, Calif., USA). RNA was immediately stored at –80°C for 1 week. To generate enough double-stranded cDNA for hybridization, we first amplified RNA (aRNA) from 1 µg of total RNA of 10 fish used in microarray using the MessageAmp II aRNA amplification kit (Applied Biosystems/Ambion, Austin, Tex., USA) following the manufacturer's instructions. Other studies have suggested that aRNA does not introduce significant bias in gene expression data [Feldman et al., 2002; Polacek et al., 2003]. Nonamplified RNA was preserved for microarray validation experiments. The aRNA was subsequently converted to double-stranded cDNA using the SuperScript® Double-Stranded cDNA Synthesis Kit (Invitrogen). cDNA was purified using the Qiaquick PCR Purification Kit (Qiagen). All samples for microarray were successfully amplified. The cDNA yields ranged from 3 to 6 µg. In accordance with NimbleGen (Madison, Wisc., USA) requirements, 2.5 µg of double-stranded cDNA were sent to the NimbleGen Microarray Service Department for hybridization.

Chip Design

The arrays were printed using the NimbleGen proprietary Maskless Array Synthesis technology on a 385 K high-density chip using both the 44,884 in silico Genscan predictions from the stickleback genome and the 27,633 gene transcripts obtained from ENSEMBL v.45.1c. Genscan predictions are based on transcriptional, translational and splicing signals as well as the length and compositional distributions of exons, introns and intergenic regions. They differ from the transcripts, which have been backed

by some experimental evidence. 60-bp probes were designed from a total of 72,517 sequences. Of these genes, all except 12 sequences had 5 probes per gene. No probes were found for 44 sequences because all of them were shorter than the minimum 60-bp probe size. Probes were designed taking into account various criteria such as nonrepetitive elements, frequency, uniqueness and melting temperature according to the NimbleGen probe design procedure.

Microarray Design and Hybridization

Ten samples were used for microarray analysis. The samples comprised 5 biological replicates in the experimental group (fish exposed to cues of predator) and 5 biological replicates in the control group (fish not exposed to cues of predator). Samples were selected to ensure even distribution among the tanks and high RNA quality. Three tanks contributed 1 individual, 2 tanks contributed 2 individuals and 1 tank contributed 3 individuals to the experiment. The cDNA-labeling (single color), hybridization, washing and scanning steps were performed in the NimbleGen Microarray Gene Expression Service Department. In addition, NimbleGen also conducted the data feature extraction and generated the XYS file format that was used in data analysis. The microarray data have been deposited in NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.gov/geo) under accession No. GSE21599, GPL10368.

Data Analysis

The microarray data were analyzed using various packages in R Bioconductor [R Development Core Team, 2009]. Although both the in silico Genescan predictions and transcripts were included in the microarray chips, we focused the analysis only on the transcripts of confirmed function because some of the Genescan predictions do not have biological function. Genescan predictions were not further considered in the analysis. Differential expression of genes between the control group and the experimental group was assessed using the package *Oligo* in R Bioconductor [Carvalho et al., 2007]. As required for the package *Oligo*, we first built a package 'pd.2007.07.05.g.aculeatus.expr', which contains the annotation information of the arrays using *pdInfoBuilder* package [Carvalho et al., 2007]. This package was then installed in R and used for analysis. Background subtraction, quantile normalization and summarization were performed using the Robust Multichip Average Algorithm. This algorithm performs three steps in the following sequence: background correction, quantile normalization and log2-transformed perfect-match value summarization, a method that averages the probe's expression intensities for each transcript [Irizarry et al., 2003]. Before exploring the differences in gene expression between the two groups, we first applied a nonspecific filtering method to the data to remove the probe sets (transcripts) with no or very low expression. This approach is commonly used to improve the detection power in microarray data analysis, particularly when testing multiple hypotheses [Calza et al., 2007; Bourgon et al., 2010]. We applied the function *rowSds* in the package *genefilter,* which calculates the standard deviation of expression intensities for each row (probe set). We then estimated the 'peak' of the distribution using the function *shorth* (the shortest interval containing half of the data). Probes with standard deviation below the shorth value were filtered out of the data. The quality of the arrays was assessed by examining the boxplots and the density plots of the log2-transformed intensity values of the probes. Hierarchical clustering was done using the function *hclust* in the package *stats* in R Bioconductor by employing the complete linkage method as the agglomeration procedure.

We used principal components analysis as a dimension reduction method to visualize the data using the *plotPCA* function in R. The experimental treatment (predator-exposed fish and control group) were the variables and the gene expression measurements served as the observations.

The log2-fold changes (logFCs) and moderated t statistics were computed to infer the differential expression of genes between the control and experimental groups using the Bayesian moderated t test in the package LIMMA (Linear Models for Microarray Data) in R Bioconductor. We used a false discovery rate (FDR) to correct for multiple testing [Benjamini and Hochberg, 1995]. The function *topTable* was used to rank the p values.

Because some fish used in the experiment were from the same tank, we conducted a post-hoc analysis of the top differentially expressed genes to detect any possible tank effect; these results are shown graphically in online supplementary figure 1 (www.karger.com/doi/10.1159/000328221).

Gene Function, GO and Pathway Analysis

Gene functional studies and GO enrichment analysis were conducted in R Bioconductor using the ENSEMBL dataset Gasterosteus_aculeatus.BROADS1.58. The annotation of the differentially expressed genes was determined using the package *BiomaRt* as implemented in R. Genes with no known function in the stickleback genome annotation were manually annotated using the BLASTX algorithm against the nonredundant protein database. The output of the file was parsed using a custom Perl script.

GO enrichment was carried out using the package *GOstat.* As a gene universe (background), we used the transcripts that were maintained after nonspecific filtering and we tested for overrepresentation of transcripts with raw p values ≤0.01 as determined by the Bayesian statistics. Both the gene universe and the target genes were first converted into ENTREZIDs using human homologues by the function *getBML* in BiomaRt because the stickleback genes have not been assigned ENTREZIDs, which are used in *GOstat.* The overrepresented biological processes (BPs), molecular functions (MFs) and cellular components (CCs) were determined by using the function hyperGtest with conditional testing, which removes the effect of child GO terms before testing parents. We also performed pathway and network analyses using the Ingenuity Pathways Analysis (IPA) software version 8.7 (Ingenuity Systems, Redwood City, Calif., USA). IPA is a proprietary application that uses data from its own repository of biological interactions and functional annotations from all species known as Ingenuity Knowledge Base, a manually curated database that is updated weekly and quarterly using annotations and findings from public and commercial databases and from information extracted from published biomedical literature. Third-party sources of database used by IPA include, for example, the NCBI Entrez Gene, Refseq and OMIM, the GWAS Database, GO annotation, Hazardous Substances Database, Human Metabolome Database, GNF Tissue Expression Body Atlas and KEGG metabolic pathway information. Since IPA does not accept stickleback transcript IDs, the differentially expressed genes were converted into ENTREZIDs using human homologues.

Table 1. List of primers used in the qPCR validation assays

Gene	Forward sequence	Reverse sequence	Amplicon size bp
GAPDH	CAAACCGTTGGTGACAGTATTTG	GCACTGAGCATAAGGACACATCTAA	69
RHO_57	ATCCCTTTCCTCTCAGGAAAAAA	TCAAGTCAGCCCACTACAGAGTTG	95
RHO_60	CCCCGAGGGCATGCA	TGTAAGTGGCGAACATGTAGATGA	95
USP-like	TGACTCACAGCGCCACATG	TGAGAGAAGCCAAAAAAAGC	71
Tr_22897	TGCGCTGGAACGAAGATG	GCCCTGAGGTCCGAAGCT	60
PLA2G12B	TCTCTCCTCAGCATGTGAAACG	AGGGTCTGCAGCCCAGAGT	71
GLUT1	GTCATGTAACCCTGATGAAAAGGA	CGCGCCGCAGACTTTCT	60
C2	AGGTGCTCACTGCGCTTACA	AAGTGCAACCGCTGTGCAT	70
CXCR1	CCCTGAGCACTGCTTTGTTTT	GGTTGACGCAGCTGTGGAA	60
KCNK12	CCACGGTCGCCCACAT	ACAATCTGCGGCCCTCAGTA	67
PCYT1B	CCTCCGTCCCTGAGCAGAA	TGGCGTAGGACAGCATTCG	64

GADPH (glyceraldehyde 3-phosphate dehydrogenase): reference gene used for normalization of the qPCR results. Target genes were RHO_57 (rhodopsin transcript variant ENSGACT-00000003757), RHO_60 (rhodopsin transcript variant ENS-GACT00000003760), USP-like (ubiquitin specific protease-like), Tr_22897 (transcript 'ENSGACT00000022897'), PLA2G12B (phospholipase 2 group XIIB), GLUT1 (glucose transporter 1), C2 (complement precursor), CXCR1 (interleukin-8 receptor alpha), KCNK12 (potassium channel), and PCYT1B (phosphate cytidylyltransferase 1, choline, beta).

Microarray Validation

Microarray validation was conducted on samples used in microarray experiment and also on 4 samples that were part of the experiment but were not included in the microarray gene expression analysis. Total RNA from each sample was reverse transcribed using the SuperScript® III First-Strand Synthesis System (Invitrogen). Quantitative real-time PCR (qPCR) reactions were performed on the Applied Biosystems 7900HT (Applied Biosystems) using the FastStart Universal SYBR Green Master (Rox) (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's recommendations. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal reference because the average expression values of this gene did not vary significantly across samples in the microarray experiment. Genes with highest fold change and lowest p values, as determined in R Bioconductor, are more likely to be differentially expressed between conditions, and were therefore selected for validation. Primers for validation were designed from each gene transcript using the Primer Express® Software v1.0 (Applied Biosystems). Primers were redesigned each time amplification across samples was not consistent. This was the case for the rhodopsin (RHO) genes, which are represented by two differentially expressed splice variants (ENSGACT00000003757, ENSG-ACT00000003760). The genes selected for validation and their respective primers are listed in table 1. Each reaction was run in triplicate and a dissociation curve was used to assess a single amplicon. No-template controls were included in the reaction to ensure the specificity of amplification. Primers were redesigned whenever primer dimers were observed in the dissociation curve. A standard curve was computed using a $10\times$ serial dilution of samples made by pooling equal quantities of all samples, including both the control and experimental samples. A standard curve was run for each gene separately. The qPCR data were analyzed using the Relative Expression Software tool (REST©) [Pfaffl et al., 2002]. To test for consistence between the samples used in microarray and the independent biological replicates, we compared the expression values of three most differentially expressed genes in these samples using a one-way ANOVA using StatPlusMac (AnalystSoft, Vancouver, B.C., Canada).

Results

As a result of nonspecific filtering, 36% of probe sets (transcripts) with standard deviations below the shorth value (0.268) were removed because they had low expression levels (fig. 1a). The target genes of these probe sets were either not expressed in the samples or their expression was not significantly higher than above background level. In total, 17,681 transcripts were maintained for further analysis.

The hierarchical clustering based upon row expression values showed that all the samples clustered according to treatment, except 1 sample (CON_1), which grouped with the experimental samples (fig. 1b). However, this sample clustered with the control group based upon differential expression (fig. 2). The principal-component analysis shown in figure 1c revealed that 74% of the variation was explained by the first principal component (PC1), suggesting that the majority of observed changes in gene expression were caused by exposure to predator cues. PC2 and PC3 explained 8.6 and 6.0% of the variation, respectively.
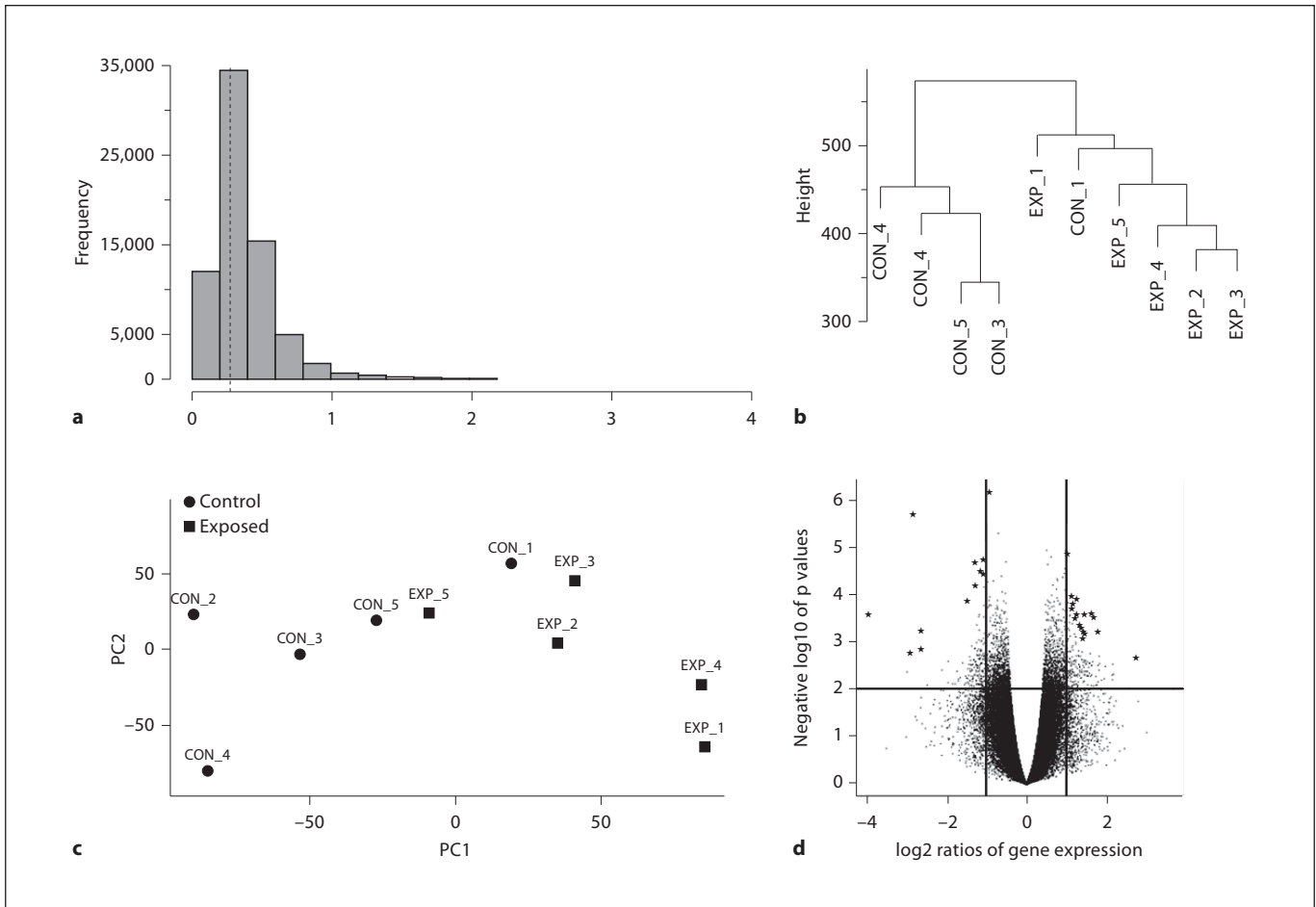
Fig. 1. Methods used in preprocessing and quality control of the arrays. **a** Histogram of standard deviations. Genes with very low standard deviations are more likely not to be differentially expressed and were removed from the data. The dotted line represents the peak of the distribution (shorth value). **b** Hierarchical clustering of the samples (arrays). Control group: CON_1–5; ex- perimental group: EXP_1–EXP_5. **c** Principal components plots used as dimension reduction of the samples (arrays). **d** Volcano plot highlighting the top 30 differentially expressed genes (★) be- tween sticklebacks exposed to predator cues and the control group.

Differential Expression

There was no evidence that any of the treatment dif- ferences were driven by specific tanks within each treat- ment. Specifically, the random effect of tank on the ex- pression of the top 15 genes was never statistically sig- nificant ($p > 0.05$); less than 0.04% of the variance could be attributed to tank effects (see online suppl. fig. 1).

A total of 735 transcripts had a logFC ≥ 2, of these, 30 had a logFC ≥ 4. Of the 17,681 transcripts, a total of 6,596 were upregulated and 11,085 were downregulated. Fig- ure 1d shows a volcano plot highlighting the top 30 dif- ferentially expressed transcripts.

Based upon the empirical Bayesian t statistics, 909 transcripts were differentially expressed at $p < 0.01$. Mul-

tiple testing on transcripts using the method of Benjami- ni and Hochberg [1995] revealed that 30 transcripts were differentially expressed at FDR = 0.15 (table 2). While some differentially expressed genes had large fold chang- es (e.g. RHO, logFC = 3.96), others appeared to be ex- pressed at lower levels (e.g. GLUT-1, logFC = –1.5). Fig- ure 2 is a heatmap generated using the top 30 differen- tially expressed transcripts with lowest p values, and reveals a clear pattern that distinguishes between fish ex- posed to predator cues and the control group.

Gene Functional Analysis: Upregulated Genes

RHO was the most upregulated gene based on p values and fold changes in fish exposed to cues of a predator.

Fig. 2. Heat map of top 30 differentially expressed genes between control (CON_1–5) and experimental (EXP_1–5) group with gene names. The Z-score represents the number of standard deviation units a specific gene expression value is from the mean (defined as 0). Red color in the heat map indicates upregulated genes, in blue are downregulated genes and in white are genes that do not change between conditions. Color-coded bars represent the clustering of samples per treatment (horizontal bar) and clusters of differentially expressed genes (vertical bar).
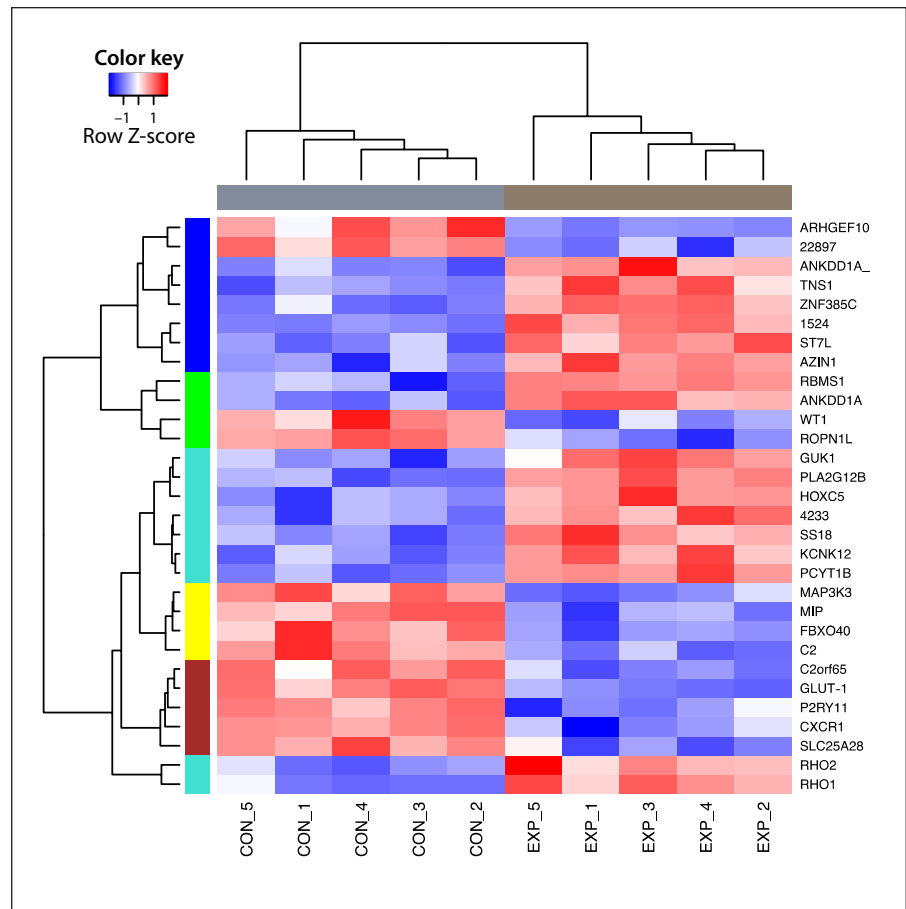
RHO is a complex of vision protein opsin and the chromofore 11-*cis*-retinal that mediates vision at low illumination. Phospholipase A2, group XIIB (PLA2G12B) was also significantly upregulated. This gene is a secreted phospholipase (sPLA2) from a family of small secreted proteins with the ability to catalyze the hydrolysis of glycolipids and release free fatty acids and lysophospholipids. The upregulated gene homeobox C5 (HOXC5) is a member of the homeobox family of genes which encodes a highly conserved family of transcription factors that provide cells with specific positional identities on the anterior-posterior axis during morphogenesis in all multicellular organisms. The ubiquitin-specific protease-like protein (USP-like), a group of serine proteases that plays a pivotal role in protein homeostasis by tagging the ubiquitin complex for degradation was also upregulated. The upregulated gene phosphate cytidylyltransferase 1, choline, beta gene (PCYT1B) is an enzyme that controls the phosphatidylcholine synthesis and is a major component of cell membranes with an important role in membrane signaling and cell growth.

Other upregulated genes included the suppressor of tumorigenicity protein 7-like protein (STL7), the RNA-inding motif (RBSMS1), the ankyrin repeat (ANKDD1A), tensin (TNS1) and the potassium channel subfamily K (KCNK12) gene (table 2).

Downregulated Genes
The most downregulated gene in fish exposed to predator cues was the transcript ENSGACT00000022897, a novel unnamed gene of undetermined function. Also downregulated was ropoporin (ROPN1), a gene most likely involved in regulating the growth, development and/or maintenance of motile cilia. The downregulation of mitoferrin-2 (MFRN2), a solute ion carrier essential for red-blood iron uptake, is also noteworthy. Of particular interest is the downregulation of the glucose transporter (GLUT1). GLUT1 is the first member of the solute carrier family (SLC2) that facilitates the transport of glucose from the bloodstream across the blood-brain barrier to the nervous system. Other downregulated genes included

Table 2. List and annotation of the top transcripts differentially expressed in the brain of sticklebacks exposed to predator cues using a p = 0.001 cutoff

Transcript ID	logFC	Ave. Exprs	p value	Adj. P.Val	Gene name	Gene description
ENSGACT00000003757	3.96	6.81	2.00E-06	0.035	RHO	rhodopsin
ENSGACT00000022897	−1.71	8.93	1.99E-05	0.105	NA	novel protein
ENSGACT00000024046	1.28	5.88	2.14E-05	0.105	PLA2G12B	group XIIB secretory phospholipase A_2-like protein
ENSGACT00000012440	1.47	5.65	2.38E-05	0.105	HOXC5	homeobox protein Hox-C5
ENSGACT00000019166	−1.82	7.59	3.21E-05	0.113	CXCR1	interleukin-8 receptor
ENSGACT00000003760	2.62	6.76	4.03E-05	0.115	RHO	rhodopsin
ENSGACT00000025444	2.91	7.90	4.90E-05	0.115	USP-like	ubiquitin-specific protease-like protein
ENSGACT00000003143	−1.28	11.00	5.18E-05	0.115	ROPN1L	ropoporin
ENSGACT00000019615	−1.45	7.05	7.88E-05	0.132	SLC25A28	mitoferrin-2 (mitochondrial iron transporter 2)
ENSGACT00000003415	1.06	6.61	8.40E-05	0.132	PCYT1B	phosphate cytidylyltransferase 1, choline, beta
ENSGACT00000016448	−1.05	7.83	8.98E-05	0.132	GLUT-1	glucose transporter 1
ENSGACT00000007884	−1.16	5.22	9.01E-05	0.132	C2	complement C2 precursor (C2b fragment; C2a fragment)
ENSGACT00000026313	−1.26	7.90	9.73E-05	0.132	C2orf65	uncharacterized protein C2orf65
ENSGACT00000004329	−1.44	10.22	0.00011	0.14	WT1	Wilm's tumor protein (WT33)
ENSGACT00000018879	1.17	9.61	0.00013	0.156	TNS1	tensin-1
ENSGACT00000008104	1.06	8.14	0.00014	0.156	ST7L	suppressor of tumorigenicity protein 7-like protein
ENSGACT00000020055	1.06	10.44	0.0002	0.157	RBMS1	RNA-binding motif, single-stranded-interacting protein 1
ENSGACT00000018168	−1.08	7.60	0.00022	0.157	P2RY11	P2Y purinoceptor 11
ENSGACT00000019149	0.96	11.46	0.00025	0.157	ANKDD1A	ankyrin repeat and death domain-containing protein 1A
ENSGACT00000012630	−1.78	7.00	0.00025	0.157	ALX1	ALX homeobox protein 1 (cartilage homeoprotein 1)
ENSGACT00000009578	−1.23	6.95	0.00026	0.157	ZFP568	zinc finger protein 568
ENSGACT00000002994	1.18	9.16	0.00028	0.157	NDRG4	protein NDRG4 (brain development-related molecule)
ENSGACT00000012508	−1.47	7.25	0.00035	0.157	CLEC4M	C-type lectin domain family 4 member M
ENSGACT00000021759	1.39	9.09	0.00036	0.157	CPLX4	complexin-4 precursor
ENSGACT00000006879	0.97	8.57	0.00036	0.157	AZIN1	antizyme inhibitor 1
ENSGACT00000002915	−1.29	5.43	0.00037	0.157	GAG	putative GAG protein
ENSGACT00000011207	1.22	7.23	0.00037	0.157	TLCD2	TLC domain-containing protein 2
ENSGACT00000015180	1.72	5.55	0.00039	0.157	MAPRE3	microtubule-associated protein RP/EB family member 3
ENSGACT00000027138	−0.97	5.55	0.00039	0.157	FBXO40	F-box only protein 40 (muscle disease-related protein)

Annotation of the transcripts based upon genome annotation in ensembl and BLASTX search. The moderated Bayesian statistics generated using the LIMMA package in R are included and the transcripts are ranked based upon their p values.

logFC = log2 of fold change; Ave. Exprs = average expression across all arrays; Adj. P.Val = p value adjusted; NA = information not available.

the complement precursor (C2), an immune gene which is part of the classical pathway of the complement system; interleukin-8 receptor alpha (CXCR1), a signal transduction protein that plays a role in inflammatory responses; the purinergic receptor P2Y G-protein coupled receptor and other genes, such as zinc finger proteins (ZFP568); the peroxisomal biogenesis factor 19 (Peroxin-19); and C-type lectin domain family 4 member M (CD209 antigen-like protein 1) (table 2).

GO and Pathway Enrichment Analysis

A hypergeometric test was used to determine the GO terms overrepresented in the brains of fish exposed to predator cues. Our study showed that 41 BPs, 17 CCs and 20 MFs were significantly overrepresented ($p < 0.05$) in transcripts differentially expressed at the $p \leq 0.01$ cutoff. The fraction of genes assigned to these GO terms among the differentially expressed transcripts is higher than expected by chance alone when they are compared to the fraction of genes that are in the universe or the background (all the transcripts after nonspecific filtration). As shown in tables 3 and 4, a wide range of biological GO terms was enriched in the brain. These terms can be grouped in several categories such as neuroplastic processes that include neurological system processes, photo-transduction and detection of light stimulus involved in

Table 3. List of GO BPs overrepresented in a set of genes differentially expressed in the brain of sticklebacks exposed to predator cues at p < 0.01

GOBPID	p value	Odds ratio	Exp. count	Count	Size	Term
GO:0019882	0.0008	5.99	1.64	7	21	antigen processing and presentation
GO:0050962	0.0009	7.13	1.26	6	16	detection of light stimulus involved in sensory perception
GO:0007602	0.0014	8.48	0.94	5	12	phototransduction
GO:0050877	0.0016	1.85	21.05	35	270	neurological system process
GO:0048704	0.0019	5.94	1.42	6	18	embryonic skeletal system morphogenesis
GO:0009584	0.0022	7.42	1.02	5	13	detection of visible light
GO:0009952	0.0027	3.97	2.52	8	32	anterior/posterior pattern formation
GO:0009416	0.0032	3.82	2.59	8	33	response to light stimulus
GO:0009581	0.0044	4.75	1.65	6	21	detection of external stimulus
GO:0009582	0.0044	4.75	1.65	6	21	detection of abiotic stimulus
GO:0046460	0.0062	Inf	0.16	2	2	neutral lipid biosynthetic process
GO:0046504	0.0062	Inf	0.16	2	2	glycerol ether biosynthetic process
GO:0032868	0.0081	11.80	0.47	3	6	response to insulin stimulus
GO:0009890	0.0108	1.75	15.50	25	197	negative regulation of biosynthetic process
GO:0045666	0.0133	8.84	0.55	3	7	positive regulation of neuron differentiation
GO:0006816	0.0149	2.80	3.30	8	42	calcium ion transport
GO:0032940	0.0156	3.42	2.10	6	27	secretion by cell
GO:0043405	0.0165	2.55	4.01	9	51	regulation of MAP kinase activity
GO:0007168	0.0176	23.54	0.24	2	3	receptor guanylyl cyclase signaling pathway
GO:0021542	0.0176	23.54	0.24	2	3	dentate gyrus development
GO:0050847	0.0176	23.54	0.24	2	3	progesterone receptor signaling pathway
GO:0015980	0.0178	23.35	0.24	2	3	energy derivation by oxidation of organic compounds
GO:0007268	0.0192	1.95	8.41	15	108	synaptic transmission
GO:0045909	0.0201	7.07	0.63	3	8	positive regulation of vasodilation
GO:0032774	0.0242	1.38	44.39	57	564	RNA biosynthetic process
GO:0007623	0.0255	4.30	1.18	4	15	circadian rhythm
GO:0006607	0.0284	5.89	0.71	3	9	NLS-bearing substrate import into nucleus
GO:0034104	0.0284	5.89	0.71	3	9	negative regulation of tissue remodeling
GO:0003001	0.0298	3.30	1.80	5	23	generation of a signal involved in cell-cell signaling
GO:0006533	0.0333	11.77	0.31	2	4	aspartate catabolic process
GO:0009405	0.0333	11.77	0.31	2	4	pathogenesis
GO:0006334	0.0359	3.11	1.89	5	24	nucleosome assembly
GO:0015674	0.0366	1.99	6.05	11	77	di-, tri-valent inorganic cation transport
GO:0045860	0.0371	1.98	6.06	11	77	positive regulation of protein kinase activity
GO:0051347	0.0371	1.98	6.06	11	77	positive regulation of transferase activity
GO:0000122	0.0377	1.91	6.83	12	87	negative regulation of transcription from RNA polymerase II promoter
GO:0008630	0.0382	5.05	0.79	3	10	DNA damage response, signal transduction resulting in apoptosis
GO:0046530	0.0382	5.05	0.79	3	10	photoreceptor cell differentiation
GO:0007601	0.0388	1.85	7.62	13	97	visual perception
GO:0000187	0.0414	2.97	1.96	5	25	activation of MAPK activity
GO:0002504	0.0496	4.42	0.87	3	11	antigen processing and presentation via MHC class II

GOBPID: GO ID of BP term; p value is the significance of enrichment; Odds ratio is the ratio of odds that a GO term is enriched in the selected category; Exp. count represents the number expected; Count represents the number of transcripts annotated for the related GO term; Size is the number of probe sets overrepresented in the universe (background).

Table 4. List of GO terms overrepresented in a set of genes differentially expressed in the brain of sticklebacks exposed to predator cues at p < 0.01

GOCCID	p value	Odds ratio	Exp. count	Count	Size	Term
Cellular component						
GO:0042612	0.0009	9.93	0.86	5	11	MHC class I protein complex
GO:0045211	0.0045	2.67	5.17	12	66	postsynaptic membrane
GO:0005751	0.0061	Infinity	0.16	2	2	mitochondrial respiratory chain complex IV
GO:0042622	0.0061	Infinity	0.16	2	2	photoreceptor outer segment membrane
GO:0005625	0.0077	2.14	8.30	16	106	soluble fraction
GO:0019717	0.0086	3.48	2.43	7	31	synaptosome
GO:0001917	0.0174	23.67	0.23	2	3	photoreceptor inner segment
GO:0000159	0.0198	7.11	0.63	3	8	protein phosphatase type 2A complex
GO:0042613	0.0198	7.11	0.63	3	8	MHC class II protein complex
GO:0005834	0.0252	4.32	1.17	4	15	heterotrimeric G-protein complex
GO:0005640	0.0280	5.93	0.70	3	9	nuclear outer membrane
GO:0005891	0.0316	3.96	1.25	4	16	voltage-gated calcium channel complex
GO:0042825	0.0330	11.83	0.31	2	4	TAP complex
GO:0045202	0.0351	1.72	10.64	17	136	synapse
GO:0019898	0.0377	2.45	3.21	7	41	extrinsic to membrane
GO:0034707	0.0480	2.83	2.04	5	26	chloride channel complex
Molecular functions						
GO:0004918	0.0063	Infinity	0.16	2	2	interleukin-8 receptor activity
GO:0005546	0.0063	Infinity	0.16	2	2	phosphatidylinositol-4,5-bisphosphate binding
GO:0048038	0.0063	Infinity	0.16	2	2	quinone binding
GO:0004691	0.0082	11.72	0.48	3	6	cAMP-dependent protein kinase activity
GO:0004871	0.0139	1.94	9.60	17	122	signal transducer activity
GO:0015226	0.0178	23.38	0.24	2	3	carnitine transporter activity
GO:0005388	0.0204	7.03	0.63	3	8	calcium-transporting ATPase activity
GO:0008601	0.0204	7.03	0.63	3	8	protein phosphatase type 2A regulator activity
GO:0008565	0.0271	2.66	3.00	7	38	protein transporter activity
GO:0022890	0.0306	3.27	1.82	5	23	inorganic cation transmembrane transporter activity
GO:0003713	0.0327	1.90	7.44	13	94	transcription coactivator activity
GO:0019905	0.0335	11.72	0.32	2	4	syntaxin binding
GO:0003711	0.0336	11.71	0.32	2	4	transcription elongation regulator activity
GO:0004571	0.0337	11.69	0.32	2	4	mannosyl-oligosaccharide 1,2-alpha-mannosidase activity
GO:0008131	0.0337	11.69	0.32	2	4	amine oxidase activity
GO:0004674	0.0372	1.61	13.97	21	177	protein serine/threonine kinase activity
GO:0005245	0.0387	5.02	0.79	3	10	voltage-gated calcium channel activity
GO:0004112	0.0403	3.61	1.35	4	17	cyclic-nucleotide phosphodiesterase activity
GO:0005216	0.0485	2.51	2.70	6	34	ion channel activity

GOCCID: GO ID of the CC term, GOMFID: GO ID of MF term; p value is the significance of enrichment; Odds ratio is the ratio of odds that a GO term is enriched in the selected category; Exp. count represents the number expected; Count represents the number of transcripts annotated for the related GO term; GO term size is the number of probe sets overrepresented in the universe (background).

sensory perception. GO terms in immune response categories involved antigen processing and presentation, detection of external stimulus while biosynthetic processes overrepresented included neutral lipid biosynthetic process, glycerol ether biosynthetic process and negative regulation of biosynthetic process. Terms associated with gene regulation were negative regulation of tissue remodeling and positive regulation of protein kinase activity. GO terms associated with development were embryonic skeletal system morphogenesis, anterior/posterior pattern formation and dentate gyrus development. Other GO terms were, for example, signal transduction resulting in induction of apoptosis. MHC class I protein complex was the most overrepresented cellular GO term with

Table 5. qPCR results of microarray data validation

Gene	Type	Fold change	STD error	p value	Regulation
GAPDH	reference	1.0			
RHO_57	target	12.1	0.573	0.038	up
RHO_60	target	6.4	0.463	0.006	up
USP-like	target	5.9	0.443	0.002	up
Tr_22897	target	1.5	1.231	0.25	NS
PLA2G12B	target	4.0	0.784	0.207	NS
GLUT1	target	0.5	0.354	0.006	down
C2	target	0.6	0.452	0.03	down
CXCR1	target	0.3	0.139	0.002	down
KCNK12	target	1.2	0.784	0.312	NS
PCYB12	target	2.7	1.289	0.046	up

GADPH (glyceraldehyde 3-phosphate dehydrogenase): reference gene used for normalization of the expression values of the target genes. Target genes were RHO_57 (rhodopsin transcript variant ENSGACT00000003757), RHO_60 (rhodopsin transcript variant ENSGACT00000003760), USP (ubiquitin-specific protease), Tr_22897 (transcript 'ENSGACT00000022897'), PLA2G12B (phospholipase 2 group XIIB), GLUT1 (glucose transporter 1), C2 (complement precursor), CXCR1 (interleukin-8 receptor alpha), KCNK12 (potassium channel), PCYT1B (phosphate cytidylyltransferase 1, choline, beta). Fold changes of the target genes are expression changes relative to the expression of the reference gene, which is by default 1. The direction of the changes in gene expression is indicated in the 'Regulation' column. up are upregulated genes, down are downregulated genes and NS represents genes whose expression is not statistically different from the reference gene.

46% genes enriched for this term followed by the postsynaptic membrane mitochondrial respiratory chain complex IV, the synaptosome and the voltage-gated calcium channel complex.

Network analysis showed that out of the 5,016 network eligible genes (genes mapped to a pathway by IPA), 348 transcripts were mapped to a network using the IPA system (p = 0.01). For example, 35 genes in a network involving cell signaling, nucleic acid metabolism, cellular assembly and organization were differentially expressed in our experiment (IPA score = 35). Similarly, 33 genes in a network involving neurological disease, developmental disorder and psychological disorders were also differentially expressed (IPA score = 30). Pathway analysis revealed that the synaptic long-term depression pathways was the most overrepresented [–log(p) = 2.84E-04], followed by the RAN signaling pathway [–log(p) = 2.62 E-04], the relaxin-signaling pathway [–log(p) = 1.98E-04] and the phototransduction pathways [-log(p) = 1.98E-04].

Microarray Validation

All the 10 genes selected for validation of the microarray results, in addition to the reference gene GAPDH, were successfully amplified by qPCR. As a first round of amplification of RHO was not consistent across samples, new primers were designed based upon the sequences of the transcript variants. For the samples that had been included on the microarray, the 10 genes were regulated in the same direction as in the microarray (table 5). Of the 10 genes selected for validation, 7 genes were significantly differentially expressed. Both RHO variants (RHO_57 and RHO_60), USP-like and PCYB12 were upregulated in qPCR as in the microarray, while GLUT1, C2, and CXCR1 were significantly downregulated at 95% confidence interval. PLA2G12B, Tr_22897 and KCNK12, which were significantly upregulated in the microarray, were also upregulated in qPCR; however, the difference between treatments was not statistically significant. Other studies have also failed to validate all genes by qPCR, particularly when the genes have low fold changes [Mukai et al., 2009]. There was no detectable difference between the samples included in the microarray experiment and in the independent biological replicates that were not included in the microarray analysis. For example, the expression values of the top differentially expressed genes did not differ significantly between these two groups (d.f. = 1, F = 0.04, p = 0.8). We also compared the expression of 6 genes in samples that had not been included on the microarray and found that the microarray and independent biological replicates showed similar patterns: 5 of the 6 genes showed the same direction of expression change (up- or downregulated) (fig. 3).

Discussion

Exposure to olfactory, visual and tactile cues of a predator for 6 days resulted in significant transcriptomic changes in the brain of sticklebacks. We found that 10% of the transcripts were differentially expressed (p ≤ 0.01) between fish exposed to cues of a predator and a control group. These patterns are consistent with other studies involving predator cues [Nanda et al., 2008; Leder et al., 2009]. For example, a 15-min exposure to ferret olfactory, visual and acoustic cues increased stress-like behavior in rats and resulted in the differential expression of 7% of genes in the amygdala 3 h after exposure to a predator [Nanda et al., 2008]. Another study found that of the 5,931 cDNA clones used in cDNA microarray, a total of 52 genes (8%) were differentially expressed in the brain of

Fig. 3. qPCR validation of microarray gene expression in the brains of sticklebacks exposed to predator cues. The microarray results are plotted next to the qPCR results for the samples used in the microarray (n = 7 experimental and n = 7 control), and the qPCR results for the independent biological replicates not on the array (n = 2 experimental and n = 2 control).
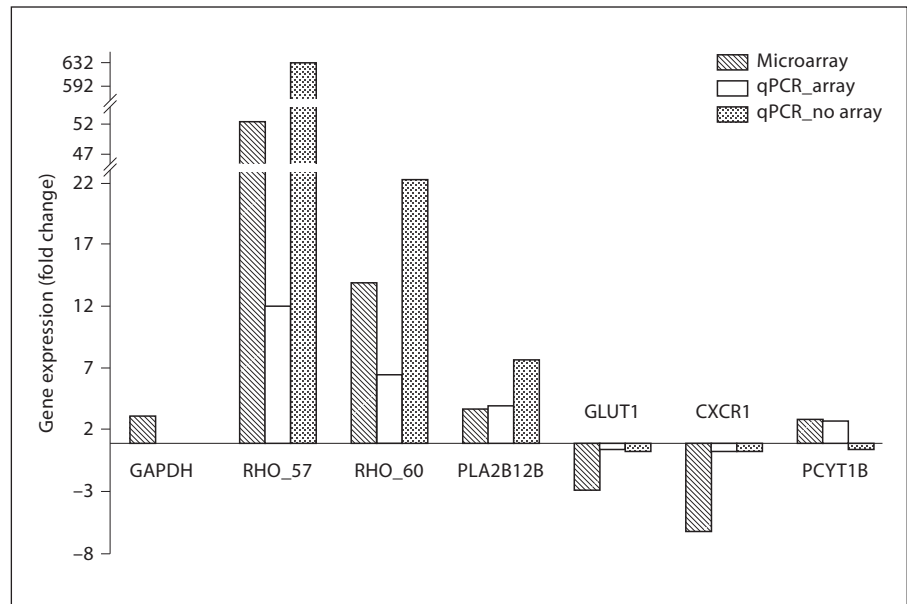
rats exposed to cats when gene expression was assessed 20 min after exposure to a predator [Wang et al., 2003].

Some of the genes and pathways that were identified in this study have also been implicated in related studies. For example, 17 out of 909 differentially expressed genes (at p < 0.01 cutoff) have been identified in previous studies. These include, for example, SAMD4A, FGFR10P2, and CD99LD, which were identified during a selection response for contextual fear conditioning in rats using quantitative trait locus (QTL) and gene expression studies [Ponder et al., 2008]; KEK4, MUB and HSPB1 were identified in fruit flies selected for aggressive behavior [Dierick and Greenspan, 2006] and PEX19 and DEDD were identified proximal to a QTL hotspot on mouse chromosome 1 that modulates behavior [Mozhui et al., 2008]. Similarly, the long-term synaptic depression (LTD) pathway, one of the most overrepresented pathways revealed in our study, has been associated with predator threat. LTD is an electrical correlate of the weakening of the connection between two neurons, thus leading to long-term reduction in the efficacy of neuronal synapsis as a consequence of a decrease in the density of postsynaptic receptors. Studies have shown that both acute and chronic stress [Yang et al., 2004; Holderbach et al., 2007], including exposure to predators [Kuzmiski et al., 2010; Kim and Diamond, 2002], can lead to LTD. LTD has also been associated with synaptic plasticity and memory formation [Bear, 1999; Malleret et al., 2010; Kim and Diamond, 2002].

Importantly, our study also identified many genes and biological pathways that have not been previously reported. For example, to the best of our knowledge PLA2G12B, RHO and GLUT1 have not yet been associated with exposure to predator stress. Similarly, our study is one of the first to report the activation of the RAN and the relaxin pathways in response to long-term exposure to predator cues. Different sets of genes in the brain respond to acute versus chronic stress [Krasnov et al., 2005], which might explain why there was some, but not complete, overlap between the differentially expressed genes found in this experiment compared to other experiments that have examined the transcriptomic response to a single, brief exposure to predation risk [e.g. Wang et al., 2003].

Although sticklebacks mounted a transcriptomic response to repeated exposure to a predator, the overall magnitude of the response, as assessed by fold change, was relatively low. While some genes, such as RHO, had a high fold change, most genes were weakly differentially expressed. Other studies have also detected subtle transcriptomic responses to behavioral challenges such as predator threat [Wang et al., 2003] or aggression [Mukai et al., 2009]. One possible explanation for the relatively small changes in expression is that transcription profiling was conducted 24 h after repeated exposure to predation risk. It is possible that the patterns we observed reflect relatively long-term, enduring changes in the brain rather than transitory responses. Alternatively, or in addition, it is possible that we measured the downstream

consequences of exposure to predation risk 24 h earlier. Detailed time-course experiments are needed to distinguish the response to single (acute) versus repeated (chronic) exposures to predation risk across short (over the course of minutes) versus long (over the course of days to months) timescales. The stickleback system offers the opportunity to frame this question from an evolutionary perspective by comparing the genes that are involved in the plastic response to predation risk within an individual's lifetime with the genes that are involved in evolutionary divergence between different stickleback populations in response to variation in predation pressure [Giles and Huntingford, 1984; Bell, 2005].

The wide range in GO terms that were overrepresented in the stickleback brain suggests that exposure to cues of a predator triggers several biological processes in the prey. Many genes related to neurological system process (n = 270), negative regulation of neurological processes (n = 197 genes), synaptic transmission (n = 108) and visual perception (n = 97) were represented in the list of differentially expressed genes; however, these genes were not necessarily the most overrepresented based upon p values of the GO terms. Intriguingly, the most overrepresented biological GO terms in the brain of sticklebacks following exposure to predator cues were related to antigen processing and presentation (p < 0.0008), which also corresponded to the top cellular component GO term: MHC class I protein complex (p < 0.0009). MHC is a cluster of genes that encode a complex of transmembrane glucoproteins expressed on the surface of all somatic cells, including neurons [Corriveau et al., 1998]. They are involved in immune response and are critical in the olfactory-based discrimination of self/nonself in many vertebrate species, including humans [Wedekind et al., 1995], mice [Egid and Brown, 1989] and fish [Milinski et al., 2005]. In sticklebacks, while MHC class II gene has been shown to play a role in mate choice [Milinski et al., 2005] and kin recognition [Olsen et al., 1998], the role of MHC class I in sticklebacks is less clear. Intriguingly, studies have shown that MHCI plays a key role in synaptic plasticity and neuron regeneration [Oliveira et al., 2004]. Other studies have demonstrated that MHCI is critical in neuronal plasticity [Goddard et al., 2007]. In addition, recent studies have implicated MHCI in memory formation and behavior [Shatz, 2009]. Some studies have shown that acute predator stress can lead to memory impairment [Diamond and Park, 2000]. The involvement of MHCI in many biological functions is correlated with its high diversity observed both intra- and interspecifically [Shiina et al., 2010]. It has been postulated that this diversity is driven by selection pressure that acts on MHC genes under various environmental pressures such as microbial threats or environmental sensory cues [Apanius et al., 1997; Bernatchez and Landry, 2003; Spurgin and Richardson, 2010]. Understanding the interactions between MHC genes, olfaction, memory and predator stress is a promising area for future research.

Our data suggest that sticklebacks strongly responded to visual cues of a predator. Both RHO splice variants were successfully validated by qPCR. This gene is expressed not only in the retina but also in the deep brain, including the pineal gland where it plays an important role in photoreception and photoperiodism [Masuda et al., 2003]. Since the eyes were separated from the brain during sample preparation, the high expression of this gene suggests its involvement in brain function. This is further supported by the overrepresentation of the GO terms related to detection of light stimulus involved in sensory perception and phototransduction and supports the hypothesis that processes associated with visual perception were important in the response to predation risk. This is consistent with previous observations that vision is an important modality for communication in sticklebacks [Huntingford and Ruiz-Gomez, 2009].

Sticklebacks in this experiment were exposed to olfactory cues of trout and dead conspecifics that contained alarm pheromones. Alarm pheromones are released from club cells when the skin is punctured and cause sticklebacks to exhibit increased antipredator behaviors [Brown and Godin, 1997] and may have originally evolved as an immune response [Chivers et al., 2007]. Interestingly, PLA2G12B, a gene that has been shown to play a regulatory role in the induction of olfactory structures in frogs [Munoz-Sanjuan and Brivanlou, 2005], was upregulated in response to our treatment, highlighting the importance of olfaction in sticklebacks.

The animals in this experiment were socially housed. Therefore, it is possible that some of the response of the sticklebacks in the experimental group was a reaction to stressed or frightened conspecifics. Observations in mammals, including humans, have shown that exposure to conspecific emotional stress elicits fear response in the amygdala [Mujica-Parodi et al., 2009]. Studies have also demonstrated that exposure to conspecific alarm hormones impedes the humoral and cellular immune response in male BALB/cJ mice [Cocke et al., 1993].

Further investigations of the gene modules and pathways herein identified might provide insight into the molecular mechanisms underlying the response of sticklebacks to predator-induced stress. Network and pathway

analyses revealed that genes differentially expressed as a result of exposure of sticklebacks to predator cues are clustered in gene modules. This was also supported by the clusters observed in the hierarchical clustering in the heat map (fig. 2). For example, gene regulatory networks that involve neurological diseases, developmental disorders and psychological disorders as well as cell signaling, nucleic acid metabolism, cellular assembly and organization were revealed using network analysis. Future studies will compare the expression of these genes across different populations of sticklebacks that vary in predation pressure, and will pharmacologically manipulate the pathways in order to infer a causal relationship between exposure to predators and behavior.

In conclusion, this study examined the gene expression pattern in the brain of sticklebacks under conditions that mimic naturally high predation pressure in the wild. We showed that repeated exposure to cues of a predator results in significant differential expression of genes involved in immune response, synaptic processes, brain metabolic processes and visual perception. Gene functional annotation and gene ontology and pathway analyses identified several biological and molecular processes that might be activated as a response to predation risk. The radiation of sticklebacks along with growing genomic resources [Kingsley and Peichel, 2007; Brown et al., 2008; Geoghegan et al., 2008; Williams et al., 2008; Leder et al., 2009] and its well-characterized biology, make this organism a suitable model system for investigating the molecular and physiological responses of the brain to ecologically relevant selection pressures that have influenced their evolution.

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