Effect of Acute Stressor and Serotonin Transporter Genotype on Amygdala First Wave Transcriptome in Mice

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Abstract

The most prominent brain region evaluating the significance of external stimuli immediately after their onset is the amygdala. Stimuli evaluated as being stressful actuate a number of physiological processes as an immediate stress response. Variation in the serotonin transporter gene has been associated with increased anxiety- and depression-like behavior, altered stress reactivity and adaptation, and pathophysiology of stress-related disorders. In this study the instant reactions to an acute stressor were measured in a serotonin transporter knockout mouse model. Mice lacking the serotonin transporter were verified to be more anxious than their wild-type conspecifics. Genome-wide gene expression changes in the amygdala were measured after the mice were subjected to control condition or to an acute stressor of one minute exposure to water. The dissection of amygdalae and stabilization of RNA was conducted within nine minutes after the onset of the stressor. This extremely short protocol allowed for analysis of first wave primary response genes, typically induced within five to ten minutes of stimulation, and was performed using Affymetrix GeneChip Mouse Gene 1.0 ST Arrays. RNA profiling revealed a largely new set of differentially expressed primary response genes between the conditions acute stress and control that differed distinctly between wild-type and knockout mice. Consequently, functional categorization and pathway analysis indicated genes related to neuroplasticity and adaptation in wild-types whereas knockouts were characterized by impaired plasticity and genes more related to chronic stress and pathophysiology. Our study therefore disclosed different coping styles dependent on serotonin transporter genotype even directly after the onset of stress and accentuates the role of the serotonergic system in processing stressors and threat in the amygdala. Moreover, several of the first wave primary response genes that we found might provide promising targets for future therapeutic interventions of stress-related disorders also in humans.

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Introduction

Acute stressors such as unexpected and potentially threatening changes of an individual’s environment usually provoke immediate stress responses to adapt, which are accompanied by and actuating a number of physiological processes [1]. However, the way an individual responds to stress may vary depending on a wide range of environmental as well as genetic factors. A promising candidate for a further elucidation of these processes is the serotonergic system which is strongly involved in stress response and adaptation. A key regulator of serotonergic activity in the central nervous system is the serotonin transporter (5-HTT) that has also been linked to inappropriate regulation of the stress response, for example in anxiety- and stress-related personality traits and neuropsychiatric disorders [2,3].

The human gene encoding the 5-HTT (Slc6A4) is modulated by a length variation of a repetitive element in its upstream regulatory region commonly composed of either 14 (short allele) or 16 (long allele) repeated elements [4,5,6]. The short variant of the polymorphism causes a reduction in the transcriptional efficiency of the 5-HTT promoter, resulting in decreased 5-HTT expression and therefore a reduced serotonin level. The low-expressing 5-HTT short variant has been implicated in higher scores of neuroticism and is associated to a heightened trait anxiety/dysphoria and an exaggerated response to fear as well as environmental stress [2,8,9,10]. The evidence of a connection between mood disorders and genetic variation of the 5-HTT led to the identification of fundamental phenotypic changes, ranging from increased anxiety- and depression-related behaviors, gene expression differences to altered dendritic morphology (reviewed by [12]). Further, a growing literature indicates altered...
stress reactivity and impaired abilities to cope with or adapt to stress in 5-HTT knockout mice [13,14], involving also differences in gene expression [15,16].

Within the central nervous system, 5-HT nerve terminals are particularly present in stress-related brain regions like amygdala, hippocampus, striatum and cortex [17]. Of these, the amygdala is one of the core systems driving emotional processes and vigilance by evaluating the significance of a stressor and triggering the most adequate emotional response based upon previous experiences as well as upon genetic predispositions [18]. A strong link exists between altered amygdala function and psychopathology, e.g. association of the short 5-HTT variant with a relative exaggeration in the response of the amygdala to anxiety-provoking or stress-related stimuli [3,18,19,20,21,22]. Further, changes in gene expression accompanying stress reactivity and coping also affect the amygdala, both after acute stress [23,24,25,26] as well as after chronic stress [27,29]. However, nothing is known about rapid changes in amygdala gene expression following immediately after an acute stressor in case of individuals with an imbalanced serotonergic system that are prone to inappropriate stress response. We hypothesized basal differences in anxiety-related behavior of mice as previously described between 5-HTT genotypes [13,29] and differences in mRNA profiles that are dependent on the condition (acutely stressed vs. control) and the genotype (5-HTT knockout vs. wild-type). Such differences between the genotypes in their first wave response might prove to be extremely valuable for explaining genotype-dependent differences in coping with stress.

The present study thus aimed to explore immediate changes in genome-wide amygdala mRNA profiles in response to an acute stressor using the 5-HTT knockout mouse model. We observed basal differences in anxiety-related behavior as well as differing transcription profiles between acutely stressed compared to control mice already a few minutes after the stressor together with differences depending on 5-HTT genotype reflecting 5-HTT level-based changes in emotion processing and stress coping.

Methods

Animals and Housing Conditions

5-HTT knockout mice were generated on a C57BL/6J background [11]. Initially a total of 16 knockout (−/−, KO) and 14 wild-type (+/+, WT) male mice, descendants from heterozygous parents from the internal stock bred at the University of Muenster, were included for behavioral analysis. Sample sizes were reduced for hormonal analysis (see below) and further cut down for microarray analysis. Genotypes were identified by gel electrophoresis of DNA-fragments of either 225 bp (WT) or 272 bp (KO) [11]. Testing animals were weaned at 21 days of age and maintained in sibling groups of 3 to 5 male mice until they were transferred to single housing at an age of 5 months, 2 months prior to behavioral testing. The housing room was maintained at a 12 h light/dark cycle (lights on at 8.00 h) at a temperature of 22±3°C.

The presented work complies with current regulations covering animal experimentation in Germany and experiments were approved by the competent local authority, North Rhine-Westphalia State Agency for Nature, Environment and Consumer Protection (LANUV NRW, Permit number: 8.87–50.10.46.08.090), and all efforts were made to minimize suffering.

Elevated Plus-maze Test (EPM)

At the age of 7 months ±14 days the mice were tested in the EPM. The plus shaped apparatus was elevated 50 cm above the ground and illuminated by an overhead bulb (200 lux in the center of the EPM). The maze comprised four arms (5 cm×30 cm) extending from a central platform (5 cm×5 cm), two opposing arms were enclosed with 20 cm high walls and the other two opposing arms were unshielded. The mice were placed for 1 min in an empty cage prior to testing to assure comparable amounts of arousal. Each mouse was placed on the central platform facing a shielded arm and was then allowed to freely explore the apparatus for 10 min. The movements of the animals were recorded by a digital tracking system (www.phenotyping.com/digital.html) assessing path-length, arm entries and time spent within the arms.

Acute Stress and Tissue Sampling

Two weeks after testing in the EPM, mice were subjected to two different conditions, either acute stress or control condition. Groups sizes were KO: nacute stress = ncontrol = 8, WT: nacute stress = 8, ncontrol = 6. Sampling was conducted in a standardized way within 9 min involving four experimenters who were coordinated with the help of an in-house developed software that prompted every single step of the time-critical procedure ("Talkative Timer", www.phenotyping.com/talktimer.html, available on demand). The protocol started with an experimenter entering the housing room and transferring the cage of the experimental animal to an adjacent room where half of the mice were left untouched in their cages for 1 min (control) and the other half was placed into a bucket filled with water (~21°C) for 1 min (acute stress). Afterwards all mice were anaesthetized by isoflurane for 40 sec, weighed, and decapitated exactly 160 sec from the start of the protocol. Trunk-blood was collected during the following 40 sec using heparinized capillaries, centrifuged (5 min at 14900 × g) and plasma was stored at −20°C for later evaluation. The brain of each mouse was dissected from the skull within 105 sec, starting 175 sec after the first step of the procedure. It was placed into ice cold (4°C) artificial cerebrospinal fluid (124 mM NaCl, 4 mM KCl, 1 mM CaCl2, 1.24 mM NaH2PO4, 1.3 mM MgSO4, 26 mM NaHCO3, 10 mM glucose; pH 7.4) for 60 sec prior to dissection of both amygdalae within 90 sec.

Homogenization of the amygdalae started after 430 sec from the protocol start by comminuting tissue in 1.5 ml eppendorf tubes using low volume filter tips (Type J, Sarstedt). After 60 sec 100 μl 2X Nucleic Acid Purification Lysis Solution (Applied Biosystems by Life Technologies) was added to further homogenize and effectively lyse tissue for 50 sec. Within this time cellular reactions were stopped, particularly all RNases were immediately inactivated and all RNAs precipitated to prevent degradation. Altogether 540 sec after the start of the protocol the procedure was finished and the samples were stored at −20°C for later RNA extraction.

Corticosterone Assay

Plasma corticosterone concentrations were determined by enzyme linked immunosorbent assay (ELISA, DE4164, Demeditec Diagnostics GmbH, Kiel, Germany) according to the manufacturer’s recommendations. All standards, samples, and controls...
were run in duplicate concurrently. The intra- and inter-assay coefficients of variation were 3.3% and 6.0%, respectively. Corticosterone titers were analyzed using the R software package (see above) by an ANOVA in a two by two factorial design with genotype and treatment as between subject factors.

**Microarray Analysis and Bioinformatics**

Total RNA was extracted from the amygdala samples using the ABI 6100 Nucleic Acid PrepStation with Total RNA Chemistry according to the manufacturer’s instructions (Applied Biosystems) including preceding Proteinase K digestion (0.4 mg/sample at RT). RNA concentrations were calculated by measurement of 260/280 nm absorbance (Eppendorf BioPhotometer; RNA program): Samples below 30 ng/µl were concentrated by Microcon centrifugal filter devices (YM-3) as recommended by the manufacturer (Millipore). RNA quantity and quality were additionally determined using Agilent Bioanalyzer 2100 with Eukaryote Total RNA Nano Chip Assays and 2100 Expert software version B.02.06 (all Agilent Technologies). RNA quantity and quality threshold was set to 30 ng/µl and RNA Integrity Number (RIN) = 8. Altogether 20 samples were chosen (5 biological replicates per genotype and condition) fitting these recommendations with averaged concentration of 60 ng/µl (range 30–84) and RIN of 9.13 (range 8.2–9.7). Three µl per RNA sample were used for subsequent gene expression analysis of 28833 genes using GeneChip Mouse Gene 1.0 ST Arrays (Affymetrix, MoGene-1_0-st). The 20 microarray datasets (CEL files) were analyzed with ArrayAssist Software (Stratagene/Agilent). exonRNA algorithm was used for summarization and normalization. After that data was variance stabilized, transformed on logarithmic scale and quality control was performed. All microarray data in this publication are MIAME compliant, have been deposited in NCBI’s Gene Expression Omnibus [30] and are accessible through GEO Series accession number GSE40393 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE40393). Differentially expressed genes were ascertained by unpaired t-tests in the four genotype by condition groups resulting in four different comparisons. Within the genotypes the conditions acute stress (stress) versus control (con) were compared: KO stress vs. con and WT stress vs. con. Further, genotype comparisons were performed within the control condition (con KO vs. WT) as well as within the acute stress condition (stress KO vs. WT). A correction for false discovery rate was not applied because this proved overly stringent given the high number of genes that were included and therefore was likely to produce false negative results. Instead, we applied a combined criterion for true positive expression differences of a fold difference >1.5 and a P-value <0.05.

Gene functional annotation and enrichment analyses were performed using the web-based tool DAVID Bioinformatics Resources 6.7 (Database for Annotation, Visualization and Integrated Discovery v6.7; http://david.abcc.ncifcrf.gov/home.jsp) [31,32]. More specifically, own gene lists of group comparisons KO stress vs. con, WT stress vs. con, con KO vs. WT, and stress KO vs. WT with gene fold differences >1.5 and P-values <0.05 were uploaded and submitted to DAVID together with identifier Affymetrix_exon_gene_ID and pre-built Affymetrix Exon Background (MoGene-1_0-st-v1). For identification of groups with similar biological meaning that are enriched in own gene lists compared to the background, data were analyzed with Functional Annotation Clustering using default setting (medium) for classification stringency. Results were narrowed down on significant annotation clusters with Group Enrichment Scores ≥1.3, EASE scores (P-values) for individual term members ≤0.05, and fold enrichment ≥1.5, as well as low family-wise false discovery rates (Benjamini multiple testing correction P-values ≤0.15). In addition, exploratory pathway analyses were performed with own gene lists of group comparisons using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database implemented in DAVID.

The validity of the microarray results was subsequently tested via commercial quantitative real-time Taqman PCR assays for several candidate genes: one or two validated genes (no pseudo or predicted genes) with fold changes >1.75 per group comparison representing different annotation clusters and pathways, respectively, and Gapdh as endogenous control. PCRs were set according to the manufacturer’s instructions (Applied Biosystems) on a Tecan Freedom EVO 150 and relative quantification was analyzed using the 7900 HT system with software SDS 2.1.1 (Applied Biosystems).

**Results**

**Elevated Plus-maze (EPM)**

General activity in the EPM measured as total number of arm entries and path length differed between WT and KO mice with the latter being less active (Wilcoxon Test, path length: W = 20, P<0.0001, Fig. 1a; total arm entries: W = 31, P = 0.0008). Consequently, anxiety-related measures of number of open arm entries and time spent in open arms of the EPM were calculated as percent. WT mice entered a significantly higher percentage of open arms (Wilcoxon Test, W = 53.3, P = 0.0077) and spent significantly more percentage of time in the open arms as KO mice (Wilcoxon Test, W = 50, P = 0.0047, Fig. 1b).

**Corticosterone**

Plasma corticosterone titers (Fig. 2) were at basal levels in the control condition in both genotype groups (mean KO: 3.87±1.67 (SEM) ng/ml, mean WT: 2.59±0.68 (SEM) ng/ml). Acute stress overall led to an increase of stress hormone concentration in both genotypes (mean KO: 8.99±2.92 (SEM) ng/ml, mean WT: 4.39±1.5 (SEM) ng/ml).

ANOVA revealed a trend for increased stress hormone concentrations in mice subjected to acute stress (F1,16 = 2.92, P = 0.063) but no effect of genotype (F1,16 = 2.56, P = 0.14) and there was no significant interaction of genotype by stress (F1,16 = 0.68, P = 0.42).

**Microarray Analysis**

Microarray analysis disclosed 80 differentially expressed genes between the conditions acute stress and control. Most of them, that is 77 genes, were upregulated immediately after the stressor, only 3 genes were downregulated. Closer inspection of the impact of acute stress vs. control in the different 5-HTT genotype groups revealed no overlap of genes in the expression profiles. About half of the genes were differentially expressed in WT mice after the acute stressor (38 genes: 36 up-, 2 downregulated) and the other half in KO mice (42 genes: 41 up, 1 downregulated). Direct comparison of both genotypes within the acute stress condition indicated 90 genes differentially expressed, 36 of them upregulated in WT mice and the other 54 upregulated in KO mice. In contrast, within the control condition only slight differences appeared between both genotypes with only 15 differentially expressed genes, 9 of them upregulated in WT and 6 in KO mice (for detailed gene lists concerning all group comparisons see Table S1).
Functional Annotation Clustering and KEGG Pathway Analysis by DAVID

In the next step the resulting microarray group gene lists were functionally categorized using DAVID. Significant annotation clusters were detected in the groups acute stress vs. control including the terms neurogenesis and cation binding as well as terms like glycoprotein, membrane, and transport (for a complete list of clusters and enriched terms in all gene groups see Table 1). Again closer inspection of the impact of acute stress within the different 5-HTT genotype groups revealed no overlap of clusters: the first terms (neurogenesis, cation binding) were observed only in WT mice and the latter terms (glycoprotein, membrane, transport) only in KO mice. The direct genotype comparison WT vs. KO within the stress condition revealed three clusters with the terms calcium ion binding, cilium/cell projection, and cell projection part. Of these, terms including ion binding (metal ion/cation/calcium binding) were enriched in upregulated genes of WT mice, while the other terms including cilium/axoneme/cilium part/cell projection part were enriched in upregulated genes of KO mice (Table 1). In contrast, no functional clusters reaching our criteria of significance were detected in the genotype comparison WT vs. KO within the control condition.

The different annotation clusters were represented by varying fractions of genes ranging from only about 5–10% up to 40–60% (overview: Table 1; details: Tables 2, 3, 4). The neurogenesis cluster, that comprised about 10% of all genes upregulated in WT stress vs. con, included genes like roundabout homolog 2 (Robo2), slit homolog 3 (Slit3), and sema domain 3E (Sema3e). All these genes were also included in the functionally highly overlapping KEGG pathway axon guidance (Table 2; details: Table 5), supporting the relevance of this cluster. The second cluster detected in WT after stress, cation binding, had marginal enrichment score but represented about 45% of this group. It included genes like cadherin 9 (Cdh9), Slit3, calcium binding protein 1 (Cabp1), parvalbumin (Pvalb) and several receptors like glutamate receptor, ionotropic, NMDA2A (Grin2a), nuclear receptors of subfamily 4, group A, members 2 and 3 (Nr4a2, Nr4a3), and ryanodine receptor 1 (Ryr1). Again similar results were obtained from KEGG pathway analysis that revealed the calcium signaling pathway containing genes like Grin2a and Ryr1, however with EASE score reaching only trend significance (Table 2, 5).

Highest enrichment scores combined with highest gene coverage were detected in the four clusters glycoprotein, plasma

Figure 1. Elevated plus-maze test. a) Activity-related measures exemplified as the path length covered during the test. Bars represent the median values and the dots represent the data of each individual animal. b) Anxiety-related behavior measured as the proportion of time spent in open arms. Serotonin transporter knockout mice (5-HTT KO, n = 17) were less active and spent a lower proportion of time on open arms indicating increased anxiety compared to wild-type (5-HTT WT, n = 14) mice. Statistics: Wilcoxon exact rank sum test: ** = P < 0.01, *** = P < 0.001.

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Figure 2. Corticosterone stress hormone concentration. Trunk blood samples were taken 130 sec after the mice were either being placed for 1 min in 21°C water (acute stress) or left untouched in the home-cage for 1 min (control). Bars represent the mean concentration, whiskers express SEM. An ANOVA revealed no significant effect of genotype (F1,26 = 2.36, P = 0.14) but a trend for increased stress hormone concentrations in mice subjected to acute stress (F1,26 = 3.21, P = 0.085).

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Table 1. Significant functional annotation clusters in different gene groups related to condition (acute stress or control) and 5-HTT genotype (WT or KO) acquired by DAVID tool.

| Cluster | Scores | Category | Enrichment Term | # (% Genes) | P-Value  |
|---------|--------|----------|-----------------|-------------|----------|
| Group: WT stress vs. con (Total: 38 genes) | 1 | 1.76 | SP_PIR_KEYWORDS | neurogenesis | 4 (10.5) | 0.003   |
| | 2 | 1.29 | GOTERM_MF_FAT | GO:0043169~cation binding | 17 (44.7) | 0.004   |
| | 2 | 1.29 | GOTERM_MF_FAT | GO:0043167~ion binding | 17 (44.7) | 0.004   |
| Group: KO stress vs. con (Total: 42 genes) | 1 | 6.03 | SP_PIR_KEYWORDS | glycoprotein | 26 (61.9) | 1.53E-09 |
| | 1 | 6.03 | SP_PIR_KEYWORDS | glycoprotein | 26 (61.9) | 1.53E-09 |
| | 2 | 2.32 | GOTERM_CC_FAT | GO:0044421~extracellular region part | 13 (31.0) | 7.92E-07 |
| | 3 | 2.27 | UP_SEQ_FEATURE | topological domain:Extracellular | 15 (35.7) | 2.80E-04 |
| | 3 | 2.27 | UP_SEQ_FEATURE | topological domain:Extracellular | 15 (35.7) | 2.80E-04 |
| | 4 | 1.75 | GOTERM_MF_FAT | GO:0005509~calcium ion binding | 11 (12.1) | 7.27E-04 |
| | 4 | 1.75 | SP_PIR_KEYWORDS | calcium | 10 (11.0) | 8.87E-04 |
| Group: con KO vs. WT (Total: 15 genes) | | | | | | |
| Group: stress KO vs. WT, upregulated in KO (Total: 54 genes) | 1 | 1.82 | GOTERM_CC_FAT | GO:0005929~cilium | 5 (9.3) | 1.64E-04 |
| | 2 | 2.21 | GOTERM_CC_FAT | GO:0044463~cell projection part | 5 (5.5) | 0.002 |
| | 3 | 1.35 | GOTERM_CC_FAT | GO:0044441~cilium part | 3 (3.3) | 0.006 |
| Group: stress KO vs. WT, upregulated in WT (Total: 36 genes) | 1 | 2.55 | GOTERM_MF_FAT | GO:0046872~metal ion binding | 11 (29.7) | 5.44E-04 |
membrane, membrane, and transport in KO mice after acute stress (KO stress vs. con), comprising on average 35% of all genes in this group (10–62% in single enrichment terms; Table 1). At the same time a great overlap existed between these four clusters with about two thirds of genes included in at least two different clusters (23 of 36 genes) and nearly half of the genes in at least three different clusters (16 of 36 genes; Table 3). Examples of genes in this category were transthyretin (Ttr), klotho (Kl), annexin A1 (Anxa1), angiotensin I converting enzyme 1 (Ace), as well as different channels and receptors like chloride intracellular channel 6 (Clic6), leptin receptor (Lepr) or prolactin receptor (Prlr).

In the direct 5-HTT genotype comparison after acute stress, stress KO vs. WT, a lower gene coverage of clusters was detected (3–12% in single enrichment terms; Table 1). The first cluster calcium ion binding represented genes like Anxa1, Cdh9, Cabp1, Pvalb, and S100 calcium binding protein A11 (S100a11). The greatly overlapping second and third clusters cilium and cell projection part represented genes, like dynein, axonemal, heavy chain 3 (Dnahc3), radial spoke head 4 homolog A (Rsph4a), Anxa1, Grin2a and Pvalb (Table 4). Of these, genes of the cluster cilium like Dnahc3, Rsph4a, or Anxa1 were upregulated in KO mice while genes of the cluster cation binding/calcium ion binding like Grin2a, Cdh9, Cabp1, or Pvalb were upregulated in WT mice (Table 4). Pathway analysis slightly supported these results disclosing two highly overlapping KEGG pathways, neuroactive ligand-receptor interaction and calcium signaling pathway, that comprised the genes adrenergic receptor alpha 2a (Adra2a), arginine vasopressin receptor 1A (Avpr1a), cholinergic receptor, muscarinic 1, CNS (Chrm1) and Grin2a (Table 5). The first two, Adra2a and Avpr1a, were found upregulated in KO mice, the latter two, Chrm1 and Grin2a in WT mice after the acute stress.

Table 1. Cont.

| Cluster | Scores | Category | Enrichment Term | # (%) Genes | P-Value |
|---------|--------|----------|-----------------|-------------|---------|
| GOTERM_MF_FAT | GO:0043169 | cation binding | 11 (29.7) | 5.90E-04 |
| GOTERM_MF_FAT | GO:0043167 | ion binding | 11 (29.7) | 6.59E-04 |
| SP_PIR_KEYWORDS | calcium | | 5 (13.5) | 0.002 |
| GOTERM_MF_FAT | GO:0005509 | calcium ion binding | 5 (13.5) | 0.008 |

Table 2. Genes included in functional annotation clusters in gene group of 5-HTT WT mice in conditions acute stress vs. control as acquired by DAVID tool.

| Cluster | Transcript | Gene Title | Symbol | FC\(^1\) | Effect | P-Value |
|---------|------------|------------|--------|---------|--------|---------|
| neurogenesis | 10440344 | roundabout homolog 2 (Drosophila) | Robo2 | 1.79 | up | 0.014 |
| neurogenesis | 10519747 | sema domain, immunoglobulin domain (Ig), short basic domain, secreted | Sema3e | 1.77 | up | 0.025 |
| neurogenesis | 10481349 | netrin G2/RIKEN cDNA 6530402F18 gene | Ntng2 | 1.7 | up | 0.003 |
| neurogenesis, cation binding | 10375175 | slit homolog 3 (Drosophila) | Slt3 | 1.57 | up | 0.012 |
| cation binding | 10586591 | carbonic anhydrase 12 | Car12 | 2.88 | up | 0.032 |
| cation binding | 10523483 | PR domain containing 8 | Pdmt8 | 2.25 | up | 0.047 |
| cation binding | 10485745 | anoctamin 3 | Ano3 | 2.09 | up | 0.006 |
| cation binding | 10423230 | cadherin 9 | Cdh9 | 2.07 | up | 0.004 |
| cation binding | 10504838 | nuclear receptor subfamily 4, group A, member 3 | Nr4a3 | 2.06 | up | 0.020 |
| cation binding | 10556381 | microtubule associated monooxygenase, calponin and LIM domain containing 2 | Mical2 | 1.81 | up | 0.033 |
| cation binding | 10437629 | glutamate receptor, ionotropic, NMDA2A (epsilon 1) | Grin2a | 1.8 | up | 0.038 |
| cation binding | 10482772 | nuclear receptor subfamily 4, group A, member 2 | Nr4a2 | 1.73 | up | 0.039 |
| cation binding | 10548940 | LIM domain only 3 | Lmo3 | 1.7 | up | 0.002 |
| cation binding | 10532956 | calcium binding protein 1 | Cabp1 | 1.59 | up | 0.023 |
| cation binding | 10561561 | ryanodine receptor 1, skeletal muscle | Ryr1 | 1.58 | up | 0.019 |
| cation binding | 10526038 | matrix metallopeptidase 17 | Mmp17 | 1.55 | up | 0.032 |
| cation binding | 10554819 | malic enzyme 3, NADP(+)-dependent, mitochondrial | Me3 | 1.51 | up | 0.029 |
| cation binding | 10364792 | polo-like kinase 5 (Drosophila) | Pik5 | 1.51 | up | 0.023 |
| cation binding | 10416800 | LIM domain only 7 | Lmo7 | 1.51 | up | 0.005 |
| cation binding | 10533751 | phosphatidylinositol transfer protein, membrane-associated 2 | Pitpm2 | 1.51 | up | 0.027 |

\(^1\) Abbreviation: FC, fold change.
In contrast to all these findings related to direct 5-HTT genotype comparison after acute stress, the control condition revealed only minor differences between the WT and KO mice (con KO vs. WT). While no functional cluster could be identified in the small group of only 15 differentially regulated genes (Table 1), KEGG pathway analysis discovered the term ribosome, which included three predicted genes or pseudogenes, respectively (GM12618, EG66485, and ribosomal protein L26 pseudogene Rpl26; Table 5).

### Table 3. Genes included in functional annotation clusters in gene group of 5-HTT KO mice in conditions acute stress vs. control as acquired by DAVID tool.

| Cluster | Transcript | Gene Title | Symbol | FC | Effect | P-Value |
|---------|------------|------------|--------|----|--------|---------|
| glycoprotein; transport | 10454192 | transthyretin | Ttr | 5.92 | up | 0.024 |
| membrane; transport | 10356403 | potassium inwardly-rectifying channel, subfamily J, member 13 | Kcnj13 | 5.82 | up | 0.007 |
| glycoprotein; plasma membrane; membrane | 10527870 | klotho | K1 | 3.45 | up | 0.015 |
| glycoprotein | 10351224 | coagulation factor V; similar to Murine coagulation factor V | F5 | 2.83 | up | 0.016 |
| glycoprotein | 10395389 | sclerostin domain containing 1 | Sostdc1 | 2.71 | up | 0.028 |
| glycoprotein; transport | 10478048 | lipopolysaccharide binding protein | Lbp | 2.38 | up | 0.040 |
| membrane | 10547191 | transmembrane protein 72 | Tmem72 | 2.1 | up | 0.033 |
| glycoprotein | 10347277 | insulin-like growth factor binding protein 2 | Igfbp2 | 2.06 | up | 0.032 |
| glycoprotein | 10569344 | insulin-like growth factor 2 | Igf2 | 2.05 | up | 0.043 |
| glycoprotein; plasma membrane; membrane; transport | 10436947 | potassium voltage-gated channel, Isk-related subfamily, gene 2 | Kcne2 | 2.04 | up | 0.035 |
| glycoprotein; plasma membrane; membrane; transport | 10495712 | ATP-binding cassette, sub-family A (ABC1), member 4 | Abca4 | 2.04 | up | 0.029 |
| glycoprotein | 10440091 | collagen, type VIII, alpha 1 | Col8a1 | 2.01 | up | 0.021 |
| plasma membrane; membrane; transport | 10436958 | chloride intracellular channel 6 | Clc6 | 1.98 | up | 0.003 |
| glycoprotein; plasma membrane; membrane | 10600024 | G-protein-coupled receptor 50 | Gpr50 | 1.95 | up | 0.050 |
| plasma membrane; membrane | 10602033 | claudin 2 | Cldn2 | 1.94 | up | 0.023 |
| glycoprotein; plasma membrane; membrane | 10428619 | ectonucleotide pyrophosphatase/ phosphodiesterase 2 | Enpp2 | 1.92 | up | 0.031 |
| glycoprotein; membrane; transport | 10362104 | solute carrier family 2 (facilitated glucose transporter), member 12 | Slc2a12 | 1.91 | up | 0.016 |
| glycoprotein; plasma membrane; membrane | 10456005 | CD74 antigen | Cd74 | 1.87 | up | 0.021 |
| glycoprotein; plasma membrane; membrane | 10506301 | leptin receptor | Lepr | 1.86 | up | 0.034 |
| glycoprotein; plasma membrane; membrane | 10450154 | histocompatibility 2, class II antigen A, alpha | H2-Aa | 1.85 | up | 0.019 |
| glycoprotein; plasma membrane; membrane | 10466606 | annexin A1 | Anxa1 | 1.85 | up | 0.023 |
| glycoprotein; plasma membrane; membrane | 10381962 | angiotensin I _raline_ne enzyme (peptidyl-dipeptidase A) | Ace | 1.81 | up | 0.016 |
| glycoprotein; membrane | 10423049 | prolactin receptor | Prlr | 1.76 | up | 0.035 |
| glycoprotein; plasma membrane; membrane | 10357870 | _raline arginine-rich end leucine-rich repeat | Prelp | 1.69 | up | 0.048 |
| glycoprotein; plasma membrane; membrane | 10362896 | CD24a antigen | Cd24a | 1.68 | up | 0.044 |
| glycoprotein | 10542993 | paraoxonase 3 | Pnpo | 1.66 | up | 0.036 |
| plasma membrane; membrane; transport | 10532839 | transient receptor potential cation channel, subfamily V, member 4 | Trpv4 | 1.65 | up | 0.039 |
| glycoprotein; plasma membrane; membrane; transport | 10538459 | aquaporin 1 | Aqp1 | 1.61 | up | 0.040 |
| membrane | 10422728 | disabled homolog 2 (Drosophila) | Dab2 | 1.59 | up | 0.026 |
| membrane | 10373588 | retinol dehydrogenase 5 | Rdh5 | 1.58 | up | 0.015 |
| glycoprotein | 10344897 | sulfatase 1 | Sulf1 | 1.57 | up | 0.034 |
| glycoprotein | 10541354 | alpha-2-macroglobulin | A2m | 1.52 | up | 0.048 |
| glycoprotein; membrane | 10511180 | matrix-remodelling associated 8 | Mxra8 | 1.52 | up | 0.006 |
| glycoprotein; membrane | 10584653 | C1q and tumor necrosis factor related protein 5 | C1qtnf5 | 1.51 | up | 0.028 |
| glycoprotein | 10536220 | collagen, type I, alpha 2 | Col1a2 | 1.5 | up | 0.034 |
| glycoprotein; plasma membrane; membrane; transport | 10366391 | potassium voltage gated channel, Shaw-related subfamily, member 2 | Kcn2 | 1.54 | down | 0.030 |

Abbreviation: FC, fold change.
doi:10.1371/journal.pone.0058880.t003
Validation of Microarray Results

For validation of the microarray results via quantitative real-time PCR genes Robo2, Cdh9, Ace, Anxa1, and Adra2a were chosen: Robo2 and Cdh9 as representatives of upregulation of functional groups, neurogenesis, axon guidance, and cation binding in WT mice after acute stress; Ace and Anxa1 as representatives of upregulation of functional groups, glycoprotein and membrane in KO mice after acute stress (Tables 2, 3, 5). Cdh9 and Anxa1 were also representatives of the cluster calcium ion/cation binding and cilium in the KO vs. WT group after stress as well as Adra2a of the pathway neuroactive ligand-receptor interaction (Tables 4, 5). Real-time PCR data confirmed the microarray data for all investigated genes as the direction of regulation was always identical between Affymetrix array and Taqman assay and resulted in similar fold change values in the above described comparisons. However, in two comparisons the genes Robo2 and Cdh9 did not reach significance in Taqman analysis in the group WT stress vs. con (for detailed results of all comparisons see supplemental Table S2).

Table 4. Genes included in functional annotation clusters in gene group of acutely stressed 5-HTT KO vs. WT mice as acquired by DAVID tool.

| Cluster | Transcript   | Gene Title | Symbol       | FC | P-Value |
|---------|--------------|------------|--------------|----|---------|
| cilia; cell projection part | 10567452 | dynein, axonemal, heavy chain 3 | Dnahc3 | 2.84 | 0.009 |
| cilia; cell projection part | 10362472 | radial spoke head 4 homolog A (Chlamydomonas) | Rshp4a | 1.99 | 0.010 |
| calcium ion binding; cilia | 10456606 | annexin A1 | Anxa1 | 1.85 | 0.030 |
| calcium ion binding | 10374777 | epidermal growth factor-containing fibrilin-like extracellular matrix protein 1 | Efemp1 | 1.74 | 0.036 |
| calcium ion binding | 10399943 | RIKEN cDNA 1110049B09 gene; Cadherin-like protein 28 | CDHR3 | 1.74 | 0.037 |
| calcium ion binding | 10362896 | CD24a antigen | Cd24a | 1.70 | 0.008 |
| calcium ion binding | 10402211 | fibulin 5 | Fbln5 | 1.67 | 0.007 |
| calcium ion binding | 10494684 | sperm associated antigen 17 | Spag17 | 1.65 | 0.003 |
| calcium ion binding | 10457640 | S100 calcium binding protein A11 (calgizzarin) | S100a11 | 1.61 | 0.047 |
| calcium ion binding | 10547227 | ret proto-oncogene | Ret | 1.59 | 0.006 |
| calcium ion binding; cilia; cell projection part | 10437629 | glutamate receptor, ionotropic, NMDA2A (epsilon 1) | Grin2a | 1.85 | 0.038 |
| calcium ion binding | 10423230 | cadherin 9 | Cd9 | 1.84 | 0.023 |
| calcium ion binding | 10485745 | anoctamin 3 | Ano3 | 1.73 | 0.001 |
| calcium ion binding | 10532956 | calcium binding protein 1 | Cabp1 | 1.58 | 0.009 |
| calcium ion binding; cilia | 10430297 | parvalbumin | Pvalb | 1.58 | 0.036 |

upregulated in KO:

| Transcript | Gene Title | Symbol       | FC | P-Value |
|------------|------------|--------------|----|---------|
| 10567452  | dynein, axonemal, heavy chain 3 | Dnahc3 | 2.84 | 0.009 |
| 10362472  | radial spoke head 4 homolog A (Chlamydomonas) | Rshp4a | 1.99 | 0.010 |
| 10456606  | annexin A1 | Anxa1 | 1.85 | 0.030 |
| 10362896  | CD24a antigen | Cd24a | 1.70 | 0.008 |
| 10494684  | sperm associated antigen 17 | Spag17 | 1.65 | 0.003 |

upregulated in WT:

| Transcript | Gene Title | Symbol       | FC | P-Value |
|------------|------------|--------------|----|---------|
| 10437629  | glutamate receptor, ionotropic, NMDA2A (epsilon 1) | Grin2a | 1.85 | 0.038 |
| 10423230  | cadherin 9 | Cd9 | 1.84 | 0.023 |
| 10386705  | ring finger protein 112 | Zfp179 | 1.75 | 0.022 |
| 10485745  | anoctamin 3 | Ano3 | 1.73 | 0.001 |
| 10556381  | microtubule associated monooxygenase, calponin and LIM domain containing 2 | Mical2 | 1.69 | 0.047 |
| 10532956  | calcium binding protein 1 | Cabp1 | 1.58 | 0.009 |
| 10403209  | parvalbumin | Pvalb | 1.58 | 0.036 |
| 10554819  | malic enzyme 3, NADP(+)-dependent, mitochondrial | Me3 | 1.57 | 0.010 |
| 10382321  | potassium inwardly-rectifying channel, subfamily J, member 2 | Kcnj2 | 1.54 | 0.019 |
| 10416800  | LIM domain only 7 | Lmo7 | 1.51 | 2.0E–005 |
| 10427389  | pol polyprotein | LOC280487 | 1.5 | 0.014 |

Abbreviation: FC, fold change.
doi:10.1371/journal.pone.0058880.t004
Discussion

Our study revealed that acute stress triggered immediate changes in the amygdala mRNA profiles compared to the control situation in mice. This is in line with an increasing number of studies reporting expression of genes as determinants for altered neuronal activity in the amygdala or other stress-related brain circuits following various stressors (e.g. [23,24,33,34,35]). A main finding of our study was, however, that even the very short period of only minutes was sufficient for a modified gene regulation following various stressors (e.g. [23,24,33,34,35]). PRGs are described to be immediately led to measurable changes in the genome-wide expression of only minutes was sufficient for a modified gene regulation finding of our study was, however, that even the very short period of minutes was sufficient for a modified gene regulation leading to measurable changes in the genome-wide expression profiles. This time span resembles that expected for immediate-neuronal activity in the amygdala or other stress-related brain circuits following various stressors (e.g. [23,24,33,34,35]). Our study thereby revealed a different and new set of PRGs involved in the mediation of acute stress in mice. However, further studies are needed to elucidate potentially regulatory mechanisms following the first wave of response to the acute stressor of 1 min exposure to water used here.

Another main finding of our study was that distinct differences existed between mice of the used 5-HTT model (on C57BL/6J background) in a genotype-dependent manner. Importantly, we could confirm previously described differences in anxiety-related behavior and activity between our WT and KO mice by means of testing in the EPM. This underlines the functional consequences of the gene deletion in challenging situations in the experimental animals. In contrast, we demonstrated that basal corticosterone titers measured under non-stressed conditions did not differ between genotypes, indicating comparable functioning of the hypothalamo-pituitary-adrenocortical stress axis. Corticosterone stress hormone concentrations were expected to increase with a delay of at least 3 min [39]. Albeit not significantly different from basal values, we found a tendency for increased corticosterone titers already within 160 sec related to the acute stress. Cortico-

Table 5. Genes included in KEGG pathways identified in different gene groups related to condition (acute stress or control) and 5-HTT genotype (WT or KO) acquired by DAVID tool.

| Group - KEGG pathway (relevance parameter) | Transcript | Gene Title | Symbol | FC | Effect | P-Value |
|------------------------------------------|------------|------------|--------|----|--------|---------|
| WT stress vs. con - Axon guidance (Gene Count in %: 7.9; EASE score: 0.036) | 10440344 | roundabout homolog 2 (Drosophila) | Robo2 | 1.79 | up | 0.014 |
| | 10519747 | sema domain, immunoglobulin domain (lg), short basic domain, secreted | Sema3e | 1.77 | up | 0.025 |
| | 10375175 | slit homolog 3 (Drosophila) | Slt3 | 1.57 | up | 0.012 |
| WT stress vs. con - Calcium signaling pathway (Gene Count in %: 7.9; EASE score: 0.066) | 10461143 | cholinergic receptor, muscarinic 1, CNS | Chrm1 | 1.87 | up | 0.011 |
| | 10437629 | glutamate receptor, ionotropic, NMDA2A (epsilon 1) | Grin2a | 1.8 | up | 0.038 |
| | 10561561 | ryanodine receptor 1, skeletal muscle | Ryr1 | 1.58 | up | 0.019 |
| stress KO vs. WT - Neuroactive ligand-receptor interaction (Gene Count in %: 4.4; EASE score: 0.024) | 10464030 | adrenergic receptor, alpha 2a | Adra2a | 1.91 | up | 0.002 |
| | 10366707 | arginine vasopressin receptor 1A | Avpr1a | 1.63 | up | 0.028 |
| | 10437629 | glutamate receptor, ionotropic, NMDA2A (epsilon 1) | Grin2a | 1.85 | down | 0.038 |
| | 10461143 | cholinergic receptor, muscarinic 1, CNS | Chrm1 | 1.71 | down | 0.005 |
| stress KO vs. WT - Calcium signaling pathway (Gene Count in %: 3.3; EASE score: 0.075) | 10366707 | arginine vasopressin receptor 1A | Avpr1a | 1.63 | up | 0.028 |
| | 10437629 | glutamate receptor, ionotropic, NMDA2A (epsilon 1) | Grin2a | 1.85 | down | 0.038 |
| | 10461143 | cholinergic receptor, muscarinic 1, CNS | Chrm1 | 1.71 | down | 0.005 |
| con KO vs. WT - Ribosome (Gene Count in %: 13.3; EASE score: 0.016) | 10562320 | predicted gene, ribosomal protein L21 pseudogene | Gm12618 | 1.5 | up | 0.012 |
| | 10466923 | ribosomal protein L26///L26 pseudogene/// | Rpl26 | 1.53 | up | 3.4E–04 |
| | 10567211 | predicted gene, EG668485 | EG668485 | 1.52 | up | 1.0E–04 |

1 Abbreviation: FC, fold change.
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steroid-binding globulin may regulate availability of free floating plasma corticosterone and thus provide a mechanism to provide corticosterone immediate after the onset of a stressor [40]. The highest corticosterone titers were found in stressed KO mice which are, in accordance with increased anxiety, possibly more primed to expect stressful situations.

In addition, different expression profiles were detected between WT and KO mice after acute stress without any overlap in the respective gene lists indicating different ways of stress response dependent on 5-HTT protein level. This is consistent with an increasing number of studies revealing altered stress response and coping abilities in 5-HTT KO mice compared to their wild-type counterparts (e.g., [13,14,15,16]) and also with our finding of different anxiety-related behavior in the challenging situation of the EPM (mentioned above). Further, in the present study different pathways and functional clusters were detected in WT and KO mice that are discussed in detail below.

Response to Acute Stressor in 5-HTT WT Mice

Axon guidance/neurogenesis was found enriched only in WTs after acute stress, including members of the Slit/Robo family (Slit3, Robo2) and the semaphorins and receptors family (Sema3e). These candidates are assumed to play a role not only in axonal pathfinding and neuronal migration during brain development [41,42] but also in neuroanatomical connectivity of functional interacting brain regions in the adult brain [43]. In addition, genes of the functional pathways or clusters calcium signaling and calcium/cation binding like Grin2a, Nr4a2, Nr4a3, Chrm1, Ryr1, Cabp1, or Pvalb were upregulated in acutely stressed WTs. Several of these genes were shown to be related to spatial memory or memory formation (e.g., Grin2a [44], Nr4a2 [45], Ryr1 [46]). Further the calcium signaling pathway on the whole has been implicated in adaptive responses to synaptic activity, synaptic plasticity, and long-lasting changes in synaptic efficacy and memory [recent review by [47]]. Rapid upregulation of all these genes – comprising about 50% of the sum of genes differentially regulated in our stressed WTs (20 of 38 genes) – might therefore reflect processes that mediate adaptation to stress involving neuroplasticity to prepare the individuals for future stressful events also in our WT mice. This is in line with findings in C57BL/6J KO mice showing an effective mobilization of plasticity genes in the amygdala which corresponded to an active behavioral response to repeated restraint stress [27].

Beside this group of genes several other appear noticeable, in particular Ntng2 and Adra2a, that were both involved in stress response, but in a somehow unexpected way. Ntng2 knockout mice performed normally in a behavioral test battery and indicated no sign of any overarching defects in the inner ear or auditory nerve but did not startle to an acoustic stimulus [48]. Increased expression of Ntng2 as observed in our WTs after acute stress in turn therefore might reflect the preparation of the organism to an increased startle response after an acute stressor which resembles a sensitization or increased arousal or fear. A comparable conclusion can be drawn for Adra2a that modulates synaptic transmission via inactivation of adenyl cyclase and decrease of cAMP in response to catecholamines in neuronal circuits that are correlated with stress in vivo [49]. A rapid downregulation as observed in our stressed WTs probably reduces these inhibitory effects, resulting in an enhanced neurotransmitter release and therefore a more vigorous arousal or fear and stress reaction. Both candidates therefore seem to strengthen or prolong the response to the acute stressor which might reflect an adaptive way adopted by the WTs that might be related with or even necessary for upregulation and mobilization of genes needed for plasticity. Support for this hypothesis comes from studies demonstrating gene expression opposite to that observed in our WT mice only in conjunction with pathophysiology: decreased Ntng2 mRNA expression in schizophrenia and bipolar disorder [50] and increased Adra2a expression after chronic psychosocial stress as trigger for depression [51]. However, future studies are required to shed more light on this speculative aspect.

Response to Acute Stressor in 5-HTT KO Mice

In KO mice functional pathways/clusters neuronal ligand receptor interaction, glycoprotein, membrane, transport, cilium, and cell projection were found enriched after acute stress, including genes like Grin2a, Chrm1, Cabp1, Pvalb, Ttr, Ki, Clic6, Pthr, Ace, Adra2a, and Anxa1. For the first four genes, all involved in calcium signaling, a lack of immediate upregulation was observed compared to stressed WT mice, leading to the assumption of reduced calcium signaling processes. Thereby KO mice might have a reduced ability to adapt to a stressor via neuroplasticity processes as it was supposed above for WTs [44,47]. Alteration or dysfunction of processes involving plasticity and learning to cope with similar situations in the future might be related to maladaptive reactions and the transition to pathological emotion processing as reviewed recently [52,53,54]. This might be reflected by our data in two ways. First, several of the genes we identified as rapidly upregulated in acutely stressed KOs are concordant to genes upregulated in chronically stressed mice, e.g. Adra2a as mentioned above [51]. Ttr, the top hit in our KO gene list with nearly six-fold upregulation is also the top hit in another study with six-fold upregulation after chronic stress in high analgesia (HA) vs. low analgesia mice [55]. Also Kl, Clic6, Pthr, and sclerostin domain containing 1 could be identified as similarly upregulated between the chronically stressed HA mice [55] and our acutely stressed KO mice. 5-HTT KO mice therefore appear to react already to an acute stressor in a comparable manner as HA mice react to chronic stress whereas no such overlap could be identified with our WT mice.

Ttr, a thyroid hormone-binding protein and carrier of thyroxin and retinol, was also demonstrated as top hit of amygdala genes upregulated after stress and fear in fear conditioning experiments in mice [56] and after chronic NMDA receptor antagonist treatment as model for schizophrenia in rats [57]. Thus, second, several of the genes we detected in stressed KO mice might indicate a possible connection with stress-related mental disorders in humans, such as anxiety disorders, depression, or schizophrenia. Indeed, polymorphisms in the Ace gene, which encodes a protein effecting blood pressure, cardiovascular and respiratory function, were shown associated with syndromal panic attacks [58] and elevated serum Ace activity and major depression [59]. Adra2a was found associated with HPA axis overdrive [60], symptoms of autonomic, sympathetic dysfunction [61], and its overexpression to be related with depression and suicidal events (review by [62]) and to be downregulated by antidepressant drug [63]. Ki was identified as rare risk candidate for schizophrenia [64] together with genes such as insulin-like growth factor binding protein 2 or ectonucleotide pyrophosphatase/phosphodiesterase 2, both upregulated in a rat model for schizophrenia [57]. Anxa1, a central player in anti-inflammatory and neuroprotective role of microglia was identified to be more strongly expressed in Alzheimer’s disease [65].

Taken together, upregulation of these genes seems to modify the pathophysiology in humans as well as to impair the way of stress response in our KO mice, in particular in combination with the above supposed reduced ability for neuroplasticity processes. This is in line with studies reporting indeed impaired coping abilities.

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5-HTT and Stress Induced Amygdala Gene Expression

Response to Acute Stressor in 5-HTT KO Mice

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Taken together, upregulation of these genes seems to modify the pathophysiology in humans as well as to impair the way of stress response in our KO mice, in particular in combination with the above supposed reduced ability for neuroplasticity processes. This is in line with studies reporting indeed impaired coping abilities.
and stress adaptation in 5-HTT KO mice [13,14,15,16,66]. Fundamental for such altered processes seems the imbalanced serotonergic system with lowered 5-HTT protein levels in our KO mice. Whether this includes acute 5-HTT dysfunction at the time of the stressor or instead developmental differences due to targeted inactivation of 5-HTT function in KO mice or an intermixture of both remains unclear. However, developmental differences resulting in different morphology and physiology of the amygdala, e.g. altered dendritic morphology and spine density [12,67] are most likely to also contribute to the distinct gene expression profile detected in our study. As described previously (review by [12]) also our EPM data indicated KO mice as showing more basal anxiety-like behavior compared to WT mice. Our study therefore revealed a new set of differentially regulated genes dependent on stressor and particularly 5-HTT genotype. Several of them reflect already known marker/candidate genes for stress-related psychiatric disorders whereas others might serve as new potential candidate genes for e.g. anxiety disorders, depression, or schizophrenia. Future studies however are needed to test for such presumable relations.

Limitations

In the present study several limitations have to be considered. First of all, no other brain regions in addition to the amygdala were investigated that might have allowed to control the identified effects as relatively specific to the amygdala. In fact, several of the differentially expressed amygdala genes discussed above actually resembled findings from other corticolimbic emotion processing regions such as the prefrontal cortex (e.g., [55]). It is likely, therefore, that other regions are also involved in the appraisal of the stressor and in driving amygdala activity and gene expression, in particular the prefrontal cortex that is functionally tightly connected with the amygdala (e.g., [54]). However, focus on the amygdala might have allowed identifying first PRGs even after few minutes of acute stress response as it is described to produce the most expression changes compared to prefrontal cortex and hippocampus in C57BL/6J mice [27]. Further, no differentiation was performed between amygdala subregions and nuclei such as the basal and lateral amygdala and central amygdaloid or medial amygdaloid nucleus, although they are known to vary in their extrinsic and intra-amygdaloid connections and stress response (review by [33]). We decided to merge all as parts as well as amygdalae from left and right hemisphere per mouse to gain enough material from each individual for one microarray-based transcriptome analysis, that is without the need to pool different animals. However, thereby we had to accept an intermixture and weakening of effects. In addition, although mice of both genotypes experienced similar life time events (e.g., weaning, group-housing prior to single housing, EPM), these events might have been perceived differentially depending upon the genotype. Thereby the observed differences may be primed by heightened state anxiety in the KO mice. Nevertheless, we identified overall low numbers of differentially regulated genes in the respective group comparisons that were lowest in the control condition (KO vs. WT: 15 genes) and highest in the genotype comparison after acute stress (90 genes). This might, admittedly, reflect a less optimal gene list as supposed by Huang et al. [31], 100–2000 genes supposed as optimal, albeit it is in the range of other studies, e.g. Matsuoka et al. [57], 39 genes. It might, in contrast, also reflect the high standardization of mice before and during the experiments and tissue preparation, that might have reduced or filtered out some of the “background-noise”. Supportive for this might be that several other aspects of our gene list indicate good quality as requested by Huang et al. [31]: many important or marker genes were detected, notable portions of differentially regulated genes are involved in certain interesting biological processes, a high proportion of enriched biology exists using DAVID analysis, and independent quantitative real-time PCR experiments confirmed the investigated marker genes. It should be noted further, that nearly no overlap existed in transcript profiles between our WT and KO mice, particularly after acute stress. Complete different ways of stress response therefore seem to be adopted dependent on 5-HTT genotype indicating adaptation processes via activated neuroplasticity in WTIs and impaired plasticity but pathophysiology-related processes in KO mice. This is in line with C57BL/6J compared to DBA/2J mice in which stress affected almost entirely different sets of genes including plasticity-related ones in C57BL/6J [27]. Another possibility might be that KO and WT mice experienced the same acute stressor in different ways, for example with distinctly higher intensity in KO mice: The slightly increased corticosterone response in KO compared to WT mice after stress might serve as subtle hint for this. However, future studies identifying also the behavior during acute stress exposure as well as faster indicators of HPA axis activity (e.g., ACTH response) are needed to elucidate this aspect.

Conclusions

Overall, time-limited procedure and microarray-based amygdala transcriptome analysis revealed a new set of genes differentially expressed in the first wave response of the amygdala to an acute stress condition in mice. Further, large differences in the mRNA profiles were detected between 5-HTT genotypes involving neuroplasticity and adaptation in WT mice but impaired plasticity and processes related to chronic stress and pathophysiology in KO mice. This indicates different coping styles dependent upon the availability of 5-HTT protein and accentuates the role of the serotonergic system in the different processing of stressors and threat in the amygdala as a core system of emotion processing. Moreover, several of the genes that we found to be differentially expressed immediate-early after the acute stress-inducing situation in mice might provide promising targets for future therapeutic interventions of stress-related disorders in humans like anxiety disorders or mood disorders.

Supporting Information

Table S1 Gene list report of differentially regulated amygdala genes in mouse groups related to condition of acute stress or control (stress or con) and 5-HTT genotype (WT or KO).

Table S2 Comparison between Array- and Taqman-based gene expression in the genes Robo2, Cdh9, Ace, Amax1, and Adra2a, showing the direction of regulation (up- or down-regulated), fold changes (FC), and P-values in the group comparisons related to condition (acute stress or control) and 5-HTT genotype (WT or KO) of mice.

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Author Contributions

Revised manuscript critically: CH AG SK EW EK OA VA KPL NS JD LL. Conceived and designed the experiments: CH AG VA KL NS JD LL. Performed the experiments: CH AG LL. Analyzed the data: CH SK EW EK OA LL. Contributed reagents/materials/analysis tools: CH AG SK EW EK VA KPL NS JD LL. Wrote the paper: CH LL.
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