Gene expression profile for different susceptibilities to sound stimulation: a comparative study on brainstems between two inbred laboratory mouse strains

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Abstract

Background: DBA/1 mice have a higher susceptibility to generalized audiogenic seizures (AGSz) and seizure-induced respiratory arrest (S-IRA) than C57/BL6 mice. The gene expression profile might be potentially related to this difference. This study aimed to investigate the susceptibility difference in AGSz and S-IRA between DBA/1 and C57BL/6 mice by profiling long noncoding RNAs (lncRNAs) and mRNA expression.

Methods: We compared lncRNAs and mRNAs from the brainstem of the two strains with Arraystar Mouse lncRNA Microarray V3.0 (Arraystar, Rockville, MD). Gene Ontology (GO) and pathway analyses were performed to determine the potentially related biological functions and pathways based on differentially expressed mRNAs. qRT–PCR was carried out to validate the results.

Results: A total of 897 lncRNAs and 438 mRNAs were differentially expressed (fold change ≥2, P<0.05), of which 192 lncRNAs were upregulated and 705 lncRNAs were downregulated. A total of 138 mRNAs were upregulated, and 300 mRNAs were downregulated. In terms of specific mRNAs, Htr5b, Gabra2, Hspa1b and Gfra1 may be related to AGSz or S-IRA. Additionally, IncRNA Neat1 may participate in the difference in susceptibility. GO and pathway analyses suggested that TGF-β signaling, metabolic process and MHC protein complex could be involved in these differences. Coexpression analysis identified 9 differentially expressed antisense IncRNAs and 115 long intergenic noncoding RNAs (lincRNAs), and 2010012P19Rik and its adjacent RNA Tnfsf12-Tnfsf13 may have participated in S-IRA by regulating sympathetic neuron function. The results of the qRT–PCR of five selected lncRNAs (AK038711, Gm11762, 1500004A13Rik, AA388235 and Neat1) and four selected mRNAs (Hspa1b, Htr5b, Gabra2 and Gfra1) were consistent with those obtained by microarray.

Conclusion: We concluded that TGF-β signaling and metabolic process may contribute to the differential sensitivity to AGSz and S-IRA. Among mRNAs, Htr5b, Gabra2, Hspa1b and Gfra1 could potentially influence the susceptibility. LncRNA Neat1 and 2010012P19Rik may also contribute to the different response to sound stimulation. Further studies should be carried out to explore the underlying functions and mechanisms of differentially expressed RNAs.

Keywords: Long noncoding RNAs, AGSz, S-IRA, SUDEP model
to audiogenic generalized seizures (AGSz), followed by seizure-induced respiratory arrest (S-IRA) [5]. This distinct feature may mimic the clinically observed sudden unexpected death in epilepsy (SUDEP) and thus makes DBA/1 mice relevant SUDEP models [6]. The underlying molecular mechanism of S-IRA following AGSz and S-IRA has not yet been clearly illustrated; however, many studies have provided valuable insights into these outcomes.

AGSz and S-IRA can be observed in many mouse strains and have been confirmed to indicate a unique form of seizure that originates in the brainstem. The physiological network in AGSz and S-IRA is thought to be common to different strains of mice, but the susceptibility to such seizures differs and may be influenced by many factors [7]. Considering only genetic background, the DBA mouse family (including DBA/1 and DBA/2 mice) show much higher susceptibility to AGSz than C57BL/6 mice, and several mutations have been found to be related to AGSz [5, 7]. However, genetic mutations cannot wholly explain the differences in susceptibility. Recent findings suggest that the serotonin (5-hydroxytryptamine, 5-HT) system plays an important role in AGSz and S-IRA. Selective serotonin reuptake inhibitors (SSRIs) were found to reduce S-IRA [8–10]. Western blotting also indicated that the tryptophan hydroxylase-2 (TPH 2) level in the DBA/1 mouse brainstem was significantly lower than that in the brainstem of the C57BL/6 mouse [11]. Another report described that optogenetic activation of 5-HT neurons on the dorsal raphe of the brainstem reduced S-IRA in DBA/1 mice [12].

Other factors, such as time after birth, also influence susceptibility. Naturally, the AGSz and S-IRA of DBA/1 mice only exist in the first 5 weeks of age. However, they can be induced by repeated audio stimulation in early life, which was termed ‘priming’ [6]. Priming endows DBA/1 mice with much greater susceptibility, which may be reestablished in later life via the same stimulation [9]. These studies have suggested that brain development and the environment participate in susceptibility, and thus, epigenetic regulation should be considered.

However, direct evidence for epigenetic regulation is limited. In this study, we aimed to compare the differences in mRNA and long noncoding RNA (lncRNA) expression profiles in the brainstem of DBA/1 and C57BL/6 mice, which may provide insights into the potential epigenetic regulation that mediates AGSz and S-IRA.

Materials and methods
In this study, we established four procedural modules, including modules for the preparation of tissues, a microarray analysis, a bioinformatics analysis, and qRT–PCR (Fig. 1).

Preparation of tissues
All experimental operations and procedures with animals were performed in accordance with the Guidelines of Animal Care and Use Committee of Sichuan University West China Hospital. The study was not preregistered. Since the AGSz response without priming in DBA/1 mice was quite stable on different postnatal days (PNDs) from 21 to 112 PNDs [13], we purchased PND 28–30 DBA/1NCrlj and C57BL/6NiIfd male mice from the Charles River Laboratories Experimental Animal Center (Beijing, China). The DBA/1 and C57BL/6 mice were housed in standard laboratory cages (4 mice per cage), and the mice had free access to water and food in a temperature-controlled room (21 °C–25°C). All animals were maintained under a 12-h light/dark cycle. We evaluated only male DBA/1 mice because 1. previous reports indicated that they are slightly more susceptible to AGSz and S-IRA than females [9] and 2. the susceptibility to seizure may be influenced by ovarian hormones in female mice [14]. Since AGSz and audio stimulation potentially affect RNA expression, these mice were housed in specific pathogen-free (SPF) conditions for 1 week without testing for AGSz. The DBA/1 (n = 8) and C57BL/6 (n = 8) mice were decapitated at the age of 5 weeks under anesthesia with isoflurane inhalation, and the whole brainstem between bregma −3 mm and bregma −9 mm was taken as described in a previous study [11]. The brainstem samples were immediately frozen in liquid nitrogen and then stored at −80°C for later use. Four samples from each strain were used for the microarray analysis, and the other 4 samples were used for verification by qRT–PCR.

RNA extraction
Total RNA was extracted from DBA/1 and C57BL/6 brainstem tissues using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA). RNA quantification and quality were evaluated with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). Standard denaturing agarose gel electrophoresis was applied for the measurement of RNA integrity.

Microarray analysis
An Arraystar mouse IncRNA Microarray V3.0 (Arraystar, Rockville, MD) was used in our study, which could detect about 35,923 IncRNAs and 24,881 coding transcripts. The microarray analysis was performed by KangChen Biotech (Shanghai, China). The IncRNAs were annotated by using authoritative public transcriptome databases (NCBI Refseq 2014, UCSC Known Gene 6.0, Ensembl 38.71 and...
Fig. 1 The flowchart of experimental procedure. The study contained four procedural modules, including preparation of tissues, microarray analysis, bioinformatics analysis, and qRT-PCR.
Genbank) and landmark publications (see Additional file 1), while coding mRNAs were collected from Collaborative Consensus Coding Sequence (CCDS) Project. Sample labeling and array hybridization were carried out on the basis of the Agilent one-color microarray-based gene expression analysis experimental scheme (Agilent Technology). First, we extracted rRNA from total RNA to obtain purified mRNA (RNA-ONLY Eukaryotic RNA Isolation Kit, Epicentre). Second, we used a random primer method to amplify and transcribe each sample into fluorescent cRNA (Arraystar flash RNA labeling kit, Arraystar). Following this, an RNeasy Mini Kit (Qiagen) was utilized to purify the labeled cRNA, the concentration and activity of which were further determined with a NanoDrop ND-1000. Then, the labeled cRNA was hybridized onto microarray slides, and the hybridized arrays were washed, fixed and scanned with an Agilent DNA Microarray Scanner (part number G2505C).

Bioinformatic analysis
The Agilent Feature Extraction software (version 11.0.1.1) and the GeneSpring GX v12.1 software package (Agilent Technologies) were applied to analyze array images and quantile normalization of the raw data, respectively. Quantile normalization was performed as follows: The expression values of specific RNAs were listed in a matrix where each row represents one RNA and each column represents one sample. For each sample, the original values of different RNAs were sorted in ascending order in the column. The mean of the sorted order across each row was obtained, and then, the value of each row was replaced by this mean. Finally, the modified matrix in the previous step was rearranged to follow the same order as the input matrix.

A hierarchical clustering map and volcano plot were created to present the profiles of differentially expressed IncRNAs and mRNAs. Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were performed to identify the potential biological functions and pathways in which the differentially expressed mRNAs were enriched.

We performed coexpression analysis to show the aberrantly expressed antisense IncRNAs with their sense mRNAs and the different expression of long noncoding intergenic RNA (lincRNA) with their nearby coding genes, which was considered important in a bioinformatics analysis [15]. We first subdivided IncRNAs into 6 subgroups, which were defined as follows:

1. Sense overlapping, the IncRNA exon overlapped a coding transcript exon in the same genomic strand;
2. Intronic, the IncRNA overlapped an intron of a coding transcript in the same genomic strand;
3. Natural antisense, the IncRNA was transcribed from the antisense strand and overlapped a coding transcript;
4. Nonoverlapping antisense, the IncRNA was transcribed from the antisense strand without overlapping an exon;
5. Bidirectional, the IncRNA was oriented head-to-head with a coding transcript within 1000 bp;
6. Intergenic, there were no overlapping or bidirectional coding transcripts near the IncRNA.

In the second part of the coexpression analysis, we included all intergenic IncRNAs and set the distance from the IncRNAs to nearby genes to be <300 kb to identify lncRNAs. As a major IncRNA subtype, a lincRNA is transcribed from intergenic regions and is involved in regulating the expression of adjacent genes [16].

Quantitative real-time PCR
Quantitative real-time polymerase chain reaction (qRT–PCR) were performed to verify different expression patterns of IncRNAs and mRNAs obtained in the microarray analysis by a SYBR green PCR kit and a ViiA 7 Real-time PCR System (Applied Biosystems). All the expression levels of IncRNAs and mRNAs were normalized to the level of the internal reference gene (GAPDH). In this study, dysregulated IncRNAs used for verification were selected primarily on the basis of fold change. We also considered homology between mice and humans and selected some IncRNAs of interest in our study. For the selection of mRNAs, we took the results of GO and KEGG analyses into account and then combined these results with potential known mechanisms that may contribute to differences in AGSz or S-IRA. The sequences of the primers are listed in Table 1.

Statistical analyses
Differentially expressed IncRNAs and mRNAs were identified as those with a fold change ≥2.0 and a P value <0.05. We performed false discovery rate (FDR) correction to minimize false-positives. Another analysis based on a fold change ≥2.0 and an FDR <0.05 was performed as a sensitivity test. The qRT–PCR results are shown as the relative expression levels. We used an independent set of 4 DBA/1 and 4 C57BL/6 mice for qRT–PCR to verify the microarray findings. By setting the expression value of the target genes in the C57BL/6 control group to 1, the expression level in the DBA/1 mouse group is reported the fold change compared with the control group.
Differentially expressed lncRNAs and mRNAs in DBA/1 mice compared with C57BL/6 mice

The microarray analysis led to the identification of a total of 897 significantly differentially expressed lncRNAs (192 up- and 705 downregulated) and 438 differentially expressed mRNAs (138 up- and 300 downregulated). The details of all these differentially expressed RNAs are showed in the supplementary material (Additional files 1 and 2). The 20 most differentially expressed lncRNAs and mRNAs between DBA/1 and C57BL/6 mice are listed in Table 2 and Table 3, respectively. Figure 2 shows the volcano plots and hierarchical clustering analysis depicting the expression levels of the distinguishable lncRNAs and mRNAs.

The detailed characteristics of the lncRNAs are also described (Fig. 3): the length distribution of the up- and downregulated lncRNAs was greatest in the 1000-2000 nt bin (24 and 27%, respectively); the most frequent transcriptional locations of up- and downregulated lncRNAs were chromosomes 2 and 4 (13 and 11%, respectively); and 43% of up- and 47% of downregulated lncRNAs were intergenic.

The results of the sensitivity test based on an FDR < 0.05 are available in the supplementary material.

GO and pathway analysis of differentially expressed mRNAs

GO analysis was performed to assess the biological functions of genes and gene products, which were classified into biological processes (BP), cellular components (CC), and molecular function (MF). The most highly enriched GO terms targeted by upregulated genes were negative regulation of peptidase activity (GO: 0010466) in BP, MHC protein complex (GO: 0042611) in CC and serine-type endopeptidase inhibitor activity (GO:0004867) in MF (Fig. 4a). The most enriched GO terms among downregulated genes were transforming growth factor beta receptor signaling pathway (GO: 0007179) in BP, cell part (GO:0044464) in CC, and hydrolase activity (GO: 0016787) in MF (Fig. 4b).

KEGG was performed to identify genes involved in different biological pathways [17–19]. This analysis revealed 28 pathways enriched with significantly differentially expressed genes, including 20 pathways corresponding to upregulated transcripts and 8 corresponding to downregulated transcripts. The most correlated pathway among upregulated genes was Type I diabetes mellitus, and among downregulated genes, it was natural killer cell-mediated cytotoxicity. The details are shown in Table 4.

Table 1 The sequences of lncRNA and mRNA primers used in the study

| Differential expression | Gene name | Primers (5′-3′)                                                                 | Amplicon size (bp) |
|------------------------|-----------|---------------------------------------------------------------------------------|-------------------|
| lncRNAs                | uc007uzp.1| F: CACCAATGCGGCTAGGACAA  
R: GGCTAAAGCGACACTGGAATC |
|                        |           | 197                                                                              |
|                        | NR_045099 | F: GCCATCCGATCTCCATCTTCT  
R: GACCCCTGTGTCGTCTCCATAA |
|                        |           | 143                                                                              |
|                        | NR_015498 | F: CAACGGGATTACTAGGGTCTG  
R: GAGGCTACGGGTGGAGGTAT |
|                        |           | 279                                                                              |
|                        | NR_033305 | F: CAAGGAATTTTGCTGTAGC  
R: AGCAATACAAACACTAGCTAACAGCA |
|                        |           | 86                                                                               |
|                        | NR_003513 | F: GGTTGTTTGTAGTGTCCTTA  
R: GGAGGGAGAAATGGTATAGT |
|                        |           | 169                                                                              |
| mRNAs                  | Hspa1b    | F: TATAGTCATGCTGCGCAAGTTTAC  
R: CAGTGCCAAAGCGTTTGT |
|                        |           | 75                                                                               |
|                        | Htr5b     | F: GGTGGTGCTCTTCGTCTACT  
R: AGTCTCCGCTGTCTCAGGA |
|                        |           | 179                                                                              |
|                        | Gabra2    | F: ACAGTCCAAGCGCAATGTC  
R: AACGGATGGAAGACGCTTGAAGT |
|                        |           | 138                                                                              |
|                        | Gfra1     | F: CCACCTCCGGATTGTCTGAT  
R: CTGAAGTTGTTTCTCTCGC |
|                        |           | 152                                                                              |
| Internal control       | GAPDH     | F: CACTGAGCAAGAGAAAGGCCGCTTAT  
R: GCAGCGGAACTTTATGATGTTT |
|                        |           | 144                                                                              |
### Table 2  Top 20 differentially expressed lncRNAs between DBA/1 and C57BL/6 mice

| Seqname | GeneSymbol | Fold Change^a | FDR |
|---------|------------|---------------|-----|
| ENSMUST00000117627 | Gm14201 | 35.75 | 0.0002 |
| uc007uzp.1 | AK038711 | 23.50 | 0.0005 |
| NR_045099 | Gm11762 | 20.65 | 0.0001 |
| ENSMUST00000173149 | H2-Bl | 17.14 | 0.0000 |
| ENSMUST00000174778 | Gm10499 | 10.86 | 0.0013 |
| uc008yry.1 | AK019984 | 10.21 | 0.0008 |
| ENSMUST00000137728 | Ajb47159 | 9.97 | 0.0002 |
| uc008mpq.1 | AK085768 | 8.95 | 0.0021 |
| ENSMUST00000161336 | Agl | 8.87 | 0.0002 |
| ENSMUST00000151051 | Gm14029 | 7.99 | 0.0010 |
| ENSMUST00000180930 | Gm26793 | 7.63 | 0.0005 |
| ENSMUST00000142000 | Itf140 | 7.03 | 0.0003 |
| ENSMUST00000129337 | Gm11508 | 6.73 | 0.0013 |
| ENSMUST00000174018 | Gm7 | 6.25 | 0.0003 |
| ENSMUST00000176545 | Aa465934 | 5.99 | 0.0009 |
| AK155705 | AK155705 | 5.85 | 0.0001 |
| AK017289 | AK017289 | 5.79 | 0.0099 |
| AK018010900181014 | D330041H03Rik | 5.40 | 0.0091 |
| AK136371 | AK136371 | 5.39 | 0.0005 |
| AK084340 | AK084340 | 5.37 | 0.0002 |

| Seqname | GeneSymbol | Fold Change^a | FDR |
|---------|------------|---------------|-----|
| uc009bwo.2 | AK005187 | 326.12 | 0.0000 |
| uc008pqw.2 | NK_015498 | 1500044A13Rik | 73.85 |
| NR_033305 | NR_033305 | 72.49 | 0.0000 |
| uc009bwo.2 | AK005187 | 326.12 | 0.0000 |
| uc009bwo.2 | AK005187 | 326.12 | 0.0000 |
| AK047380 | AK047380 | 66.64 | 0.0000 |
| NR_033305 | NR_033305 | 66.92 | 0.0000 |
| NR_033305 | NR_033305 | 66.92 | 0.0000 |
| AK047380 | AK047380 | 66.64 | 0.0000 |
| NR_033305 | NR_033305 | 66.92 | 0.0000 |

### Table 3  Top 20 differentially expressed mRNAs between DBA/1 and C57BL/6 mice

| Seqname | GeneSymbol | Fold Change^a | FDR |
|---------|------------|---------------|-----|
| NM_001037713 | Xaf1 | 112.48 | 0.0159 |
| NM_001163810 | Tesc1 | 20.19 | 0.0008 |
| NM_001142938 | Ak010878 | 16.23 | 0.0001 |
| NM_00114141 | Slpi | 12.71 | 0.0004 |
| NM_009247 | Serpinale | 11.51 | 0.0123 |
| NM_001083918 | Gm13139 | 10.97 | 0.0005 |
| NM_001111119 | Cnb1ip1 | 9.30 | 0.0000 |
| NM_001001490 | Oxl1 | 8.66 | 0.0027 |
| NM_001037713 | Xaf1 | 112.48 | 0.0159 |
| NM_001015000 | Ier5 | 48.72 | 0.0000 |
| NM_001161411 | Trappc12 | 34.68 | 0.0004 |
| NM_0024472 | Gltpd1 | 31.90 | 0.0000 |
| NM_00103033149 | Tcm9 | 22.04 | 0.0652 |
| NM_001030533 | Pdxd1 | 20.65 | 0.0005 |
| NM_001145899 | Slc15a2 | 16.47 | 0.0006 |
| NM_001161411 | Trappc12 | 34.68 | 0.0004 |
| NM_00103033149 | Tcm9 | 22.04 | 0.0652 |
| NM_00103033149 | Tcm9 | 22.04 | 0.0652 |
| NM_00103033149 | Tcm9 | 22.04 | 0.0652 |
| NM_00103033149 | Tcm9 | 22.04 | 0.0652 |

| Seqname | GeneSymbol | Fold Change^a | FDR |
|---------|------------|---------------|-----|
| NM_001037713 | Xaf1 | 112.48 | 0.0159 |
| NM_001015000 | Ier5 | 48.72 | 0.0000 |
| NM_001161411 | Trappc12 | 34.68 | 0.0004 |
| NM_0024472 | Gltpd1 | 31.90 | 0.0000 |
| NM_00103033149 | Tcm9 | 22.04 | 0.0652 |
| NM_001030533 | Pdxd1 | 20.65 | 0.0005 |
| NM_001145899 | Slc15a2 | 16.47 | 0.0006 |
| NM_001161411 | Trappc12 | 34.68 | 0.0004 |
| NM_00103033149 | Tcm9 | 22.04 | 0.0652 |
| NM_00103033149 | Tcm9 | 22.04 | 0.0652 |
| NM_00103033149 | Tcm9 | 22.04 | 0.0652 |
Fig. 2 Differentially expressed lncRNAs and mRNAs in DBA/1 mice compared with C57BL/6 mice. The Volcano Plots of lncRNA (a) and mRNA (b) expression; Hierarchical clustering of differentially expressed lncRNA (c) and mRNA (d). 'red' indicates high relative expression, and 'green' indicates low relative expression. 'C' and 'D' respectively represent C57BL/6 and DBA/1 group (each group with four mice)
Fig. 3  a The percentage of the length distribution of differentially expressed lncRNAs; (b) The percentage of the chromosome distribution of differentially expressed lncRNAs; (c) The types of differentially expressed lncRNAs. Upregulated lncRNAs were showed in the inner circle, and downregulated lncRNAs were described in the outer circle.
Fig. 4 GO analysis comparing DBA/1 group with C57BL/6 group. a Top 10 enriched GO terms from upregulated mRNAs in biological process, cellular component, and molecular function. b Top 10 enriched GO terms from downregulated mRNAs in biological process, cellular component, and molecular function.
The sensitivity test for GO and KEGG was showed in the supplemental files based on a fold change ≥ 2.0 and an FDR < 0.05.

**Coexpression analysis**

In this study, nine differentially expressed antisense lncRNAs were found between the DBA/1 group and the C57BL/6 control group. Among these lncRNAs, 2010012P19Rik was the most downregulated lncRNA (fold change = 6.09, FDR < 0.001) with its nearby gene, Tnfsf12-Tnfsf13 (fold change = 2.04, FDR = 0.025). Other differentially expressed antisense lncRNAs and their paired sense mRNAs are shown in Table 5.

A total of 115 long intergenic noncoding RNAs (lincRNAs) were found to be differentially expressed. Among them, approximately 68.5% of the lincRNAs and their adjacent coding genes were changed in the same direction (which means that both lincRNAs and mRNAs were upregulated or that both were downregulated), including 12.9% upregulated and 55.6% downregulated pairs. Eight percent of dysregulated lincRNAs were upregulated and their paired mRNAs were downregulated, while 22.5% of lincRNAs were downregulated, with their nearby mRNAs upregulated. Fold-changes of differential expression among lincRNA-gene pairs are presented in a scatter plot (Fig. 5a). The length distribution of differentially expressed lincRNAs was greatest in the 1000-2000 nt bin (30.4%), and these differential lincRNAs were most frequently transcribed from chromosome 4 (27%). The details are shown in Fig. 5b and Fig. 5c, respectively. The 20 most differentially expressed lincRNAs and their adjacent mRNAs are presented in Table 6.

A sensitivity test was also performed on the basis of an FDR < 0.05, and the results are also given in the supplementary material.
**Table 5** Differentially expressed antisense lncRNAs and nearby coding gene

| Seqname of lncRNA | Gene symbol | Fold change<sup>a</sup> (lncRNAs) | Regulation of lncRNA | Genome relationship | Nearby gene seqname | Nearby gene Symbol | Fold change<sup>a</sup> (mRNAs) | Regulation of mRNA |
|-------------------|-------------|-------------------------------|----------------------|---------------------|---------------------|-------------------|-------------------|-------------------|
| ENS-MUST00000145435 | 2010012P19Rik | 6.0917526 | down | natural antisense | NM_001034097 | Trfsl2-Trfsl3 | 2.0415635 | down |
| AK017289 | AK017289 | 5.792853 | up | natural antisense | NM_001267808 | H2-L | 2.8392743 | up |
| AK017289 | AK017289 | 5.792853 | up | natural antisense | NM_010380 | H2-D1 | 2.6094529 | up |
| AK155933 | AK155933 | 4.4353006 | down | intronic antisense | NM_146191 | Lnk1 | 2.53359 | down |
| AK087052 | AK087052 | 3.4286643 | up | intronic antisense | NM_001035242 | Trpm3 | 2.0411979 | down |
| AK087052 | AK087052 | 3.4286643 | up | intronic antisense | NM_001035243 | Trpm3 | 2.0100955 | down |
| AK149710 | AK149710 | 3.163844 | down | natural antisense | NM_008850 | Pitpna | 2.4984672 | down |
| AK158573 | AK158573 | 2.5693914 | down | natural antisense | NM_028803 | Gbe1 | 2.5839838 | down |
| ENS-MUST00000148180 | Gm15396 | 2.5550359 | down | natural antisense | NM_008437 | Napsa | 4.8824943 | down |
| AK007047 | AK007047 | 2.510748 | down | natural antisense | NM_001200023 | Zfp963 | 3.3589015 | up |
| ENS-MUST00000124513 | Gm15247 | 2.064782 | down | natural antisense | NM_183151 | Mid1 | 3.2269554 | down |

Notes: lncRNAs, long non-coding RNAs. * DBA/1 mice vs. C57BL/6 mice

Validation of differentially expressed lncRNAs and mRNAs

Differentially expressed genes were selected to be analyzed by qRT–PCR, including five lncRNAs (two upregulated and three downregulated) and four mRNAs (three upregulated and one downregulated). According to the qRT–PCRs, the expression of AK038711 (uc007uzp.1) and Gm11762 (NR_045099) was upregulated (Fig. 6a), whereas that of 1500004A13Rik (NR_015498), AA388235 (NR_033305) and Neat1 (NR_003513) was downregulated (Fig. 6b) in DBA/1 mice compared with C57BL/6 mice. This result was consistent with the microarray assay.

Through a GO and KEGG analysis, we identified 4 potentially related transcripts for validation according to known mechanisms that are potentially related to AGSz or S-IRA. They were Hsp1a, Htr5b, Gabra2 and Gfra1. In our enrichment analysis, Hsp1a was found to be involved in multiple process of regulation on multiple enzyme activity (GO) and stress responses (GO), while Gfra1 was located on axon (GO) and exert molecular function as binding (GO). Both Htr5b and Gabra2 participated in neurotransmitter-related functions (GO) and neuroactive ligand–receptor interactions (KEGG). Compared to the C57BL/6 group, qRT–PCR showed that the expression levels of these genes (Hsp1a, Htr5b and Gabra2) in the DBA/1 group were significantly higher (Fig. 6c), while Gfra1 expression was significantly lower in DBA/1. The results of qRT-PCR were consistent with that in microarray analysis.

Discussion

DBA/1 mice were more susceptible to AGSz and S-IRA than C57BL/6 mice. Previous studies have explored the influence of genetic background [7] but the differences were not completely explained. The gene expression profiles of these mice were not fully understood. In the present study, using microarray analysis, we investigated the potential roles of mRNAs and lncRNAs in the different AGSz and S-IRA susceptibilities of these two strains and identified 897 lncRNAs and 438 mRNAs that were dysregulated in the brainstems of DBA/1 and C57BL/6 mice. GO and KEGG analyses revealed that the significantly differentially expressed mRNAs were involved in many biological functions. Comparing DBA/1 mice with C57BL/6 mice, the most highly enriched biological process in GO analysis was transforming growth factor beta (TGF-β) receptor signaling pathway for downregulated genes. In this process, the cluster of genes included TDGF1, RASL11B, SNX6, HTRA3, ADAM9, BMPR1B, HPGD, and ARRB. The TGF-β signaling pathway is involved in the dysfunction of neuronal, glial cell and blood–brain barrier (BBB) via alteration of ion channels, adenosine, glutamate, and GABA receptors [20]. Thus, disruption to the TGF-β signaling pathway can lead to changes in neuronal excitability and increase the risk of seizures [21, 22]. TGF-β signaling is also related to inflammation, which plays an important role in epileptogenesis [23]. In this study, the enrichment of downregulated genes in this term seems to contradict the epileptogenesis and thus
Fig. 5  a Fold change of significantly dysregulated lincRNA and their differentially expressed adjacent mRNAs for DBA/1mice vs. C57BL/6 mice; (b) The percentage of the length distribution of differentially expressed lincRNAs; (c) The percentage of the chromosome distribution of differentially expressed lincRNAs.
Table 6  Top 20 differentially expressed lincRNAs and adjacent mRNAs

| Seqname of lncRNA | Gene symbol | Fold changea (lncRNAs) | Regulation of lncRNA | Genome relationship | Nearby gene seqname | Nearby gene Symbol | Fold changea (mRNAs) | Regulation of mRNA |
|------------------|-------------|------------------------|----------------------|---------------------|---------------------|-------------------|---------------------|---------------------|
| NR_033305        | AA38235     | 66.9199947             | down                 | downstream          | NM_010386           | H2-Dma            | 2.170267           | up                  |
| NR_033305        | AA38235     | 66.9199947             | down                 | upstream            | NM_019420           | B3gal1h          | 5.7973092          | down                |
| AK047372         | AK047372    | 41.8748727             | down                 | downstream          | NM_001163042       | Haus8             | 2.1908769          | down                |
| AK047372         | AK047372    | 41.8748727             | down                 | downstream          | NM_029865           | Ocel1             | 7.5104853          | up                  |
| AK143879         | AK143879    | 41.7014068             | down                 | upstream            | NM_001193667       | Gmj1987           | 2.8931939          | down                |
| AK143879         | AK143879    | 41.7014068             | down                 | upstream            | NM_001277167       | Gmj1249           | 5.3971264          | down                |
| TCONS_00025043   | XLOC_018501 | 28.0948375             | down                 | upstream            | NM_008861           | Pkd2              | 3.0839338          | up                  |
| AK053990         | AK053990    | 24.5521064             | down                 | upstream            | NM_030707           | Fcrs              | 7.1055169          | down                |
| ENS-MUST0000178906 | Gm10593   | 23.884969             | downstream           | NM_001193667       | Gmj1987           | 2.8931939          | down                |
| ENS-MUST0000178906 | Gm10593   | 23.884969             | downstream           | NM_01277167        | Gmj1249           | 5.3971264          | down                |
| uc007uzp.1       | AK038711    | 23.495669             | up                   | downstream          | NM_001001490       | Dgkr1             | 8.6586855          | up                  |
| AK037460         | AK037460    | 19.6676915             | down                 | upstream            | NM_001008232       | Asap3             | 2.3508325          | down                |
| uc029usn.1       | Gm5859      | 19.0222774             | down                 | upstream            | NM_001085530       | Gmj13298          | 3.895603           | down                |
| AK037363         | AK037363    | 15.2204905             | down                 | upstream            | NM_008911           | Ppox              | 3.5903657          | down                |
| AK136314         | AK136314    | 14.6596357             | up                   | downstream          | NM_108861           | Pkd2              | 3.0839338          | up                  |
| NR_040401        | C920006011Rik | 13.1823016            | down                 | downstream          | NM_025274           | Dppa5a            | 5.3029739          | down                |
| uc029urz.1       | DQ551946    | 12.8034244             | down                 | downstream          | NM_001085530       | Gmj13298          | 3.895603           | down                |
| AK141495         | AK141495    | 12.6885049             | down                 | downstream          | NM_001193667       | Gmj1987           | 2.8931939          | down                |
| AK141495         | AK141495    | 12.6885049             | down                 | downstream          | NM_001277167       | Gmj1249           | 5.3971264          | down                |
| ENS-MUST0000151374 | Snhg3     | 12.1370958             | down                 | downstream          | NM_001081651       | Rab42             | 3.2691229          | down                |
| ENS-MUST0000151374 | Snhg3     | 12.1370958             | down                 | upstream            | NM_001081211       | Praf              | 2.3332466          | up                  |
| ENS-MUST0000151374 | Snhg3     | 12.1370958             | down                 | upstream            | NM_001161797       | Phactr4           | 3.4588717          | down                |
| ENS-MUST0000151374 | Snhg3     | 12.1370958             | down                 | upstream            | NM_026039           | Med18             | 2.3377675          | down                |
| ENS-MUST0000121728 | Gm1301    | 11.4196096             | down                 | upstream            | NM_001085530       | Gmj13298          | 3.895603           | down                |
| ENS-MUST0000178043 | Gm3892    | 11.2523847             | down                 | upstream            | NM_001085530       | Gmj13298          | 3.895603           | down                |
| ENS-MUST0000174778 | Gm1049    | 10.8643014             | up                   | downstream          | NM_008207           | H2-T24            | 2.5411856          | up                  |
| ENS-MUST0000107991 | Gm3892    | 10.8301037             | down                 | upstream            | NM_001085530       | Gmj13298          | 3.895603           | down                |
| AK052053         | AK052053    | 10.6618917             | down                 | downstream          | NM_001193667       | Gmj1987           | 2.8931939          | down                |
| AK052053         | AK052053    | 10.6618917             | down                 | downstream          | NM_01277167        | Gmj1249           | 5.3971264          | down                |

Notes: lincRNAs, long intergenic noncoding RNAs. a DBA/1 mice vs. C57BL/6 mice

Fig. 6 The qRT-PCR validation of differentially expressed lncRNAs and mRNAs between DBA/1 mice with C57BL/6. a The qRT-PCR results of up-regulated lncRNAs; (b) The qRT-PCR results of down-regulated lncRNAs; (c) The qRT-PCR results of differentially expressed mRNAs. By setting the expression value of target genes in C57BL/6 control group at 1, the expression level of which in DBA/1 mice group was the fold change relative to control group. Significant levels were indicated by * (P < 0.05). The results of qRT-PCR were consistent with that in microarray analysis (n = 4 animals/group).

(See figure on next page.)
Fig. 6 (See legend on previous page.)
higher susceptibility to AGSz. However, we found one study reporting that the C57BL/6 strain was resistant to TGF-β- or IL-6-induced seizures [24]. This previous finding hints to the fact that seizure in DBA/1 mice is induced by sound and is not spontaneous, and therefore, we hypothesized that lower expression of TGF-β-related genes in DBA/1 mice might represent instability due to external influences on this biological process, especially sound priming, making these mice vulnerable to AGSz. Further study is still needed.

Another important BP enriched with downregulated genes was metabolic process, with 145 genes differentially expressed. An early study demonstrated that metabolic dysfunction is evident in and may even directly cause epilepsy [25]. More specifically, one study reported that in C57BL/6 mice, a soy protein-containing diet was associated with higher susceptibility to AGSz [7]. Similarly, two recent publications indicated that a high tryptophan or ketogenic diet reduced the risk for S-IRA [26, 27]. Although one of these studies attributed these changes to gut microbes [26], combined with our data, recent evidence strongly suggests that metabolic process differences between these two strains may play an important role in terms of susceptibility to AGSz and S-IRA.

Serine-type endopeptidase inhibitor activity and hydrolase activity showed the highest enrichment score (the former due to upregulated genes and the latter due to downregulated genes) in molecular function in the GO analysis. However, the relation of these results to AGSz or S-IRA is largely unknown.

The most highly enriched cellular component terms were major histocompatibility complex (MHC) class protein complexes, enriched with upregulated genes. MHC molecules participate in negatively regulation of synaptic plasticity [28–31], and overexpression of MHC complex I protein can lead to a decreased ability to form synapses, which has been linked to several central nervous system (CNS) disorders, including autism spectrum disorders (ASDs) and schizophrenia [32, 33]. MHC may exert its effects by interacting with inflammatory cytokines that are involved in epilepsy [33, 34]. These findings suggest that MHC has the potential to be involved in AGSz and S-IRA in DBA/1 mice, although no direct evidence is currently available to support this possibility.

KEGG pathway analysis revealed that Type I diabetes mellitus (T1DM) (upregulated genes) was one of the most differentially expressed pathways. Energy metabolism through glycolysis is related to hereditary susceptibility to epileptic seizures. A previous study reported that maintenance of low blood glucose levels exerted seizure-protecting effects [35]. On the other hand, another study noted that the common mechanism in seizures and T1DM is autoimmune dysfunction [36]. In our study, other immune-mediated pathways were differentially expressed between DBA/1 and C57BL/6 mice, including antigen processing and presentation, allograft rejection and graft-versus-host disease. These findings, combined with the findings from the GO analysis (i.e., TGF-β receptor signaling pathway, MHC, etc.) implied that a broad spectrum of immune pathways might play a role in the susceptibility to AGSz in DBA/1 mice.

When considering the results of GO and KEGG together, we found that the following transcripts required extensive investigation: Htr5b, Gabra2, and Hspa1b.

The Htr5b mRNA level in DBA/1 mice was higher than that in the C57BL/6 mice, with a fold change = 6.76 (P < 0.05). As mentioned, serotonergic neurons were the focus of a recent study on the mechanism of S-IRA and SUDEP. Intervention to activate 5-HT neurons has been proven to reduce S-IRA in DBA/1 mice [8–10]. An electrophysiological study revealed that serotonergic neuron function was suppressed during and after seizures [37]. Since the serotonin system in the brainstem plays a key role in the regulation of breathing and arousal [38, 39], our finding on Htr5b may be related to AGSz-induced S-IRA.

We found that Gabra2 mRNA was more highly expressed in DBA/1 mice than in C57BL/6 mice (fold change = 4.15, P < 0.05). Other studies performed on DBA/2 mice have found that gamma-aminobutyric acid (GABA) and its receptors were related to AGSz and S-IRA. Elevated brain GABA concentrations protected against AGSz [40]. However, DBA/2 mice showed lower K+-induced GABA release on PND 30, which may have been related to susceptibility to AGSz [41]. Thus, we assumed that similar to DBA/2, the increased expression of the GABA receptor might be compensate for the lower GABA level in the brainstem of the DBA/1 mice compared to that in the C57BL/6 mice at certain time point. However, this hypothesis requires further examination. A previous study pointed out that the sequence and expression of the 2 subunits (gamma-1 and alpha-4) of the GABA receptor in DBA/2 mice did not differ from those in the C57BL/6 strain [42, 43]. However, these two studies were performed on the cortex and cerebellum, and the expression of the GABA receptor in DBA/1 mice had not been previously tested. Our findings suggest that, similar to those in the DBA/2 mice, the GABA receptors in the DBA/1 strain may have potentially influenced AGSz and S-IRA.

Hspa1b, which translates into heat shock protein 70 (HSP70), was expressed at significantly higher levels in the DBA/1 group than in the C57BL/6 group (fold change = 6.04, P < 0.05). HSP70 is an indicator of the
stress response, which can be induced by various stimuli, including ischemia, traumatic injury and seizure [44–47]. A prolonged stress response is related to cell injury in neurological disease and may lead to damage to the brainstem [48]. Hence, the elevated Hspa1b expression level in DBA/1 mice may represent a high stress state in the DBA/1 strain and may be related to S-IRA susceptibility. A recent proteomic and RNA-seq study on human SUDEP cases indicated increased HSP70-positive neurons in the hippocampus, which was considered to be related to antemortem neuronal injury, such as seizures prior to death [49]. However, in our study, we found higher expression of Hspa1b in DBA/1 mice without seizures than in normal C57BL/6 mice. These results suggest that HSP70, may be more than a biomarker. It may be involved in the mechanism of AGSz, S-IRA and even SUDEP. Studies on stress are potential new prospects for mechanistic research on S-IRA and SUDEP.

In the Leitner et al. study on high-risk SUDEP patients, Gfra1 was identified as the most downregulated mRNA in the hippocampus compared to that in low-risk patients [49]. Similarly, Gfra1 was also expressed at low levels in DBA/1 mice compared to C57BL/6 mice in our study (DBA/1 vs. C57BL/6, fold change = 1.81, downregulated). Gfra1 binds to glial cell-derived neurotrophic factor (GDNF), influencing the survival and differentiation of GABAergic interneurons [50]. Decreased Gfra1 also affects the release of GDNF, resulting in more seizure activities and thus a higher risk for SUDEP [49, 51]. Combined with our findings on Gabra2, these previous findings point to the GABA system as an important modulator for both S-IRA and SUDEP.

Among IncRNAs, nuclear paraspeckle assembly transcript 1 (Neat1) is highly conserved in mammals and participates in various developmental and pathological processes. In our study, we found that the expression of Neat1 in the brainstem of DBA/1 mice was significantly lower than that in the brainstem of C57BL/6 mice (fold change = 7.51, P < 0.001). It has been reported that seizures can lead to a transient downregulation of Neat1, providing a scaffolding function for regulating ion channels and thus was believed to act as a protective mechanism for restoring neuron functionality after seizure [52]. We presume that Neat1 had the ability to participate in forming an electrical barrier against spreading depolarization, which has been found to be closely related to S-IRA and SUDEP [37, 53], by increasing the threshold for propagating. The relatively low Neat1 level in the brainstem of DBA/1 mice represents a deficiency in response to seizures, making these mice vulnerable to postictal electroencephalogram suppression and leading to their high susceptibility to S-IRA.

A number of antisense IncRNAs (n = 9) and lncRNAs (n = 115) were found to be dysregulated in a coexpression analysis. Among the differentially expressed IncRNAs, intergenic IncRNAs were the most common, while exon sense-overlapping IncRNAs ranked second. Among these IncRNAs, Gm14201 (ENSMUST00000117627) was the most markedly upregulated IncRNA, with a fold change = 35.75, and AK005187 (uc009bwo.2) was the most notably downregulated IncRNA, with a fold change of 326.12. The functions of these IncRNAs remain unclear. However, with such marked fold changes, these IncRNAs may have critical functions in the regulation of development and may be potential biomarkers for certain physiological processes.

In our coexpression analysis, 2010012P19Rik was the most differentially expressed antisense IncRNA and was associated with the significantly downregulated adjacent coding gene Tnfsf12-Tnfsf13. Tnfsf12-Tnfsf13 is also known as the TWE-PRIL gene. The gene is, in fact, a hybrid transcript of TWEAK and APRIL. One study reported that knocking down TWE-PRIL enhanced axonal growth of sympathetic neurons [54]. Although sympathetic neuron function in AGSz and S-IRA has not yet been studied in DBA/1 mice, a clinical case reported that SUDEP patients could present with sympathetic hyperactivity [55]. Thus, it is reasonable to deduce that downregulation of Tnfsf12-Tnfsf13 enhanced sympathetic neuron function by stimulating axon growth, leading to hyperactivity related to S-IRA.

A number of differentially expressed lncRNAs were detected. Our analysis showed that a majority of lncRNAs and their nearby coding genes shared the same direction of expression change. The most differentially expressed lncRNA was AA388235, with the upregulated downstream mRNA H2-Dma and the downregulated upstream mRNA B3galt4. The determination of the biological functions and detailed regulatory mechanisms of these lncRNAs requires further exploration.

Limitation
There are several limitations to this research. First, the sample lacked anatomical precision. In this study, the whole brainstem was used to extract RNA, leading to high heterogeneity in cell types and nuclei. Thus, further separation of tissue via anatomical methods or single-cell sequencing of certain nuclei is recommended. Second, the DBA/1 mice were not tested for AGSz or S-IRA. This choice was based on a consideration that seizure itself, as well as S-IRA, which potentially influence the expression of RNA. Notably, the susceptibility of DBA/1 mice to S-IRA increased after priming by daily stimulation with
Conclusions
Our findings showed that a number of lncRNAs and mRNAs were differentially expressed between the brainstems of DBA/1 and C57BL/6 mice. We found TGF-β signaling and metabolic process may contribute to the differential sensitivity to AGSz and S-IRA. Also, many differentially expressed mRNAs such as Htr5b, Gabra2, Hspa1b and Gfra1 could potentially influence the susceptibility. Finally, current evidence suggested that IncRNA Neat1 and 2010012P19Rik might exert effect on AGSz and S-IRA. These findings provide new directions in the study of AGSz, S-IRA and even SUDEP.

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Authors’ contributions
XL and DC performed the experiments. LNZ carried out data analysis and prepared the figures and tables. LNZ and DC wrote the manuscript. LL designed the experiment and critically revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets presented in this study can be found in online repositories and have been deposited with the Gene Expression Omnibus (GEO) under the project accession number GSE152931 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE152931).

Declarations

Ethics approval and consent to participate
The study was approved by the Ethics Committee of Sichuan University West China Hospital. All experiments were performed in accordance with relevant guidelines and regulations, which were also in accordance with ARRIVE guidelines.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Supplementary Information
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Additional file 1. The details of all differentially expressed lncRNAs.

Additional file 2. The details of all differentially expressed mRNAs.

Additional file 3. Supplementary Table based on fold change ≥ 2.0 and FDR<0.05.

Additional file 4. Supplementary Figure based on fold change ≥ 2.0 and FDR<0.05.

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