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Dysregulation of long non-coding RNAs in mouse models of localization-related epilepsy

Doo Young Lee [a, 1], Jangsup Moon [a, 1], Soon-Tae Lee [a, b], Keun-Hwa Jung [a, b], Dong-Kyu Park [a], Jung-Seok Yoo [a], Jun-Sang Sunwoo [a, b], Jung-Ick Byun [a, b], Jung-Ah Lim [a, b], Tae-Joon Kim [a], Ki-Young Jung [a, b], Manho Kim [a, b, c], Daejong Jeon [a], Kon Chu [a, b, *], Sang Kun Lee [a, b, **]

[a] Department of Neurology, Laboratory for Neurotherapeutics, Biomedical Research Institute, Comprehensive Epilepsy Center, Seoul National University Hospital, Seoul, South Korea
[b] Program in Neuroscience, Neuroscience Research Institute of Seoul National University Medical Research Council, College of Medicine, Seoul National University, Seoul, South Korea
[c] Protein Metabolism Medical Research Center, College of Medicine, Seoul National University, Seoul, South Korea

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ABSTRACT

Genome-wide profiling has revealed that eukaryotic genomes are transcribed into numerous non-coding RNAs. In particular, long non-coding RNAs (lncRNAs) have been implicated in various human diseases due to their biochemical and functional diversity. Epileptic disorders have been characterized by dysregulation of epigenetic regulatory mechanisms, and recent studies have identified several lncRNAs involved in neural development and network function. However, comprehensive profiling of lncRNAs implicated in chronic epilepsy has been lacking. In this study, microarray analysis was performed to obtain the expression profile of lncRNAs dysregulated in pilocarpine and kainate models, two models of temporal lobe epilepsy commonly used for studying epileptic mechanisms. Total of 4622 lncRNAs were analyzed: 384 lncRNAs were significantly dysregulated in pilocarpine model, and 279 lncRNAs were significantly dysregulated in kainate model compared with control mice ($\geq$3.0-fold, $p < 0.05$). Among these, 54 and 14 lncRNAs, respectively, had adjacent protein-coding genes whose expressions were also significantly dysregulated ($\geq$2.0-fold, $p < 0.05$). Majority of these pairs of lncRNAs and adjacent genes shared the same direction of dysregulation. For the selected adjacent gene-lncRNA pairs, significant Gene Ontology terms were embryonic appendage morphogenesis and neuron differentiation. This was the first study to comprehensively identify dysregulated lncRNAs in two different models of chronic epilepsy and will likely provide a novel insight into developing lncRNA therapeutics.

1. Introduction

Epilepsy is a common neurological disease that affects more than 50 million people worldwide [1]. People with epilepsy have increased risk of psychosocial disorders, neurodegenerative conditions, and systemic diseases [2]. Multiple antiepileptic drugs (AEDs) have been developed for treatment of seizures, but 30–40% of epileptic patients remain refractory to treatment, and the search for more tolerable AEDs with improved pharmacokinetics has been ongoing [3,4]. Animal models of acute seizures such as maximal electroshock seizure (MES) and pentylenetetrazole (PTZ) models have widely been used for AED discovery, as these models are suitable for testing a large number of potentially anticonvulsant drugs in relatively short time [3]. However, to develop therapeutics with better efficacy for pharmacoresistant epilepsy, investigation into the pathophysiological basis of the disease is necessary, using animal models of chronic epilepsy [5,6].

* Corresponding author. Department of Neurology, Seoul National University Hospital, 101, Daehangno, Jongno-gu, Seoul, 110-744, South Korea. Fax: +82 2 2072 7424.
** Corresponding author. Department of Neurology, Seoul National University Hospital, 101, Daehangro, Jongro-Gu, Seoul 110-744, South Korea. Fax: +82 2 3672 4949.
E-mail addresses: stemcell.snu@gmail.com (K. Chu), sangkun2923@gmail.com (S.K. Lee).
[1] These authors contributed equally to this work.

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Long non-coding RNAs (lncRNAs) are a subgroup of non-coding RNAs longer than 200 nucleotides. Constituting the largest proportion of mammalian non-coding transcriptome, lncRNAs have been shown to be involved in diverse tasks such as chromatin modulation, post-transcriptional and post-translational regulation, protein complex organization, and cell-to-cell signaling [7]. Long intergenic non-coding RNAs (lincRNAs), a major subcategory of lncRNAs, are transcribed from intergenic regions to modulate the expression of adjacent genes [8].

Because of their biochemical and functional diversity, lncRNAs are likely to play a significant role in neural development and neurological disorders. Precise temporal and spatial patterns of lncRNA expression in the brain support this possibility [9]. In fact, multiple lncRNAs have already been implicated in various neural processes. To illustrate, lncRNA Dlx1as regulates neuronal differentiation by modulating the expression of neighboring homeobox genes [10], while Evf2 modulates transcription of homeodomain transcription factors in the developing mouse forebrain, facilitating the proper formation of GABA-dependent neuronal circuitry [11]. Because lncRNAs and mRNAs maintain intricate balance in the neural regulatory networks of the brain, studying the mechanisms underlying lncRNAs' functions might shed light on the molecular basis of neurological disorders [9]. Despite these findings, detailed investigations into lncRNAs involved in epilepsy have been lacking.

In this study, we profiled the expression of lncRNAs in two popular mouse models of chronic epilepsy using microarray analysis. Systemic administration of pilocarpine or kainic acid has been known to induce status epilepticus and, with time, lead to spontaneous recurrent seizures and clear behavioral phenotypes [12]. By assessing comprehensive profiles of dysregulated lncRNAs in these chronic epilepsy models, we tried to identify lncRNAs that might function as crucial mediators of the disease. Epigenetic regulatory mechanisms have been implicated in neural development, neural network function, and homeostasis, the processes that underlie the complex states of epileptic disorders [13]. As part of the diverse epigenetic regulatory processes that occur in the epileptic brain [4], lncRNAs are likely involved in modulating the molecular pathophysiology of epilepsy. Thus, identification of dysregulated lncRNAs in epilepsy and investigation into their potential functions might provide an important step towards developing drugs based on novel therapeutic targets.

2. Materials & methods

2.1. Preparation of tissues

Two different mouse models of epilepsy were used for the experiment. Pilocarpine model was generated by a single systemic injection of pilocarpine (280 mg/kg, intraperitoneal; Sigma) to 8-week-old C57BL/6 mice [14,15]. Methyl-scopolamine (1 mg/kg, intraperitoneal; Sigma) was administered to mice 30 min before pilocarpine injection to minimize peripheral muscarinic effects. Approximately 30 min after the onset of status epilepticus (SE), mice were treated with Diazepam (5 mg/kg, intraperitoneal). Kainate model was generated by a systemic injection of kainic acid (30 mg/kg, intraperitoneal; Sigma) followed by three serial injections of kainic acid (10 mg/kg) 2 h after the first injection, using 8-week-old C57BL/6 mice. Previous protocol of a single systemic injection of 30–40 mg/kg kainic acid [16,17] was slightly modified in our study to reduce mortality. All the animals exhibited convulsive seizures (≥Racine stage 4) after the fourth injection of kainic acid.

Brain samples were taken 60 days after the induction of SE. Each group consisted of 4 mice, leading to a total of 12 mice brain samples. All of the mice were males. Upon acquisition of whole brain, olfactory bulb and cerebellum were removed, and the remainder of the brain was immediately stored at −80 °C. All procedures in animal experiments were approved by the Institutional Animal Care and Use Committee and performed in compliance with the set guidelines.

2.2. Microarray analysis

Total RNA was extracted from the brain samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Quantification and quality check were performed with Nanodrop and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. Synthesis of target cRNA probes and hybridization were performed using Agilent's Low RNA Input Linear Amplification kit (Agilent Technologies, Santa Clara, CA, USA). Transcription of dsDNA was performed by adding the transcription master mix to the dsDNA reaction samples. Amplified and labeled cRNA was purified on cRNA Cleanup Module (Agilent Technologies, Santa Clara, CA, USA). Then, labeled cRNA target was quantified using ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA), and cRNA was fragmented. The fragmented cRNA was resuspended with 2X hybridization buffer and directly pipetted onto assembled Agilent's microarray. The arrays were hybridized and then washed. SurePrint G3 Mouse GE 8 × 60 K Microarray contained 28,609 Entrez gene targets and 4622 lincRNA targets. The microarray analysis was carried out by ebiogen, Seoul, South Korea.

2.3. Data acquisition and analysis

The hybridized images were scanned using Agilent's DNA microarray scanner and quantified with Feature Extraction Software (Agilent Technologies, Santa Clara, CA, USA). All data normalization and selection of fold-changed genes were performed using GeneSpringGX 7.3 (Agilent Technologies, Santa Clara, CA, USA). Student's t-test was employed to identify statistically significant genes that were differentially expressed between groups. Benjamini-Hochberg procedure with False Discovery Rate (FDR) of <0.05 was further applied to correct for multiple comparisons. Functional annotation of genes was performed according to Gene OntologyTM Consortium (http://www.geneontology.org/index.shtml) by GeneSpringGX 7.3. Gene classification was based on DAVID (http://david.abcc.ncifcrf.gov/) [18].

2.4. Gene Ontology and pathway analysis

We focused our analysis on lincRNAs, which represent a major subtype of lncRNAs. Because previous studies on lincRNAs have indicated that lincRNAs might function by affecting adjacent protein-coding genes [8], Gene Ontology (GO) and pathway analyses were applied to significantly dysregulated protein-coding genes that were adjacent to significantly dysregulated lincRNAs to identify categories that these genes fall into and thereby conjecture about the putative roles of lincRNAs. Genes that overlapped with the lincRNAs or were located within 100 kb from the center of the lincRNAs in chromosomal location were classified as lincRNA-associated genes [19]. GO and pathway analyses were also applied to differentially expressed mRNAs independent of dysregulated lincRNAs. Fisher's exact test was used to test the significance of each categorization. GO and pathway terms with $p < 0.05$ were considered significant.

3. Results

3.1. Pilocarpine model vs. control

Expression levels of lincRNAs were measured in 4-month-old pilocarpine model relative to 4-month-old control mice, using microarray analysis (Supplementary Fig. 1A). We identified 384 significantly dysregulated lincRNAs: 370 were upregulated, while 14 were downregulated in the pilocarpine model (≥3.0-fold, $p < 0.05$). These lincRNAs were characterized based on their lengths and chromosomal distribution: the 10,000–20,000 nt bin was most heavily populated (21.35%), and the lincRNAs were most frequently transcribed from chromosome 3 (7.55%) (Fig. 2A and B). Clustering analysis revealed the relationship among lincRNA expression patterns in different samples (Fig. 1A).

Expression levels of mRNAs were also compared between pilocarpine model and control mice, using microarray analysis (Supplementary Fig. 1B). We identified 392 significantly dysregulated mRNAs: 374 were upregulated, while 18 were downregulated in the pilocarpine model (≥3.0-fold, $p < 0.05$). Dendrogram was constructed to assess the relationship among mRNA expression patterns in different samples (Fig. 1B).

3.2. Kainate model vs. control

Expression levels of lincRNAs were measured in 4-month-old kainate model relative to 4-month-old control mice, using microarray analysis (Supplementary Fig. 1C). We identified 279 significantly dysregulated lincRNAs: 268 were upregulated, while 11 were downregulated in the kainate model (≥3.0-fold, $p < 0.05$). These lincRNAs were characterized based on their lengths and chromosomal distribution: the 10,000–20,000 nt bin was most heavily populated (25.81%), and the lincRNAs were most frequently transcribed from chromosome 8, 9, 14, and 15 (each 7.17%) (Fig. 2C and D). Clustering analysis revealed the relationship among lincRNA expression patterns in different samples (Fig. 1C).

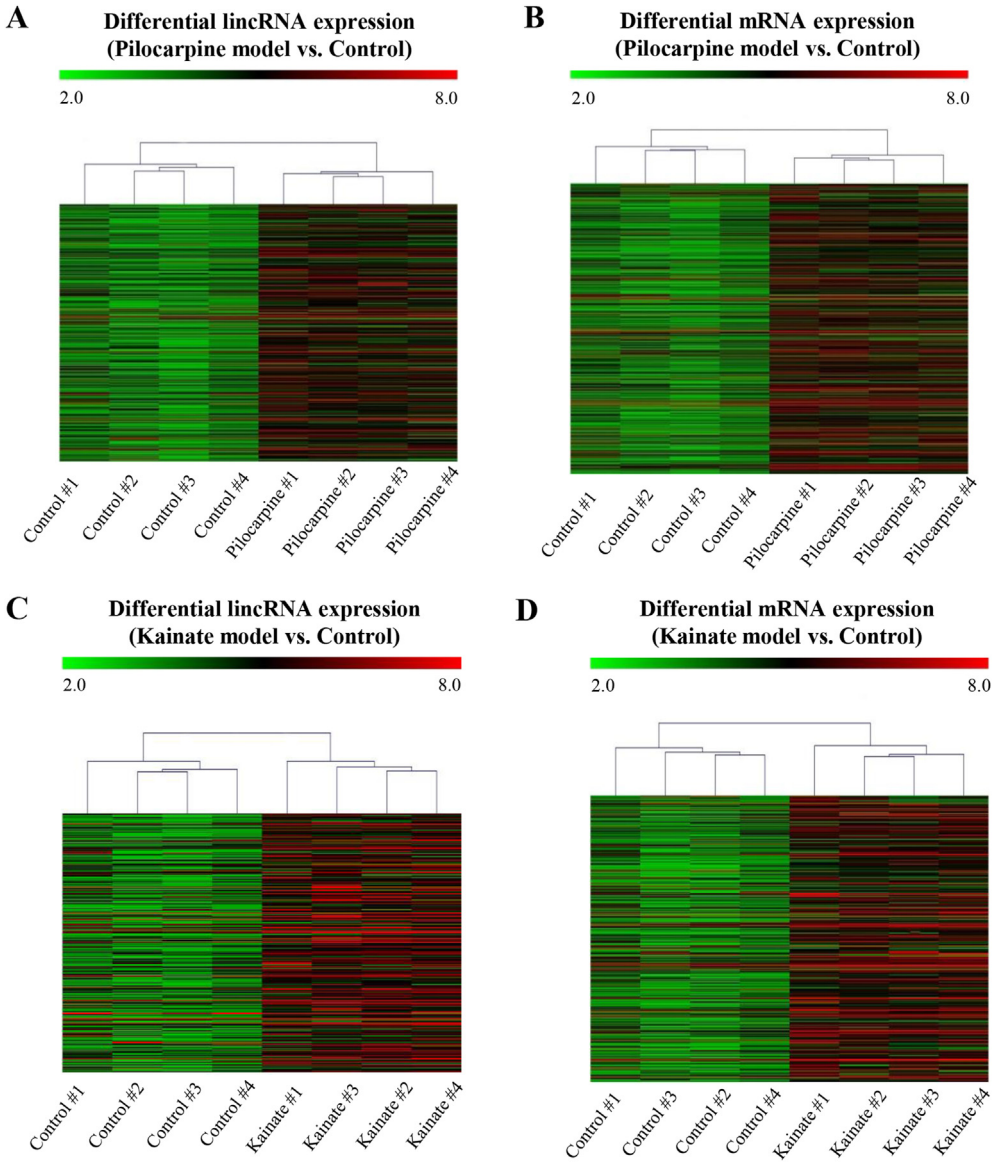

Fig. 1. Differences in RNA expression profiles between epilepsy model and control. Hierarchical clustering analysis of dysregulated lincRNAs (A) and dysregulated mRNAs (B) for pilocarpine model vs. control. Hierarchical clustering analysis of dysregulated lincRNAs (C) and dysregulated mRNAs (D) for kainate model vs. control. Samples were arranged into groups based on their lincRNA or mRNA expression levels, and dendrograms were subsequently constructed.

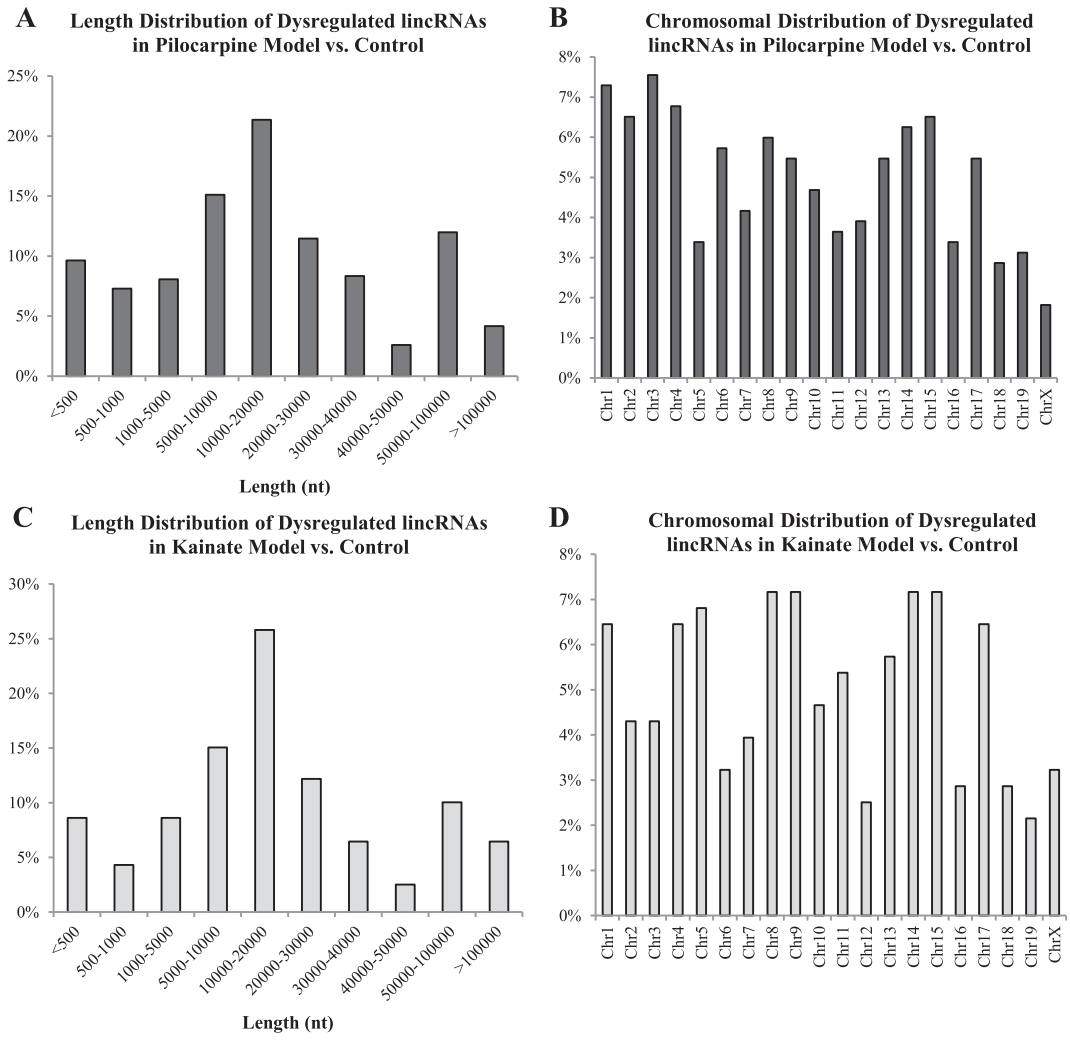

Fig. 2. Characterization of dysregulated lincRNAs. (A) Length distribution of lincRNAs dysregulated in pilocarpine model vs. control. The 10,000–20,000 nt bin was most heavily populated. (B) Chromosomal distribution analysis indicated that dysregulated lincRNAs were most frequently transcribed from chromosome 3. (C) Length distribution of lincRNAs dysregulated in kainate model vs. control. The 10,000–20,000 nt bin was most heavily populated. (D) Chromosomal distribution analysis indicated that dysregulated lincRNAs were most frequently transcribed equally from chromosome 8, 9, 14, and 15.

Expression levels of mRNAs were also compared between kainate model and control mice, using microarray analysis (Supplementary Fig. 1D). We identified 251 significantly dysregulated mRNAs: 241 were upregulated, while 10 were downregulated in the kainate model (≥3.0-fold, $p < 0.05$). Dendrogram was constructed to assess the relationship among mRNA expression patterns in different samples (Fig. 1D).

3.3. GO and pathway analysis (pilocarpine model vs. control)

A total of 384 significantly dysregulated lincRNAs in pilocarpine model were analyzed to identify protein-coding genes associated with these lincRNAs. Among the 384 dysregulated lincRNAs, 51 upregulated lincRNAs and 3 downregulated lincRNAs had adjacent protein-coding genes that were significantly dysregulated (≥2.0-fold, $p < 0.05$). Significantly dysregulated genes ($n = 50$) adjacent to the 54 lincRNAs were presented (Fig. 3A). Not all lincRNAs had one-to-one correspondence with adjacent genes as some of the lincRNAs were associated with multiple adjacent genes and vice versa (Supplementary Table 1). Majority of these lincRNAs and their adjacent genes shared the same direction of dysregulation (94.4%) (Fig. 4A). To evaluate enrichment of the significant adjacent genes in biological processes, cellular components, and molecular functions, GO analysis was performed. Embryonic appendage morphogenesis, plasma membrane, and cytokine activity were the GO terms most highly enriched in the significantly dysregulated, lincRNA-associated genes (Fig. 3C). Embryonic appendage morphogenesis comprised Hoxc10, Mecom, and Alx4, while cytokine activity, although not statistically significant, consisted of Il17b, Clcf1, and Gdf7. Other genes such as Sytl3 and Anxa4 were associated with calcium-dependent phospholipid binding.

Then, GO analysis was performed on 392 significantly dysregulated mRNAs from the microarray. Highly enriched GO terms included cell surface receptor linked signal transduction, intrinsic to membrane, and olfactory receptor activity. Pathway analysis revealed olfactory transduction to be the most correlated pathway for the dysregulated mRNAs (Fig. 3D).

3.4. GO and pathway analysis (kainate model vs. control)

A total of 279 significantly dysregulated lincRNAs in kainate model were analyzed to identify protein-coding genes associated with these lincRNAs. Among the 279 dysregulated lincRNAs, 14 upregulated lincRNAs had adjacent protein-coding genes that were

A

Dysregulated genes adjacent to dysregulated lincRNAs (Pilocarpine model vs. Control)				
Adam24	Cbx7	Fxyd4	Hsf2bp	Rab44
Adcy10	Ccdc27	Gcnt7	Il17b	Rex2
Ahdc1	Cdk15	Gdf7	Mecom	Rtp2
Alx4	Cdkn1a	Gm10362	Mgam	Sctr
Ankrd33	Clcf1	Gm6792	Mos	Slc12a3
Anxa4	Cpn2	Gpr152	Mrgprh	Sytl3
Apobec3	Ephx3	Gzmk	Ndst1	Tead4
Banf2	Fam160a1	Hoxc10	Nlrp14	Tmem54
Best2	Foxd4	Hoxc12	Ntsr1	Wnt8b
Cass4	Fut4	Hrnr	Pabpc6	2310002L13 Rik

[a] All of the adjacent genes were significantly upregulated.

B

Dysregulated genes adjacent to dysregulated lincRNAs (Kainate model vs. Control)	
Ablim1	Hoxa6
B4galnt2	Il12rb2
Bahcc1	Neurog1
Cbx7	Nkx1-1
Cpn2	Nlrp4c
Crb1	Slc25a5
Gm4461	Slc38a10

[a] All of the adjacent genes were significantly upregulated.

C

	Dysregulated genes adjacent to dysregulated lincRNAs	
	Pilocarpine model	Kainate model
Biological Process	Embryonic appendage morphogenesis*	Neuron differentiation*
Cellular Component	Plasma membrane*	N/A
Molecular Function	Cytokine activity	N/A
Correlated Pathway	N/A	N/A

[a] * indicates $p<0.05$, N/A=Not Available

D

	Dysregulated mRNAs (independent of lincRNAs)	
	Pilocarpine model	Kainate model
Biological Process	Cell surface receptor linked signal transduction*	Cell surface receptor linked signal transduction*
Cellular Component	Intrinsic to membrane*	Intrinsic to membrane*
Molecular Function	Olfactory receptor activity*	Olfactory receptor activity*
Correlated Pathway	Olfactory transduction*	Olfactory transduction*

[a] * indicates $p<0.05$

Fig. 3. Categorization of significantly dysregulated, lincRNA-adjacent genes. The list of significantly dysregulated genes that were adjacent to significantly dysregulated lincRNAs for pilocarpine model vs. control (A) and kainate model vs. control (B). All of the adjacent genes were significantly upregulated. (C) These significant adjacent genes were categorized using GO and pathway analyses. (D) Independent of lincRNAs, significantly dysregulated mRNAs in pilocarpine and kainate models were also categorized using GO and pathway analyses. * indicates $p < 0.05$, N/A = Not Available.

significantly dysregulated (≥2.0-fold, $p < 0.05$). Significantly dysregulated genes ($n = 14$) adjacent to the 14 lincRNAs were presented (Fig. 3B). Not all lincRNAs had one-to-one correspondence with adjacent genes as some of the lincRNAs were associated with multiple adjacent genes and vice versa (Supplementary Table 1). All of these lincRNAs and their adjacent genes shared the same direction of dysregulation (Fig. 4B). Neuron differentiation was the GO term most highly enriched in the significantly dysregulated, lincRNA-associated genes (Fig. 3C). Neuron differentiation comprised Ablim1, Crb1, and Neurog1. Other genes such as Nkx1-1, Hoxa6, and Cbx7 were associated with transcriptional regulation.

Then, GO analysis was performed on 251 significantly dysregulated mRNAs from the microarray. Highly enriched GO terms included cell surface receptor linked signal transduction, intrinsic to membrane, and olfactory receptor activity. Pathway analysis revealed olfactory transduction to be the most correlated pathway for the dysregulated mRNAs (Fig. 3D).

3.5. Analysis of RNAs that were dysregulated in common

LincRNAs that were significantly dysregulated in both pilocarpine and kainate models of epilepsy were analyzed to identify those that might play an important role in induction of spontaneous seizure. A total of 118 lincRNAs were dysregulated in common: 110 were upregulated, while 8 were downregulated in both models (≥3.0-fold, $p < 0.05$). These lncRNAs were characterized based on their lengths and chromosomal distribution (Fig. 4C and D). Among the 118 commonly dysregulated lincRNAs, 2 upregulated lincRNAs had adjacent protein-coding genes that were also significantly dysregulated in both epilepsy models: lincRNA:chr16:30170306-30223193 forward strand with carboxypeptidase N, polypeptide 2 (Cpn2), and lincRNA:chr15:79764481-79809909 forward strand with chromobox homolog 7 (Cbx7). Cpn2 and Cbx7 were significantly upregulated in both models.

A total of 116 mRNAs were dysregulated in common: 113 were upregulated, while 3 were downregulated in both epilepsy models (≥3.0-fold, $p < 0.05$). GO and pathway analyses of these dysregulated genes revealed cell surface receptor linked signal transduction, intrinsic to membrane, olfactory receptor activity, and olfactory transduction to be the most enriched categories.

4. Discussion

This was the first study to identify significantly dysregulated lincRNAs in two popular mouse models of chronic epilepsy. For pilocarpine model vs. control, the biological process most enriched

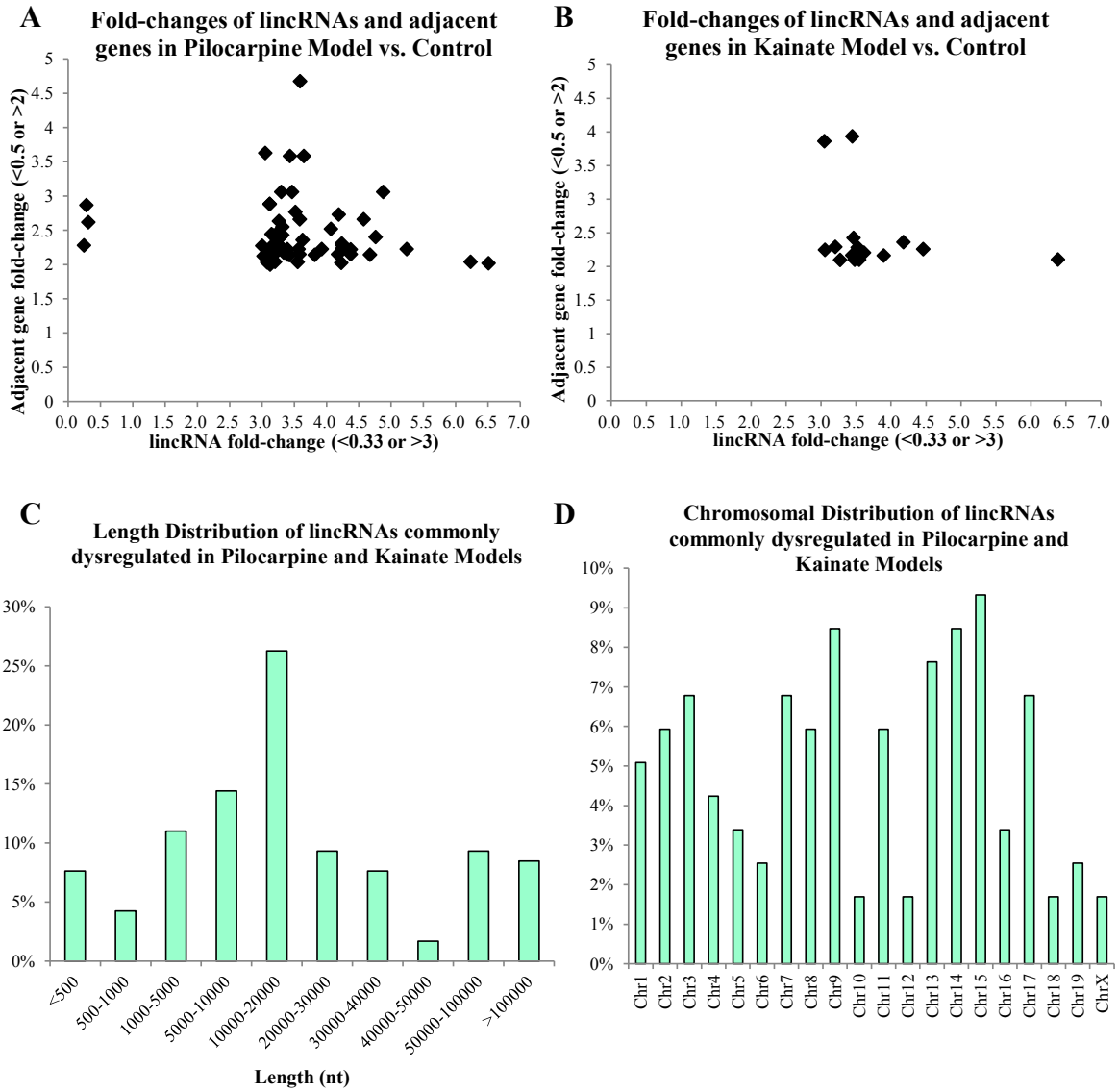

Fig. 4. Scatter plots of significantly dysregulated lincRNA-gene pairs and characterization of lincRNAs dysregulated in common. Fold-changes of significantly dysregulated lincRNAs and their significantly dysregulated adjacent genes for pilocarpine model vs. control (A) and kainate model vs. control (B) were plotted. X-coordinate represents fold-change values of lincRNAs. Y-coordinate represents fold-change values of adjacent protein-coding genes. (C) Length distribution of lincRNAs commonly dysregulated in pilocarpine and kainate models. The 10,000–20,000 nt bin was most heavily populated. (D) Chromosomal distribution analysis indicated that commonly dysregulated lincRNAs were most frequently transcribed from chromosome 15.

in the 50 significantly dysregulated genes that were adjacent to 54 significantly dysregulated lincRNAs was embryonic appendage morphogenesis. Adjacent genes in this category included homeobox gene (Hoxc10) and homeodomain transcription factor (Alx4). Associations between homeobox genes and epilepsy have been suggested by previous studies: mutations in X-chromosome-linked Aristaless-related, homeobox gene contributed to X-linked mental retardation and epilepsy [20], while the absence of orthodenticle homeobox 1 gene led to spontaneous epileptic behavior in mouse [21]. Most enriched molecular function category was cytokine activity, indicating that lincRNAs might facilitate the progression of epilepsy by modulating immune response. In fact, innate immune responses alone were sufficient to produce focal seizures by enhancing brain excitability [22], and pilocarpine model was previously characterized by upregulated expression of genes related to inflammation [15].

For kainate model vs. control, the biological process most enriched in the 14 significantly dysregulated genes that were adjacent to 14 significantly dysregulated lincRNAs was neuron differentiation. Previously, differentiation of nascent granule cells was suggested to be the basis for aberrant network reorganization observed in epilepsy [23], indicating a pro-epileptogenic role of seizure-induced neurogenesis in forming an epileptic hippocampus [24].

Among the 118 lincRNAs commonly dysregulated in both pilocarpine and kainate models, only 2 lincRNAs had adjacent genes that were also significantly dysregulated in both models. These genes were Cpn2 and Cbx7, which were significantly upregulated in the two epilepsy models. Previous studies have indicated association between carboxypeptidases and seizure. To illustrate, inhibition of glutamate carboxypeptidase II activity led to increased threshold for electroconvulsions [25], and transcription of

carboxypeptidase H was transiently upregulated in the granule cell layer of rat hippocampus after kainic acid-induced seizures [26]. Because carboxypeptidase cleaves a peptide bond at the carboxy-terminal, it can function in diverse contexts, and upregulation of Cpn2, mediated by a dysregulated lincRNA, might facilitate the progression of epilepsy. On the other hand, Cbx7, which resides within polycomb repressive complex I, was previously shown to interact with the antisense noncoding RNA in the INK4 locus (ANRIL). Function of Cbx7 is activated upon recognition of methylated H3K27 and ANRIL binding, and the activated Cbx7 subsequently regulates senescence by suppressing INK4b/ARF/INK4a locus [27]. Because Cbx7 is a chromatin-associated factor that recognizes and interacts with methylated H3K27 to facilitate gene silencing [27], upregulation of Cbx7 transcription might play a role in induction of epileptic seizures.

Majority of the significantly dysregulated lincRNAs and significantly dysregulated adjacent genes shared the same direction of dysregulation, indicating that most of the lincRNAs were involved in positive regulation of their adjacent genes. However, lincRNAs in these pairs constituted a fraction of total significantly dysregulated lincRNAs, suggesting that the remaining lincRNAs require further investigation to elucidate their potential functions. As the functions of the remaining lincRNAs are unveiled, enrichment categories might be adjusted accordingly.

For significantly dysregulated mRNAs, highly enriched GO categories included cell surface receptor linked signal transduction and olfactory receptor activity, and most correlated pathway was olfactory transduction for both pilocarpine and kainate models. These results were obtained mainly due to upregulated expression of olfactory receptors in the two epilepsy models. Intriguingly, some cases of epilepsy have been accompanied by olfactory auras [28], and single administration of pilocarpine was sufficient for improved olfactory discrimination in rats, suggesting the participation of muscarinic receptors in regulating olfactory functions [29]. On the other hand, ectopic expression of olfactory receptor genes in non-olfactory tissues and its functional implications have previously been studied [30,31], indicating that olfactory receptor transcripts might play distinct roles in different tissues. Considering that olfactory bulbs were completely removed from our brain samples prior to microarray analysis, upregulated olfactory receptor transcripts in the remaining brain might be involved in non-olfactory functions. Further studies will be required to evaluate the roles of these upregulated olfactory receptor transcripts in epilepsy.

To conclude, differential expression of lncRNAs was observed in pilocarpine and kainate models. LncRNAs of varying lengths transcribed from different chromosomal regions were dysregulated in the epilepsy models. Embryonic appendage morphogenesis and neuron differentiation were the GO categories principally enriched in lncRNA-associated genes. Further investigations into molecular mechanisms underlying the actions of these lncRNAs will be required for full elucidation of their functions. Nevertheless, this study profiled lncRNAs dysregulated in epilepsy and illuminated their potential functions. Delving into these lncRNAs might facilitate elucidation of pathophysiological mechanisms underlying epilepsy.

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.149.

Transparency document

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