Chapter 5: Global Analysis of the Effect of Densin Knockout on Gene Transcription in the Brain

Introduction

High throughput methods for analyzing transcriptional changes in response to deletion or mutation of a gene has provided modern biologists with a powerful tool for identifying candidate genes involved in observed phenotypes. Methods such as sequencing of expressed sequence tags (ESTs), microarray analysis, whole-genome tiling arrays, and other high throughput platforms have provided a wealth of insight into many biological processes. However, they have not been without limitations, chief among them are a need for prior knowledge of the sequences being investigated, poor discrimination of highly similar sequences, poor reproducibility of results across laboratories and platforms, and the semi-quantitative nature of the signals, especially of low abundance species [1, 2]. RNA-seq, a new, ultra-high throughput sequencing method for quantifying and annotating transcriptomes aims to alleviate many of these technical limitations. However, one major limitation still exists, we don't know how best to analyze these large datasets to distill biologically relevant information that can inform future experimentation.

In this chapter I present some preliminary findings from the first application of RNA seq technology to characterize changes in gene transcription from a gene knockout mutation.

Material and Methods

5.1 Construction of forebrain and hippocampal RNA seq libraries

The generation of forebrain and hippocampal RNA seq libraries was previously described (Chapter 2.6)

5.2 Analysis of RNA seq data sets

A simple analysis scheme was used to identify the transcripts that differed between brains of wild type and Densin knockout mice. Analysis was based on the RPKM counts generated from the "unique read" output. Only transcripts in which one of the RPKM values was >1 was considered. The percent difference in RPKM counts was calculated for all transcripts of a wt:ko sibling pair. For further analysis, the direction of change (increase or decrease) had to be the same for both sets of sibling pairs.

The top 150 transcripts were then compared between the forebrain and hippocampus. Transcripts that were on both lists were further analyzed.

Gene ontology information was obtained by submitting my list of most affected transcripts to the web-based Ingenuity Pathway Analysis (IPA) bioinformatics software program (Ingenuity Systems, Redwood City, CA). I then identified the transcripts that seemed most relevant to the phenotypes observed in the Densin knockout mouse.

Results

5.3 The Densin knockout animal does not exhibit gross changes in transcriptional activity

The RPKM counts were compared for each transcript to determine if there was any global changes in transcription. The degree of relationship between the wild type and knockout sibling pairs was determined by calculating the correlation value (Fig 5.1). Wild type and knockout forebrain transcript levels are highly correlated and significant; r^2 values of 0.9963 (pair 1; Fig 5.1a) and 0.9928 (pair 2; data not shown); $p<0.0001$ for both pairs. Hippocampal transcript levels are also highly correlated between the wild type and knockout; r^2 values of 0.9727 (pair 1; Fig 5.1b) and 0.9963 (pair 2), $p<0.0001$ for both pairs. This means that most transcript levels do not change between the wild type and knockout animals. Such a high correlation suggest that the loss of Densin does not result in large scale changes in transcriptional activity.

5.4 Transcription levels do not change for PSD proteins with decreased levels of protein in the knockout

Relatively small changes in the forebrain were detected for all six of the PSD proteins whose protein levels were significantly decreased in the forebrain of knockout mice (Fig 5.2). In the hippocampus, of these six proteins only the transcript level for Erbin showed a large change, +22.6%. These results suggest that the decrease in protein levels are not due to changes in gene transcription.

5.5 Immediate early gene transcripts show large changes in the Densin knockout mice

The transcript levels of eight immediate early genes (Arc, Egr1-4, Fos, Fos-b, and Junb) are decreased in the forebrain (Fig 5.3). Interestingly, seven of these transcripts show a concomitant increase in the hippocampus. Four of these IEGs (Egr1/ zif268, Egr2, Egr3, and Arc) have symmetrically changed transcript levels.

Transcript	% Change Forebrain	% Change Hippocampus
Egr1/ Zif268	-25.68103818	24.57384221
Egr2	-53.74	52.59259259
Npas4	-46.10	7.581967213
Fos	-41.25	18.49529781
Junb	-35.39	61.3592233
Egr4	-34.08	47.10312862
Fosb	-29.38	38.53727145
Egr3	-27.27	26.63920357
Arc	-46.55	47.56240576

Such results suggest that Densin may play a different mechanistic role in the hippocampus compared to the forebrain.

5.6 Comparison of transcript levels of PSD protein genes in the forebrain and hippocampus

76 transcripts were analyzed for their relative enrichment in the hippocampus verses the forebrain of wild type animals (Table 5.1). Enrichment was defined as a two-fold (100%) or more increase in hippocampal expression compared to the forebrain. A similar table was generated for changes in the relative enrichment of transcripts in the

hippocampus of Densin knockout mice. Transcripts were manually grouped into functional families based on published reports.

In wild type mice, transcripts for Pak6 and Ras-GRF 2 are highly enriched in the hippocampus, exhibiting an enrichment of +476% and +671% of forebrain levels, respectively. The transcript of Pak3, another cytoskeletal remodeling protein, is also enriched in the hippocampus. Transcripts for glutamate receptor subunits NR2A, NR2B, GluR1 and GluR2, and the high voltage-dependent R-type α1E Ca^{2+} channel are also enriched in the hippocampus relative to the forebrain. Finally, Maguin-1, an adaptor protein that links Densin to PSD-95, and likely to the NMDA receptor complex, shows a marked hippocampal enrichment (+129%). Transcripts enriched in the forebrain relative to the hippocampus are Rac3, PKCδ, Hras1, LIM Kinase 1, and the inward rectifying K^+ channel Kcnj4.

One interesting find concerns the transcript levels of GKAP. GKAP is a major adaptor protein linking the PSD-95/ NMDA receptor complex to the Shank sub-matrix. In the wild type animal GKAP is not expressed in the hippocampus. This result is supported by in situ hybridization reported in the Allen Brain Map (Fig 5.4). Interestingly, the GKAP transcript is turned on in the hippocampus of the knockout animal.

5.7 Candidate genes for the seizure phenotype

Npas4 and Gabra2 (GABA-A α2) show two of the largest absolute changes in the forebrain (Fig 5.5); neither exhibit large changes in the hippocampus (Npas4, +7.6%, GABA(A) α2). In the forebrain, Npas4 decreases by 46%, while $GABA_A$ α2 increases

by 80.8%. These are appealing candidate transcripts for the seizure phenotype because $GABA_A$ α2 is one of four $GABA_A$ receptor subunits that can mediate the action of benzodiazepine drugs [3, 4], and Npas4 directly regulates the development of inhibitory synapses [5].

References

1. Wang, Z., M. Gerstein, and M. Snyder, *RNA-Seq: a revolutionary tool for transcriptomics.* Nat Rev Genet, 2009. **10**(1): p. 57-63.
2. Shendure, J., *The beginning of the end for microarrays?* Nat Methods, 2008. **5**(7): p. 585-7.
3. Rudolph, U., et al., *Benzodiazepine actions mediated by specific gamma-aminobutyric acid(A) receptor subtypes.* Nature, 1999. **401**(6755): p. 796-800.
4. Low, K., et al., *Molecular and neuronal substrate for the selective attenuation of anxiety.* Science, 2000. **290**(5489): p. 131-4.
5. Lin, Y., et al., *Activity-dependent regulation of inhibitory synapse development by Npas4.* Nature, 2008. **455**(7217): p. 1198-204.

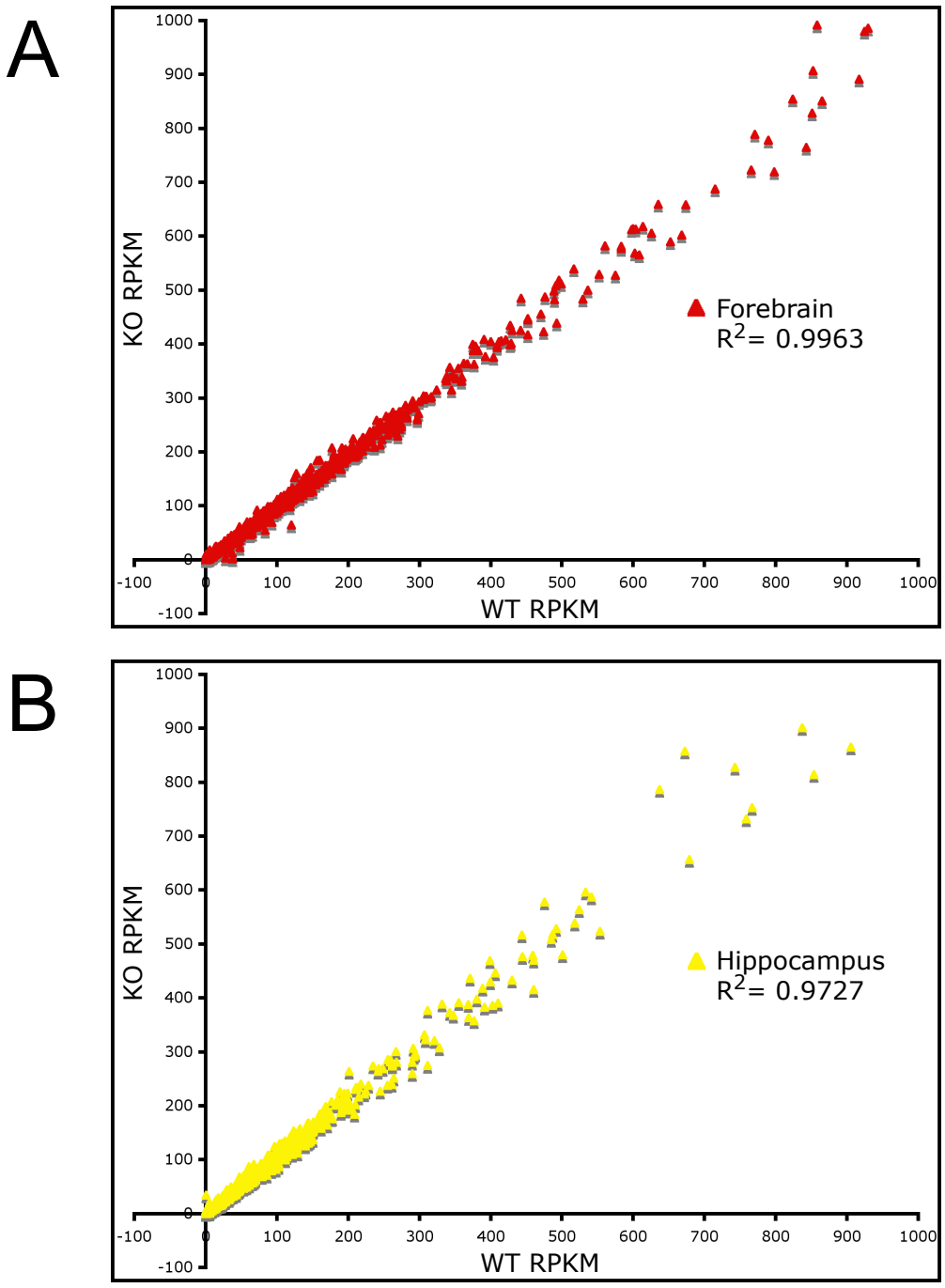

Figure 5.1 Comparison of wild type and ko transcriptional activity (as measured in reads per kilobase of exon per million mapped reads; RPKM) demonstrates that the loss of Densin does not result in gross changes in gene expression in either the forebrain or hippocampus. (A) Comparison of RPKM values from RNA seq libraries generated from sibling pair wt and ko animal forebrain tissue (red) demonstrate a positive linear correlation (R^2= 0.9963, p<0.0001). (B) Comparison of RPKM values from RNA seq libraries generated from sibling pair wt and ko animal hippocampal tissue (yellow) demonstrate a positive linear correlation (R^2= 0.9727, p<0.0001). Intra-genotype comparisons (wt vs. wt or ko vs. ko) of forebrain or hippocampus tissue RNA seq libraries (R^2 >0.97, p<0.0001 for all libraries compared; data not shown) demonstrates reproducibility, linearity, and sensitivity of the RNA seq method.

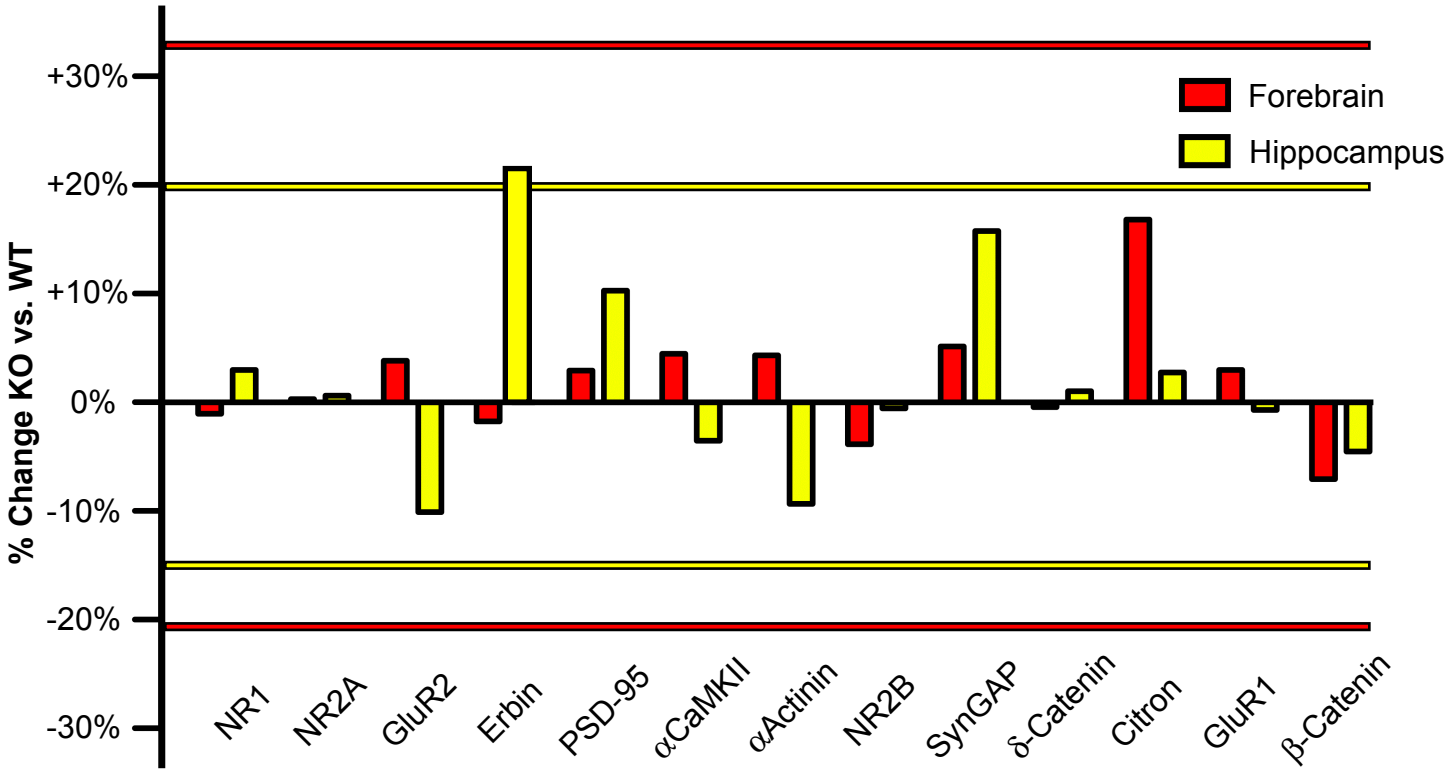

Figure 5.2 Decreased changes in gene transcription are not the likely cause of the decreased protein expression levels observed for the PSD proteins studied by immunoblot (Fig 4.5). Relatively small changes in the ko animal forebrain or hippocampus are observed for transcript levels of the PSD proteins studies by quantitative immunoblots. Erbin is the only PSD transcript that shows a relatively large increase in its level of expression (+22.6%). Bars represent average percent change between wild type and knockout RPKM values (red, forebrain; yellow, hippocampus) . Yellow horizontal lines represent the 5% (-16.97%) and 95% (19.72%) percentile ranges for changes in the hippocampal. Red horizontal lines represent the 5% (-21.56%) and 95% (32.42%) percentile ranges for changes in the forebrain.

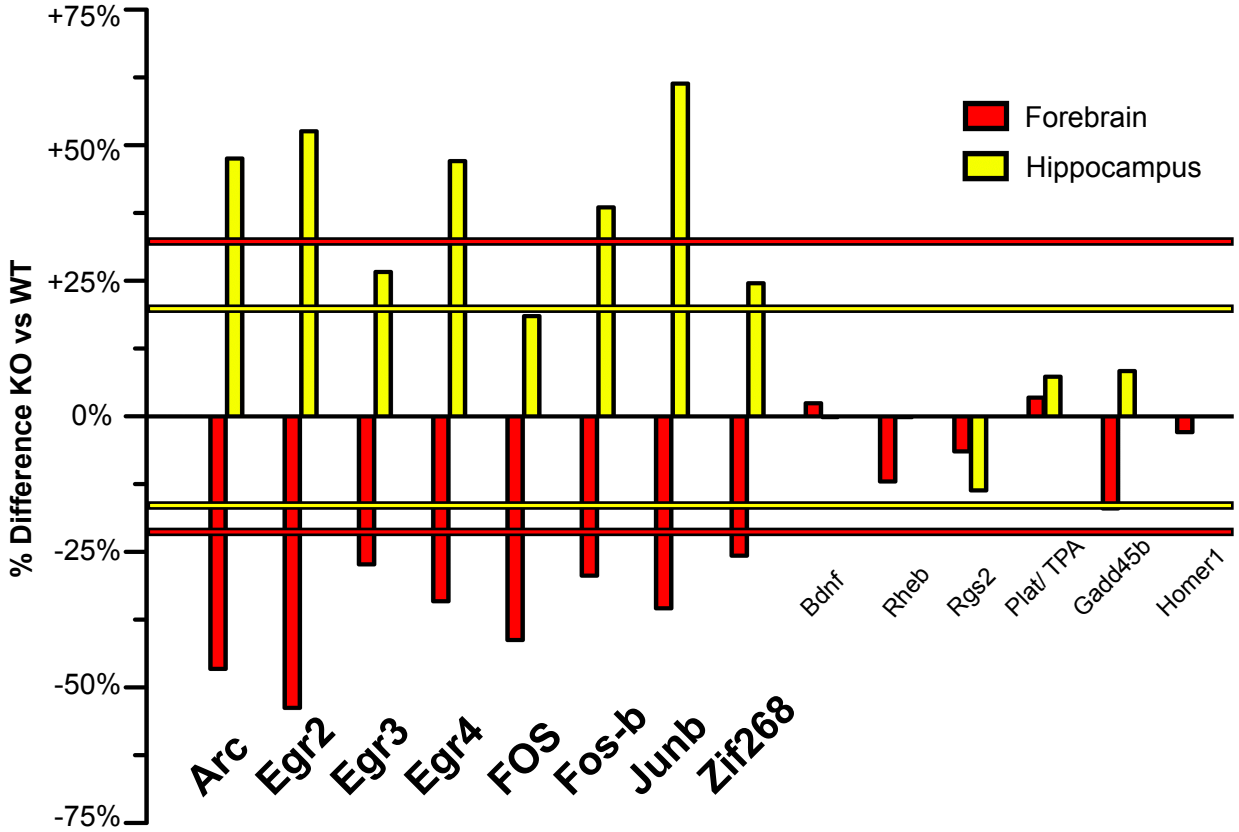

Figure 5.3 A subset of Immediate Early Gene (IEG) transcripts (large, bold text) show altered expression levels in both the forebrain and the hippocampus of Densin knockout mice. Expression of Arc, Egr1-4 (Egr1 = Zif268), Fos, Fos-b, and Junb decrease in the forebrain (red). Expression of Arc, Egr1-4, Fos-b and Junb increase in the hippocampus (yellow). Half of the IEGs show highly similar symmetric changes in opposite directions (Arc, -46.5% fb, +47.5% hip; Egr3, -27.3% fb, +26.6% hip; Egr2, -53.7% fb, +52.6% hip; Zif268, -25.7% fb, +24.6% hip). IEGs in small type are shown as a comparison. Bars represent average percent change between wild type and knockout RPKM values (red, forebrain; yellow, hippocampus) . Yellow horizontal lines represent the 5% (-16.97%) and 95% (19.72%) percentile ranges for changes in the hippocampal. Red horizontal lines represent the 5% (-21.56%) and 95% (32.42%) percentile ranges for changes in the forebrain.

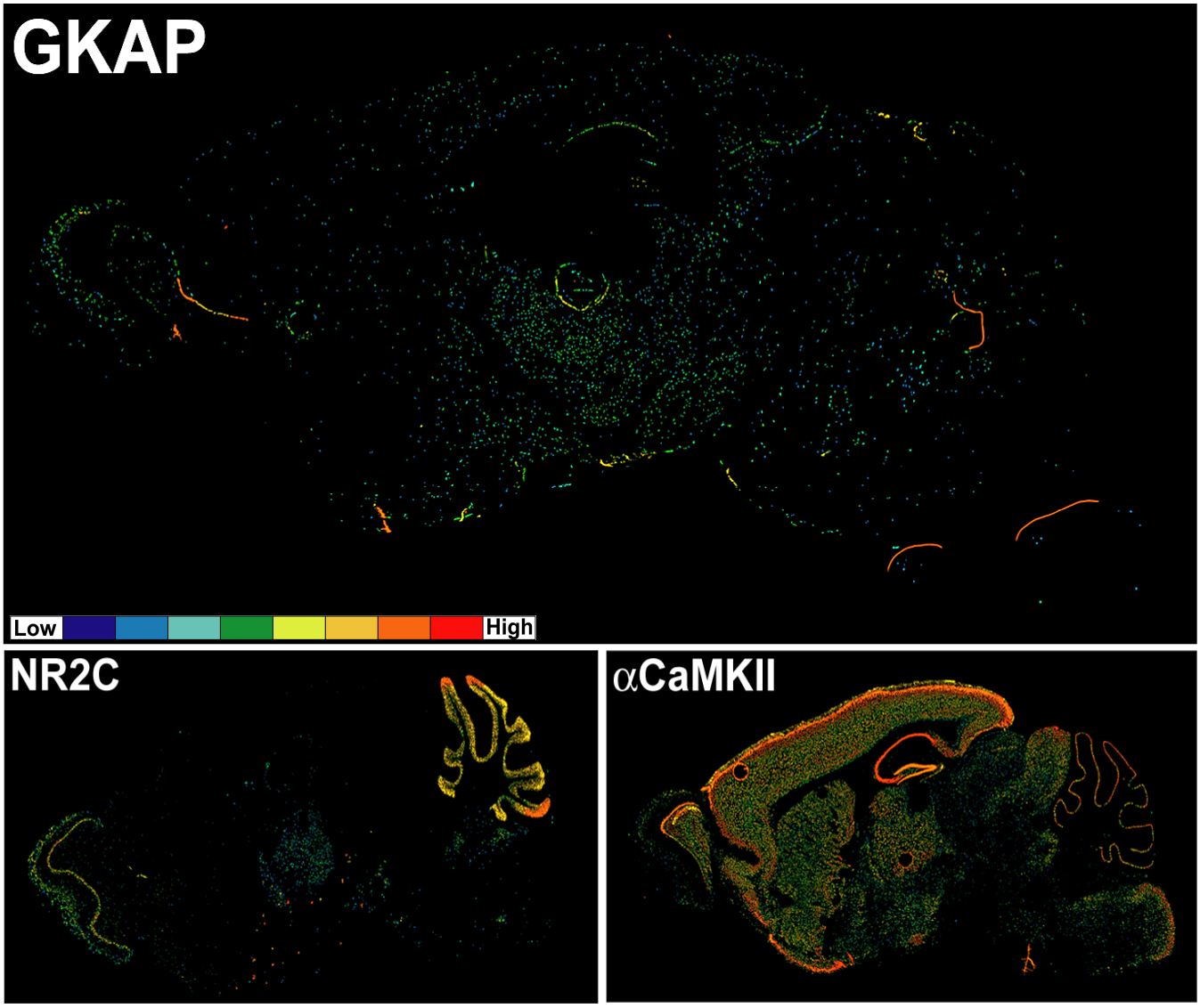

Figure 5.4 In situ hybridization heat maps of GKAP, NR2C and αCaMKII at saggital level 12-13 are shown. GKAP gene expression is low in all regions of the brain and is shown in comparison to NR2C (low gene expression in forebrain) and αCaMKII (high gene expression in forebrain). GKAP expression results are supported by RNA seq analysis which shows low expression in the forebrain and no expression in the hippocampus. Heat map color scale indicates level of gene expression.

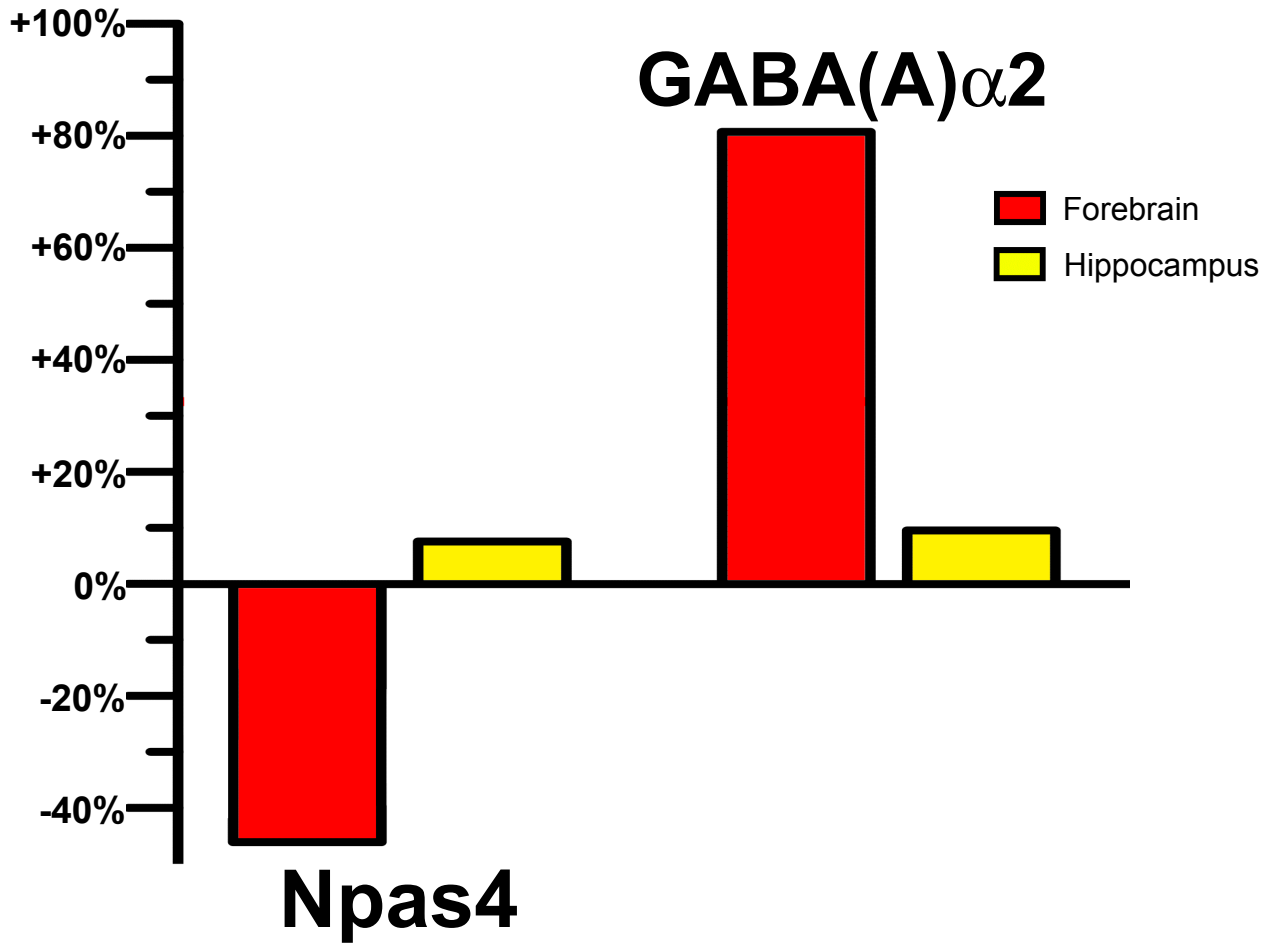

Figure 5.5 Npas4 and GABA(A)α2 are candidate genes whose expression could influence the seizure phenotype. Forebrain expression (red bars) of Npas4 and the GABA(A)α2 subunit are significantly altered in the Densin knockout mouse. The Npas4 transcript decreases by 46.1% and the GABA(A)α2 transcript increases by 80.8%. Neither exhibit large changes in the hippocampus (yellow bars; Npas4, +7.6%; GABA(A)α2, +9.5%).

	Wild type			Knockout		
	Forebrain (RPKM)	Hipp. (RPKM)	% Change (Hipp./ Forebrain)	Forebrain (RPKM)	Hipp. (RPKM)	% Change (Hipp./ Forebrain)
Glutamate Receptors						
NR1	162	125	-23%	161	129	-20%
NR2A	3	9	169%	3	9	170%
NR2B	7	13	104%	6	13	111%
NR2C	14	10	-30%	14	10	-26%
NR2D	7	4	-42%	7	5	-36%
GluR1	70	178	153%	72	177	144%
GluR2	45	94	110%	47	85	82%
GluR3	24	41	0%	24	37	0%
GluR4	15	11	-24%	17	10	-39%
Stargazin	21	18	-14%	21	21	2%
mGluR1	13	12	-10%	13	12	-12%
mGluR2	23	22	-7%	28	24	-15%
mGluR3	19	13	-32%	20	11	-46%
mGluR4	14	5	-61%	15	5	-67%
mGluR5	19	30	57%	20	28	39%
mGluR7	13	13	4%	13	14	3%
Scaffold Proteins						
Shank-1	35	64	81%	37	73	101%
Shank-2	15	24	53%	15	26	69%
Shank-3	39	34	-13%	39	36	-6%
Homer1	15	15	1%	15	15	6%
Homer2	9	13	42%	9	13	53%
GKAP	4	0	-91%	4	5	20%
GRIP-1	4	4	5%	5	5	3%
SAP-97	26	22	-14%	24	20	-17%
SAP-102	38	45	17%	37	42	13%
Chapsyn-110	46	41	0%	47	43	0%
PSD95	262	212	-19%	270	234	-13%
Gephyrin	23	19	0%	23	18	0%
Maguin-1	20	45	129%	23	42	83%
Signalling						
Calmodulin 1	790	679	-14%	778	657	-16%
Calmodulin 2	553	411	-26%	529	390	-26%
Calmodulin 3	1087	743	-32%	1060	827	-22%
SynGAP	55	55	0%	58	64	10%
Rac1	78	73	-6%	76	72	-5%
Rac2	1	1	-17%	1	0	-39%
Rac3	23	11	-50%	23	13	-43%
Kinases/ Phosphatases						
αCaMKII	496	759	53%	518	732	41%
βCaMKII	258	293	14%	264	292	10%
δCaMKII	15	10	-38%	14	8	-44%
γCaMKII	69	48	-31%	68	47	-32%
PKCα	19	33	70%	22	34	58%
PKCβ	84	117	39%	90	111	24%
PKCδ	34	16	-53%	33	13	-61%
Citron	10	9	-5%	12	10	-16%
CDK5	49	34	-30%	49	37	-25%
Adhesion Proteins						
δ-catenin	42	37	-11%	41	37	-10%
β-catenin	182	149	-18%	169	142	-16%
N-Cadherin	18	19	7%	18	18	-1%

	Wild type			Knockout		
	Forebrain (RPKM)	Hipp. (RPKM)	% Change (Hipp./ Forebrain)	Forebrain (RPKM)	Hipp. (RPKM)	% Change (Hipp./ Forebrain)
Densin	7	10	53%	9	12	29%
Regulatory and Cytoskeletal Associated Proteins						
Kalirin	34	42	25%	38	44	15%
α-actinin	8	5	-45%	9	4	-52%
Cofillin-1	163	89	-45%	149	96	-36%
Cofilin-2	18	18	-1%	17	15	-12%
Hras1	197	90	-54%	195	105	-46%
N-ras	7	9	26%	7	9	30%
Pak1	147	100	-32%	141	102	-28%
Pak2	9	11	25%	9	12	30%
Pak3	7	17	126%	8	15	80%
Pak6	3	16	476%	3	18	537%
Ras-GRF 2	15	117	671%	14	111	685%
LIM Kinase 1	26	13	-51%	25	14	-43%
LIM Kinase 2	14	11	-21%	15	12	-21%
RhoV	6	3	-39%	7	4	-45%
Rap1, GAP1	92	45	-51%	97	50	-48%
γ-Actin	0	0	0%	0	0	0%
Drebrin	92	83	-9%	93	100	8%
Cortactin	27	30	10%	26	32	21%
Ion Channels						
Calcium Channels						
P/Q-type Ca Channel	19	19	2%	20	21	6%
Voltage-dependent N-type α1B	8	11	37%	9	11	26%
Voltage-dependent L-type α1C	7	9	37%	6	9	33%
Voltage-dependent L-type- α1D	4	5	22%	4	5	21%
Voltage-dependent R-type α1E	10	24	145%	10	23	131%
Voltage-dependent T-type α1G	12	8	-32%	13	8	-38%
Potassium Channels						
Inward Rectifying K+ Channel	89	32	-64%	95	33	-66%
Ca^{2+} activated K^+ Channel 3	3	2	-24%	3	2	-31%
Ca^{2+} activated K^+ Channel 4	0	0	0%	0	0	0%
Immediate Early Genes						
Homer1	15	15	0%	15	15	0%
Egr1/ Zif268	93	33	-64%	69	41	-40%
Bdnf	6	7	34%	6	7	30%
Rheb	58	45	-23%	51	45	-12%
Rgs2	32	17	-46%	30	15	-50%
Plat/ TPA	15	12	-17%	15	13	-13%
Ptgs2	3	5	38%	2	4	71%
Egr2	7	1	-90%	3	1	-67%
Npas4	11	2	-77%	6	3	-54%
Fos	33	5	-85%	19	6	-71%
Junb	84	18	-79%	54	29	-46%
Egr4	26	9	-66%	17	13	-25%
Fosb	8	4	-53%	5	5	-8%
Gadd45b	16	8	-49%	13	9	-34%
Egr3	48	29	-39%	35	37	5%