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## The connexin43-dependent transcriptome during brain development: Importance of genetic background

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### Abstract

Use of null mutant mice is a powerful way to evaluate the role of specific proteins in brain function. Studies performed on knockout mice have revealed some unexpected roles of the gap junction proteins (connexins). Thus, analyses of gene expression in connexin43 (Cx43) null brains indicated that deletion of a single gene (*Gja1*) induced expression level change of numerous other genes located on all chromosomes and involved in a wide diversity of functional pathways. The significant overlap between alterations in gene expression level, control and coordination in Cx43 knockout and knockdown astrocytes raised the possibility that *Gja1* represents a transcriptomic node of gene regulatory networks. However, conditional deletion of *Gja1* in astrocytes of two mouse strains resulted in remarkably different phenotypes. In order to evaluate the influence of the genetic background on the transcriptome, we performed microarray studies on brains of GFAP-Cre:Cx43<sup>fl/f</sup> C57Bl/6 and 129/SVEV mice. The surprisingly low number of Cx43 core genes (regulated in all Cx43 nulls regardless of strain) and the high number of differently regulated genes in the two Cx43 conditional knockouts indicate high influence of mouse strain on brain transcriptome.

### Keywords

Gap junction; mouse strain; gene array; astrocytes

## 1. INTRODUCTION

We have previously characterized transcriptomic alterations in brains and hearts of Cx43 null mice (Iacobas et al, 2003, 2005a,b; Kardami et al, 2007; Spray and Iacobas, 2007). These studies revealed a surprisingly high variety of genes whose expression was altered in the knockout mice; some of these changes might be due to developmental compensation. However, our comparison of astrocytes from Cx43 null mice and from knock-down (Cx43 siRNA) studies revealing a very substantial overlap of alteration in gene expression level, control and coordination suggested that the widespread changes more likely reflect connexin-dependent gene regulatory networks rather than developmental compensation for the missing gene (Iacobas et al, 2008).

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It was recently reported that targeted deletion of Cx43 in astrocytes resulted in markedly different phenotypes from two different mouse strains (Weincken-Barger et al, 2007). Thus, in the C57Bl/6 background, hGFAP-Cre:Cx43<sup>fl/fl</sup> mice showed reduced Cx43 expression in astrocytes but no obvious behavioral or anatomical phenotype, whereas in a mixed 129/SVEV:C57Bl/6 background, GFAP targeted deletion of the *Gjal* gene in some instances led to grossly deranged organization of the hind brain in the so-called shuffler phenotype of profoundly disturbed gait.

In order to examine more closely this strain dependence of gene expression alterations following astrocyte deletion of Cx43, we have used gene expression microarrays to compare brain transcriptomes of wild-type mice and those with astrocyte-specific deletion of Cx43 in both C57Bl/6 and 129/SVEV strains at perinatal and adult time points. Our results indicate a surprisingly divergent set of gene expression profiles when brains of the two mouse strains are compared, that numerous differences between gene expression patterns result from Cx43 deletion in both strains, and that only very small numbers of “core” genes appear to be biomarkers for the phenotype of conditional deletion of Cx43 across these two mouse strains.

## 2. RESULTS

The microarray study was performed according to the standards of the Microarray Gene Expression Data Society (MGED) and data complying with the “Minimum Information About Microarray Experiments” (MIAME, Brazma et al. 2001) have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database and are accessible at <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE37239>

Analyses of brain transcriptome alterations of neonatal and adult C57Bl/6 and 129/SVEV mice in which the gap junction *Gjal* gene was deleted in astrocytes revealed that the genetic background plays a major role in defining the contribution of Cx43 to the phenotype.

### 2.1. Genes differentially expressed in the two mouse strains during brain development

Comparison between the transcriptomes of brains obtained from wild-type (WT: Cx43<sup>f/f</sup>) neonatal (P0) 129/SVEV and C57Bl/6 mice revealed that 5.9% (895 genes of the 15,204 non-redundant genes analyzed in the oligonucleotide microarrays) were expressed at different levels in these two mouse strains (Fig. 1; Supplementary Table 1). During development this value changed, increasing to 7.9% (1,190 of 15,144 total genes) in the mature brain (6–9 month old mice) (Fig. 1; Supplementary Table 2). The most differently expressed genes in the neonatal brains of 129/SVEV compared to those of C57Bl/6 were suppressor of Ty16 homolog (21.82 fold higher in 129/SVEV), synaptotagmin IV (19.98 fold higher in C57Bl/6), RNA binding motif protein45 (9.50 fold higher in 129/SVEV) and serine threonine kinase 25 (9.04 fold higher in C57Bl/6).

In the adult brain, we found that the most differently expressed genes between the two mouse strains were peptidylglycine alpha-amidating monooxygenase (29.69 fold higher in 129/SVEV), synaptotagmin IV (19.63 fold higher in C57Bl/6), suppressor of Ty16 homolog (9.22 fold higher in 129/SVEV) and the regulator of G-protein signaling 5 (9.07 fold higher in C57Bl/6). This overlap in strain differences in genes expressed at different developmental times extended to the overall transcriptomes of the two mouse backgrounds, where a common set of 92 regulated genes in the neonatal brains was also regulated in the adult brains (Fig. 1; Table 1). We term this set of genes that are changed similarly during development in brains of both mouse strains, “core” genes of brain development; because they change similarly, they are unlikely to contribute to the difference in phenotype between the two mouse backgrounds.

## 2.2. Analysis of pathways differentially regulated in neonatal and adult brains with respect to mouse strain

Comparison of gene expression levels in neonatal brains of the two strains revealed that expression within certain pathways was higher in 129/SVEV than C57Bl/6, notably regulation of innate immune response (*Ereg*, *Kirk1*), monovalent inorganic anion homeostasis (*Stc1*, *Slsn6*), oogenesis and sexual reproduction (*Ereg*, *Nanos3*), midbrain/hindbrain boundary development (*engrailed1*, *En1*), and TGFbeta receptor complex assembly. Pathways with higher expression of component genes in WT C57Bl/6 neonatal brain included carboxypeptidase activity (*Abhd2*, *Acot2*, *Med12*, *Nlgn2*), voltage gated calcium channel complex (*Cacna1b*, *Cacnb1*), dendrite (*Abl2*, *Cacna1b*, *Dpysl2*, *Freq*, *Sympo*) and cAMP kinase.

When the transcriptome of the adult WT 129/SVEV mouse was compared with that of C57Bl/6, numerous genes were found to be differentially expressed in the two genotypes. Notable categories of pathways showing higher expression in 129/SVEV were metalloprotease activity (*Supt16h*, *Nepps*, *Psc5*, *Mebsp2*; whereas two other metalloproteases were expressed at lower levels: *Lnpep*, *Nep11*), hydrolase and catalase pathways, mitosis in particular, M phase genes (*Chfr*, *Nek9*, *Wee1*, *Zc3HCl*, *Smcl*); although other mitosis genes were expressed at lower levels: *Cdk2*, *Txno4*, *Ptlg1*), neuronal migration (*Ndn*, *Pex5*, *Chl1*; whereas other migration genes were lower: *Lmx1b*, *Myb10*, *Twist1*), G-protein coupled kinase activity (*Grk6*), learning (*Accn2*, *Atpla2*; whereas *EphB2*, *Nos3*, *Atpa3* where lower), sodium ion transport (*Accn2*, *Atp1a2*, *Slc8a8*; whereas *Atpa3*, *Hcn3*, *Slcra7*, *Slc5a11*, *Slc5a7*, *Nos3* were reduced), neurotransmitter uptake (the ATPase *Atpla2*), vesicle fusion (*Plgn*), dendrite (*Chl1*, *Dpyl5*; whereas *Abl2*, and *Myh10* were both lower), glial cell migration (*Ndn*), and NGF signaling (also *Ndn*). Genes within pathways that were significantly lower in adult WT 129/SVEV mice included the connexin family of gap junction proteins (*Gja6* encoding Cx33, *Gjd3* encoding Cx30.2, *Gjb2* encoding Cx26, and *Gjc3* encoding Cx29), sensory perception of smell (several olfactory receptors), intermediate filaments other than GFAP (*Krt15*, *Krt18*, *Krt2*, *Krt33a*, *Krt7*, *Krtab6.2*, *Krt14*, *Krtap4.7*, *Krtap5.4*, *Nerf*; notably C57Bl/6 mice have reduced *Krt42*, *Krt73*, *Krt10*), inorganic anion transport (primarily phosphate and chloride channel genes, *Clcc1*, *Clcn5*, *Clc1*, *Gabrg2*, *Sla12a7*, *Clb12*, *Clqlnf1*, *Clqtnf6*, *Col5c1*, *Colla1*, *Col9a1*, *Col4a5*, *Col9a3*, *Mpst*), the myelin sheath (*Mbp*, *Gjc3*), cell junctions (the connexins mentioned above and *Fnm1*, *Abi2*, *Cd1*, *Tjp1*, *Csk*, *Pelhg5*, *Cacng8*), mid-brain development (*Otx1*, *Pitx3*, *Shh*), receptors (*Bdkrb2*, *Ccr111*, *Ffar2*, *GprB2*, *P2ry2*, *Adora2a*), eye development (*Myh10*), and cholinergic transmission (*Chrn*, *S2c5a7*).

## 2.3. How much is Cx43 down-regulated during brain development in the conditional knockout mice?

GFAP expression is not maximal until after birth, and we thus expected that Cx43 expression in brain of the conditional knockout (cKO) mice (hGFAP-Cre:Cx43<sup>f/f</sup>) would decrease during 2–3 weeks postnatal development. A 55% and a 45% decrease in *Gja1* gene expression was found when we analyzed microarrays probing transcripts from neonatal (P0) brains of C57Bl/6 and 129/SVEV cKO (GFAP-Cre:Cx43<sup>f/f</sup>) mice, respectively. This decrease in gene expression levels was paralleled by a 29% decrease of Cx43 protein expression level in the 129/SVEV cKO neonatal brains (Fig. 2). To evaluate the extent of Cx43 reduction in the GFAP positive cell population, we performed western blots on astrocytes cultured for 1 week that were derived from WT and cKO E14 brains of C57Bl/6 mice. As indicated in Figure 3, we found a 67% decrease in Cx43 protein level in cultured astrocytes. These results are similar to those previously reported using the mouse GFAP promoter to drive the Cre-recombinase to delete the floxed Cx43 gene in C57Bl/6 mice (Lutz et al., 2009). Although we don't know the reason for such low level of deletion, it is

possible that this is related to the fact that the astrocyte population matures over the two first weeks after birth. To test this possibility we compared Cx43 expression levels in brains and cerebellum of 129/SVEV and C57Bl/6 WT and cKOs mice 2–3 weeks after birth. Indeed, western blot analyses of Cx43 expression during brain development (P14–P21) of mice from both strains indicate a substantial loss of this gap junction protein two weeks after birth, with virtually total disappearance after the third postnatal week (Fig. 4).

#### 2.4. Deletion of Cx43 in two mouse strains leads to different transcriptomes

Marked differences between strains were found when transcriptomes of neonatal brains derived from WT and cKO mice were compared. We found 286 genes with altered expression levels in the cKO compared to WT C57Bl/6 neonatal brains and 193 genes differently regulated in the cKO compared to WT 129/SVEV neonatal brains (Fig. 5; Supplementary Tables 3 and 4). When we eliminated those genes with altered expression levels between the two WT mouse strains, practically no overlap of regulated genes was found between the cKOs of the two strains; there was a common set of only 19 genes that were regulated in the cKO brains of the two strains, including *Gja1* (Table 2).

In the adult brain, where *Gja1* expression levels decreased by 91% in C57Bl/6 and by 92% in 129/SVEV cKOs compared to WT, respectively, we found a very different set of genes regulated compared with those differentially expressed in the neonatal brain. Compared to WT, C57Bl/6 cKO adult brains displayed 979 genes (Supplementary Table 5) with altered expression levels, whereas only 39 genes (Supplementary Table 6) had altered expression in adult 129/SVEV mouse brains (Fig. 5). Differently from neonatal brains, we found a common set of only 4 genes that were regulated in the adult brains of C57Bl/6 and 129/SVEV cKO mice [breast carcinoma amplified sequence 1 (*Brca1*); chemokine ligand 12 (*Cxcl12*), gap junction membrane channel protein alpha 1 (*Gja1*), and tubby-like protein 3 (*Tulp3*)].

#### 2.5. Analysis of pathways differentially regulated in neonatal and adult brains with respect Cx43 deletion

When we compared pathways encoded by genes up-regulated in the cKO C57Bl/6 neonatal mouse, most prominent were those associated with mRNA polyadenylation (*Cstf2t*, *Palpc2*), latent viral infection (*Hcfc1*), myoblast cell fate/determination (*Myod1*), leukocyte adhesion (*Itga1*), carboxypeptidase activity (*Folh1*), epithelial cell differentiation (*Hgpat6*) and organic cation transport (*Slc22a2*). Genes that were down-regulated in the neonatal cKO C57Bl/6 brains spanned a wide range of categories, including regulation of adaptive immune response (*Spn*, *Bcl6*, *Traf6*), keratinocyte differentiation (*Lor*, *Madcam1*, *Map2k1*, *Sfn*, *Tsg101*), cation transporter (*Sfxn2*, *Sfxn3*, *Slc30ab*), inward potassium channel (*Kcnj4*, *Kcnj6*), extracellular matrix (*Lama3*, *Adamts14*, *Hspg2*, *Thsd4*, *Sgcd*, *Col5a1*, *Spn*, *Cola1*, *Col6a1*, *Col15a1*, *Col14a1*, *Madcam1*, *Med12*, *Nlgn2*, *Pgm5*, *Rs1*), regulation of cell proliferation (*Bmp10*), astrocyte activation and establishment of blood-brain barrier (*Agt*), tube development (*Agt*, *Wnt5*, *B4galt1*, *Bmp10*, *Pax3*), receptor binding (*Angpt1*, *Lama3*, *Msn*, *Ubqln1*, *Wtn4*), and fatty acid metabolism (*Acaa1b*, *Acadv1*, *Acot3*, *Ptges3*, *Agt*).

When the adult 129/SVEV wild-type was compared to the cKO, the primary pathways found to be upregulated in the knockout were integrin mediated intracellular signaling (*Abants1*, *Abants4*, *Gab2*, *Itga1*, *Lat*), glycosaminoglycan binding (*Adamts1*, *Hbegf*, *Hapln3*, *Stab1*) and female gamete genes. The gene pathways that were most prominently down-regulated in the cKO 129/SVEV strain were those related to function of the chemokine CXCL12 (chemotaxis, motor axon guidance, axon projection, cell migration, and growth factor).

When the adult WT C57Bl/6 mouse was compared to the cKO, genes that were up-regulated in the cKO were those associated with learning (*Gabra5*, *Aehb1*), amino acid permease (*Slc7a5*, *Slc7a7*), sulphuric ester hydrolase (*Sulf4*), response to light stimulus (*Apvb1*, *Tub*), water homeostasis (*Aqp4*), actin cytoskeleton (*Coro2b*, *Grif1*, *Gsn*, *Lasp1*), vesicular fraction (*Ephs1*), fibroblast growth factor 20 (*Fgf20*), neurotransmitter uptake (*Atp1a2*), behavioral fear response (*Gabra5*), and cell communication (*Sh3pxd2a*). Genes that were down-regulated in the cKO C57Bl/6 adult mice included G-protein coupled receptors (*Cdsr1*, *Eltld1*, *Tas2r102*) and memory perception of smell which depends on the G-protein coupled receptors, the intermediate filament genes (*Krt10*, *Krt42*, *Krt72*), hyperosmotic response (*Pkn1*, *Rac1*), thromboxane receptor activity and cardioblast differentiation (*Bmp10*, *Tdgl1*).

## 2.6. Chromosomal location of regulated genes

Frequency distribution of regulated genes according to their chromosome location indicated that deletion of Cx43 in both mouse strains altered gene expression of genes located in all chromosomes (Fig. 6) and not only on the chromosome (Ch10) on which *Gjal* is located. However, chromosomes 4 and 11 contained different numbers of regulated genes when cKO and WT genotypes were compared (Fig. 6A). Comparison of the frequency distribution of regulated genes according to their chromosome location between mouse strains indicated that chromosomes 2, 3, 5, and 13 contained more than 50% of the differences in numbers of regulated genes between mouse strains (Fig. 6B).

## 2.7. Correlation between gene and protein expression levels

As mentioned above, we found that for a 50% decrease in *Gjal* expression level in the neonatal brains, a significant 29% decrease in Cx43 expression levels was detected by western blot (Fig. 1). When we analyzed PAM (peptidylglycine alpha-amidating monooxygenase) expression level by western blot, a significant 1.83 fold increase was detected in brains from C57Bl/6 compared to those of 129/SVEV cKO mice (Fig. 7A;  $P = 0.015$ , t-test), while microarray analysis indicated a 35.9 fold increase among the two strains. For vimentin, which by microarray analysis was found to be 1.6 fold up regulated in the cKO compared to WT 129/SVEV, no significant difference at the protein levels was detected by western blots (Fig. 7B). Such differences between levels of transcripts and their encoded proteins are likely resultant from various post-transcriptional regulations, although contribution of other factors related to the affinity of antibodies, normalization methods (GAPDH x background fluorescence), stability of mRNA, and sensitivity of detection methods, among others cannot be ignored. Nevertheless, there is a very good correspondence between the two techniques, at least in terms of direction of changes (1.3 fold increase in Synaptotagmin IV protein levels compared to a 9.5 fold increase at the transcript levels between SVEV and C57Bl/6 cKOs and 37% decrease in Glypican4 protein levels for a 71% decrease in transcript levels between SVEV and C57Bl/6 cKOs, data not shown).

## 3. DISCUSSION

Among the most exciting current topics in translational research is that of susceptibility factors by which gene expression alteration, environmental changes, and other stimuli bias in favor or against acquisition of a phenotype. In the gap junction field, it was initially surprising that expression of numerous other genes was affected in Cx43 null brain, heart and astrocytes (Iacobas et al., 2002, 2003; Naus et al., 2000). Moreover, such pervasive transcriptomic changes provide the expectation that phenotype resulting from knockdown of Cx43 might be highly variable with regard to strain, age or sex or other environmental conditions. In support of this hypothesis, we have found major sex dependent differences in



gene expression changes in heart ventricles and atria (Iacobas et al., 2010), and it was reported that transgenic deletion of the astrocyte-specific gap junction gene *Gja1* (encoding Cx43) in a GFAP-targeted strategy led to markedly different morphological and behavioral phenotypes in two different mouse strains (Wiencken-Barger et al, 2007). Moreover, these results were reminiscent of the profound destruction of the midbrain-hindbrain region obtained when the mitogen Wnt1 was deleted (Melloy et al, 2005); strikingly, when the authors crossed Wnt-1 heterozygotes into a Cx43 overexpressing mouse line, the expression of anatomically correct cerebellum was rescued in a substantial percentage of offspring (Melloy et al., 2005). Thus, the loss of one signaling molecule can be compensated by overexpression of a gap junction protein. A similar result was recently reported in *C. elegans*, where a patterning defect caused by loss of expression of a gap junction protein could be rescued by overexpression of an intercellular adhesion molecule (Chuang et al, 2007).

We have speculated that such compensatory changes are the result of transcriptomic interlinkage, such that alteration of a single gene affects many others and that certain genes have partners with similar connectivity (Spray and Iacobas, 2007; Iacobas et al. 2007a). If so, this connectivity might be exploited as novel therapies where gene expression is lost (Iacobas et al. 2007b).

The large number of genes whose expression was altered in the cKO mice of both genetic backgrounds and at both developmental stages confirms our previous conclusion that Cx43 constitutes a highly interconnected transcriptomic node. However, only a small number of genes were affected similarly in both strains (“Cx43 core” genes). In the immature brain, we found a common set of 19 “Cx43 core” genes that had their expression levels altered in the cKO compared to wild-type of both mouse strains. Intriguingly, in the adult brain, a different and much smaller set of 4 “Cx43 core” genes was found. Given that there are major gene expression alterations in both strains but with little overlap in which genes are affected, we conclude that Cx43-controlled pathways are strongly dependent on strain and are profoundly remodeled during development. Thus, the phenotype of transgenic mice will likely be determined in part by functions both of the altered gene and the altered genomic network, which may be distinct for each strain.

## 4. EXPERIMENTAL PROCEDURE

### 4.1. Animals

Conditional knockout (hGFAP-Cre:Cx43<sup>f/f</sup>) mice on the C57Bl/6 and SVEV backgrounds used in this study were generated in Dr. Kenneth McCarthy’s laboratory (Wiencken-Barger et al, 2007) and housed at our animal facility at Albert Einstein College of Medicine. All studies used protocols approved by the Einstein Animal Care and Use Committee.

### 4.2. Tail PCR

PCR of tails from offspring of Cx43<sup>f/f</sup> x GFAP-Cre:Cx43<sup>f/f</sup> parents was performed as previously described (Dermietzel et al., 2000) but using the following primers: (Cx43<sup>f/f</sup> – Forward) – GGTGATGCAACGAGTGATGAGG; (Cx43<sup>f/f</sup> – Reverse) – GCTAAGTGCTTCTCTACACCTGCG; (hGFAP-Cre – Forward) – CTTTGACTCTGATTACAGAGCTTAA; (hGFAP-Cre–Reverse) – GTCTCACTGTTACTTAACAGCTTGA.

### 4.3. Tissues

For gene expression and western blot analyses (see below), we used brains from neonatal and adult wild-type (Cx43<sup>f/f</sup>) and cKO (hGFAP:Cre:Cx43<sup>f/f</sup>) C57Bl/6 and SVEV mice. Four

brain samples of each of the genotypes, mouse strains and ages used for the microarray studies were also used in some experiments to evaluate protein expression levels.

**4.3.1. Brain tissues**—To remove brains, animals were anesthetized with isoflurane prior to decapitation. Brains from neonatal and adult mice were removed from the skull and placed in ice cold Dulbecco's phosphate buffered saline (PBS; Cellgro, Herndon, VA, USA). The two hemispheres and cerebellum were separated with a razor blade and tissues immediately placed at  $-80^{\circ}\text{C}$  Revco until processed for RNA and protein extractions. In some experiments, brain tissues from juvenile mice (postnatal days 14–21) were used and processed as described above.

**4.3.2. Astrocyte cultures**—Brain cortices of neonatal (P0) mice were minced in ice cold  $\text{Ca}^{2+}$ -free PBS and tissue digested with 0.05% trypsin (Cellgro) for 5 min at  $37^{\circ}\text{C}$ , after which digests were centrifuged at 1,500 RPM for 5 min. Supernatants were removed and cells re-suspended in Dulbecco's minimal essential medium (DMEM, Invitrogen, NY, USA) containing 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin antibiotics (Invitrogen). Cells were grown for 1–2 weeks in plastic tissue culture dishes (BD Falcon, NJ, USA) and maintained at  $37^{\circ}\text{C}$  in a humidified 5%  $\text{CO}_2$  incubator.

#### 4.4. Gene Micro-arrays

For RNA extraction and hybridization, 20  $\mu\text{g}$  total RNA extracted in Trizol from brains of each of the 32 mice (4 per condition) was reverse transcribed in the presence of fluorescent Alexa Fluor®\_647 and Alexa Fluor®\_555-aha-dUTPs (Invitrogen, CA) to obtain labeled cDNAs. Red- and green-labeled samples of biological replicas were then co-hybridized ("multiple yellow" strategy, Iacobas et al. 2010) overnight at  $50^{\circ}\text{C}$  with mouse MO36k oligonucleotide arrays printed by Duke University Microarray Facility with Operon Mouse Oligo Set, version 4.0 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL8928>). After washing (0.1% SDS and 1% SSC) to remove the non-hybridized cDNA, each array was scanned with a GenePix 4000B scanner (MDS, Toronto, Canada) and images were primarily analyzed with GenePixPro 6.0 (Axon Instruments, CA).

**4.4.1. Data processing (Iacobas et al, 2008)**—All spots affected by local corruption, or with saturated pixels, or with foreground red or green fluorescence less than twice the background level on any slide were removed from the analysis. Normalized relative expression levels were determined as average values of individual spots probing the same gene (redundancy groups). Detection of differentially expressed genes relied on both absolute  $>1.5\text{x}$  fold-change and  $<0.05$  p-value of the heteroscedastic t-test applied to the means of the background subtracted normalized fluorescence values in the four biological replicas of the compared transcriptomes. The p-values were computed with a Bonferroni type correction applied to the redundancy groups (Iacobas et al. 2008). GenMAPP (Dahlquist et al. 2002) and MappFinder software ([www.genmapp.org](http://www.genmapp.org), Doniger et al. 2003) and databases were used to identify the GO (Gene Ontology) categories affected by Cx43 deletion in the different genetic backgrounds. These statistically significant Gene Ontology categories are described in which there are at least ten nested members at the node.

#### 4.5. Westerns Blots

Homogenates from brain tissues (cortices and cerebellum) were prepared as follows. Frozen cortices and cerebellum were placed in lysis buffer (50 mM NaCl, 25 mM Tris-HCl, 5 mM EDTA, 1% NP-40, 0.25 mM Na-deoxycholate, pH 7.5) and tissue sonicated. Samples of whole cell lysates in Laemmli buffer (20  $\mu\text{g}$  total protein) were electrophoresed in 4–20% SDS-PAGE (Bio-Rad, Hercules, CA) and then transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). Immunoblots were performed after overnight

incubation of membranes with blocking solution (5% dry nonfat milk and 0.4% polyoxyethylenesorbitan monolaurate: Tween-20; Sigma) using primary antibodies. After several washes with 1X PBS-Tween-20, membranes were incubated with HRP-conjugated secondary antibodies (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Detection of bands was performed on X-ray films (Kodak, Rochester, NY, USA) following incubation with enhanced chemiluminescence reagents (Amersham Pharmacia Biotechnology, Piscataway, NJ, USA). Quantification of protein expression levels was performed by densitometric analysis (Scion-NIH Image software) using GAPDH as an invariant control against which the protein of interest was normalized.

The following primary antibodies were used: rabbit polyclonal Glypican-4 (1:500; Abcam Inc.); mouse monoclonal anti-vimentin (1:200; Abcam Inc.); rabbit polyclonal anti-Connexin43 (1:2000; Sigma-Aldrich); goat polyclonal anti-Synaptotagmin IV (1:100; Santa Cruz Biotech.); goat polyclonal anti-Peptidyl-glycine alpha-amidating monooxygenase (PAM; 1:200; Santa Cruz Biotech.); GAPDH (1:5000; Fitzgerald).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

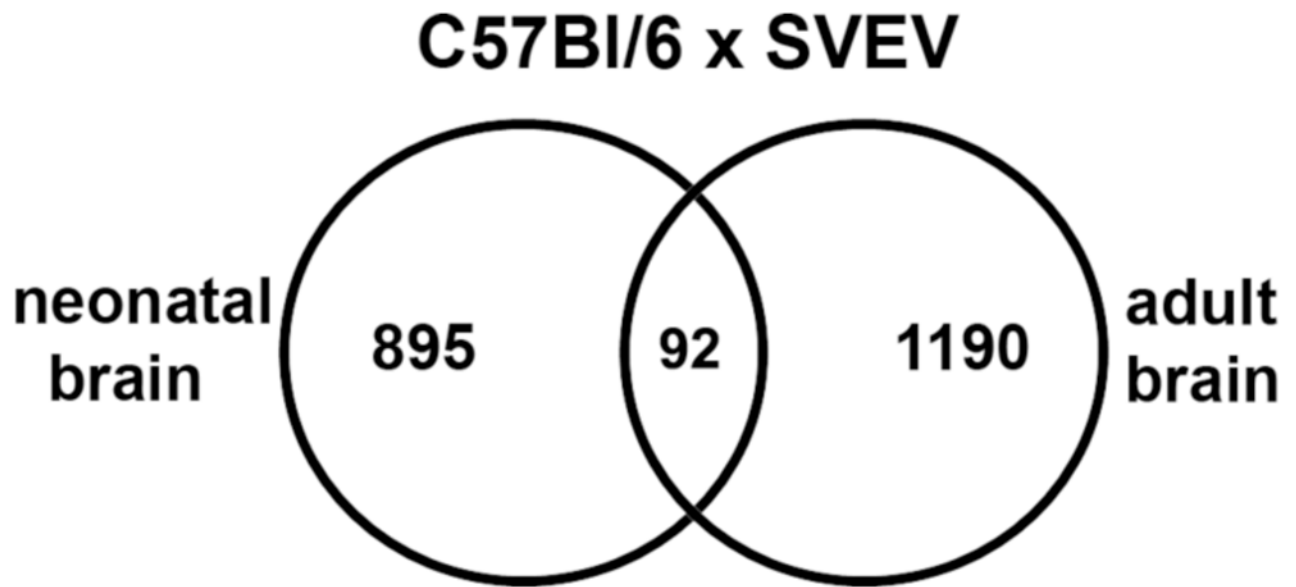
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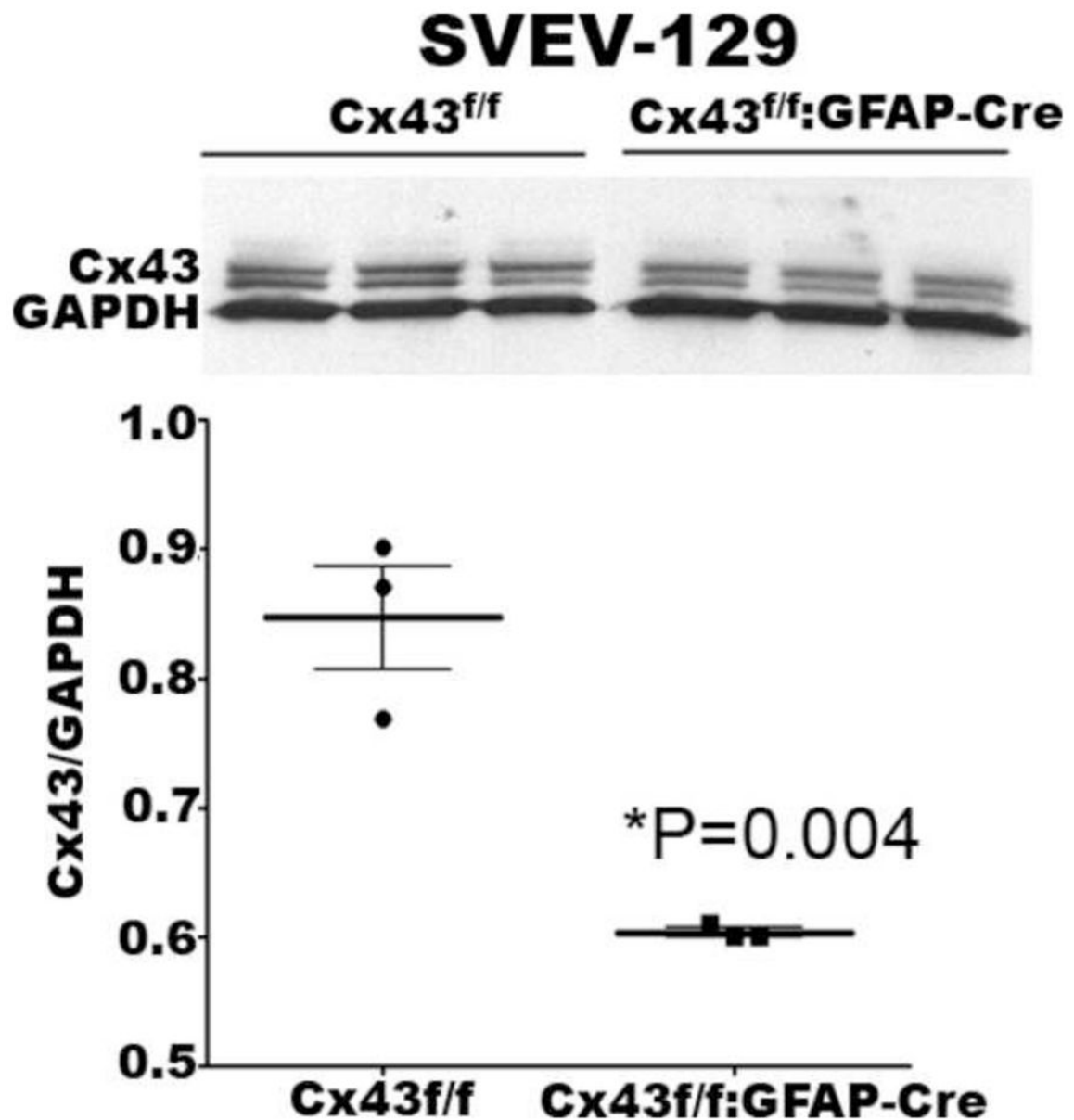


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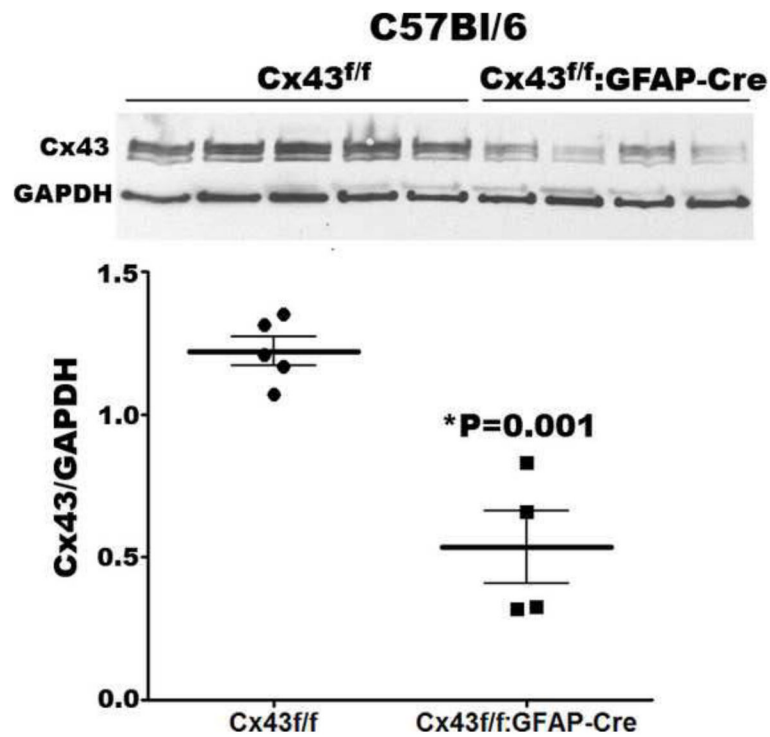
**Figure 1. Developmentally regulated genes differ between mouse strains**

Venn diagram showing regulated genes obtained by comparing transcriptomes of wild-type C57Bl/6 and SVEV neonatal brains as well as from adults. Note the 10% overlap of regulated genes in the two mouse strains; this common set of genes that are differently regulated in both mouse strains and are independent of brain developmental stages are likely to contribute the phenotypic differences between the two backgrounds.



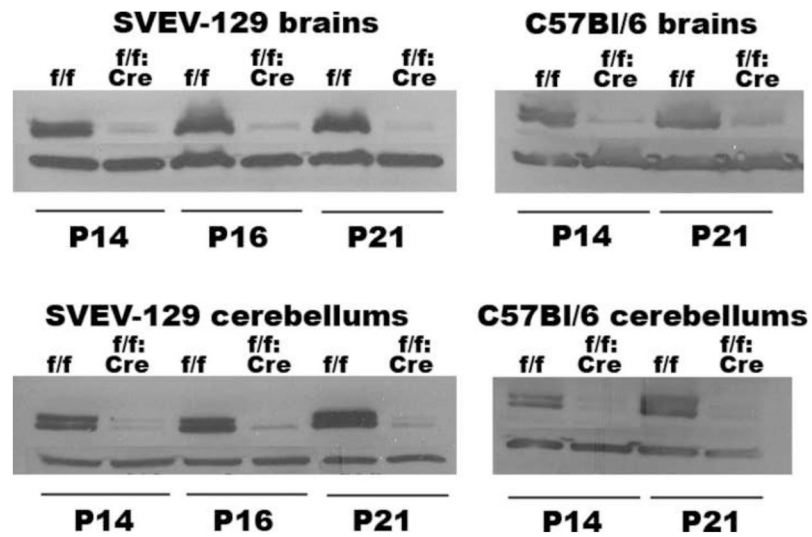
**Figure 2. Incomplete down-regulation of Cx43 in neonatal brains of C57Bl/6 and SVEV cKO mice**

Mean  $\pm$  SE values of Cx43 expression levels obtained for neonatal brains of SVEV-129 “wild-type” (Cx43<sup>f/f</sup>) and Cx43 cKO (Cx43<sup>f/f</sup>:GFAP-Cre) mice using Western blot analyses (inset). Note that at this stage of development there is no complete deletion of Cx43. Statistical significance obtained using unpaired T-test (N=3 animals per group).



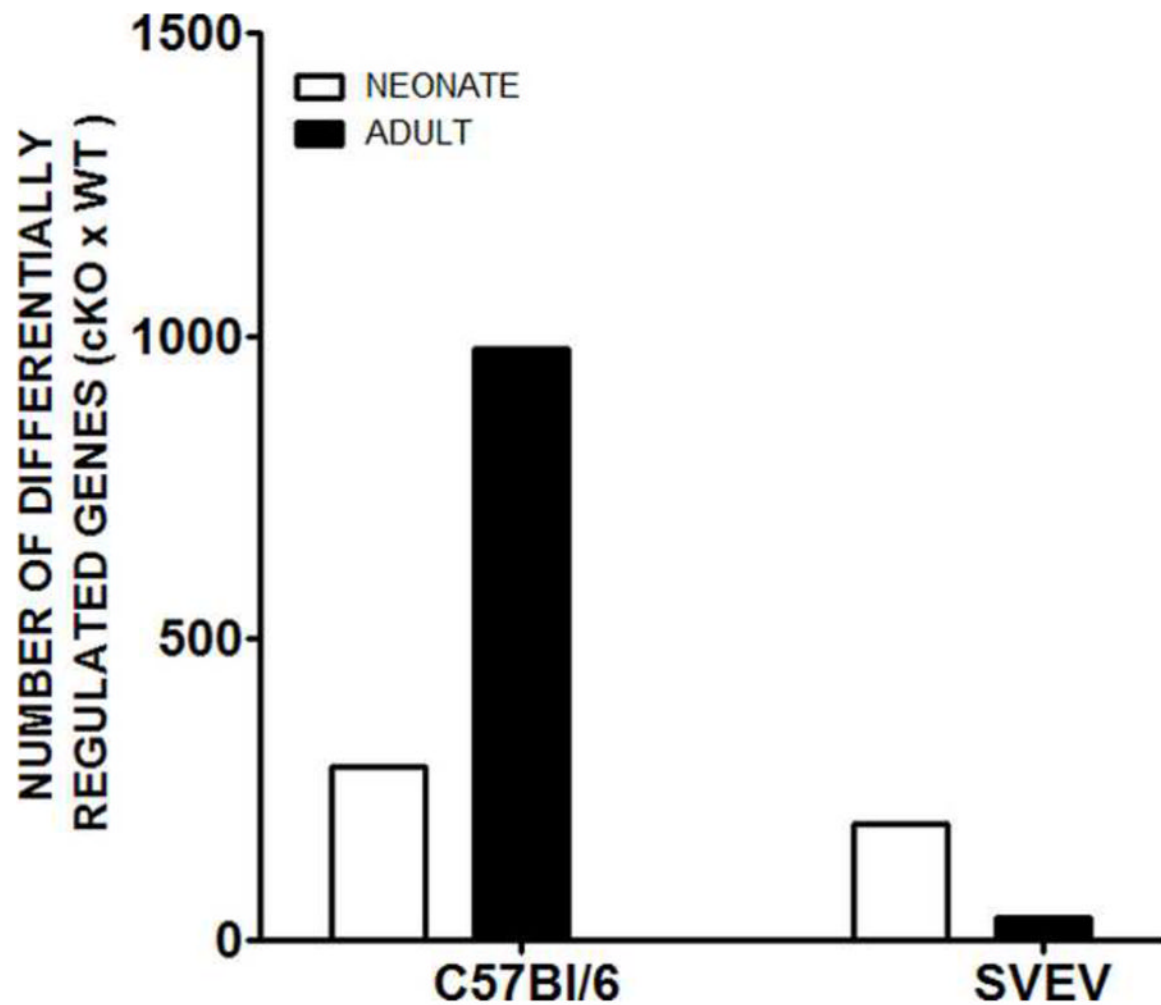
**Figure 3. Incomplete down regulation of Cx43 in cultured Cx43 cKO astrocytes**

Mean  $\pm$  SE values of Cx43 expression levels obtained for cultured astrocytes derived from neonatal brains of C57Bl/6 “wild-type” (Cx43<sup>f/f</sup>) and Cx43 cKO (Cx43<sup>f/f</sup>:GFAP-Cre) using Western blot analyses (inset). Note that after 2 weeks in culture deletion of Cx43 from the astrocyte population is incomplete. Statistical significance obtained using unpaired T-test (N=5 wild-type and 4 cKOs animals).



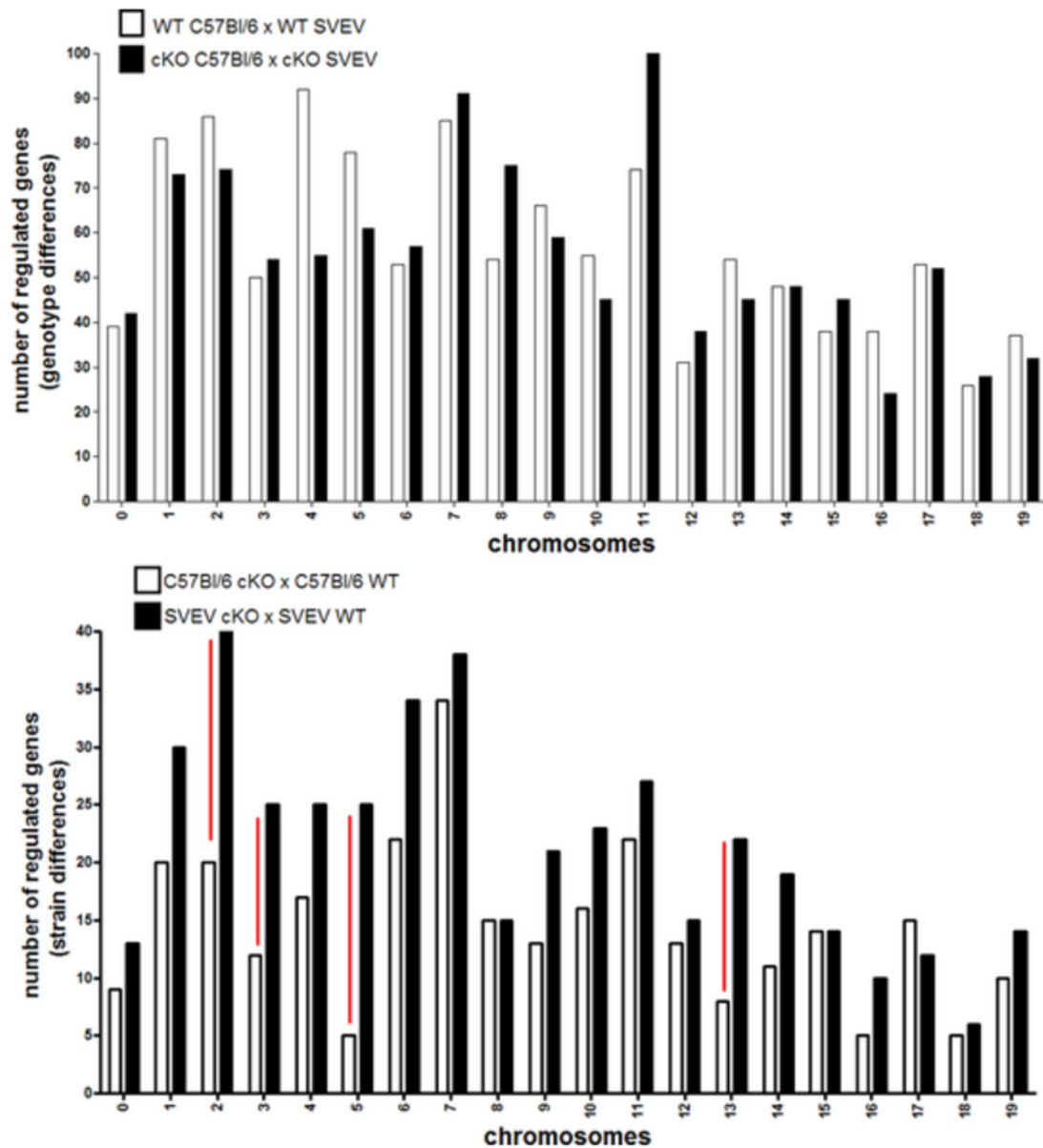
**Figure 4. Substantial knockdown of Cx43 in CNS from juvenile cKOs mice**  
 Examples of western blots showing the expression levels of Cx43 in brains and cerebellums of wild-type (f/f) and Cx43 cKOs (f/f:Cre) SVEV-129 and C57Bl/6 juvenile mice.





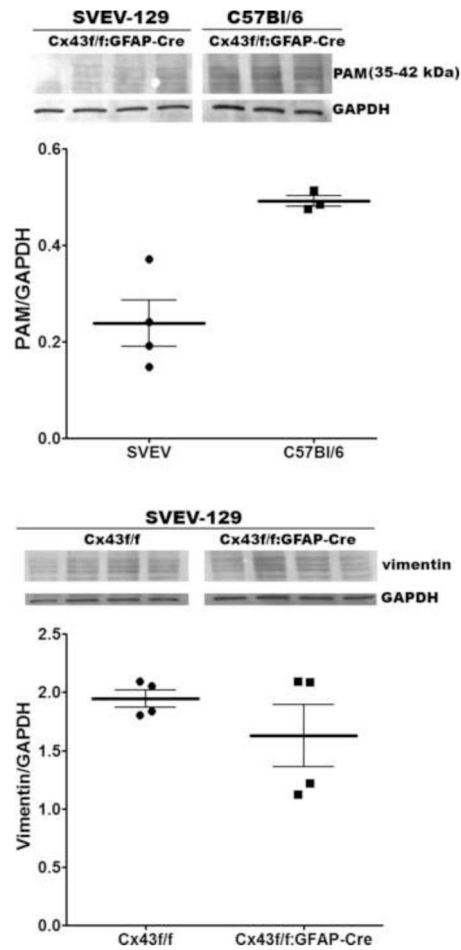
**Figure 5. Impact of mouse strain on the transcriptome of Cx43 cKO brains**

Analysis of regulated genes obtained by comparing brain transcriptome of Cx43 cKOs and WT mice of two strains revealed a substantially higher number of regulated genes in the adult brains of C57BL/6 than in SVEV mice. Similar number of regulated genes was detected in the neonatal brains of C57BL/6 and SVEV mice when transcriptomes of WT and Cx43 cKOs were compared.



**Figure 6. Chromosome location of regulated genes**

Frequency distribution of regulated genes according to their chromosome location obtained from transcriptome analysis performed between (A) mouse strains and (B) mouse genotypes. (A) The frequency distribution of chromosome location of regulated genes obtained from transcriptome analysis between WT C57Bl/6 and SVEV mice (white bars) was similar to that obtained from similar analysis performed between Cx43 cKO C57Bl/6 and SVEV mice (black bars). (B) Except for chromosomes 2, 3, 5, and 13, the frequency distribution of chromosome location of regulated genes obtained from brain transcriptome analysis between Cx43 cKO and WT C57Bl/6 mice (white bars) was similar to those of SVEV mice (black bars). Note the 50% difference (red lines) in terms of number of regulated genes in chromosomes 2, 3, 5, and 13 in SVEV compared to C57Bl/6 mice.



**Figure 7. Protein expression levels validate transcriptome**

Scatter plot with mean  $\pm$  SE values of (A) PAM and (B) vimentin protein expression levels relative to that of GAPDH obtained from brains of (A) SVEV and C57Bl/6 Cx43<sup>f/f</sup>:GFAP-Cre and (B) WT (Cx43f/f) and cKO SVEV mice.

**Table 1**  
**List of a common set of genes that were regulated in both mouse wild-type (W) strains during brain development**

Genes highlighted in green showed significantly lower expression in SVEV than C57Bl/6 brains; red highlights indicate significantly higher expression in SVEV brains. Numbers represent fold changes. In all cases, differences are statistically significant ( $p < 0.05$ ).

Name	neonatal W-SVEV/W-C57	adult W-SVEV/W-C57
3-hydroxy-3-methylglutaryl-Coenzyme A reductase	-4.97	-3.27
6-pyruvoyl-tetrahydropterin synthase	-1.7	-2.13
Abhydrolase domain containing 2	-2.68	-1.59
Abl-interactor 2	-2.54	-1.65
Adaptor-related protein complex AP-4, mu 1	2.28	2.19
AF4/FMR2 family, member 4	-2.06	-2.61
Amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 13 (human)	-1.54	-1.68
ATPase, H <sup>+</sup> transporting, lysosomal V1 subunit C1	1.68	1.52
ATP-binding cassette, sub-family B (MDR/TAP), member 10	-3.8	-2.26
Beta-2 microglobulin	-2.84	-2.35
Biglycan	-1.61	-1.51
Bisphosphate 3'-nucleotidase 1	-2.77	-2.05
CAP, adenylate cyclase-associated protein 1 (yeast)	2.63	6.82
Caspase recruitment domain family, member 10	1.69	1.73
CDC42 effector protein (Rho GTPase binding) 2	-1.56	-1.52
CDGSH iron sulfur domain 2	-1.61	-2.08
Centrosomal protein 27	-3.57	-2.3
Cholinergic receptor, muscarinic 3, cardiac	-1.92	-2.2
Chromobox homolog 3 (Drosophila HP1 gamma)	-5.59	-2.24
Complement component 1, q subcomponent, C chain	-1.58	-1.97
DCMP deaminase	-2.28	-1.83
Diacylglycerol kinase, epsilon	-2.72	-2.21
Dihydropyrimidinase-like 2	-1.82	-2.2
D-tyrosyl-tRNA deacylase 1 homolog (S. cerevisiae)	-1.82	-1.53
Ectodermal-neural cortex 1	-2.09	-1.97
Esterase D/formylglutathione hydrolase	-2.71	-1.86
Exosome component 9	-1.9	-1.87
Exotoses (multiple)-like 2	-1.59	-1.82
F-box protein 42	-1.78	-1.79
G protein-coupled receptor 180	-1.87	-1.96
GA repeat binding protein, alpha	-2.21	-2.36
Gamma-aminobutyric acid (GABA-A) receptor, subunit alpha 2	1.72	2.27
Gamma-aminobutyric acid (GABA-A) receptor, subunit gamma 2	-1.67	-1.8
Glial cell line derived neurotrophic factor family receptor alpha 1	-1.66	-2
Glutamate receptor, ionotropic, NMDA2B (epsilon 2)	-2.36	-1.81

Name	neonatal	adult
	W-SVEV/W-C57	W-SVEV/W-C57
Glutathione S-transferase, mu 6	-2.62	-1.7
Glyoxalase 1	2.56	2.3
Growth factor receptor bound protein 2-associated protein 2	-1.99	-1.74
Guanine nucleotide binding protein, beta 1	4.3	3.97
Guanine nucleotide binding protein-like 3 (nucleolar)-like	-1.52	-2.15
HD domain containing 3	4.54	2.87
Histone cluster 1, H4c	-2.1	-1.84
Histone cluster 1, H4d	-2.63	-2.57
Histone cluster 1, H4k	-3.03	-1.71
Iduronidase, alpha-L-	-1.67	-1.56
Interleukin-1 receptor-associated kinase 2	-1.77	-2.03
Limb expression 1 homolog (chicken)	-2.51	-1.94
Lysophospholipase-like 1	2.4	2.67
Macrophage scavenger receptor 2	-1.52	-4.14
Mitogen activated protein kinase kinase 7	-1.51	-2.3
Multiple EGF-like-domains 9	-2.2	-3.12
MutL homolog 1 (E. coli)	-2.2	-2.07
Olfactory receptor 679	-2.96	-3.32
Papillary renal cell carcinoma (translocation-associated)	-1.96	-1.73
Paralemmin 2	-1.74	-1.76
Phosphatidylinositol-5-phosphate 4-kinase, type II, beta	-2.45	-1.98
Plasma glutamate carboxypeptidase	-1.69	-1.68
Plasmalemma vesicle associated protein	2.27	1.76
Polyadenylate binding protein-interacting protein 1	2.3	3.05
Prenylcysteine oxidase 1 like	-1.64	-2.38
Procollagen, type I, alpha 1	-1.56	-1.58
Proteasome (prosome, macropain) 26S subunit, non-ATPase, 9	-1.99	-2.04
Protein phosphatase 1M	-1.96	-1.66
REC8 homolog (yeast)	-2.33	-3.62
Regulator of G-protein signaling 5	-5.47	-9.07
Regulatory factor X, 4 (influences HLA class II expression)	-2.53	-2.02
Retinoblastoma 1	1.61	1.54
Rho-related BTB domain containing 3	-1.94	-1.68
Ring finger protein 168	-1.55	-2.32
RIO kinase 3 (yeast)	1.5	1.76
Serine (or cysteine) peptidase inhibitor, clade A, member 3C	2.29	4.81
Serine (or cysteine) peptidase inhibitor, clade A, member 3N	4.06	4.62
Serine/threonine kinase 25 (yeast)	-9.04	-5.46
Solute carrier family 1 (glial high affinity glutamate transporter), member 2	-2.14	-1.64
Solute carrier family 27 (fatty acid transporter), member 1	-3.36	-4.56
Solute carrier family 6 (neurotransmitter transporter, creatine), member 8	2.45	1.54
Splicing factor, arginine/serine-rich 14	1.67	1.9



Name	neonatal	adult
	W-SVEV/W-C57	W-SVEV/W-C57
Sterol-C5-desaturase (fungal ERG3, delta-5-desaturase) homolog ( <i>S. cerevisiae</i> )	-5.39	-2.5
Synaptotagmin IV	-19.98	-19.63
Syndecan 3	-2.51	-2
Syntaxin binding protein 2	-1.94	-2.18
TAF15 RNA polymerase II, TATA box binding protein (TBP)-associated factor	-1.81	-1.82
Tight junction protein 2	-1.81	-1.66
Transmembrane and tetratricopeptide repeat containing 4	2.24	1.63
Transmembrane protein 74	-2.26	-1.65
Tweety homolog 2 ( <i>Drosophila</i> )	-1.8	-1.88
Ubiquitin fusion degradation 1 like	-2.02	-1.75
Xylosylprotein beta1,4-galactosyltransferase, polypeptide 7 (galactosyltransferase I)	-3.55	-2.72
Zinc finger CCCH type containing 13	-1.87	-1.64
Zinc finger protein 40	-2.15	-1.78

**Table 2**  
**List of a common set of genes that were regulated in the cKO (K) neonatal brains of the two mouse strains (C57 and SVEV)**

Shading indicates whether gene was up-regulated (red) or down-regulated (green) in cKO brain compared to that of wild-type (W). Note instances where sign of regulation was different. Numbers represent fold changes. In all cases, differences are statistically significant ( $p < 0.05$ ).

Name (Stanford, dec10-2007 from refSeq (black), Riken (brown))	K/W-C57	K/W-SVEV
Hexamethylene bis-acetamide inducible 1	-2.86	-1.50
Cyclin N-terminal domain containing 1	-2.55	-3.22
Deoxynucleotidyltransferase, terminal	-2.36	-3.45
Bone morphogenetic protein 10	-2.31	-3.76
Gap junction membrane channel protein alpha 1	-2.24	-1.84
Sialophorin	-2.08	-3.49
RAS-like, family 10, member A	-1.99	-1.86
Ankyrin repeat and SOCS box-containing protein 2	-1.70	-2.02
Protein kinase N3	-1.59	2.24
Ubiquitin domain containing 2	-1.58	-1.59
Tripartite motif-containing 54	-1.50	-2.21
Cathelicidin antimicrobial peptide	1.52	-3.09
Vacuolar protein sorting 33B (yeast)	1.53	-1.62
Zinc finger protein 273	1.53	-1.55
Peptidylprolyl isomerase A	1.63	-1.74
Cytochrome P450, family 4, subfamily b, polypeptide 1	1.63	-1.59
Mediator complex subunit 13	2.09	-1.94
Olfactory receptor 362	2.40	2.70