ARCHIVAL REPORT

Dysfunction of Astrocyte Connexins 30 and 43 in Dorsal Lateral Prefrontal Cortex of Suicide Completers

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Background: Suicide is an important public health problem that results from the interaction of both psychosocial and biological factors. Although it is known that particular neurobiological processes underlie suicidal ideation and behavior, there still remains limited knowledge about the specific factors involved.

Methods: To explore the neurobiology of suicide we generated microarray data from dorsal lateral prefrontal cortex (DLPFC) in each of 28 male French-Canadian subjects (20 suicide completers). These results were followed up in a larger French-Canadian sample ($n = 47$, 38 suicide completers) and in microarray data available from the Stanley Foundation ($n = 100$, 36 suicide completers). To investigate the molecular mechanisms of this finding, we performed RNA interference and electrophoretic mobility shift assays. Animal behavioral experiments were done to control for drug and alcohol effects.

Results: We found reduced expression of Cx30 and Cx43 in DLPFC of suicide completers. We identified a previously unknown function for Sox9 as a transcription factor affecting expression of Cx30 in brain.

Conclusions: These results suggest that alterations of astrocyte connexins might be involved in the suicide process and provide further evidence implicating astrocytes in psychopathology.

Suicide is the leading cause of death in young adults, affecting people regardless of race, gender, or socioeconomic status. In a 2000 report the World Health Organization estimated that over 1 million deaths occurred by suicide in that year, a statistic overshadowed by the rate of suicide attempts, estimated to occur at a rate 25× that of suicide completion (1,2). Studies aiming to understand the biology of suicide have proceeded since the mid-1960s, when it became clear that suicide was not just a reactive response to a life event (3) but also associated with underlying biological processes (4). To date, different brain systems and their corresponding genes have been associated with suicide (reviewed in Ernst et al. [5]), yet independent and consistent replication of most findings remains elusive.

Traditionally, astrocytes have been considered as the support cells of the central nervous system, although more recently studies indicate that astrocytes play a number of different functional roles. For example, astrocytes express functional neurotransmitter receptors, respond to synaptic activity, and form part of the tri-partite synapse where they can modify communication between neurons (6). A growing number of studies have suggested that astrocytes might also be implicated in psychiatric disorders. In schizophrenia, astrocyte numbers are reported to be reduced (7,8) independently of the possible effects of antipsychotic medication, whereas other studies suggest glial cell dysfunction without a reduction in actual cell counts (9–11). In major depressive disorder, studies have suggested astrocytic cell count alterations in a number of different brain regions (12–15). Astrocytic dysfunction might also be directly involved in major depression (MD). For example, glia-derived factors stimulate synaptogenesis (16–18), where altered synaptic connections could underlie the pathology of depression. In addition, recent important evidence indicates that riluzole, a drug thought to upregulate the (SLC1A3/A2) glial glutamate receptors (18), is effective in the treatment of MD (19).

In the current study, we used samples from Quebec and an independent replication sample to study the neurobiology of suicide. As a starting point, we took advantage of microarray data from dorsal lateral prefrontal cortex (DLPFC) from suicide completers and control subjects to assess brain gene expression changes. Through a series of complementary studies, here we present evidence of reduced expression of astrocyte connexins Cx30 and Cx43 in DLPFC of suicide completers. We further identified a transcription factor of previously unknown function in astrocytes, Sox9, as a potential regulator of Cx30.

Methods and Materials

French-Canadian Subjects

Brain tissue for this study was obtained from the Quebec Suicide Brain Bank (QSBB) (http://www.douglasrecherche.qc.ca/suicide). A case was considered a suicide when classified as such by the office of the coroner. Control subjects were individuals who died suddenly and, identically to suicides, could not have undergone any resuscitation procedures or other type of medical intervention. All subjects were male persons of French-Canadian origin (identified by determining whether both sets of grandparents were born in Quebec and spoke French) (Table 1).

Brains that are part of the QSBB are collected in partnership with the Quebec Coroner's Office and undergo a psychological autopsy to retrieve phenotypic information. Briefly, brains are collected after consent is obtained from next-of-kin, and samples from brain tissue, peripheral blood, and urine are collected for toxicologic analysis. Immediately after a death, families are contacted, and the person

Table 1. Descriptive Variables of French-Canadian Subjects

	Microarray Sample		Validation Sample	
	Suicide $n = 20$	Non-Suicide $n = 8$	Suicide $n = 38$	Non-Suicide $n = 9$
Age, yrs	36.8 ± 14.1	42.1 ± 13.7	45.4 ± 14.3	49.1 ± 22.3
Gender (M/F)	20/0	8/0	38/0	9/0
PMI (h)	27.3 ± 7.2	23.1 ± 4.9	54.7 ± 19.4	58.6 ± 24.2
Brain pH	6.57 ± .24	6.51 ± .19	6.70 ± .26	6.53 ± .27
Toxicology[a]	1/3/4/0	0/0/0	6/3/3/2	0/0/0/0
Death	13/1/3/3/0/0[b]	8[c]	27/8/0/0/1/2[b]	9[c]
DSM-IV[d]	3/1/12/2	0/0/0/0	8/3/8/1	0/0/0/0

The continuous variables represent the mean ± SD; no significant differences were found between any variables.
DSM-IV, Diagnostic and Statistical Manual of Mental Disorders, 4th edition; F, female; M, male; PMI, postmortem interval.
[a]Antidepressant present/cocaine present/ethanol present /diazepam present.
[b]Alcohol dependence/cocaine dependence/major depression/bipolar I.
[c]Hanging/intoxication/gunshot/asphyxiation/drowning/jumping.
[d]Accidental death.

best acquainted with the deceased is recruited to undergo a series of structured interviews. The interviews are supplemented with information from archival material obtained from hospitals, the Coroner's Office, and social services. After the interviews, clinical vignettes are produced and assessed by a panel of clinicians to generate DSM-IV diagnostic criteria. All procedures in this study were approved by the ethics review board of our institution.

Stanley Foundation Subjects

The Stanley external replication sample consisted of 36 suicides and 64 non-suicides (Table 2). There were 44 subjects with bipolar disorder, 45 subjects with schizophrenia, and 11 subjects with MD. There were 22 suicides in the bipolar disorder sample, 10 suicides in the schizophrenia sample, and 4 in the MD sample. Brain tissue was dissected from DLPFC, as described here (http://www.stanleyresearch.org/brain/).

Neuroanatomy

Brains were analyzed, processed, and dissected into different regions on the basis of histological maps (20,21) and gyri/sulci landmarks at the QSBB. We define the DLPFC as encompassing Brodmann areas 9, 10, 11, 44a, 45, 46, and 47, and only gray matter was used from the left hemisphere. Three different cortical regions

Table 2. Descriptive Variables of the External Validation Sample of Subjects From the Stanley Foundation

	Suicide $n = 36$	Non-Suicide $n = 64$	p
Age, yrs	40.4 ± 10.3	45.6 ± 9.9	.01
Gender (M/F)	19/17	43/21	—
PMI	35.3 ± 18.4	32.5 ± 15.5	ns
Brain pH	6.4 ± .3	6.4 ± .3	ns
Alcohol[a]	6/9/5/3/4/9	15/15/6/7/7/13	—
Drug abuse[b]	12/5/5/4/5/5	33/4/4/5/6/10	—
Antipsychotic[c]	25/11	51/13	—

The continuous variables represent the mean ± SD.
F, female; M, male; ns, not significant; PMI, postmortem interval.
[a]Alcohol; little or none/social/moderate drinking in past/moderate drinking in present/heavy drinking in past/heavy drinking in present.
[b]Drug abuse; little or none/social/moderate drug use in past/moderate drug use in present/heavy drug use in past/heavy drug use in present/unknown.
[c]Antipsychotic treatment; Yes/No.

(from the lateral occipital gyrus, postcentral gyrus, and middle temporal gyrus) were also sectioned and Nissl-stained to detect any signs of pathology. After dissection, brain sections were flash frozen in isopentene and stored at −80°C. Brain pH measurements were performed as described (22).

Microarray Analysis

Microarray analysis was performed with the Affymetrix Human Genome (HG)-U133 Plus 2.0 chip in DLPFC and processed with robust multi-array averages (RMA). No RNA extracted from human brain was used with an RNA integrity number value < 6 (23). In Stanley foundation samples, RMA-normalized microarray data from four independent studies performed in DLPFC were downloaded from the Stanley Medical Research Institute database (http://www.stanleyresearch.org/brain/). Microarray data from the same platform, Affymetrix Human Genome U133 Set A (HG-U133A), were used to avoid platform-to-platform variation. Two to three microarray chip datasets were generated from each Stanley patient sample ($n = 100$ total subjects). Duplicate microarray datasets were treated as technical replicates. Expression data were analyzed with Genesis 2.0 (GeneLogic, Gaithersburg, Maryland) and AVADIS (Strand Genomics, Redwood City, California). Several RNA integrity measures were used in the screen to detect samples with poor RNA quality before final analysis. Microarray quality control parameters included: noise (RawQ), consistent scale factors, and consistent β-actin and glyceraldehyde-3-phosphate dehydrogenase 5′/3′ signal ratios (> .3 and > .5 for all probes, respectively). Microarray data were filtered by fold change (> 2) and p value (< .01). False Discovery Rate analysis was performed with BRB-array tools (http://linus.nci.nih.gov/BRB-ArrayTools.html) on the default setting ($p < .1$).

Animal behavioral experiments, western blot details, and RNA analyses methods can be found in Supplement 1.

Sox9 Knockdown Experiments

Short hairpin RNA (shRNA) plasmids directed at rat Sox9 were assayed in a rat astrocyte cell line (CCL-107; ATCC) maintained in F-12K medium containing 2.5% fetal bovine serum, 15% horse serum, and 1% penicillin-streptomycin. We used Sure_silencing shRNA (Invitrogen, Carlsbad, California) directly from the manufacturer to transiently knockdown Sox9. The clone with the most efficient knockdown capabilities of Sox9 had the cloneID: GAGCGA-CAACTTTACCAGTTT. All transfections were performed with Lipo-

fectamine 2000 over a 24-hour period, unless otherwise noted. In all experiments, a plasmid that generated a nonspecific shRNA molecule (negative control) was used, and all investigations were done in triplicate. The RNA was extracted from cells 24 hours after transfection and measured relative to levels of the genes of interest in knockdown and negative control transfected wells. We used β-actin as an internal control for semiquantitative polymerase chain reaction (PCR) analysis.

Electrophoretic Mobility Shift Assays (EMSA)

We followed guidelines from Pierce (Thermo Scientific, Wilmington, Massachusetts) to perform nonradioactive EMSAs. For probe design, we scanned target DNA downloaded from the UCSC human genome database (http://www.genome.ucsc.edu/) for potential Sox9 binding sites. Upon identification of a potential Sox9 site, we generated a 30-base pair (bp) oligo with the target site flanked by 10 bps and a 5′-biotin tag. Control probes were identical to wild type probes, except for a randomly scrambled consensus site. Nuclear extracts were prepared from HEK-293 cells (transfected with Sox9 plasmid), with NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Pierce). Binding reactions were incubated for 30 min at room temperature and then loaded on a 6% nondenaturing polyacrylamide DNA retardation gel (Invitrogen) and run at 100 V for 2 hours. After the gel run, we transferred the gel products to a nitrocellulose membrane at 365 mA for 30 min. After transfer to nitrocellulose membrane, the nitrocellulose membrane was exposed to 15 min of UV light to crosslink the DNA to the membrane. Membranes were then blocked and assessed by avidin-biotin immune-reaction following standard western blot protocols.

Statistical Analysis

All statistics were performed with program R (version 2.12.1). For microarray data, we performed t tests from $\log_2$ transformed data to generate p values. To control for confounding variables (pH, age, and postmortem interval [PMI]), we performed analyses of covariance, following standard procedures. Pearson correlations were performed between two variables of interest and r^2 values are quoted throughout. All error bars throughout the manuscript represent standard error of the mean. Quantitation of band intensity was performed with Alphaimager (Cell Biosciences, Santa Clara, California), by integration of the area under the curve (with factory settings).

Results

A flow chart outlining the experimental procedures of this study can be found in Figure S1 in Supplement 1.

The RMA-processed microarray data from DLPFC from all subjects ($n = 28$ subjects; 20 suicide completers) (Table 1) from the QSBB were stringently filtered according to fold change and p value. Probe sets ($n = 54,676$) from Affymetrix HG_133 plus 2 arrays were available for analysis. Of these probe sets, 1206 were differentially expressed in the brains of suicide completers ($p < .01$); thus we observed > twice as many probe sets as would be expected by chance. Of 54,676 probe sets, 89 showed a negative fold-change > 2 between suicides and control subjects and had p values < .01 (Table S1 in Supplement 1). An independently performed False Discovery Rate analysis confirmed this finding.

Gene ontology analysis (http://www.bioinformatics.ubc.ca/ermineJ/) suggested an overrepresentation of genes expressed in glia. Follow-up analysis to this result with PubMed searches revealed a number of probe sets were expressed specifically in astrocytes without ubiquitous expression in multiple cell types, including CXCR4, AQP4, NTRK2_T1, SLC1A3, GJB6, GJA1, SOX9, and EDNRB.

We opted to follow-up the gap-junction (GJ) genes, because they have been recently related to behavior in mouse knockout (KO) studies and are known to bind to each other in the extracellular space. That two binding partners emerged from an unsupervised microarray screen is not likely to be random. The GJB6 (a.k.a. Cx30) KO mice demonstrate abnormal behavior and altered monoaminergic processing, whereas GJA1 (a.k.a. Cx43) astrocyte KO mice demonstrate increased exploratory behavior and altered acetylcholine levels (24,25). In brain, Cx30 and Cx43 are expressed almost exclusively in astrocytes, although Cx43 is expressed to a minor extent in the choroid plexus (26). Accordingly, we chose to pursue the hypothesis that Cx30 and Cx43 are downregulated in the DLPFC of suicide completers.

To determine whether this selection strategy was justified, we performed an initial pilot experiment to see whether Cx30 and Cx43 were reduced in suicide cases compared with control subjects. To perform this pilot experiment, we selected the four suicide cases with the lowest expression of Cx30 and Cx43 (identical subjects) on the basis of microarray data and matched to two control subjects each on the basis of pH, PMI, and age. We performed semiquantitative PCR to see whether there was as profound a difference as suggested by the microarray data, followed by western blots to determine whether protein levels of both Cx30 and Cx43 were also affected (semiquantitative PCR gel images can be found in the upper right panel of Figures 1A and 1B; Western blot experiments: Figures 1C, 1D, 1E, and 1F). We found significant reductions in Cx30 and Cx43 in DLPFC of suicide completers; thus we next validated the full sample of microarray subjects by quantitative PCR (bar graphs in Figures 1A and 1B). With commercially available TaqMan assays for Cx30 and Cx43 we found that the expression was reduced in brains of suicide completers (Cx30: $t = 2.34$; $p = .028$; Cx43: $t = 2.39$; $p = .025$).

We next screened a new sample of brains from the QSBB for Cx30 and Cx43 expression (subjects not included in the initial microarray experiment). Nine sudden death control subjects and 38 suicide completers comprised this validation sample (Table 1). Expression of these genes in DLPFC was decreased in suicide cases compared with control subjects (Cx43: $t = 2.82$, $p = .007$; Cx30: $t = 2.13$, $p = .038$) (Figure 2).

Confounders

Suicide is sometimes associated with comorbid substance use disorders, particularly cocaine and alcohol dependence. Although there was no evidence from the clinical data that suggested that this was playing a role (Table 1), we treated rats with either cocaine or alcohol in different regimens to more definitively rule out the possibility that low Cx30/Cx43 gene expression resulted from differential substance use. To perform these experiments, we treated rats in acute and chronic conditions with cocaine or ethanol. After a period, we killed the animal, removed the frontal cortex and extracted RNA to assess the expression level of connexins, compared with control animals. We found no direct effect of cocaine or ethanol on Cx30 or Cx43 expression in frontal cortex (Figure S2 in Supplement 1).

We detected different psychotropic medications in brain tissue from suicide completers used in this study (Table 1). Studies in rodents suggest that chronic fluoxetine increases the expression of connexin 43 (13), and our data show a downregulation of this gene. Therefore, we compared suicide completers with negative toxicology ($n = 32$) with those with positive toxicology of antidepressants ($n = 6$; selective serotonin reuptake inhibitor: three subjects; serotonin norepinephrine reuptake inhibitor: two subjects; tricyclic antidepressant: one subject) on levels of Cx30 and Cx43 expression.
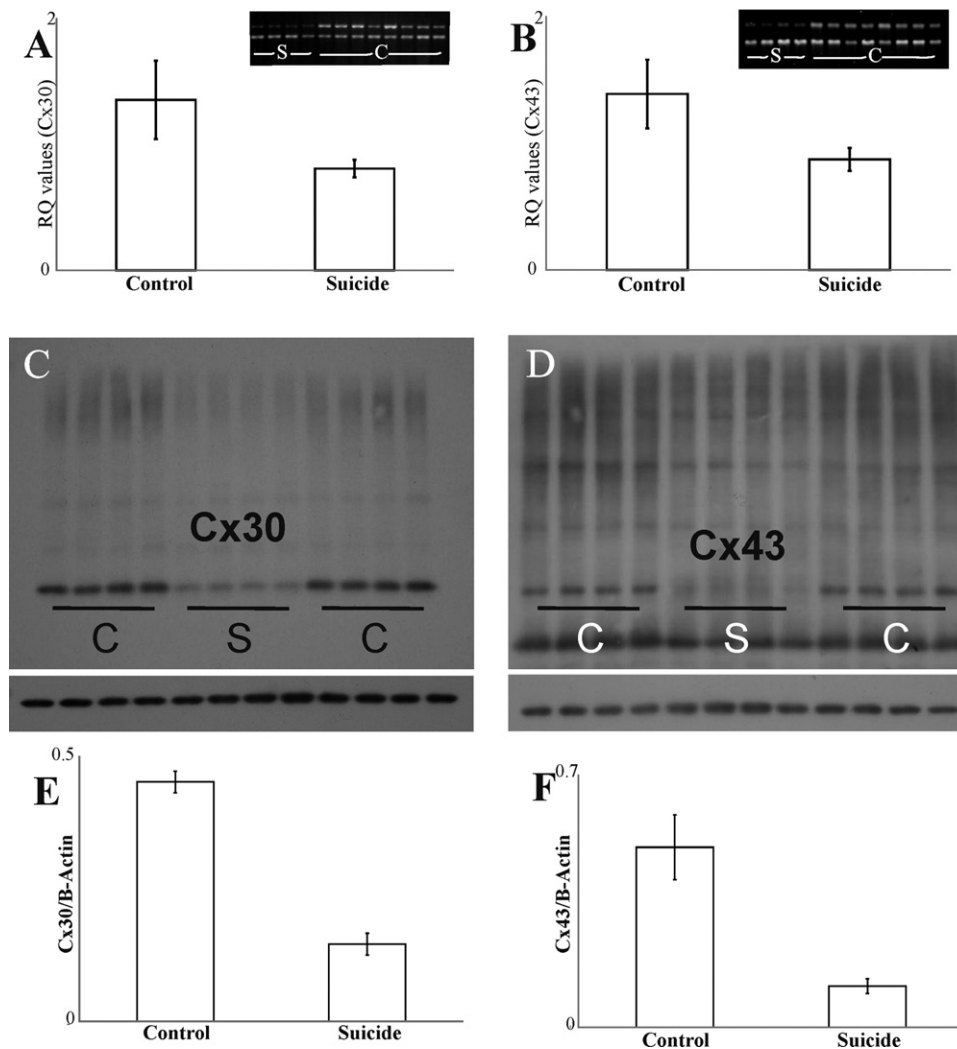
Figure 1. Cx30 and Cx43 show reduced expression in dorsal lateral prefrontal cortex from suicide completers compared with control subjects ($n = 28$). Quantitative polymerase chain reaction (PCR) data from Cx30 **(A)** and Cx43 **(B)** from 20 suicide completers and 8 control subjects, with two housekeeping genes and four replicates used for each. Images in upper right corners illustrate semiquantitative PCR data from 4 suicide completers and 8 matched control subjects (pilot sample [$n = 12$]; 2 control subjects/suicide case). Western blot images from Cx30 **(C)** and Cx43 **(D)** demonstrating profound reduction in protein levels of Cx30 and Cx43 in the pilot sample of suicide cases and control subjects. Quantitation of band intensity of western blot images for Cx30 **(E)** and Cx43 **(F)** with β-actin used as a loading control. C, control; RQ, relative quantification; S, suicide case.

However, no significant differences were observed (Cx30: $t = 1.51$, $p = .15$; Cx43: $t = .56$, $p = .58$). Furthermore, we show boxplots of these data to demonstrate visually that these subjects do not drive the observed effect of reduced Cx30 and Cx43 expression in suicide completers (Figure S3 in Supplement 1). To understand how DSM diagnosis affected Cx30 and Cx43 expression, we performed a similar analysis with depression status as a factor. We found no significant difference in Cx30/Cx43 expression level according to psychopathology (Cx30: $t = .64$, $p = .53$; Cx43: $t = 1.01$, $p = .31$).

Brain pH, postmortem interval, and subject age can also affect results. We found no significant difference between cases and control subjects in this regard (Table 1); nor was the Pearson correlation significant for any of these variables with expression of either Cx30 or Cx43. Finally, to determine whether age, pH, or PMI was driving Connexin 30/Connexin 43 expression, we performed an analysis of covariance (Table 3). Differences in expression between cases and control subjects with respect to Cx30 and Cx43 remained significant after factoring out these confounders.

Reduced Cx43 in Suicide Cases from the Stanley Foundation

We sought external validation of our findings in microarray datasets with publicly available data from the Stanley Foundation (Table 2). Because the Stanley Foundation microarray data were processed on Affymetrix HG-U133A chips, we note the compatibility of our own Affymetrix microarray data with that of the Stanley foundation. In the Stanley datasets, 243 total genes were differentially expressed. Of note, Cx43 was among the most significant differentially expressed genes between suicide and non-suicide subjects in the Stanley Foundation dataset. We note that Cx30 is present only on the Affymetrix HG-U133B chip; thus it was not expected to be identified in the Stanley dataset.

Potential Mechanism for Reduced Cx30 and Cx43 in Suicide Brain

To elucidate a mechanism of reduced connexin expression in suicide brain, we reexamined the filtered microarray list (Table S1 in Supplement 1) and asked whether any potential regulators of con-
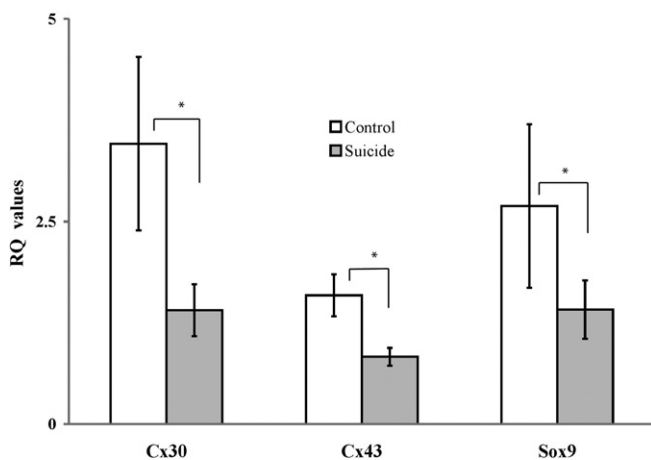
Figure 2. Cx30, Cx43, and Sox9 show reduced expression in dorsal lateral prefrontal cortex from suicide completers, compared with control subjects in a different sample of subjects ($n = 47$). The RQ values represent average of quadruplicates of each gene after subtraction of housekeeping genes (β-actin/glyceraldehyde-3-phosphate dehydrogenase). Error bars represent SEM. RQ, relative quantification.

nexin genes were present. We identified the transcription factor Sox9 from this list as a potential modifier of connexin gene expression in astrocytes (27). Notably, the structurally similar transcription factor Sox10 is known to regulate similar connexin genes, Cx47 and Cx32, in oligodendrocytes (28,29). Real-time-PCR of Sox9 in the sample of 38 suicide brains and 9 control subjects further suggested that Sox9 is reduced in suicide brain ($t = 2.88$; $p = .006$) (Figure 2). Furthermore, the probe set interrogating Sox9 was also identified as significantly different between suicides and non-suicides in the Stanley Foundation microarray data. We note that Sox9 expression strongly correlated ($r^2 = .82$) with Cx30 expression (Table S2 in Supplement 1 lists all genes that correlate strongly with Cx30 expression). We hypothesized that Sox9 bound DNA elements near the connexin genes and that a reduction of Sox9 in suicide brain led to the reduction of Cx30 and Cx43.

Knockdown of Sox9 in Rat Astrocytes Alters the Expression of Cx30

We used a rat astrocyte cell line to investigate whether transiently reducing Sox9 expression could affect the expression of Cx30 and Cx43. The RNA was extracted from cells 24 hours after shRNA transfection and measured relative to levels of the connexins in knockdown and negative control transfected wells. First, we tested which of four shRNA gave the best knockdown of Sox9 and which concentration of shRNA was optimal. Our transfection protocol was able to knock down Sox9 by > 50% in astrocytes (Figure 3A). We established a concentration of 120 pmol shRNA as optimal with very little cell death and maximal knockdown (Figure S4 in Supplement 1). Extraction of RNA 24 hours after shRNA transfection resulted in minimal decrease in Cx43 (Figure S5 in Supplement 1) but a drastic decrease in Cx30 (Figure 3B, and Figure S5 in Supplement 1). We further explored this decrease in Cx30 after Sox9 knockdown. We transfected anti-Sox9 shRNA into primary rat astrocytes in culture and proceeded to extract RNA at 4, 8, and 24 hours (Figure 3C). We found a systematic reduction in Cx30 over time even within 4 hours. Finally, we investigated whether the effects of transfection of shRNA Sox9 on Cx30 RNA levels are translated at the protein level. We observed a large reduction in Cx30 protein levels when shRNA Sox9 was transfected into the rat astrocyte cell line (Figure 3D), as would be expected from the RNA experiments.

Sox family members have similar DNA sequences, particularly in those regions coding for important domains. To ensure the specificity of our anti-Sox9 shRNA, we assessed the levels of Sox genes expressed in neurons and astrocytes (Figure S6 in Supplement 1). We found no differences in the expression level of any other Sox genes when the anti-Sox9 shRNA was compared with a negative control plasmid.

Sox9 Protein Binds Cx30 In Vitro

The DNA consensus site for Sox9 binding is 10 bases, and Sox9 can bind in either monomer or dimer (30). The Sox consensus site is C(A/T) TTG(A/T) (A/T), with flanking bases conferring specificity to unique Sox genes. The Sox9 preferred binding site is AGAACAATGG. We scanned Cx30 for potential Sox9 binding sites and found one possible binding site (Figure 4A). Encouragingly, the potential Sox9 binding site was an exact 10-bp match, the genomic location (in an intron immediately preceding the final exon) is in a similar structural region as the known Sox9 binding site in *Col2a1* (31), and the core binding motif seems to be conserved across species (Figure S7 in Supplement 1). We performed EMSAs with short (30 bps) biotin-tagged oligonucleotides representing the binding site for Sox9 (Table S3 in Supplement 1) and incubated these with Sox9 protein. We used a consensus-site scrambled probe as a nonspecific control (referred to as MUT probe in Figure 4), where the flanking bases were identical to the wild type probes. We observed the presence of a band in the lane where Sox9 protein was incubated with short oligos with the consensus site but not in scrambled control subjects. Reducing proteins in the extract with B-mercaptoethanol resulted in the abolition of this band.

Discussion

We found that astrocyte connexins 30 and 43 were downregulated in brains from suicide completers and that Sox9 can affect the expression of Cx30. These findings suggest that reduced expression of Cx30 and Cx43 in the cortex could be a mediating factor in suicide and further implicate astrocytes in the suicide process (15).

Our data suggest that astrocyte connexins are downregulated in the suicide brain, a finding consistent with a recent gene expression study performed in locus coeruleus of depressed subjects who died mostly from suicide (32). Connexins are a multigene family of cell membrane proteins that oligomerize in either a homomeric or heteromeric fashion to form a connexin—six connexins aligned radially around a central pore. In the extracellular space, connexins align between two cells forming a channel and allow molecules under 1 kDa to pass (33). How might astrocyte connexin expression affect human mood and behavior? We postulate that decreased expression of Cx30 and Cx43 might affect calcium wave propagation in astrocytes, a process mediated by Cx43 and Cx30 (34). Calcium transients in astrocytes are a form of long-range glial communication in the brain and have been associated with both stroke (35) and migraine headache (36). Further support for a role of connexins

Table 3. Analysis of Covariance Results in the Microarray Sample and the Validation Sample

	Gene	pH	PMI	Age
Microarray	Cx30	.028	.025	.033
	Cx43	.016	.009	.014
Validation	Cx30	.039	.04	.038
	Cx43	.008	.004	.008

Significance levels after factoring out each of pH, postmortem interval (PMI), and age on Cx30 and Cx43 expression.
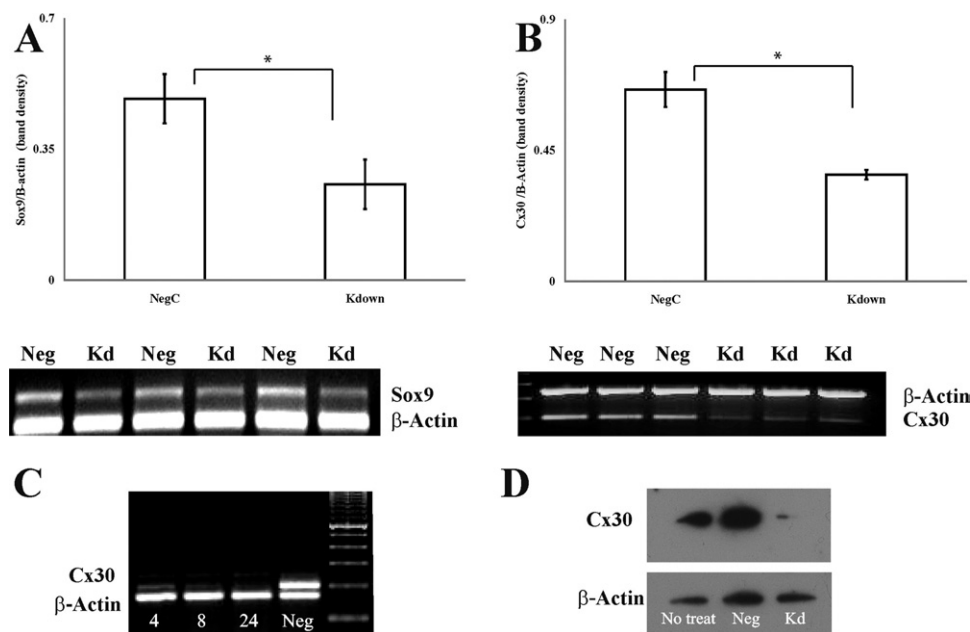
Figure 3. Knockdown (Kdown) of Sox9 reduces the expression of Cx30. **(A)** Effective knockdown of Sox9 in a rat astrocyte cell line. Bar graph is generated from triplicate image underneath graph. **(B)** Semiquantitative polymerase chain reaction (PCR) with primers directed at Cx30 after transfection of rat astrocyte cells with shRNA targeting Sox9. Graph generated from image shown below. **(C)** Time-course experiment where rat astrocyte cells transfected with shRNA targeted against Sox9 are harvested 4, 8, and 24 hours after transfection. Note how Cx30 band is gradually reduced over time. **(D)** Reducing Sox9 in astrocytes reduces Cx30 protein. Neg, negative control; kd, short hairpin RNA [shRNA] targeted to Sox9 transcript.

in mood more generally is that the anti-malarial drug mefloquine (know to induce temporary psychosis in some people) is a potent blocker of Cx36 and Cx50 (37), two neuronally expressed connexins.

Although our results cannot establish causality, there is evidence implicating connexins in altered mood and antidepressant

Figure 4. Evidence for Sox9 binding to Cx30 in a cell-free system. **(A)** Depiction of Cx30 gene with black boxes representing exons and a white box to represent the 5′ untranslated region. The probe used in the electrophoretic mobility shift assay experiment is shown with the Sox9 consensus site underlined, with matched lines indicating the genomic position. **(B)** Chemiluminescent assay detecting biotin-labeled probes. Arrow notes presence of Sox9/probe complex. WT, wild-type probe; β-ME, β-mercaptoethanol; MUT, probe with scrambled Sox9 binding site.

response. For example, reduced expression or deletion of Cx30 and Cx43 in mouse astrocytes has a behavioral phenotype. Astrocyte-specific, single KO experiments demonstrate altered reactivity to novel environments and important changes in brain chemistry, specifically those involving serotonin and acetylcholine, neurotransmitters that are thought to play a role in depression and suicide (24,25). Double KO animals show white matter tract deficits and swollen astrocytes, all of which likely lead to impairments in spatial memory and anxiety-related behaviors (38). More pertinent to the current study, Cx30 and Cx43 are known to upregulate in response to antidepressant treatments. In a comprehensive analysis of all major classes of psychotropic medications, Fatemi *et al.* (39) showed that prefrontal cortex astrocyte Cx43 is upregulated after chronic administration of fluoxetine and clozapine and downregulated by haloperidol and lithium. Finally, calcium wave velocity in astrocytes and Cx43 expression is increased in brain sections exposed to serotonin (40,41). Thus, connexin expression is known to be affected by mood-altering drugs.

Sox9 was significantly downregulated in DLPFC in both microarray samples and the follow-up sample. Given that Sox9 is expressed in astrocytes and it operates as a transcription factor, we hypothesized that Sox9 could be a regulator of Cx30 and Cx43 expression. The Sox family of genes is a family of transcription factors defined by an approximately 80-amino-acid high mobility group region whose function is to bend DNA. In vitro suppression of Sox9 in rat astrocyte cells had a profound effect on Cx30 expression, even within 4 hours of Sox9 knockdown, yet Cx43 expression was largely spared. The presence of a conserved Sox9 binding element in the final intron of Cx30 further supports this result. No perfect match was found in *Cx43*, possibly explaining why less reduction was observed in knockdown experiments with this gene product. We were surprised that a partial block of Sox9 expression of approximately 50% led to a reduction in Cx30 of essentially 100%. Although it is not necessarily expected that a given number of transcription
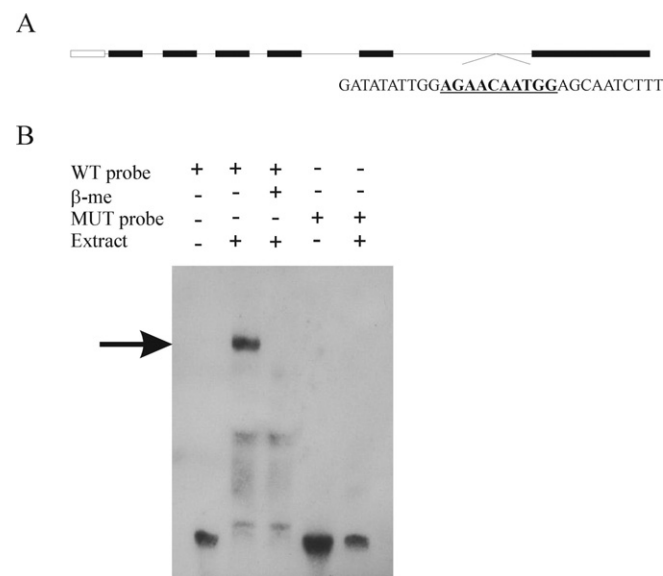
factors would lead to an equal number of transcribed molecules, it seems that some level of critical mass of Sox9 is required in astrocytes to initiate Cx30 transcription. This is similar to the concept of haploinsufficiency; for example, absence of one copy of the MBD5 gene leads to microcephaly and intellectual disability (42).

Any postmortem study in suicide encumbers the daunting challenge of confounding factors, including phenotyping with proxy-based interviews, postmortem delays (that is, disentangling premortem brain chemistry from postmortem effects), complex medication history and compliance, and unknown environmental triggers. By design, this study did not control for method of death, and one prevalent method of death is hanging, which could suggest that these results are a product of hypoxia. A literature review suggests that hypoxic conditions increase connexin expression, including in human brain (43,44). Inversely, Cx43 gap junction channels are thought to be blocked and downregulated in response to inflammation, as in multiple sclerosis or Alzheimer's disease (45). In our study, brains were analyzed for evidence of neuropathology, including MS and AD, as part of collection procedures by a trained neuropathologist. Because all subjects were free of inflammatory diseases and hypoxic conditions are thought to increase expression of Cx43, we suggest that the final common pathway of hypoxia does not account for the severe decrease in connexin observed in suicide brains. Another limitation of our study is that we focused our analyses on the prefrontal cortex. It is possible that our results are dependent on the region investigated. Future studies should investigate generalizability of our findings to other brain regions.

Supplementary material cited in this article is available online.

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