Repression of Astrocytic Connexins in Cortical and Subcortical Brain Regions and Prefrontal Enrichment of H3K9me3 in Depression and Suicide

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Abstract

Background: Major depressive disorder has been associated with dysfunctional astrocytic networks. The underlying causes, extent, and consequences of such dysfunctions remain to be characterized. Astrocyte-astrocyte communication occurs principally through gap junction channels primarily formed by connexin 30 and 43 (CX30 and CX43). We previously reported decreased connexin expression in the prefrontal cortex of depressed suicides. In the present study, we investigated whether these changes are mediated by epigenetic regulation, and expanded gene expression quantifications to other cortical and subcortical regions to assess the regional distribution of connexion disruptions in depressed suicides.

Methods: The expression of CX30 and CX43 was measured by real-time PCR in samples of neocortex (Brodmann areas 4 and 17), cerebellar cortex, mediodorsal thalamus, and caudate nucleus of 22 depressed suicides and 22 matched sudden-death controls. Chromatin immunoprecipitation was used to measure enrichment levels of the repressive chromatin mark H3K9me3 in the prefrontal cortex.

Results: We found a consistent downregulation of connexin genes in all regions examined, except in the cerebellum where an increase in the expression of CX30 was measured and using chromatin immunoprecipitation we observed an enrichment of H3K9me3 for both Cx30 and Cx43 in the prefrontal cortex.

Conclusions: Our study shows widespread astrocytic CX gene repression in depressed suicides that is mediated, at least in part, through epigenetic mechanisms. Taken together, these findings support the notion of widespread cerebral astrocytic dysfunction in major depressive disorder.

Keywords: chromatin modification, astrocytes, connexions, depression, suicide
Significance Statement

The research presented here shows a widely distributed dysregulation of the astrocytic connexin genes CX30 and CX43 across the brain of depressed suicides. Disruption to connexins may have important consequences for astrocytes given their numerous and significant roles in normal astrocytic function. We have also shown that the decrease in CX30 and CX43 expression is likely mediated by an epigenetic mechanism in the form of chromatin conformational changes, as we found significant increases in the repressive H3K9me3 histone mark. These findings are in line with the growing body of literature that implicates aberrant astrocytic function in the pathophysiology of major depression.

Introduction

Glial cells have been implicated in major depression for nearly 20 years (Ongur et al., 1998), with much of the data pointing toward astrocytes as the main glial contributor (Rajkowska and Stockmeier, 2013). Of all the cells in the brain, astrocytes exhibit the highest level of connexin (Cx) expression (Giaume and Naus, 2013). Cxs form the base unit of gap junction channels (GJs), and it is estimated that each cell will form up to 30 000 GJs (Rohlmann and Wolff, 1996). These specialized channels facilitate intercellular communication through direct transfer of molecules between adjacent cells (Rash et al., 2012), allowing for the regulation of metabolic and homeostatic functions (Pannasch and Rouach, 2013) and propagating Ca2+ waves (Verkhratsky and Kettenmann, 1996). GJs are comprised of 2 hemichannels (connexons) that can be composed of 6 identical connexons (homomeric) or of multiple connexin iso-types (heteromeric). Each of the 2 participating cells contribute one hemichannel, and each Cx combination (heter- or homo- meric or type) endows unique physiological properties to the channel (Kumar and Gilula, 1996). Though mRNA for Cx26, Cx30, Cx32, Cx40, and Cx43 have been detected by single-cell RT-PCR using astrocytes from brain slices (Blemstrand et al., 2004), the major Cxs have been identified as Cx30 and Cx43 (Giaume and McCarthy, 1996; Rouach and Giaume, 2001), which form either homotypic or heterotypic gap junctions (Nagy et al., 1999). The high expression levels of Cx30 and 43 throughout the brain (Nagy et al., 2004) and the fact that Cx30/Cx43 double-knockout mice lack GJs (Wallraff et al., 2006) both support these as the main 2 Cxs iso-types of astrocytes. Astrocytic connexins have previously been implicated in depression by our group (Ernst et al., 2011; Nagy et al., 2015) and others (Bernard et al., 2011; Miguel-Hidalgo et al., 2014). Similarly, studies using animal models of depression have shown decreased expression and function of Cx43, with recovery using antidepressants such as fluoxetine, duloxetine, or the tricyclic amitriptyline (Li et al., 2010; Sun et al., 2012b; Morikota et al., 2014; Mostafavi et al., 2014; Quesseveur et al., 2015). Given their abundance, role in communication, and association with antidepressant response, these proteins represent markers that may provide insight into the functional status of astrocytes in depression.

Astrocytes are known to be both sensitive and reactive to various microenvironmental cues, such as those arising from traumatic brain injury or autoimmune disorders (Sofroniew, 2009). Likewise, epigenetic modifications, such as histone modifications, constitute molecular mechanisms that respond to environmental changes. Histone modifications have previously been identified as a form of gene regulation in mood disorders, including major depression (for review, see Sun et al., 2013). Chromatin conformation can be altered so that DNA becomes more or less accessible to transcription factors or other DNA binding proteins. Modifications to the histone tail, often at a lysine, are known to create either an open/active state or a closed/repressive state. Histone 3 lysine 9 trimethylation (H3K9me3), for example, is almost exclusively associated with repressed chromatin states and is often found near the promoter region of a gene (Bannister and Kouzarides, 2011). H3K9me3 in particular has been associated with stress regulation (Hunter et al., 2009) and antidepressant response (Jiang et al., 2010), making it an interesting target in the context of depression.

We previously reported a robust downregulation of Cx30 and Cx43 in the prefrontal cortex (PFC) (BA8/9 and BA10) of people died by suicide (Nagy et al., 2015). In the present study, we set out to determine if disrupted Cx gene expression in depression and suicide is confined to these cortical areas, or if it is a more widespread phenomenon in the brain. As the motor, visual, and cerebellar cortex are not typically implicated in mood disorders, they were selected to test if altered connexin expression is a general phenomenon in the brain, or if it is localized to regions implicated in mood disorders. Additionally, to determine the subcortical involvement of astrocytic connexin dysregulation, the mediodorsal thalamus and caudate nucleus were selected given their known connectivity with neocortical regions involved in mood regulation. Furthermore, we sought to determine in PFC samples whether decreased astrocytic Cx expression is associated with an altered chromatin state. We found both Cx30 and Cx43 were downregulated across a number of brain regions and that PFC tissue showed an enrichment of the repressive histone mark H3K9me3 in cases compared with controls.

Materials and Methods

Subjects

This study was approved by the Douglas Hospital Research Ethics Board, and written informed consent from next-of-kin was obtained for each subject. Postmortem brain samples were provided by the suicide section of the Douglas-Bell Canada Brain Bank (www.douglasbrainbank.ca). Frozen gray matter samples were dissected from the left hemisphere of 22 male depressed suicides and 22 matched sudden-death controls for the mediodorsal thalamus (mdThal), caudate nucleus (CN), cerebellar cortex (Brodmann areas [BAs] 4 and 17). Suicides were selected from a recently characterized large cohort of subjects based on extreme low expression of astrocytic genes GFAP, ALDH1L1, SOX9, GLUL, SLC1A3, GJA1, and GJB6 in 2 prefrontal cortical areas (BA8/9, BA10), as previously described (Nagy et al., 2015).

Overall, a significant decrease in the expression of all these genes was found in suicides within a subgroup displaying extremely low expression defined by those found in the bottom quartile of at least 5/7 genes (Nagy et al., 2015). For each individual, the cause of death was ascertained by the Quebec Coroner’s office, and psychological autopsies were performed by proxy-based interviews, as described previously (Dumais et al., 2005). Cases met criteria for major depressive disorder, Depressive Disorder Not Otherwise Specified, or had evidence of depressive symptoms at the moment of death. Controls were individuals who died suddenly and did not have evidence of any axis I disorders (Table 1). Postmortem interval (PMI) represents the delay between a subject’s death and
collection and processing of the brain. There were no statistical differences found for age (t=0.40, P = .70), tissue pH (t = 1.64, P = .11), or PMI (U = 198.0, P = .31) between groups.

### Absolute Quantification of Gene Expression

Total RNA was extracted from 40 mg of frozen tissues using the RNeasy Lipid Tissue Mini Kit (Qiagen Inc., Mississauga, Canada) with DNase digestion following the manufacturer’s instructions. Total RNA content was quantified using Nanodrop spectrophotometer (NanoDrop technologies, Rockland, DE) and RNA integrity was determined with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The average RNA Integrity Number for all brain regions was 6.99±0.11 for all subjects.

cDNA was synthesized from 500 ng of total RNA using 400 U M-MLV reverse transcriptase (Gibco BRL Life Technologies, Burlington, ON, Canada) and oligo-deoxymethylidene-16 according to the instructions of M-MLV manufacturer. To reduce variability in cDNA synthesis, 2 independent plates of cDNA were simultaneously synthesized, and the end product was combined and used for Real-Time PCR. The GJB6 (CX30) Taqman probesets (TaqMan assay id: Hs00917676_m1) and GAPDH (TaqMan assay id: Hs00748445_s1) were labelled with FAM targets all transcripts for each gene. Endogenous controls β-actin (TaqMan assay id: Hs99999903_m1) and GAPDH assay id: Hs00772679_m1) and GAPDH (TaqMan assay id: Hs00748445_s1) labelled with VIC targets were designed to detect enrichment of chromatin modification. Enrichment was quantified by normalizing immunoprecipitated sample over an input sample from the same dounced, sheared chromatin not subjected to antibodies. It was further normalized to a negative control region of the gene GAPDH. The results were presented as a fold change of cases to controls.

### Statistical Analyses

Statistical analyses were performed using SPSS 18 (Statistical Product and Service Solutions, Chicago, IL) and Prism 6 (GraphPad Software, Inc., La Jolla, CA). All measurements were expressed as mean ± SEM, and P ≤ 0.05 was considered significant in all statistical tests. Normality was assessed using Shapiro-Wilk tests. Two-tailed Student’s t tests were used for nonnormally distributed data and Student’s t tests otherwise. ROUT test (Q = 1.0%) was applied for multiple comparisons. Statistical analyses were performed using SPSS 18 (Statistical Product and Service Solutions, Chicago, IL) and Prism 6 (GraphPad Software, Inc., La Jolla, CA). All measurements were expressed as mean ± SEM, and P ≤ 0.05 was considered significant in all statistical tests. Normality was assessed using Shapiro-Wilk tests. Two-tailed Student’s t tests were used for nonnormally distributed data and Student’s t tests otherwise. ROUT test (Q = 1.0%) was applied for multiple comparisons. All correlations were subjected to Bonferroni correction for multiple comparisons.

### Results

#### Regional Expression of Astrocyte-Specific Connexin Genes

All brain regions investigated in this study showed differences between cases and controls (Figure 1a-j) (P values ranging between .05 and .0001). The cerebellum was the only region to display an increase of either connexin, with CX30 being increased in cases compared with controls [F2,91 = 2.51, P=.0082] (Figure 1c). Given that Cx30 and Cx43 colocalize, as confirmed by immunolabelings observed with electron and fluorescence microscopy (Nagy and Rash, 2000), we expected their expression patterns to correlated with each other. Interestingly, in controls, correlations were...
found in all brain regions [BA4: \( r_{\text{gG}} = 0.70, P = 0.005 \) BA17: \( r_{\text{gG}} = 0.73, P = 0.0009 \)]; cerebellum: \( r_{\text{gG}} = -0.54, P = 0.05 \); mdThal: \( r_{\text{gG}} = 0.92, P = 0.0001 \) but the CN \( r_{\text{gG}} = 0.54, P = 0.10 \) (Figure 2a), whereas in cases, we observed a correlation in only the subcortical regions, mdThal \( r_{\text{gG}} = 0.75, P = 0.0007 \), and caudate \( r_{\text{gG}} = 0.74, P = 0.002 \) (Figure 2b). Evidence in animals shows that antidepressant treatment can reverse the effects of downregulated Cx43 (Sun et al., 2012a). Here, we found no significant correlation between antidepressant use and Cx43 expression, though all the \( r \) values suggest an inverse relationship (data not shown).

### Enrichment of Histone Methylation in the PFC

Since cases demonstrated a strong depletion in the expression of astrocytic connexins, we sought to investigate the underlying molecular mechanism. Epigenetic mechanisms explain, at least in part, altered gene expression and have previously been reported to play an important role in the depressed brain (Fiori et al., 2011; Fiori and Turecki, 2011; Turecki, 2014). We previously looked at DNA methylation and found no site-specific methylation changes associated with either Cx30 or Cx43 (Nagy et al., 2015). We therefore hypothesized that chromatin rearrangement could be responsible for this gene repression. As patterns of expression were similar across regions with the exception of the cerebellum, we opted to conduct chromatin immunoprecipitation studies in the PFC (BA8/9), a representative region with pronounced changes to the astrocytic connexin genes. ChIP was performed to enrich for H3K9me3 modifications representative of hetero- or inaccessible chromatin. We found that for both CX genes, the case group showed enrichment in H3K9me3 (Cx30 \( t_{\text{gH}} = 4.036, P = 0.0002 \)) and Cx43 \( t_{\text{gH}} = 9.3, P = 0.048 \) (Figure 3a-c).

To determine a possible mechanism for the strong repression of connexin genes, we examined the PFC, a region canonically implicated in depression (Koenigs and Grafman, 2009) that is strongly associated with the subcortical regions (Mega and Cummings, 1994) examined in this study. We found that the repressive histone mark H3K9me3 is enriched in cases compared to controls for both astrocytic CX genes and that this increase is inversely correlated with expression of Cx30. Cx43 histone methylation did not correlate with expression, but this could be due to a lack of power, as the enrichment was more subtle within this gene. Though there was an inverse relationship between enrichment and expression, it was not significant.

### Discussion

We recently reported that the astrocyte-specific marker glial-fibrillary acidic protein (GFAP) is downregulated in subcortical (Torres-Platas et al., 2016) and prefrontal cortical (Nagy et al., 2015), areas implicated in mood disorders, but not in the other neocortical regions examined (BA4, BA17) nor in cerebellar cortex. Here we sought to determine whether the astrocyte-specific CX30 and Cx43 would display a similar pattern of expression. In samples from depressed suicides relative to psychiatrically healthy controls, we found that the expression of both CX30 and Cx43 was strongly repressed in all regions examined, except for Cx30 in the cerebellum, where it was significantly increased in cases. It thus appears that astrocytic connexin dysregulation in the depressed brain is not limited to regions known to be implicated in mood regulation and is thus more globally affected than GFAP.

As expected from the colocalization of these 2 connexins using electron microscopy (Nagy and Raash, 2000), these genes correlate well with each other across most regions, showing proportional expression in the control group. However, this relationship was found to be highly altered when examining cases alone, where the only correlation observed was subcortical and in a different pattern than controls. This indicates that in the normally functioning neocortex, astrocyte connexins are proportionally expressed, and dysregulation in depressed suicides interferes with this relationship.

In the animal literature, there is evidence that decreases of Cx43 in animals subjected to chronic unpredictable stress can be reversed by antidepressant drug treatment (Sun et al., 2012b). Additionally, treating primary mouse astrocytes with the antidepressant amitriptyline increases levels of mRNA, protein, and gap junction intercellular communication (Morikawa et al., 2014). This was shown to affect only Cx43 but not Cx30 expression.
Figure 2. Correlations of connexin 30 and 43 across brain regions. P values for Spearman correlations between connexins for each region in (a) controls and (b) cases. P values defined by color scale, and all values are Bonferroni corrected for multiple testing.

(Morioka et al., 2014), indicating that restoring levels of this Cx may be critical to antidepressant effects. Though we found no significant relationship between antidepressant use and level of CX43 gene expression, it is possible that we do not have enough power to detect such a change, as the presence of antidepressants by toxicological reports was found in only 5 cases at the time of death.

Previous human postmortem brain studies have described a significant decrease in the expression of both CX43 and CX30 in brain samples from depressed individuals relative to controls (Bernard et al., 2011; Ernst et al., 2011; Nagy et al., 2015). In fact, Ernst and colleagues (2011) described a robust decrease of both astrocytic connexin genes in a widely encompassing prefrontal cortical region including BAs 9,10,11, 44a, 45,46, and 47, and further demonstrated a strong protein decrease in the PFC. Here, we found a strongly significant decrease in CX30 expression in BA4, BA17 mdThal, and a milder but still significant decrease in the CN. Somewhat surprisingly, we found an increase in CX30 expression in the cerebellum of cases compared to controls. Certainly, this still represents a decoupling of CX30 with CX43, as the latter displays a strong downregulation in this same brain region in the same samples. This is not the first report of inverse expression of astroglial connexins in the cerebellum. Mice infected with Borna Disease Virus show increased Cx43 together with decreased Cx30 expression which, in turn, resulted in changes to the functional coupling of these proteins (Koster-Patzlaff et al., 2007).

A number of studies have described a reduction of glial density and/or glial number (Ongür et al., 1998; Rakowska et al., 1999; Rajkowska, 2000; Cotter et al., 2001, 2002; Rajkowska and Stockmeier, 2013) and molecular markers of glia (Choudary et al., 2005; Chandley et al., 2013), commonly concluding that reduced GFAP expression or immunoreactivity reflects a loss of astrocytic cells in particular (Webster et al., 2005). However, GFAP is not always a reliable indicator of astrocyte numbers, as not all astrocytes express GFAP and not all GFAP expression is from astrocytes (Khakh and Sofroniew, 2015). Animal studies using models of depression report a reduction of GFAP-IR cells but no change to other markers (Gosselin et al., 2009) or cell counts (Leventopoulos et al., 2007; Tynan et al., 2013). The growing research in animal models of depression has shown a certain malleability of GFAP as many have shown a drug effect on GFAP-IR (Czech et al., 2006; Banasr and Duman, 2008; Banasr et al., 2010; Ye et al., 2011; Sun et al., 2012a). These data open the door to the possibility of molecular alterations or even cellular atrophy rather than a loss in astrocytic cell numbers per se. In line with the idea of malleable GFAP, connexins are known to interact with the cytoskeleton, for example astrocytes with altered Cx43 expression have been shown to differ in their morphology (Olk et al., 2009). In an in situ study using hippocampal brain slices from Cx43 KO mice, astrocytes were found to have increased somal size, though no difference in GFAP immunolabelling astrocytes was reported (Chever et al., 2014). Taken together, this suggests a link between connexins and cell shape through cytoskeletal reorganization.

In addition to their role in the formation of GJCs and cytoskeletal interactions, connexins have an important role in the maintenance of ion homeostasis (Waller et al., 2006), minimizing damage in the CNS (Lin et al., 2008), extracellular exchanges through hemichannels, as well as channel-independent functions involving cell adhesion /signalling (Theis et al., 2005; Elias et al., 2007; Olk et al., 2009), cell proliferation (Cheng et al., 2004), migration (Scemes et al., 2003), and differentiation (Duval et al., 2002). In the context of adult hippocampal neurogenesis, a double knockout model of Cx30/Cx43 seemed to suggest Cx- 30 and 43 affect neurogenic processes in an opposing manner, where Cx43 promotes survival of newborn neurons and Cx30 restricts their survival (Liebmann et al., 2013). This is interesting in the context of major depression, since there have been links made between adult hippocampal neurogenesis and the etiopathogenesis of depression (Lee et al., 2013; Mahar et al., 2014).

Astrocytic connexins have also been shown to altered oligodendrocytic functions (Lutz et al., 2009). Cx30 and Cx43 are known to form heterotypic GJCs with the somata and lamellae of oligodendrocytes (Kamasawa et al., 2005), double knockout of these Csxs resulted in widespread edema and vacuolation in white matter resulting in oligodendrocyte and myelin pathology, while astrocytes were found to have structural changes. In the gray matter, astrocytes were found to be swollen, though even a single allele of either Cx was sufficient to protect against glial edema, suggesting a partial redundancy in these connexins (Lutz et al., 2009). Hypertrophic astrocytes have previously been described in the white matter of brains from depressed suicides, but no such changes were found in gray matter.
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As white matter astrocytes are devoid of Cx30 (Nagy et al., 1999), the potential for connexin compensation is lost, suggesting a possible link between the globally strong decreases of CX43 found here and the morphological changes to white matter astrocytes previously described. This is, however, an extrapolation of our results as only gray matter expression was examined in the present work. More studies are therefore required.

One of the mechanisms shown to regulate Cx expression is inflammatory responses through microglial activation. It was previously demonstrated that microglial activation decreases Cx43 expression resulting in a loss of functional coupling between astrocytic cells (Faustmann et al., 2003; Hinkerohe et al., 2005; Meme et al., 2006). A recent study examining cerebral inflammatory markers in depressed suicides revealed increased microglial/macrophage activity in the anterior cingulate cortex of cases relative to controls (Torres-Platas et al., 2014). Interestingly, these changes were found in the same region as the morphological changes mentioned above (Torres-Platas et al., 2011). Macrophage metabolic status has been linked to the activity of chromatin-modifying enzymes (Baardman et al., 2015), and a number of studies have implicated epigenetic modifications as a regulatory mechanism in depression (for review, see Sun et al., 2013). Based on our current findings, whatever may be triggering a downregulation of gene expression seems to be mediated through altered chromatin state, an epigenetic regulatory factor.

Our study is not without limitations. In particular, epigenetic modifications are cell and tissue specific; therefore, the altered histone marks in the prefrontal cortex may not apply to all brain regions studied here. Additionally, given the strong evidence that antidepressants can alter the expression of connexins, it would have been interesting to have access to more samples from individuals using antidepressants at the time of death.

In conclusion, our study shows that CX genes specific to astrocytes are widely dysregulated in depression and suicide. The findings in this study implicate the involvement of an epigenetic molecular mechanism in the regulation of CX gene expression. Our data are correlational and do not discount the possibility of cell loss but are suggestive of something beyond cell loss as being involved in the astrocytic abnormalities seen in major depressive disorder.

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Statement of Interest
None.

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Figure 3. Enrichment of the repressive chromatin mark H3K9me3 is increased in cases compared to controls. (a) Enrichment of H3K9me3 in CX30 is increased in cases (t(40) = 4.036, P = .0002) and inversely correlate with gene expression (b) Pearson correlation (r = 0.48, P = .0018). (c) CX43 also shows an increase in H3K9me3 enrichment (U(35) = 93, P = .048) though the fold change was not as strong and (d) the correlation though inverse, was not significant (Spearman Rho = -0.17, P = .36).
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