Published in final edited form as:

Schizophr Res. 2015 December ; 169(0): 381–385. doi:10.1016/j.schres.2015.10.002.

Increased Extracellular Clusterin in the Prefrontal Cortex in Schizophrenia

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Abstract

The expression of the gene that encodes clusterin, a glycoprotein that has been implicated in the regulation of many cellular processes, has previously been found in gene expression profiling studies to be among the most significantly differentially expressed genes in pyramidal and parvalbumin-containing inhibitory neurons in the cerebral cortex in subjects with schizophrenia. In this study, we investigated whether clusterin may also be dysregulated at the protein level in schizophrenia subjects. We found that, although the intracellular amount of clusterin may be unchanged, the level of extracellular, secreted clusterin appears to be significantly increased in schizophrenia subjects. It is speculated that this finding may represent a neuroprotective response to pathophysiological events that underlie schizophrenia.

Clusterin, also known as apolipoprotein J, is a rather ubiquitous multifunctional glycoprotein that has been shown to be involved in a multitude of cellular processes, such as maturation, differentiation, remodeling, transportation, proliferation, survival and death, in multiple organ systems, including the brain (Jones and Jomary, 2002; Klock et al., 2009; Rosenberg and Silksen, 1995). In addition, clusterin dysregulation has been implicated in the pathogenesis of various human disorders, from tumorigenesis to neurodegenerative diseases, such as Alzheimer's disease, by acting to either promote or inhibit, amongst other events, oxidative stress, apoptosis, and synaptic plasticity (Charnay et al., 2012; Koltai, 2014; Park et al., 2014; Yu and Tan, 2012).

Clusterin exists in at least two sets of isoforms that exhibit distinct cellular and subcellular localization (Rizzi et al., 2009; Yu and Tan, 2012). The secreted forms (~75-80 kDa) of clusterin, which are partially to heavily glycosylated, exhibit pro-survival functions and,

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Conflicts of Interest and Financial Disclosure: None.

Authors' Contributions: KMA and SLM carried out the all of the experiments. KMA performed statistical analysis and helped write the manuscript. TUWW conceived of the study, participated in its design and data interpretation, and wrote the manuscript.

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additionally, have been shown to promote neuronal network complexity (Kang et al., 2005; Rizzi and Bettuzzi, 2010; White et al., 2001; Wicher et al., 2008; Yu and Tan, 2012). Conversely, the non-secreted, nuclear forms (~45-60 kDa) of unglycosylated clusterin appear to exert pro-apoptotic functions (Han et al., 2001; Kim and Choi, 2011; Kim et al., 2012; Rizzi and Bettuzzi, 2010).

Recent gene expression profiling studies suggest that clusterin is one of the most differentially regulated genes in schizophrenia. Specifically, we have found that clusterin gene expression appears to be upregulated by more than 2-fold in both pyramidal and parvalbumin-containing inhibitory neurons in the cerebral cortex in subjects with schizophrenia (Pietersen et al., 2014a; Pietersen et al., 2014b). In this study, we investigated whether clusterin protein expression might also be increased in this disorder. We found that although the density of cells that contained clusterin was not altered, the amount of the secreted, extracellular clusterin was in fact significantly increased in schizophrenia subjects. Given the pro-survival functions of the secreted clusterin, this increase may represent a homeostatic consequence of cellular injury mediated by, among other possible events, oxidative stress, which has recently been strongly implicated in the pathophysiology of schizophrenia (Bitanhirwe and Woo, 2011; Do et al., 2009; Do et al., 2015; Fournier et al., 2014; Gysin et al., 2007; Monin et al., 2014).

Materials and Methods

Postmortem human brain tissue

A total of 30 postmortem human brains were included in this study. Liquid nitrogen vapor fresh-frozen issue blocks containing Brodmann's area 9 of the prefrontal cortex from 15 schizophrenia and 15 normal control subjects, matched for age, sex and postmortem interval (PMI), were obtained from the Harvard Brain Tissue Resource Center (HBTRC) at McLean Hospital in Belmont, MA (Table 1). Postmortem human brain collection procedures at the HBTRC have been approved by the Partners Human Research Committee. Written informed consent for use of each of the brains for research has been obtained by the legal next-of-kin. The diagnosis of schizophrenia was made by two Board-certified psychiatrists by reviewing medical records and an extensive family questionnaire that included medical, psychiatric and social history. All of the brains included in this study were also examined by a Board-certified neuropathologist to rule out any neurological conditions. In addition, the fact that none of the subjects had any history of active substance abuse or dependence disorder was confirmed by toxicological analysis.

Immunohistochemistry

Tissue blocks were sectioned at 20 μ m, mounted on gelatin-subbed slides, and post-fixed in 4% paraformaldehyde for 20 minutes at room temperature. Sections were then incubated in endogenous enzyme block (1% H₂O₂, 10% MeOH) for 15 minutes and additionally blocked using 2% bovine serum albumin (BSA) with 10 % normal goat serum (Life Technologies, 16210-064, Grand Island, NY) at room temperature for 1 hour, followed by incubation in an anti-clusterin antibody produced in rabbit (1:100, SAB3500199, lot #38560504, Sigma-Aldrich, St. Louis, MO) at 4°C overnight. Sections were then incubated at room temperature

in biotinylated anti-rabbit IgG antibody produced in goat (1:500, BA-1000, Vector Labs, Burlingame, CA), followed by a 2-hour incubation in horseradish peroxidase-conjugated streptavidin (1:5000, Zymed, San Francisco, CA) made in 1 Mol/L of phosphate buffer (PB) at room temperature, and finally in nickel-enhanced diaminobenzidine/peroxidase reaction (0.02% diaminobenzidine, 0.08% nickel-sulphate, 0.006% hydrogen peroxide in 1 Mol/L PB). Sections were finally counterstained with cresyl violet, dehydrated and coverslipped.

Clusterin-immunoreactive elements comprised morphologically identifiable pyramidal, nonpyramidal, and glial cells. For each section, clusterin-immunoreactive cell within a 250 µm-wide cortical traverse containing all six cortical layers were quantified (Figure 1). Quantification was performed in a blind fashion by one investigator (KA) using StereoInvestigator (MBF Bioscience, Williston, VT) and BioQuant (Nashville, TN) software.

Western blot

Tissue blocks consisting of only the grey matter were sectioned at 100 µm. Protein was isolated with radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% Na deoxycholate, 0.1% SDS, 10 mM EDTA, 100× Halt Protease Inhibitor Cocktail), sonicated 4 times at 30 Hz for 3 seconds with 15 seconds resting on ice, centrifuged at 13.2 RPM for 10 minutes at 4°C, and frozen at -80°C. A Bradford assay was completed in order to determine protein concentrations of each sample. Tissue samples were then run using NuPAGE 12% Bis-Tris Gel 1.0mm × 10 well (NP0341BOX, lot #14072571, Carlsbad, CA) and transferred to immobilon-FL transfer membrane (IPFL00010, Lot #K4JA7438C, EMD Millipore, Billerica, MA), using powerease 500 supply (Invitrogen, Life technologies, Singapore).

Blots were blocked using with LI-COR blocking buffer (927-4000, LI-COR Biosciences, Lincoln, NE) and 0.01 Mol/L PBS with 0.05% Tween for 1 hour at room temperature (1:1) before co-incubating overnight at 4°C in an anti-clusterin antibody produced in rabbit (1:1000, SAB3500199, lot #38560504, Sigma-Aldrich, St. Louis, MO) and loading control anti- α -tubulin-containing protein antibody made in mouse (VCP; 1:2500, ab11433, ABCAM, Cambridge, MA). Membranes were washed in 0.01 Mol/L PBS with 0.05% Tween before co-incubating for 2 hours at room temperature in the secondary goat anti-rabbit antibody (IRDye 800CW, 827-08365, lot #C40902-01, LI-COR Biosciences, Lincoln, NE) and donkey anti-mouse antibody (IRDye 680RD, 926-68072, lot #404910-05, LI-COR Biosciences, Lincoln, NE). Individual bands from all subjects were measured using Odyssey 3.0 analytical software (LI-COR Biosciences, Lincoln, NE) and normalized to VCP as a loading control. VCP has previously been shown to be unaltered in schizophrenia subjects (Bauer et al., 2009, Mueller et al., 2014, Stan et al., 2006). Clusterin (secreted form) (SRP8004, Sigma- Aldrich, St. Louis, MO) was included as a standardized control on each blot to control for inter-blot variability. For quantification, the intensity of clusterin in each lane was normalized to the VCP in that lane (clusterin protein intensity/VCP intensity). Each sample was then normalized to the secreted clusterin protein (which was also initially normalized to VCP) in that blot in order to analyze across blots (clusterin sample intensity/ secreted clusterin intensity).

Statistical analysis

The densities of clusterin-immunoreactive cells for each cortical layer and the level of clusterin protein expression determined by immunoblot were compared between schizophrenia and normal control subjects using Student's t-test. We also used correlation analysis to evaluate any potential effect of each of the numerical covariates (i.e. age, PMI, antipsychotic exposure in terms of chlorpromazine equivalent) on the cell density measures. The possible effect of sex on each of the density measures was evaluated by comparing cell densities between the two sexes within each of the two subject groups.

Results

Densities of cells containing Intracellular clusterin are unaltered in schizophrenia

Clusterin appears to be widely distributed across cell types in the prefrontal cortex. Approximately 35%, 79% and 100% of pyramidal, non-pyramidal and glial cells were clusterin-immunoreactive. Because our recent gene expression profiling studies suggest that clusterin gene expression is upregulated in pyramidal and parvalbumin-containing inhibitory neurons in the cerebral cortex in schizophrenia, we investigated in this study whether this increase may also be reflected at the protein level. Contrary to our prediction, we found that the average densities of clusterin-immunoreactive pyramidal neurons, nonpyramidal neurons and glial cells in the prefrontal cortex were unaltered in schizophrenia (mean $\pm$ SE=18.3 $\pm$ 1.2, 180.4 $\pm$ 80.9, 140 $\pm$ 35.8 cells/mm², respectively), compared to normal control (mean $\pm$ SE=3.4 $\pm$ 1.1, 182.4 $\pm$ 135.0, 110.5 $\pm$ 24.7 cells/mm², respectively) subjects (Figure 2). In addition, the densities of cells for each of the layers also did not differ between the two subject groups (data not shown; see Figure 1). Nevertheless, we cannot rule out the possibility that the amount of clusterin expression per cell for any of these cell types may still be altered but the detection of this is beyond the sensitivity of immunohistochemical labeling. Finally, none of the potential confounds appear to have influenced our findings (Table 2).

Extracellular clusterin is increased in schizophrenia

Because clusterin is a secreted glycoprotein, we reasoned that even though the densities of cells that contained intracellular clusterin might not be altered, the amount of extracellular clusterin could still be dysregulated. To address this, we quantified the amount of clusterin in homogenized cortical tissue, which included both the intracellular and extracellular forms of the protein. Using this approach, we found that the level of the extracellular clusterin was in fact significantly ($p=0.014$) increased by $\sim 29\%$ in subjects with schizophrenia (mean $\pm$ SD=1.25 $\pm$ 0.08), compared to normal control subjects (mean $\pm$ SD=0.97 $\pm$ 0.07; Figure 3).

Discussion

Our recent gene expression profiling studies have shown that clusterin gene expression appears to be significantly upregulated in both pyramidal and parvalbumin-containing inhibitory neurons in the cerebral cortex in schizophrenia (Pietersen et al., 2014a; Pietersen et al., 2014b). Findings of the present study suggest that, at the protein level, although the

intracellular content of clusterin in various cell types may be unchanged, the extracellular, secreted clusterin appears to be significantly upregulated in schizophrenia subjects.

The pathophysiological basis of the increase in extracellular clusterin is unclear at the present time. However, because oxidative stress, which is associated with the upregulation of clusterin (Trogakos, 2013), has recently been strongly implicated in the pathophysiology of schizophrenia (Bitanirwe and Woo, 2011; Do et al., 2009; Fournier et al., 2014; Gysin et al., 2007; Monin et al., 2014), one possibility is that our finding may be a consequence of oxidative stress-mediated cellular insult. Specifically, because secreted clusterin exerts pro-survival properties (Rizzi and Bettuzzi, 2010; Yu and Tan, 2012), the upregulation of secreted clusterin may represent a homeostatic response to mitigate the pathophysiological sequelae of oxidative insult. In previous studies, we and others have found that TGF(β)-transforming growth factor-β signaling appears to be upregulated in both pyramidal and inhibitory neurons in schizophrenia (Benes, 2011; Benes et al., 2007; Pietersen et al., 2014a; Pietersen et al., 2014b), which may represent an additional compensatory response to oxidative stress (Chou et al., 2006; Dhandapani and Brann, 2003; Gabriel et al., 2003; Klempt et al., 1992; Petegnief et al., 2003). Of interest, TGF-β signaling has also been shown to positively modulate clusterin gene expression (Jin and Howe, 1997, 1999; Laping et al., 1994; Reddy et al., 1996). Thus, clusterin expression upregulation may in part mediate the presumed neuroprotective effects of increased TGF-β signaling. If this is the case, maximizing the availability of extracellular clusterin may prove to have therapeutic benefits. For instance, increased clusterin could help restore cortical circuitry connectivity deficits by promoting the integrity of dendritic, axonal and synaptic structures, as clusterin has been shown to exhibit the properties of enhancing neurite growth and extension (Kang et al., 2005; White et al., 2001; Wicher et al., 2008).

Many neurons in the prefrontal cortex are ensheathed by extracellular matrix that forms perineuronal nets, which play a critical role in synaptic plasticity (Berretta et al., 2015; Beurdeley et al., 2012; Bitanirwe and Woo, 2014; Dityatev et al., 2010; Kwok et al., 2011). The density of perineuronal nets have been shown to be decreased not only in the prefrontal cortex but also in other brain structures in schizophrenia (Mauney et al., 2013; Pantazopoulos et al., 2015; Pantazopoulos et al., 2010), which may contribute to the structural and functional instability of brain circuitry (Woo, 2014). The formation and degradation of perineuronal nets are regulated by a large number of enzymes; among them the matrix metalloproteinases (MMPs) contribute to their degradation. The enzymatic activity of one of the MMPs, MMP-9, has been shown to be inhibited by clusterin (Jeong et al., 2012). Thus, increased clusterin expression may help promote the integrity of perineuronal nets.

It is not possible at this point in time to draw any explicit conclusion in regards to the specific cell types that are associated with the observed upregulation of extracellular clusterin. However, because the expression of the clusterin gene appears to be upregulated in both pyramidal and parvalbumin-containing inhibitory neurons (Pietersen et al., 2014a; Pietersen et al., 2014b), it may be reasonable to assume that these two cell types are likely to be involved. In fact, the expression of genes that are associated with cell death and cellular injury has been found to be altered in both of these cell types (Pietersen et al., 2014a;

Pietersen et al., 2014b). In addition, oxidative stress has been shown in animal studies to induce changes in parvalbumin-containing inhibitory neurons that resemble deficits that have been observed in postmortem brains from subjects with schizophrenia (Cabungcal et al., 2013a; Cabungcal et al., 2013b; Do et al., 2009; Steullet et al., 2014). Interestingly, our findings suggest that the amount of intracellular clusterin in nonpyramidal neurons, which include parvalbumin-containing neurons, appears to be markedly higher than that in pyramidal neurons (Figure 2). If the amount of extracellular clusterin is more or less correlated with the amount of the intracellular form, increased secretion of clusterin from nonpyramidal neurons is likely to contribute to a significantly larger degree to our observation of increased level of clusterin in the extracellular domain. Finally, we found that clusterin was also detectable in glia. However, at present, it is not possible to estimate the contribution, if any, of these cells to our finding of increased extracellular clusterin.

Acknowledgments

This study was supported by NIH grant MH076060.

Role of the Funding Source: The NIH played no role in the design and execution of this study.

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Figure 1.

A. Photomicrograph showing the distribution of clusterin-immunoreactive cells in the human prefrontal cortex in a control subject. Scale bar=50 μ m. **B.** Cell plots showing that the densities of cluster-immunoreactive cells are unaltered in a schizophrenia (right) compared a normal control (left) subjects. Blue circles= non-pyramidal cells, yellow circles= glia, and green circles=pyramidal cells.

Figure 2. Densities of clusterin-immunoreactive pyramidal cells (PYR), nonpyramidal interneurons (IN) and glia (GL) in the prefrontal cortex are unchanged in schizophrenia subjects.

Figure 3.

A. The amount of extracellular clusterin as measured by fluorescent intensity is significantly ($p=0.014$) increased in schizophrenia subjects ($\text{mean} \pm \text{SD} = 1.25 \pm 0.08$) compared to normal control subjects ($\text{mean} \pm \text{SD} = 0.97 \pm 0.07$). **B.** Western blot showing averaged fluorescent intensities of extracellular and intracellular forms of cluster in schizophrenia (SZ) and control (C) subjects. Band 1=extracellular form of clusterin, band 2 and band 3=intracellular forms of clusterin.

Table 1

Cases included in this study

Diagnosis	Age	Sex	PMI
Schizophrenia	60	M	22.17
Schizophrenia	77	M	25.33
Schizophrenia	48	F	29.92
Schizophrenia	32	M	38.43
Schizophrenia	56	F	26.55
Schizophrenia	58	M	25.33
Schizophrenia	47	F	31.85
Schizophrenia	65	M	21.06
Schizophrenia	59	M	29.67
Schizophrenia	69	F	23.08
Schizophrenia	58	F	25
Schizophrenia	63	M	26.16
Schizophrenia	66	M	21.75
Schizophrenia	60	F	17.13
Schizophrenia	64	F	18.5
	58.8±10.5	M:F=8:7	25.4±5.5
Control	62	M	26.07
Control	76	M	23.92
Control	47	F	25.83
Control	46	M	28.83
Control	53	F	34.5
Control	55	M	23.92
Control	51	F	30.62
Control	65	M	20.92
Control	57	M	35.52
Control	65	F	27.17
Control	61	M	21
Control	60	F	21.67
Control	62	M	17.92
Control	58	F	25.7
Control	63	F	23.5
	58.7±7.7	M:F=8:7	25.8±4.9

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Table 2
Lack of significant effects of potential confounding factors on cell densities

		Age	PMI	CPZ	Sex
P-value	Pyramidal cells	0.44	0.62	0.65	0.68
	Nonpyramidal cells	0.34	0.83	0.72	0.78
	Glial cells	0.91	0.63	0.43	0.89