Published in final edited form as:

Synapse. 2008 July ; 62(7): 501–507. doi:10.1002/syn.20519.

Expression of astrocytic markers aquaporin 4 and connexin 43 is altered in brains of subjects with autism

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Abstract

Neuroanatomical studies have revealed extensive structural brain abnormalities in subjects with autism. Recently, studies have provided evidence of neuroglial responses and neuroinflammation in autism. The current study investigated whether two astrocytic markers: aquaporin 4 and connexin 43 are altered in brains from subjects with autism. Postmortem brain tissues from Brodmann's Area 40 (BA40, parietal cortex), Brodmann's Area 9 (BA9, superior frontal cortex), and cerebella of subjects with autism and matched controls were subject to SDS-PAGE and western blotting. Connexin 43 expression was increased significantly in BA9. Aquaporin 4 expression was decreased significantly in cerebellum. These data suggest that changes are apparent in markers for abnormal glial-neuronal communication (connexin 43 and aquaporin 4) in brains of subjects with autism.

Keywords

astroglia; aquaporin 4; connexin 43; cerebellum; BA9; BA40

INTRODUCTION

Neuroanatomical studies have revealed structural abnormalities throughout the brain of subjects with autism including frontal (Brodmann's Area 9 (BA9)) and parietal (Brodmann's Area 40 (BA40)) cortices and cerebellum (for review, see Bauman and Kemper, 1994, 2005). Cerebellar structural abnormalities include loss of granular and Purkinje cells (Ritvo et al., 1986; Bauman and Kemper, 1994) and atrophy of Purkinje cells (Fatemi et al., 2000). The cerebellar abnormalities may be responsible for the dysfunctions within the motor system associated with autism (reviewed in Nayate et al., 2005). Several studies also indicate that the parietal cortex may be abnormal in autism (Courchesne et al., 1993; Saitoh and Courchesne, 1998). Courchesne et al., (1993) reported on the reduction in volumes of the parietal lobes in some autistic subjects. Abnormalities of the parietal cortex in autism may be associated with disturbances of visuospatial-integration, impaired language, and slowed attention shift between and within modalities (Townsend et al., 1996; Haas et al., 1996). Abnormalities of the frontal cortex, including early growth abnormalities (Carper et al., 2002; Carper and Courchesne, 2005) and minicolumn maldevelopment (Casanova et al., 2002; Buxhoeveden et al., 2004),

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are likely to contribute to the serious deficiencies in cognition, language, and emotional functions associated with autism (reviewed in Courchesne and Pierce, 2005).

While earlier studies did not show evidence of astrogliosis or microglial activation (Bauman and Kemper, 1994) more recent work has suggested a role for neuroglial activation and neuroinflammation in autism (for review, see Laurence and Fatemi, 2005; Pardo et al., 2005). A role for neuroglial activation in autism is suggested by altered expression of glial fibrillary acidic protein (GFAP), a marker of astroglial activation, in brain (Ahlsen et al., 1993; Vargas et al., 2005) and cerebral spinal fluid (Ahlsen et al., 1993; Rosengren et al., 1992) of subjects with autism. Furthermore, antibodies against GFAP have been shown to be increased in the plasma of subjects with autism (Singh et al., 1997). Vargas et al (2005) also demonstrated an altered pattern of cytokine expression in brains from subjects with autism including increased expression of pro-inflammatory chemokines macrophage chemoattractant protein-1 and thymus and activation regulated chemokine and pro-inflammatory cytokines including interleukin-6 and interleukin-10 (Pardo et al., 2005) suggesting neuroinflammation is also associated with autism.

In addition to GFAP, altered expression of two other glial markers have been associated with brain pathology. Aquaporin 4 (AQP4), a transmembrane water channel protein (Badaut et al., 2002) shows altered expression in response to a number of pathological conditions marked by astrocytic activation and/or blood-brain barrier changes including glioma, stroke and HIV-related dementia (Papadopoulos et al., 2002; St. Hilarie et al., 2005; Taniguchi et al., 2000). Connexin 43 (CX43) is a major component protein in astrocytic gap junctions (Giaume et al., 1991) that has been shown to be increased as a result of ischemia (Nakase et al., 2006) and to be elevated in the hippocampus of subjects with epilepsy (Collignon et al., 2006).

In the current study we build upon on our experiments with GFAP to compare the protein levels of two additional astrocytic markers: aquaporin 4 and connexin 43, in subjects with autism and matched controls in three brain regions: BA9, BA40 and cerebellum. We hypothesized that CX43 and AQP4, like GFAP, would also be increased in all three brain regions.

MATERIALS AND METHODS

Brain Procurement

The Institutional Review Board of the University of Minnesota-School of Medicine approved this study. Postmortem blocks of parietal cortex (Brodmann's area 40), superior frontal cortex (Brodmann's area 9), and cerebellum (lobar origin unknown) were obtained from the Autism Research Foundation and their contributing brain banks (Harvard Brain Research Center, University of Miami Brain Bank, University of Maryland Brain Bank). These samples, which have been used by our laboratory previously, are some of the most well-characterized and well studied brain collections used by multiple groups (for review, see Palmen et al., 2004). Before being frozen, donated brains were sectioned in half, dissected by anatomists and placed in labeled bags. Consent from next of kin was given to the respective institutions. DSM-IV diagnoses were established prior to death by neurologists and psychiatrists using information from all available medical records and from family interviews. Details regarding the subject selection, diagnostic process, and tissue processing were collected by the Autism Research Foundation. Demographics are tabulated in Table 1. Samples were derived from three groups of subjects (cerebellum: N=8 from subjects with autism, N=10 from control subjects; BA9: N=6 from subjects with autism, N=6 from control subjects; BA40: N=8 from subjects with autism, N=6 from control subjects). Samples were matched for age, gender, and postmortem interval (PMI). All samples were stored at -86°C until use. Information obtained from next of kin showed that none of the controls had any known history of neuropsychiatric disorders or drug abuse except for one control who had a history of alcohol abuse.

SDS-PAGE and Western Blotting

Brain tissue (~40 mg per subject) was cut and placed on ice in lysis buffer (3 μ l/mg of tissue) [20mM Tris, pH 8.0, 0.2 mM EDTA, 150 mM NaCl, 3% Igepal.NP40 (v/v), 1% sodium deoxycholate (w/v), 0.1% SDS (w/v), 50 μ l/ml leupeptin, 0.2 mM PMSF, 1 mM sodium orthovanadate, and aprotinin (Sigma, St. Louis, MO; A6279, 30 μ l/ml buffer)]. Tissue samples were homogenized using a Kontes hand pestle (Kimble-Kontes, Vineland, NJ) while the temperature was maintained at 4°C. Following homogenization, an additional 1 μ l of PMSF (0.2 mM) was added to each sample, and the samples were incubated on ice for 30 min. The homogenates were centrifuged for 20 min at 10,000 X g at 4°C. Supernatants were collected and assayed for total protein using the Bradford method (BioRad, Richmond, CA). Samples were stored at -86°C until used. Samples were mixed with denaturing SDS sample buffer (20% glycerol, 100 mM Tris, pH 6.8, 0.05% w/v Bromophenol blue, 2.5% SDS (w/v), 5% β -mercaptoethanol) and denatured by heating at 100°C for 5 minutes. SDS polyacrylamide gels were prepared with standard Laemmli solutions (BioRad) (resolving: 6%, stacking: 5% for CX43 and β -actin; resolving: 12%, stacking: 5% for AQP4). Sixty μ g of protein per lane was loaded onto the gel and electrophoresed for 15 min at 75V followed by 55 min at 150V at Room Temperature (RT). The proteins were electrotransferred onto nitrocellulose membranes for 2 hr at 300 mA at 4°C. Protein blots were blocked with 0.2% I-Block (Tropix, Bedford, MA, USA) in PBS with 0.3% Tween 20 overnight at 4 °C (CX43 and β -actin) or for 1 hour at RT (AQP4). The blots were then incubated with mouse anti- β -actin (Sigma A5441, 1:5000), mouse anti-connexin 43 (Fred Hutchinson Cancer Research Center, 1:10,000), or rabbit anti-aquaporin 4 (Chemicon AB3594, 1:1000) for 1 hr at RT (CX43 and β -actin) or overnight at 4 °C (AQP4), washed with 0.3% Tween-PBS for 30 minutes, then incubated in secondary antibody for 1 hr at RT (CX43 and β -actin: Sigma, rabbit-anti-mouse IgG 1:80,000; AQP4: Sigma, goat-anti-rabbit IgG, 1:80,000). Blots were washed for 2 $\times$ 15 minutes with 0.3% Tween-PBS. The immune complexes were visualized using the ECL Plus detection system (Amersham Pharmacia Biotech, Arlington Hts., IL) and exposed to Hyperfilm ECL (Amersham Pharmacia Biotech). Sample densities were analyzed using a BioRad densitometer and the BioRad Multi Analyst software. The densities of approximately 42kDa β -actin, 43 and 41kDa CX43, and 34 kDa AQP4 immunoreactive bands were quantified with background subtraction. Results obtained are based on 4–6 independent experiments.

Statistical Analysis

Differences of the protein levels (AQP4 and CX43) between subjects with autism and controls were normalized against β -actin and analyzed using multivariate analysis of covariance (MANCOVA). Potential confounding factors (age, PMI, sex, and race) were entered in the model as covariates to control for their effects on the protein levels. Using MANCOVA allowed us to avoid multiple testing and to take into account of the correlations that exist between the two proteins. In case of significance, follow-up univariate ANCOVA was conducted for each protein. Significant differences were defined as those with a p value < 0.05. Group differences in demographic variables were examined using chi-square tests (sex, race) and t-tests (age, PMI). Confounder effects on protein levels were examined through Pearson Product-Moment correlations (age, PMI) and t-tests (sex, race).

RESULTS

All AQP4 and CX43 western blotting experiments were normalized against β -actin and are shown as ratios of the various proteins to β -actin (Table 2). MANCOVAs detected a significant group effect between subjects with autism and controls in cerebellum [$F(2, 7) = 4.54, p = .05$] and in area 9 [$F(2, 2) = 58.52, p = .017$] after controlling for age, PMI, sex, and race while there was no significant group effect for area 40. Follow-up univariate ANCOVAs conducted on AQP4 and CX43 ratios for cerebellum and area 9 revealed that the groups were significantly

different for AQP4 in cerebellum [$F(1, 8) = 10.11, p = .013$ (Figure 1, Table 2)] and significantly different for CX43 in area 9 [$F(1,3) = 35.07, p = .010$ (Figure 2, Table 2)] after controlling for age, PMI, sex, and race (Table 2). There was no change in β -actin expression between subjects with autism and controls in any of the regions studied (data not shown).

Mean age, PMI, sex and race did not vary significantly between subjects with autism and control subjects in all three brain regions (Table 2). Finally, there was no significant confounder effect except for a significant age effect on Aquaporin 4 in BA40 ($p < 0.009$) and significant age effect on β -actin in cerebellum ($p < 0.03$). Further inspection of data showed that these associations were not meaningful.

DISCUSSION

We observed that AQP4 expression was decreased in cerebellum of subjects with autism. CX43 expression was increased in BA9 of subjects with autism. The changes in expression of CX43 and AQP4 in the brains of subjects with autism appeared to be specific since levels of β -actin did not vary between groups significantly.

Several recent reports show evidence for astroglial activation in cerebellum, frontal, and cingulate cortex of autistic subjects (Fatemi et al., 2002a; Laurence and Fatemi, 2005; Vargas et al., 2005), adding to the weight of pathologic evidence in favor of immune dysregulation in brains of autistic subjects. Vargas et al (2005) also showed that activation of astroglia in autistic brains accompanied a marked increase in macrophage chemoattractant protein-1 and tumor growth factor β -1 suggesting neuroinflammation was also present in subjects with autism. Pardo et al (2005) suggest that while it is unclear how and when microglia and astroglia become activated it may result from intrinsic disturbances in neuroglial function or neuronal-neuroglial interactions during brain development or from extrinsic effects resulting from unknown factors that disturb prenatal or postnatal development (Pardo et al., 2005).

A potential extrinsic effect could be viral infection during pregnancy which could lead to either enhanced expression of pro-inflammatory cytokines in the fetal environment or a direct viral effect on the fetus or both. Our previous work showed elevations in GFAP which could indicate reactive astrogliosis and signify injury/insult to brain tissue (Laurence and Fatemi, 2005). A correlation can be seen in an animal model for autism where prenatal influenza infection in mice causes an upregulation in gene for GFAP in postnatal brains similar to what is observed in autism (Fatemi et al., 2002b), pointing to potential shared mechanisms for brain injury.

Comparison of our current results with our previous examination of GFAP expression shows both similarities and differences. GFAP was significantly increased in all three regions studied (Laurence and Fatemi, 2005). Interestingly, CX43 was similarly increased in BA9 which is an important finding as dysfunction in this region may be responsible for deficits in cognition, language and social function observed in autism (Courchesne and Pierce, 2005). Although non-significant, there were increases of 39% for AQP4 and 54% for CX43 in BA40 suggesting that like GFAP, other astrocytic markers are increased in this region. These increases may potentially contribute to dysfunctions observed in subjects with autism such as language and visuospatial integration, functions associated with BA40 (Haas et al., 1996; Townsend et al., 1996). In contrast, AQP4 was significantly decreased in cerebellum while CX43 showed no change. We had expected to observe similar increases in both proteins given the motor system abnormalities associated with autism (Nayate et al., 2005). Taken together, while GFAP is increased in all three brain areas in subjects with autism, the expression patterns of AQP4 and CX43 are more selective.

In the brain, AQP4 is strongly expressed in astrocytes and ependymal cells (Yang et al., 1995). Expression is strongest at sites of fluid transport including the pial and ependymal

surfaces in contact with the cerebrospinal fluid (CSF), in the subarachnoid space, and the ventricular system (Nielsen et al., 1997; Rash et al., 1998) suggesting a role for AQP4 in movement of water between brain and CSF compartments (Verkman et al., 2006). Additionally, a role for AQP4 in the movement of water between blood and brain is suggested by polarized AQP4 expression found in astrocytic foot processes in direct contact with blood vessels (Verkman et al., 2006).

Increased AQP4 expression has been demonstrated in response to a number of pathological conditions marked by astrocytic activation and/or blood-brain barrier changes including glioma (Papadopoulos et al., 2002), stroke (Taniguchi et al., 2000; Papadopoulos et al., 2002), and HIV-related dementia (St. Hilarie et al., 2005). Alternatively, changes observed in AQP4 in subjects with autism may also reflect alterations in cell morphology, and potential instability in the F-actin molecule (Nicchia et al., 2005). In contrast to the above studies we observed a decrease in AQP4 expression. As with GFAP, however, a correlation can be seen in our animal model for autism where prenatal influenza infection in mice resulted in a downregulation in AQP4 expression in the offspring at P0 (birth) (Fatemi et al., 2005) which persists at P35 (adolescence) (Fatemi et al., 2007). Decreased AQP4 expression may mean that cell structure, cell volume and ionic homeostasis are compromised. Nicchia et al (2005) showed that cultured astrocytes from AQP4 knockdown mice had altered morphology and reduced osmotic permeability.

Glial connexins have important functions in the brain (Theis et al., 2004) including gap-junction-mediated intercellular communication between astrocytes, transport of nutrients from blood to neurons, K^+ spatial buffering, glutamate uptake and dissipation (Theis et al., 2004). Additionally, connexins - especially CX43 - help in several other important functions such as regulation of cell growth, interaction with β catenin, localization to nucleus and affecting gene expression, and cell-cell adhesion (Theis et al., 2004). CX43 is widely present in the astrocytes of the adult brain (Sohl et al., 2005). Gap junctions containing CX43 decrease the propagation of spreading depression (a pathophysiological event leading to neuronal inactivation) by facilitation of uptake of glutamate and K^+ (Theis et al., 2004). CX43 may have a neuroprotective role in focal brain ischemia (Theis et al., 2004). Additionally, CX43 upregulation has been identified in several neurological abnormalities such as Parkinson's, Huntington's, Alzheimer's, seizure/epilepsy, transient brain ischemia and facial nerve lesion (Rouach et al., 2002). Nicchia et al (2005) observed that there was a decrease in CX43 expression in cultured astrocytes from AQP4 knockdown mice with a concomitant reduction in cell-cell coupling (Nicchia et al., 2005). We didn't observe a similar decrease in CX43 expression in the brains of subjects with autism, which may reflect a difference between the two systems. An increase in CX43 expression in brains of subjects with autism may signify increased glial-neuronal signaling, potentially being responsible for enhancement of cell-cell communication in frontal lobe, an area for executive function. This may help explain defects in sensory processing observed in subjects with autism (Kern et al., 2006, 2007; Tomchek et al., 2007).

Our results demonstrated significant changes in two astrocytic markers in brains from subjects with autism. These changes suggest abnormal glial-neuronal communication in brains of subjects with autism.

Acknowledgements

We wish to thank the TARF and affiliated brain banks (Harvard University Brain Bank, Universities of Maryland and Miami Brain Banks) for the gift of brain specimens. We wish to thank L. Iversen for secretarial assistance. Grant support by National Institute of Child Health and Human Development (#1R01HD052074-01A2) to SHF is gratefully acknowledged.

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Figure 1.

Mean AQP4/ β-actin ratios for autistic (filled histogram bars) and control subjects are shown for cerebellum (a), A9 (b), and A40 (c). Levels of AQP4/ β-actin were significantly decreased in cerebellum ($p < 0.013$).

Figure 2.

Mean CX43/ β -actin ratios for autistic (filled histogram bars) and control subjects are shown for cerebellum (a), A9 (b), and A40 (c). Levels of CX43/ β -actin were significantly increased in A9 ($p<0.010$).

Table 1
Demographic information on brain specimens obtained from the Autism Research Foundation

Case	Diagnosis	Sex	Age	PMI	Ethnicity	Cause of Death	Brain Areas
1	Control	M	29	18.3	C	Renal Failure	A9
2	Control	M	36	20	C	MI	Cer, A9
3	Control	M	22	24.3	C	MVA	A9
4	Control	M	26	20	AA	MVA	Cer
5	Control	M	30	22	AA	Cardiomyopathy	Cer, A40
6	Control	M	28	24	C	IDC	Cer, A9, A40
7	Control	M	20	16	C	Accident	Cer, A9, A40
8	Control	F	20	21	C	MVA	Cer
9	Control	M	30	20	AA	MVA	A9
10	Control	M	24	5	Unknown	Gun shot wound	Cer, A40
11	Control	M	19	21	AA	Epiglottitis	Cer, A40
12	Control	M	21	9	C	MVA	Cer, A40
13	Control	M	19	17	C	Accident, chest injuries	Cer
14	Autistic	M	22	14.3	C	Asphyxia	A40
15	Autistic	M	28	16.3	C	Cardiac Arrest	Cer, A40
16	Autistic	M	56	19.5	C	Asphyxia	Cer, A9,
17	Autistic	M	27	8.3	C	Drowning	Cer, A40
18	Autistic	F	21	20.6	C	Pneumonia, Sepsis	Cer, A9, A40
19	Autistic	M	20	15	C	Asphyxia	Cer, A9, A40
20	Autistic	M	19	9.5	C	Seizure	Cer, A9, A40
21	Autistic	M	29	15	C	Hit by train	Cer, A9, A40
22	Autistic	M	30	28.4	C	Shock, Acute Pancreatitis	Cer, A9, A40

A9, Brodmann area 9; A40, Brodmann area 40; AA, African American; C, Caucasian; Cer, cerebellum; IDC, idiopathic dilated cardiomyopathy; MI, myocardial Infarction; MVA, motor vehicle accident

Table 2
Expression of Aquaporin 4 and Connexin 43 in subjects with autism vs. controls

Area	Protein	Control	Autistic	Change	p Value
Cerebellum	Aquaporin 4/ β -Actin	0.036 $\pm$ 0.021	0.017 $\pm$ 0.009	53% $\downarrow$	p=0.013^a
	Connexin 43/ β -Actin	0.59 $\pm$ 0.20	0.60 $\pm$ 0.22	2% $\uparrow$	p=0.61 ^a
	Age $\pm$ SD	25.91 $\pm$ 10.65	24.33 $\pm$ 4.89	6% $\downarrow$	p=0.73
	PMI $\pm$ SD	17.27 $\pm$ 6.19	16.13 $\pm$ 7.46	7% $\uparrow$	p=0.84
	Male %	100	86	14% $\uparrow$	p=0.27
	Caucasian %	86	100	14% $\downarrow$	p=0.30
Area 9	Aquaporin 4/ β -Actin	0.0060 $\pm$ 0.0051	0.0058 $\pm$ 0.0064	3% $\downarrow$	p=0.43 ^a
	Connexin 43/ β -Actin	0.70 $\pm$ 0.22	1.19 $\pm$ 0.20	70% $\uparrow$	p=0.010^a
	Age $\pm$ SD	27.73 $\pm$ 10.21	28.85 $\pm$ 12.77	4% $\uparrow$	p=0.21
	PMI $\pm$ SD	18.41 $\pm$ 6.21	16.61 $\pm$ 6.93	10% $\downarrow$	p=0.16
	Male %	100	75	25% $\downarrow$	p=0.28
	Caucasian %	75	100	25% $\uparrow$	p=0.28
Area 40	Aquaporin 4/ β -Actin	0.018 $\pm$ 0.019	0.025 $\pm$ 0.026	39% $\uparrow$	p=0.47^a
	Connexin 43/ β -Actin	0.61 $\pm$ 0.32	0.94 $\pm$ 0.37	54% $\uparrow$	p=0.47 ^a
	Age $\pm$ SD	23.67 $\pm$ 4.5	24.14 $\pm$ 4.67	2% $\uparrow$	p=0.96
	PMI $\pm$ SD	16.17 $\pm$ 7.67	17.01 $\pm$ 5.99	5% $\uparrow$	p=0.82
	Male %	100	83	13% $\downarrow$	p=0.30
	Caucasian %	80	100	25% $\uparrow$	p=0.25

^a ANCOVA results