Dysregulation of fragile X mental retardation protein and metabotropic glutamate receptor 5 in superior frontal cortex of individuals with autism: a postmortem brain study

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Abstract

Background: Fragile X syndrome is caused by loss of function of the fragile X mental retardation 1 (FMR1) gene and shares multiple phenotypes with autism. We have previously found reduced expression of the protein product of FMR1 (FMRP) in vermis of adults with autism.

Methods: In the current study, we have investigated levels of FMRP in the superior frontal cortex of people with autism and matched controls using Western blot analysis. Because FMRP regulates the translation of multiple genes, we also measured protein levels for downstream molecules metabotropic glutamate receptor 5 (mGluR5) and γ-aminobutyric acid (GABA) A receptor β3 (GABRβ3), as well as glial fibrillary acidic protein (GFAP).

Results: We observed significantly reduced levels of protein for FMRP in adults with autism, significantly increased levels of protein for mGluR5 in children with autism and significantly increased levels of GFAP in adults and children with autism. We found no change in expression of GABRβ3. Our results for FMRP, mGluR5 and GFAP confirm our previous work in the cerebellar vermis of people with autism.

Conclusion: These changes may be responsible for cognitive deficits and seizure disorder in people with autism.

Background

Autism and fragile X syndrome (FXS) are two disorders that share several commonalities, including reduced cerebellar volume, altered dendritic spine morphology [1-4], presence of seizures, mental retardation and social anxiety [2-5], as well as other behavioral abnormalities [5,6]. People with both FXS and autism have been shown to have lower IQ scores [7], lower adaptive skills [7], lower expressive language skills [8] and greater autonomic dysfunction and hyperarousal [9] than people with FXS alone. The rate of FXS in people with autism varies from 2% to 8% [10,11]. The prevalence of autism in people with FXS has been estimated to be anywhere from 25% to 47% [5,12,13].

All diagnoses of FXS require loss of function mutation of the fragile X mental retardation 1 (FMR1) gene [14]. The product of the FMR1 gene, fragile X mental retardation protein (FMRP), has roles in multiple intranuclear and posttranscriptional events [15,16] and has been shown to bind to up to 4% of the mRNA expressed in the brain [17]. FMRP is thought to be involved in multiple developmental events, including normal differentiation, migration of subpopulations of neuronal cells and the development of cortical circuits [18]. FMRP may also have roles in synaptic pruning [19], and the results of multiple studies have supported a role for FMRP in synaptic plasticity [17,20]. All of these functions are relevant to the pathology of autism. Any abnormality in FMRP function could affect multiple genes and pathways [21]. For example, reduced FMRP expression has been shown to negatively affect the expression of γ-aminobutyric acid A (GABA_A) receptors in FXS animal models [22-24], and work done at our laboratory has...
shown significant reductions in protein for both FMRP and the GABA_A, β3 subunit (GABRβ3) in vermis of adults with autism [25].

In vermis of people with autism, our laboratory has recently demonstrated the following: (1) reduction in levels of FMRP and GABRβ3 in adults with autism, (2) increased expression of metabotropic glutamate receptor 5 (mGluR5) in children with autism and (3) increased glial fibrillary acidic protein (GFAP) in children and adults with autism [25]. In the current study, we have expanded our research to measure protein levels for the same molecules in the superior frontal cortex (Brodmann’s area 9 (BA9)). We hypothesized that FMRP would be reduced, potentially explaining deficits that are common in autism spectrum disorder and FXS.

Results
All values derived using Western blot analysis were normalized against two housekeeping proteins: NSE and β-actin. Previous studies of FMRP have shown that two to five distinct bands of FMRP may appear on SDS-PAGE (55% to 50%, 46 kDa for GFAP, and the FMRP/NSE ratio (55%, P < 0.017) and the FMRP/β-actin ratio (50%,
| Case  | Dx    | Sex | Age | PMI, hours | Ethnicity         | Medication History                          | Cause of Death         | Seizure | MR   |
|-------|-------|-----|-----|------------|-------------------|---------------------------------------------|------------------------|---------|------|
| 4670  | Control | M   | 4   | 17         | Caucasian         | None                                        | Commotio Cordis        | No      | No   |
| 1674  | Control | M   | 8   | 36         | Caucasian         | None                                        | Drowning               | No      | No   |
| 4787  | Control | M   | 12  | 15         | African American  | Montelukast, Albuterol, Prednisone, Loratadine | Asthma                 | No      | No   |
| 1823  | Control | M   | 15  | 18         | Caucasian         | None                                        | MVA                    | No      | No   |
| 6396  | Control | M   | 18  | 19.83      | Unknown           | None                                        | Unknown                | No      | No   |
| 1846  | Control | F   | 20  | 9          | Caucasian         | None                                        | MVA                    | No      | No   |
| 6316  | Control | F   | 32  | 28.92      | Unknown           | None                                        | Unknown                | No      | No   |
| 1169  | Control | M   | 33  | 27         | African American  | Metoclopramide, Loratadine                  | Dilated Cardiomyopathy (morbid obesity) | No      | No   |
| 1376  | Control | M   | 37  | 12         | African American  | None                                        | ASCVD                  | No      | No   |
| 6420  | Control | M   | 41  | 30.4       | Unknown           | None                                        | Heart Attack           | No      | No   |
| 7002  | Autism | F   | 5   | 32.73      | Asian             | None                                        | Drowning               | No      | No   |
| 5569  | Autism | M   | 5   | 39         | Caucasian         | None                                        | Drowning               | No      | No   |
| 1174  | Autism | F   | 7   | 14         | Caucasian         | None                                        | Seizure disorder       | Yes     | No   |
| 5666  | Autism | M   | 8   | 22.16      | Caucasian         | None                                        | Cancer                 | Yes     | No   |
| 4231  | Autism | M   | 8   | 12         | African American  | Olanzapine, Galantamine                      | Drowning               | No      | Yes  |
| 4925  | Autism | M   | 9   | 27         | Caucasian         | Clonidine, Sodium valproate, Phenytoin, Lamotrigine, Methylphenidate, Carbamazepine | Seizure disorder       | Yes     | No   |
| 4899  | Autism | M   | 14  | 9          | Caucasian         | None                                        | Drowning               | No      | No   |
| 6184  | Autism | F   | 18  | 6.75       | Caucasian         | None                                        | Seizure disorder       | Yes     | No   |
| 5144  | Autism | M   | 20  | 23.66      | Caucasian         | Erythromycin gel, Minocycline                | MVA                    | No      | Yes  |
| 1638  | Autism | F   | 20  | 50         | Caucasian         | None                                        | Seizure disorder       | Yes     | Yes  |
| 6337  | Autism | M   | 22  | 25         | Caucasian         | Aripiprazole, Lamotrigine, Zonisamide        | Aspiration             | Yes     | No   |
| 6994  | Autism | M   | 29  | 43.25      | Caucasian         | Fexofenadine, Ziprasidone HCl, Carbamazepine | Seizure (suspected)    | Yes     | No   |
| 6640  | Autism | F   | 29  | 17.83      | Caucasian         | Fluvoxamine                                  | Seizure disorder       | Yes     | No   |
| 6677  | Autism | M   | 30  | 16.06      | Caucasian         | None                                        | Heart Failure (congestive) | No      | Yes  |
| 5173  | Autism | M   | 30  | 20.33      | Caucasian         | Cisapride, Clorazepate, Sodium valproate, Phenytoin, Folic Acid, Primidone, Phenobarbital, Omeprazole, Cisapride, Metoclopramide | Gastrointestinal bleeding | Yes     | No   |
| 5027  | Autism | M   | 37  | 26         | African American  | None                                        | Obstruction of bowel due to adhesion        | No      | No   |
| 6401  | Autism | M   | 39  | 13.95      | Caucasian         | None                                        | Cardiac Tamonade       | No      | No   |
| 5562  | Autism | M   | 39  | 22.75      | Caucasian         | None                                        | Sudden unexpected death | Yes     | Yes  |
| 6469  | Autism | F   | 49  | 16.33      | Caucasian         | Wafarin, Venlafaxine, Erythromycin, Lansoprazole, Risperidone, Meflofin, Gabapentin, Propranolol, Levothyroxine | Pulmonary Arrest       | No      | Yes  |
| 4498  | Autism | M   | 56  | 19.48      | Caucasian         | Benztrapine mesylate, Haloperidol, Lithium, Chlorpromazine, Alprazolam | Anoxic encephalopathy  | Yes     | No   |

| Children | Control | Autistic | Change | P value | Cohen’s d |
|----------|---------|----------|--------|---------|-----------|
| Age ± SD (years) | 11.4 ± 5.55 | 9.25 ± 4.53 | ↓18.9% | ns | – |
| PMI ± SD (years) | 212 ± 8.47 | 186 ± 9.47 | ↓12.3% | ns | – |
| Gender | 5M | 5M, 3F | – | – | – |
Table 1 Demographic data for people with autism and healthy controls (Continued)

| Adults | Control | Autistic | Change | P value | Cohen’s d |
|--------|---------|---------|--------|---------|-----------|
| Age ± SD (years) | 32.6 ± 7.89 | 33.3 ± 11.2 | ↓21.1% | ns | – |
| PMI ± SD (years) | 21.5 ± 10.1 | 24.6 ± 11.1 | ↓14.4% | ns | – |
| Gender | 3M:2F | 9M:3F | – | – | – |

*aDx, diagnosis; PMI, postmortem interval; MVA, motor vehicle accident; MR, mental retardation; ns, not significant.

P < 0.042) in BA9 of adults with autism compared with controls (P < 0.026) (Figure 1 and Tables 2 and 3). In BA9 of children with autism, there were significant increases in the dimerized mGluR5/NSE ratio (159%, P < 0.013) and total mGluR5/NSE ratio (165%, P < 0.014) (Figure 2 and Table 2). Similarly, there were significant increases in both the dimerized mGluR5/β-actin ratio (168%, P < 0.023) and the total mGluR5/β-actin ratio (191%, P < 0.011) (Table 3). We previously observed a significant difference in dimerized mGluR5 vs. total mGluR5 in vermis of children with autism [25]; however, a similar result was not seen in BA9 (Table 2). GFAP was found to be significantly elevated in BA9 of both children (136%, P < 0.012) and adults (58%, P < 0.033) with autism (Figure 3 and Tables 2 and 3). We observed no significant differences in protein levels for NSE (Figure 3 and Table 2) or β-actin (Figure 3 and Table 3), suggesting that the observed changes were not due to changes in neuronal cell numbers between people with autism and matched controls. Finally, there was no significant change in GABRβ3 between people with autism and matched controls (Figure 4 and Tables 2 and 3), despite large effect sizes.

There were no significant differences between individuals with autism and controls related to age or postmortem interval (PMI) (Tables 2 and 3). We examined the confounding effects of age and PMI on our results and found no impact of age on the levels of any proteins (data not shown). There was an impact of PMI on the FMRP/β-actin ratio. However, as there was no significant difference in PMI between the two groups, the impact of PMI on the FMRP/β-actin ratio was not meaningful.

The effect of gender was examined in both children and adults. For children, there were no girls in the control group. However, when control boys were compared with boys with autism, the results for FMRP and GABRβ3 remained nonsignificant and total mGluR5 and GFAP remained significantly elevated, while the dimerized mGluR5/β-actin ratio lost significance (data not shown). In adults, it was impossible to evaluate the effect of gender on levels of FMRP and GFAP owing to low power, and mGluR5 and GABRβ3 remained nonsignificant (data not shown).

Mental retardation, seizures and use of anticonvulsants were all confounded within the autism group. Neither seizure disorder nor anticonvulsant use affected the Western blot analysis results (data not shown). In comparing people with autism who had seizure disorder with people with autism without seizure disorder, we found that there were no significant differences for FMRP in children, while this comparison could not be tested in adults because of the low sample size. However, there was a 47% reduction in FMRP in adults with autism and seizure disorder compared with adults with autism without seizure disorder (data not shown). In only one instance was mental retardation a significant predictor of differences in Western blot analysis results, and that was for the GFAP/β-actin ratio in adults. When that individual (subject 6469) was removed from the analysis, the difference remained significant between people with autism and controls.

Effect sizes were calculated for the comparisons using Cohen’s d statistic where an effect size >0.8 is considered a large effect [32]. In children, the comparisons of mGluR5 dimers/NSE ratio, mGluR5 total/NSE ratio, GABRβ3/NSE ratio, mGluR5 dimers/β-actin ratio, mGluR5 total/β-actin ratio, GABRβ3/β-actin ratio and GFAP/β-actin ratio displayed large effect sizes (Tables 2 and 3). In adults, the comparisons of FMRP/NSE ratio, mGluR5 total/NSE ratio, FMRP/β-actin ratio, mGluR5 dimers/β-actin ratio, mGluR5 total/β-actin ratio and GFAP/β-actin ratio between the two groups consisted of large or very large effect sizes (Tables 2 and 3).

**Discussion**

In the current study, we found reduced protein expression of FMRP in BA9 of adults with autism, increased expression of mGluR5 in children with autism, increased expression of GFAP in both children and adults with autism and no significant difference in GABRβ3 expression. With the exception of no change in GABRβ3 expression, these changes in the prefrontal cortex (PFC; that is, BA9) mirror what we have previously observed in cerebellar vermis of people with autism [25].

We are the first to demonstrate significant changes in protein for FMRP in BA9 of adults with autism. Recent imaging studies have shown reduced activation of the
PFC during cognitive performance tasks in individuals with FXS [35-37]. Hoeft et al. [36] found reduced activation in the right ventrolateral PFC in adolescent boys with FXS during a go vs. no go executive function task. Similarly, in a study of females with FXS, there was reduced activation of the PFC and striatum during a working memory task [35]. Reduced activation of the PFC has also been shown in females with FXS during mathematical processing [37]. Taken together, these studies indicate impairment of multiple PFC-mediated functions in people with FXS. The reduction in FMRP in BA9 in adults with autism may result in similar changes in brain activation and cognitive processing in individuals with autism.

Dopamine plays a critical role in cognitive functions in the PFC [38,39], and recent studies involving Fmr1-knockout mice have shown a role of FMRP in dopamine modulation in the PFC [40,41]. Such a role for FMRP may help to explain learning and memory deficits in people with FXS [40,41]. Our finding of reduced FMRP expression in BA9 of adults may help to explain cognitive deficits in people with autism.
In contrast to our studies of vermis, we did not observe a significant reduction in GABRβ3 in BA9 of adults with autism. However, previous experiments in our laboratory using a different set of BA9 tissue from controls and individuals with autism also failed to show any significant difference in protein expression of GABRβ3 (SHF and TJ Reutiman, unpublished data). These results may reflect regional differences in GABRβ3 protein expression in the brains of people with autism.

Our finding regarding GFAP confirms our earlier findings of significant elevation of GFAP in BA9 of a different set of people with autism [42]. This increased level of GFAP is consistent with previous findings of astrogial activation in the brains of individuals with autism [43-45], suggesting dysregulation of the immune system. Coexpression of GFAP and FMRP has been demonstrated by immunocytochemical studies in the hippocampi of embryonic and postnatal mice, but not in adults [46,47]. Significantly increased GFAP has been observed in the cerebellum [48] and in the striatum, hippocampus, and cerebral cortex [49] of fragile X-knockout mice. Ellegood et al. [48] suggested that the loss of FMRP and increase in cerebellar GFAP could contribute to anatomical changes in the cerebellum.

The significant elevation of mGluR5 protein in children with autism is an interesting finding in light of the connection between group 1 mGluRs and FMRP in FXS, which are believed to act in opposition to one another. It has been proposed that in the absence of FMRP, unchecked mGluR-dependent protein synthesis leads to the pathology of FXS [50,51]. A number of recent findings in Fmr1-knockout mice have shown that

Table 2 Western blot analysis results for FMRP, mGluR5, GABRβ3 and NSE and their ratios in BA9

| Children |  |  |  |
|----------|---|---|---|
| FMRP/NSE | 0.08 ± 0.057 | 0.123 ± 0.069 | 153% | 0.013b | 1.93b | ns | 0.66 |
| mGluR5 dimer/NSE | 0.158 ± 0.175 | 0.409 ± 0.101 | 159% | ns | 0.014b | 1.90b | 0.66 |
| mGluR5 total/NSE | 0.167 ± 0.182 | 0.443 ± 0.123 | 165% | 0.014b | 1.90b | 0.66 |
| mGluR5 dimer/mGluR5 total | 0.867 ± 0.154 | 0.93 ± 0.042 | 17.2% | ns | 0.014b | 1.90b | 0.66 |
| GABRβ3/NSE | 0.095 ± 0.052 | 0.161 ± 0.086 | 169% | 0.014b | 1.90b | 0.66 |
| NSE | 17.4 ± 0.642 | 19.20 ± 2.67 | 10.4% | ns | 0.66 |

Table 3 Western blot analysis results for FMRP, mGluR5, GABRβ3, GFAP and β-actin and their ratios in BA9

| Children |  |  |  |
|----------|---|---|---|
| FMRP/β-actin | 0.073 ± 0.054 | 0.119 ± 0.062 | 153% | 0.017b | 1.74b | ns | 0.66 |
| mGluR5 dimer/β-actin | 0.186 ± 0.099 | 0.309 ± 0.188 | 160% | 0.013b | 1.74b | ns | 0.66 |
| mGluR5 total/β-actin | 0.191 ± 0.099 | 0.367 ± 0.187 | 192% | 0.013b | 1.74b | 0.50 |
| mGluR5 dimer/mGluR5 total | 0.974 ± 0.023 | 0.982 ± 0.011 | 10.8% | 0.013b | 1.74b | 0.50 |
| GABRβ3/β-actin | 0.177 ± 0.082 | 0.186 ± 0.103 | 15.1% | 0.013b | 1.74b | 0.50 |
| GFAP/β-actin | 1.75 ± 3.17 | 18.8 ± 2.3 | 11.3% | ns | 0.66 |

Table 2 Western blot analysis results for FMRP, mGluR5, GABRβ3 and NSE and their ratios in BA9

| Children |  |  |  |
|----------|---|---|---|
| FMRP/NSE | 0.24 ± 0.094 | 0.107 ± 0.068 | 155% | 0.017b | 1.74b | ns | 0.66 |
| mGluR5 dimer/NSE | 0.186 ± 0.099 | 0.309 ± 0.188 | 160% | 0.013b | 1.74b | ns | 0.66 |
| mGluR5 total/NSE | 0.191 ± 0.099 | 0.367 ± 0.187 | 192% | 0.013b | 1.74b | 0.50 |
| mGluR5 dimer/mGluR5 total | 0.974 ± 0.023 | 0.982 ± 0.011 | 10.8% | 0.013b | 1.74b | 0.50 |
| GABRβ3/NSE | 0.177 ± 0.082 | 0.186 ± 0.103 | 15.1% | 0.013b | 1.74b | 0.50 |
| NSE | 17.5 ± 3.17 | 18.8 ± 2.3 | 11.3% | ns | 0.66 |

Table 3 Western blot analysis results for FMRP, mGluR5, GABRβ3, GFAP and β-actin and their ratios in BA9

| Children |  |  |  |
|----------|---|---|---|
| FMRP/β-actin | 0.175 ± 0.075 | 0.087 ± 0.055 | 150% | 0.042b | 1.43b | ns | 0.66 |
| mGluR5 dimer/β-actin | 0.136 ± 0.069 | 0.267 ± 0.175 | 190% | 0.033b | 1.46b | ns | 0.66 |
| mGluR5 total/β-actin | 0.139 ± 0.069 | 0.272 ± 0.178 | 190% | 0.033b | 1.46b | ns | 0.66 |
| mGluR5 dimer/mGluR5 total | 0.136 ± 0.069 | 0.159 ± 0.087 | 17% | 0.033b | 1.46b | ns | 0.66 |
| GFAP/β-actin | 1.93 ± 0.762 | 3.04 ± 0.758 | 158% | 0.033b | 1.46b | ns | 0.66 |
| β-actin | 232 ± 1.97 | 217 ± 3.28 | 65.5% | ns | 0.66 |

*FMRP, fragile X mental retardation protein; mGluR5, metabotropic glutamate receptor 5; GABRβ3, γ-aminobutyric acid (GABA) A receptor β3; NSE, neuron-specific enolase; BA9, Brodmann’s area 9; ns, not significant; *statistically significant.
Figure 2 Expression of mGluR5 in superior frontal cortex of people with autism and matched controls. (A) Representative samples of metabotropic glutamate receptor 5 (mGluR5) from controls (C) and people with autism (A). (B) Mean mGluR5 dimer and monomer/neuron-specific enolase (NSE) ratios for people with autism (filled histogram bars) and controls are shown for Brodmann’s area 9 (BA9). (C) Mean mGluR5/β-actin ratios for people with autism (filled histogram bars) and controls are shown for BA9. Error bars express standard error of the mean. **P < 0.05.
the use of mGluR5 inhibitors, such as 2-methyl-6-(phe-nylethynyl)-pyridine (MPEP), rescues FXS phenotypes [52,53]. Recent experiments by Silverman et al. [54,55] have shown that MPEP was useful in the treatment of repetitive grooming behavior in the BTBR mouse model of autism by antagonizing mGluR5 receptors. This new model adds to the previous animal models for autism and FXS and helps to expand our understanding of the role of mGluR5 dysfunction.

The increased expression of mGluR5 in children with autism suggests pathologic activation of mGluR5 which may be prenatal or early postnatal. This activation may lead to seizures, cognitive problems and perhaps brain morphological abnormalities in children with autism and FXS. While FMRP levels are similar in children with autism and controls, overactive mGluR5 signaling may be strong enough to overcome FMRP’s inhibitory effect. In adults, there continue to be elevated levels of dimerized mGluR5 (66% increase for mGluR5/NSE ratio and 92% increase for mGluR5/β-actin ratio) and total mGluR5 (96% increase for both mGluR5/NSE and mGluR5/β-actin ratios), although they do not reach the level of statistical significance. At the same time, adults with autism have significantly lower levels of FMRP. This reduction in FMRP reduces its inhibitory effect on mGluR5 signaling, resulting in a continuation of the phenotypes observed in children with autism and FXS.

The interaction between FMRP and mGluR5 also presents a potential means of treatment using allosteric modulators of mGluR5. In a pilot study, treatment of patients with FXS with lithium, which reduces mGluR-activated translation, led to improved scores on the

Figure 3 (A) Representative samples of glial fibrillary acidic protein (GFAP), neuron-specific enolase (NSE) and β-actin from controls (C) and people with autism (A). (B) Mean GFAP/β-actin ratios for people with autism (filled histogram bars) and controls are shown for Brodmann’s area 9 (BA9). Error bars express standard error of the mean. **P < 0.05.
Aberrant Behavior Checklist-Community Edition and was well-tolerated [56]. Additionally, fenobam, an anxiolytic drug that acts as an inhibitor of mGluR5 [57], has been shown to correct prepulse inhibition deficits in people with FXS in a recent open-label pilot study [58]. Norbin is a positive modulator of endogenous mGluR5 that enhances mGluR5 signaling [59]. Reduction in norbin has been shown to lead to a consequent reduction in mGluR5 expression, and norbin-knockout mice show enhanced locomotor activity similar to that of mice treated with MPEP [59]. It may be the case that allosteric modulators of mGluR5 or targeting of naturally occurring modulators of mGluR5 could have similar effects on people with autism and warrant future exploration. A further avenue of treatment may be the use agents which raise the levels of GABA in the brains of individuals with autism. Recently, Heulens et al. [60] reported on ganaxolone as a potential GABA<sub>A</sub> receptor agonist in the treatment of people with FXS. Certainly this agent, as well as GABA<sub>B</sub> receptor agonists such as arbaclofen, could be investigated in the treatment of autism.

**Figure 4** Expression of GABR<sub>B</sub>3 in superior frontal cortex of people with autism and matched controls. (A) Representative samples of γ-aminobutyric acid (GABA) A receptor β3 (GABR<sub>B</sub>3) from controls (C) and people with autism (A). (B) Mean GABR<sub>B</sub>3/neuron-specific enolase (NSE) ratios for people with autism (filled histogram bars) and controls are shown for Brodmann’s area 9 (BA9). (C) Mean GABR<sub>B</sub>3/β-actin ratios for people with autism (filled histogram bars) and controls are shown for BA9. Error bars express standard error of the mean.
Conclusions

We observed reduced expression of FMRP in BA9 of adults with autism, and increased expression of mGluR5 in children with autism, compared with matched controls. These findings mirror results observed in our laboratory in cerebellar vermis of people with autism. These observed changes in protein expression of FMRP in adults and mGluR5 in children are likely to contribute to cognitive deficits and the presence of comorbid seizure disorder in individuals with autism. The interaction between FMRP and mGluR5 provides a potential avenue for treatment through the use of allosteric modulators.

Acknowledgements

Human tissue was obtained from the National Institute of Child Health & Human Development (NICHD) Brain and Tissue Bank for Developmental Disorders, University of Maryland, Baltimore, MD, USA. (The role of the NICHD Brain and Tissue Bank is to distribute tissue, and therefore it cannot endorse the studies performed or the interpretation of results.) Human tissue was also obtained from the Harvard Brain Tissue Resource Center, which is supported in part by Public Health Service R24 MH086855, the Brain Endowment Bank, which is funded in part by the National Parkinson Foundation, Inc., Miami, FL, USA; and the Autism Tissue Program, and all are gratefully acknowledged. Grant support by NICHD grants SRO1HD052074-01A2 and SRO1HD052074-03S1 to SHF is gratefully acknowledged. Information regarding FMRP from Dr J Damell, Rockefeller University, New York, NY, USA, is greatly appreciated. Assistance with statistical analyses provided by Dr P Thuras is greatly appreciated. A brief summary of some of the findings presented here dealing with FMRP, mGluR5 and GABAA receptor expression in children with autism is previously presented in a review article [61] with permission from Elsevier.

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Authors’ contributions

SHF conceived of the study, participated in its design and contributed to the drafting of the manuscript. TDF performed the Western blot analysis, was involved in statistical analyses and contributed to the drafting of the manuscript. Both authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 15 October 2010 Accepted: 6 May 2011
Published: 6 May 2011

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doi:10.1186/2040-2392-2-6

Cite this article as: Fatemi and Folsom: Dysregulation of fragile X mental retardation protein and metabotropic glutamate receptor 5 in superior frontal cortex of individuals with autism: a postmortem brain study. Molecular Autism 2011 2:6.