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mRNA and protein expression for novel $GABA_A$ receptors θ and ρ2 are altered in schizophrenia and mood disorders; relevance to FMRP-mGluR5 signaling pathway

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Fragile X mental retardation protein (FMRP) is an RNA-binding protein that targets ~5% of all mRNAs expressed in the brain. Previous work by our laboratory demonstrated significantly lower protein levels for FMRP in lateral cerebella of subjects with schizophrenia, bipolar disorder and major depression when compared with controls. Absence of FMRP expression in animal models of fragile X syndrome (FXS) has been shown to reduce expression of gamma-aminobutyric acid A ($GABA_A$) receptor mRNAs. Previous work by our laboratory has found reduced expression of FMRP, as well as multiple $GABA_A$ and $GABA_B$ receptor subunits in subjects with autism. Less is known about levels for $GABA_A$ subunit protein expression in brains of subjects with schizophrenia and mood disorders. In the current study, we have expanded our previous studies to examine the protein and mRNA expression of two novel $GABA_A$ receptors, theta (GABRθ) and rho 2 (GABRρ2) as well as FMRP, and metabotropic glutamate receptor 5 (mGluR5) in lateral cerebella of subjects with schizophrenia, bipolar disorder, major depression and healthy controls, and in superior frontal cortex (Brodmann Area 9 (BA9)) of subjects with schizophrenia, bipolar disorder and healthy controls. We observed multiple statistically significant mRNA and protein changes in levels of GABRθ, GABRρ2, mGluR5 and FMRP molecules including concordant reductions in mRNA and proteins for GABRθ and mGluR5 in lateral cerebella of subjects with schizophrenia; for increased mRNA and protein for GABRρ2 in lateral cerebella of subjects with bipolar disorder; and for reduced mRNA and protein for mGluR5 in BA9 of subjects with bipolar disorder. There were no significant effects of confounds on any of the results.
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Introduction

Impairment of the gamma-aminobutyric acid (GABA) signaling system is believed to partially account for behavioral and cognitive deficits associated with schizophrenia and mood disorders.[1,2] Reduction of $GABA_A$ mediated signal transmission has also been associated with anxiety, panic, impaired learning and memory.[3–5] $GABA_A$ receptors are responsible for mediating the fast inhibitory action of GABA,[6] and are important sites for clinical action of a number of drugs including benzodiazepines, barbiturates and anesthetics. Recent work has suggested that proper GABAergic neurotransmission is required for network oscillations that facilitate the processing of information both in and between various brain regions and that this may be required for normal cognition.[1] Altered expression of $GABA_A$ receptor subunits could impair these oscillations and result in improper cognitive function. Little is currently known about $GABA_A$ receptor subunit expression in schizophrenia and mood disorders, although it is likely that changes in $GABA_A$ receptor expression would result in reduced GABAergic transmission.

Recent evidence[7–9] provides a linkage between GABA neurotransmission and fragile X mental retardation protein (FMRP). FMRP is an RNA-binding protein that has been estimated to regulate translation of 842 transcripts in the brain.[10] In animal models of fragile X syndrome (FXS), the absence of FMRP is accompanied by reduced mRNA expression of $GABA_A$ receptor subunits including alpha 1 (α1), α3, α4, beta 1 (β1), β2, delta (δ), gamma 1 (γ1) and γ2 in frontal cortex, whereas there was no change in the cerebellum.[7–9] A functional consequence of this reduced expression has been observed in fragile X mental retardation 1 (Fmr1)-knocked out mice that display impaired GABAergic signaling in striatal neurons, as measured by increased frequency of spontaneous and miniature inhibitory postsynaptic currents and reduced paired pulse ratio of inhibitory postsynaptic currents.[11]

FMRP normally represses metabotropic glutamate receptor 5 (mGluR5) signaling, whereas the absence of FMRP has been hypothesized to lead to unregulated mGluR5 signaling, and ultimately result in the various abnormal phenotypes

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associated with FXS.[12] Animal studies using antagonists of the mGluR5 receptor have rescued learning and behavioral deficits associated with FXS, and reduced seizures in FMR1-knock out mice.[13–17]

Recently, we reported on reduced protein expression of FMRP in lateral cerebellum from subjects with schizophrenia, bipolar disorder and major depression.[18] These results are novel, as gene association studies have not identified fragile X mental retardation 1 (*FMR1*), the gene that codes FMRP, as a candidate gene for schizophrenia.[19–20] However, a recent study verified our earlier study, finding reduced FMRP expression in peripheral blood lymphocytes from subjects with schizophrenia.[21] Kovács *et al.*[21] found that age of onset and IQ predicted FMRP levels, but chlorpromazine-equivalent antipsychotic dose did not. Importantly, none of the study subjects showed the CGG triplet expansion that normally causes silencing of the *FMR1* gene in subjects with FXS.[21] Combined with our findings of reduced FMRP expression in the cerebellar vermis and prefrontal cortex of subjects with autism,[22–23] who were not comorbid for FXS, reduction of FMRP expression may be a hallmark of multiple psychiatric disorders.

Based on the evidence from animal models that decrease in FMRP expression results in reduced expression of $GABA_A$ receptor subunit mRNA and our finding of significantly reduced FMRP in cerebella of subjects with schizophrenia and mood disorders,[18] we hypothesized that we would observe reduced expression of the $GABA_A$ receptor subunits in lateral cerebella from the same diagnostic groups. An initial screen of several GABA receptor subunits in lateral cerebella of subjects with schizophrenia, bipolar disorder and major depression found reductions in $GABA_B$ receptor subunits one and two (GABBR1 and GABBR2).[24] Here, we report novel findings regarding alterations in levels of mRNA and protein for $GABA_A$ receptor theta (GABRθ) and $GABA_A$ receptor rho 2 (GABRρ2), as well as mGluR5 and FMRP levels in the lateral cerebellum and Brodmann Area 9 (BA9) of subjects with schizophrenia and mood disorders. These results demonstrate the disruption of the GABAergic and FMRP-mGluR5 signaling systems in subjects with schizophrenia and mood disorders.

Materials and methods

Brain procurement. The Institutional Review Board of the University of Minnesota—School of Medicine has approved this study. Post-mortem lateral cerebella were obtained from the Stanley Foundation Neuropathology Consortium under approved ethical guidelines. Post-mortem superior frontal cortex (BA9) was obtained from the McLean 74 Cohort, Harvard Brain and Tissue Resource Center. DSM-IV diagnoses were established prior to death by psychiatrists using information from all available medical records and family interviews. Details regarding the subject selection, demographics, diagnostic process and tissue processing were collected by the Stanley Medical Research Foundation, and the Harvard Brain and Tissue Resource Center. The Stanley collection consisted of 15 subjects with schizophrenia, 15 with bipolar disorder, 14 with major depression without psychotic features and 14 normal controls (Table 1). The McLean 74 Cohort consists of 20 subjects with schizophrenia, 19 with bipolar disorder and 29 normal controls (Table 2). All groups were matched for age, sex, race, post-mortem interval and hemispheric side.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blotting. Brain tissue was prepared as previously described.[18,23–28] For lateral cerebellum, 60 μg of tissue was used, whereas for BA9, 30 μg of tissue was used. For mGluR5 and FMRP, we used 6% resolving gels, whereas for GABRθ, GABRρ2, neuronal-specific enolase (NSE) and β-actin we used 10% resolving gels. We minimized interblot variability by including samples from subjects of each group (control, schizophrenia, bipolar disorder and major depression) on each gel. Samples were run in duplicate. Samples were electrophoresed for 15 min at 75 V, followed by 55 min at 150 V. Samples were then electroblotted onto nitrocellulose membranes for 2 h at 300 mAmp at 4 °C. Blots were blocked with 0.2% I-Block (Tropix, Bedford, MA, USA) in phosphate-buffered saline with 0.3% Tween 20 for 1 h at room temperature (RT), followed by an overnight incubation in primary antibodies at 4 °C. The primary antibodies used were anti-GABRθ

Table 1 Demographic information for the four diagnostic groups from Stanley Medical Research Institute

	Bipolar	*Control*	*Schizophrenia*	*Depression*	*F or* χ^2	P
Age	42.33 (11.72)	46.64 (9.46)	44.53 (13.11)	46.57 (9.66)	0.49[a]	0.69
Sex	6F, 9M	5F, 9M	6F, 9M	6F, 8M	0.15[b]	0.99
Race	14W, 1B	13W, 1B	12W, 3A	14W	14.12[b]	0.12
PMI	32.53 (16.12)	24.5 (9.85)	33.67 (14.62)	27.57 (11.13)	1.51[a]	0.22
pH	6.18 (0.23)	6.26 (0.25)	6.16 (0.26)	6.18 (0.23)	0.51[a]	0.68
Side of brain	7L, 8R	7L, 7R	9L, 6R	9L, 5R	1.20[b]	0.75
Brain weight	1441.2 (171.5)	1511 (165.4)	1471.7 (108.2)	1443.57 (127.56)	0.71[a]	0.55
Family hx	0.93 (0.8)	0.13 (0.52)	1.13 (0.83)	0.73 (0.46)	27.19[b]	**0.0001**
Suicidal death	9 (5 Violent)	0	6 (2 Violent)	9 (4 Violent)	14.04[b]	**0.029**
Drug/Alc hx	0.8 (0.77)	0.36 (0.74)	0.53 (0.74)	0.43 (0.64)	5.08[b]	0.45
Age of onset	21.47 (8.35)	—	23.2 (7.96)	33.36 (13.68)	3.63[a]	**0.001**
Duration of illness	20.13 (9.67)	—	21.67 (11.24)	12.29 (11.37)	2.47[a]	**0.018**
Severity of substance abuse	1.93 (1.98)	0.14 (0.54)	1.20 (1.86)	1.15 (2.04)	22.21[b]	0.10
Severity of alcohol abuse	2.27 (1.98)	1.14 (1.03)	1.47 (1.59)	1.93 (2.02)	9.52[b]	0.85
Fluphenazine (lifetime)	20 826.67 (24 015.96)	—	52 266.67 (62 061.57)	—	6.21[a]	**0.019**

[a]ANOVA. [b]χ^2 test.
Bold values indicate significant ($P<0.05$) values.

Table 2 Demographic information for the three diagnostic groups from the McLean 74 **Cohort**

	Bipolar	*Control*	*Schizophrenia*	*F,* t *or* χ^2	P
Age	61.75 (19.20)	58.59 (15.12)	60.71 (12.07)	0.24[a]	0.79
Sex	4M:15F	17M:12F	14M:6F	10.38[b]	**0.006**
PMI	22.25 (5.39)	21.76 (3.85)	23.86 (7.20)	0.92[a]	0.41
pH	6.45 (0.76)	6.45 (0.17)	6.49 (0.30)	0.07[a]	0.93
Side of brain	10L:9R	14L:15R	10L:10R	0.087[b]	0.96
Suicidal death	4 (1 violent)	0	0	10.96[b]	**0.027**
Drug/Alc hx	0.42 (0.51)	0.69 (0.66)	0.30 (0.47)	6.84[b]	0.15
Severity of substance abuse	0.58 (1.39)	0.41 (0.95)	0.70 (1.45)	0.33[b]	0.72
Severity of alcohol abuse	0.95 (1.43)	0.86 (1.73)	0.60 (1.23)	0.39[b]	0.75
Age of onset	23.53 (7.67)	—	20.52 (3.52)	1.45[c]	0.16
Duration of illness	39.69 (18.27)	—	40.18 (12.09)	0.091[c]	0.93
Use of MS	9	—	1	9.17[b]	**0.002**

Abbreviation: MS, mood stabilizer.
[a]ANOVA. [b]χ^2 test. [c]*t*-test for bipolar versus schizophrenia.
Bold values indicate significant ($P<0.05$) values.

(ab49188, Abcam (Cambridge, MA, USA) 1:1000), anti-GABRρ2 (ab83223, Abcam, 1:500), anti-FMRP (MAB2160, Millipore (Temecula, CA, USA), 1:500), anti-mGluR5 (ab53090, Abcam, 1:300), anti-NSE (1:2000; Abcam) and anti-β actin (A5441, Sigma Aldrich (St Louis, MO, USA), 1:5 000). Blots were washed for 30 min in phosphate-buffered saline supplemented with 0.3% Tween 20 (PBST) for 30 min at RT, and were subsequently incubated in the proper secondary antibodies. Secondary antibodies were goat anti-mouse IgG (A9044, Sigma Aldrich, 1:80 000) and goat anti-rabbit IgG (A9169, Sigma Aldrich, 1:80 000). Blots were washed twice in PBST for 15 min each. Following the second wash, bands were visualized using the ECL-plus detection system (GE Healthcare, Buckinghamshire, UK) and exposed to CL-Xposure film (Thermo Scientific, Rockford, IL, USA). The molecular weights of ~224 (dimer) and 112 kDa (monomer) for mGluR5; 73 kDa (FMRP); 70 kDa (GABRθ); 54 kDa (GABRρ2); 46 kDa (NSE) and 42 kDa (β-actin) immunoreactive bands were quantified with background subtraction using a Bio-Rad GS-800 Calibrated Densitometer (Bio-Rad, Hercules, CA, USA) and Quantity One 1-D Analysis software (Bio-Rad). Sample densities were analyzed blind to nature of diagnosis. Results obtained are based on at least two independent experiments.

Quantitative real-time polymerase chain reaction (qRT-PCR). We performed qRT-PCR as previously described.[28] Raw data were analyzed as previously described[28] using the Sequence Detection Software RQ Manager (ABI, Foster City, CA, USA), whereas relative quantitation using the comparative threshold cycle (C_T method) was performed in Bioconductor using the ABqPCR package in Microsoft Excel (ABI Technote no. 2: Relative Gene Expression Quantitation). Calculations were done assuming that 1 delta C_T equals a two-fold difference in expression. Significance values were determined using unpaired *t*-tests. The probe IDs used were: (1) $GABA_A$ receptor theta (*GABRQ*): Hs00610921_m1; (2) $GABA_A$ receptor rho 2 (*GABRR2*): Hs00266703_m1; (3) fragile X mental retardation 1 (*FMR1*): Hs00924547_m1; (4) metabotropic glutamate 5 (*GRM5*): Hs00168275_m1; (5) beta actin: Hs99999903_m1 and (6) glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*): Hs99999905_m1.

Statistical analysis. All protein measurements for each group were normalized against β-actin and NSE, and expressed as ratios of GABRθ/β-actin, GABRρ2/β-actin, FMRP/β-actin, mGluR5/β-actin, GABRθ/NSE, GABRρ2/NSE, FMRP/NSE and mGluR5/NSE. Statistical analysis was performed as previously described,[18,23,25] with $P<0.05$ considered significant. Group comparisons were conducted using analysis of variance (ANOVA). Follow-up independent *t*-tests were then conducted if the results were significant. Group differences on possible confounding factors were explored using χ^2 tests for categorical variables, and ANOVA for continuous variables. Where group differences were found, analysis of covariance was used to explore these effects on group differences for continuous variables, and factorial ANOVA with interaction terms for categorical variables. All analyses were conducted using SPSS v.17 (SPSS, Chicago, IL, USA).

Results

Western blotting results for GABRθ, GABRρ2, FMRP and mGluR5 in the lateral cerebellum. All protein measurements were normalized against β-actin or NSE. In lateral cerebella, ANOVA identified group differences for GABRθ/β-actin ($F(3,48)=5.49$, $P<0.003$), GABRθ/NSE ($F(3,48)=5.61$, $P<0.002$), GABRρ2/β-actin ($F(3,40)=2.90$, $P<0.047$), GABRρ2/NSE ($F(3,40)=3.05$, $P<0.039$), mGluR5 monomer/β-actin ($F(3,46)=4.15$, $P<0.011$) and mGluR5 monomer/NSE ($F(3,46)=5.18$, $P<0.004$) (Figure 1; Table 3). There was a group difference for FMRP/NSE ($F(3,50)=4.93$, $P<0.004$) (Figure 1; Table 3).

Follow-up *t*-tests found significant reductions in protein for GABRθ/β-actin ($P<0.001$), GABRθ/NSE ($P<0.001$), mGluR5 monomer/β-actin ($P<0.050$), mGluR5 monomer/NSE ($P<0.030$) and FMRP/NSE ($P<0.001$) in subjects with schizophrenia (Table 3; Figures 2 and 3). In lateral cerebella from subjects with bipolar disorder, there were significant reductions in GABRθ/β-actin ($P<0.012$), GABRθ/NSE ($P<0.005$), mGluR5 monomer/β-actin ($P<0.001$), mGluR5 monomer/NSE ($P<0.004$) and FMRP/NSE ($P<0.003$), and significant increased expression of GABRρ2/β-actin ($P<0.0044$) and GABRρ2/NSE ($P<0.009$) (Table 3;

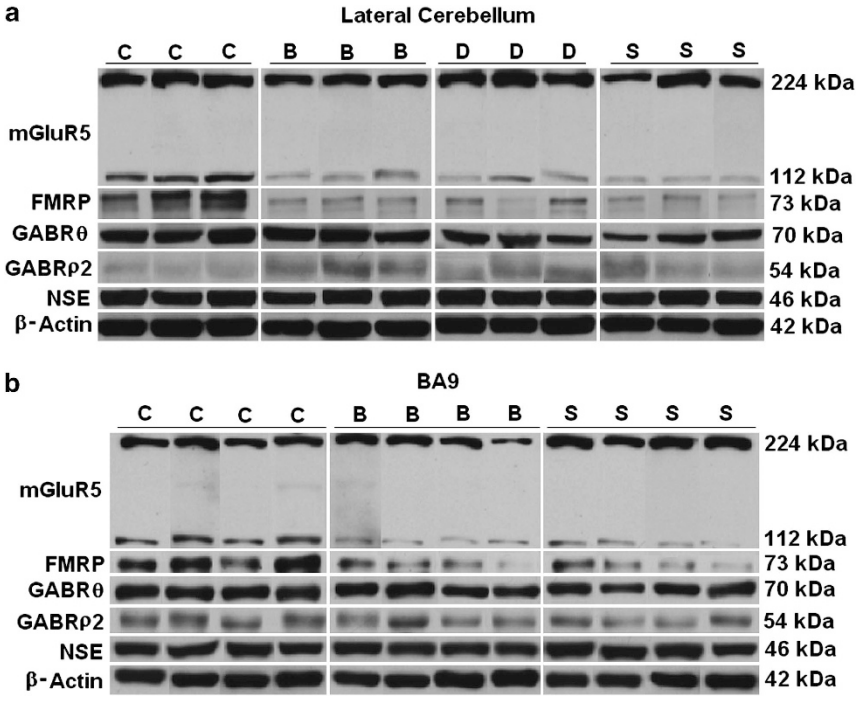

Figure 1 Representative bands for mGluR5, FMRP, GABRθ, GABRρ2, NSE and β-actin in lateral cerebellum (**a**) and BA9 (**b**) of subjects with schizophrenia and mood disorders. B, bipolar disorder; C, control; D, major depression; S, schizophrenia. FMRP and β-actin images for lateral cerebellum reprinted from Fatemi *et al.*,[18] with permission from Elsevier.

Table 3 Western blotting results for FMRP, GABRθ, mGluR5 and GABRρ2 values expressed as ratios to β-actin and neuronal-specific enolase (NSE) in lateral cerebellum

	ANOVA		*Control*		*Schizophrenia*		*Bipolar disorder*		*Major depression*	
	F	P	*Protein*	P	*Protein*	P	*Protein*	P	*Protein*	P
GABRθ/β-actin	5.49	**0.003**	0.425 ± 0.105	RG	0.231 ± 0.136	**0.001**	0.297 ± 0.090	**0.012**	0.301 ± 0.138	**0.014**
GABRρ2/β-actin	2.90	**0.047**	0.023 ± 0.013	RG	0.048 ± 0.042	NC	0.047 ± 0.014	**0.0044**	0.058 ± 0.038	**0.0085**
mGluR5 dimer/β-actin	2.14	NC	0.176 ± 0.111	RG	0.125 ± 0.10	NC	0.127 ± 0.101	NC	0.211 ± 0.064	NC
mGluR5 monomer/β-actin	4.15	**0.011**	0.038 ± 0.030	RG	0.015 ± 0.023	**0.05**	0.0086 ± 0.0098	**0.001**	0.019 ± 0.020	NC
FMRP/β-actin[a]	6.22	**0.001**	0.070 ± 0.052	RG	0.017 ± 0.029	**0.039**	0.029 ± 0.033	**0.014**	0.023 ± 0.021	**0.005**
β-actin	0.83	NC	25.8 ± 2.07	RG	25.2 ± 2.07	NC	26.9 ± 2.91	NC	26.1 ± 4.38	NC
GABRθ/NSE	5.61	**0.002**	0.87 ± 0.18	RG	0.48 ± 0.30	**0.001**	0.58 ± 0.20	**0.005**	0.62 ± 0.24	**0.012**
GABRρ2/NSE	3.05	**0.039**	0.045 ± 0.025	RG	0.1 ± 0.094	NC	0.096 ± 0.052	**0.009**	0.13 ± 0.085	**0.006**
mGluR5 dimer/NSE	1.99	NC	0.32 ± 0.20	RG	0.26 ± 0.23	NC	0.26 ± 0.22	NC	0.44 ± 0.12	NC
mGluR5 monomer/NSE	5.18	**0.004**	0.08 ± 0.48	RG	0.032 ± 0.049	**0.03**	0.017 ± 0.021	**0.004**	0.041 ± 0.041	**0.047**
FMRP/NSE	4.93	**0.004**	0.092 ± 0.07	RG	0.023 ± 0.04	**0.001**	0.042 ± 0.05	**0.003**	0.033 ± 0.033	**0.001**
NSE	0.93	NC	20.2 ± 3.34	RG	20.1 ± 1.67	NC	20.2 ± 3.24	NC	21.6 ± 2.87	NC

Abbreviations: ANOVA, analysis of variance; NC, no change; RG, reference group.
[a]FMRP data reprinted from Schizophrenia Research, 124(1–3):246-247, Fatemi, S.H., Kneeland, R.E., Liesch, S.B., Folsom, T.D., Fragile X mental retardation protein levels are decreased in major psychiatric disorders, page 246, Copyright (2010), with permission from Elsevier.
Bold values indicate significant ($P<0.05$) values.

Figures 2 and 3). In subjects with major depression, follow-up *t*-tests found significant reductions in GABRθ/β-actin ($P<0.014$), GABRθ/NSE ($P<0.012$), mGluR5 monomer/NSE ($P<0.047$) and FMRP/NSE ($P<0.001$), and significantly increased expression of GABRρ2/β-actin ($P<0.0085$) and GABRρ2/NSE ($P<0.006$) (Table 3; Figures 2 and 3). There were no significant changes in protein levels of mGluR5 dimer in lateral cerebella.

Western blotting results for GABRθ, GABRρ2, FMRP and mGluR5 in BA9. In BA9, ANOVA identified group differences for GABRθ/β-actin ($F(2,64)=4.04$, $P<0.022$), GABRθ/NSE ($F(2,62)=4.54$, $P<0.014$), mGluR5 monomer/β-actin ($F(2,63)=10.72$, $P<0.001$), mGluR5 monomer/NSE ($F(2,63)=8.14$, $P<0.001$), FMRP/β-actin ($F(2,59)=3.85$, $P<0.027$) and FMRP/NSE ($F(2,60)=4.26$, $P<0.019$) (Figure 1; Table 4). Follow-up *t*-tests found significant reductions of GABRθ/β-actin, mGluR5 monomer/β-actin and FMRP/β-actin ($P<0.017$, $P<0.001$ and $P<0.018$, respectively), and GABRθ/NSE, mGluR5 monomer/NSE and FMRP/NSE ($P<0.019$, $P<0.003$ and $P<0.029$, respectively) in BA9 of subjects with schizophrenia (Table 4, Figures 4 and 5). In subjects with bipolar disorder, follow-up *t*-tests found significant reductions in GABRθ/β-actin,

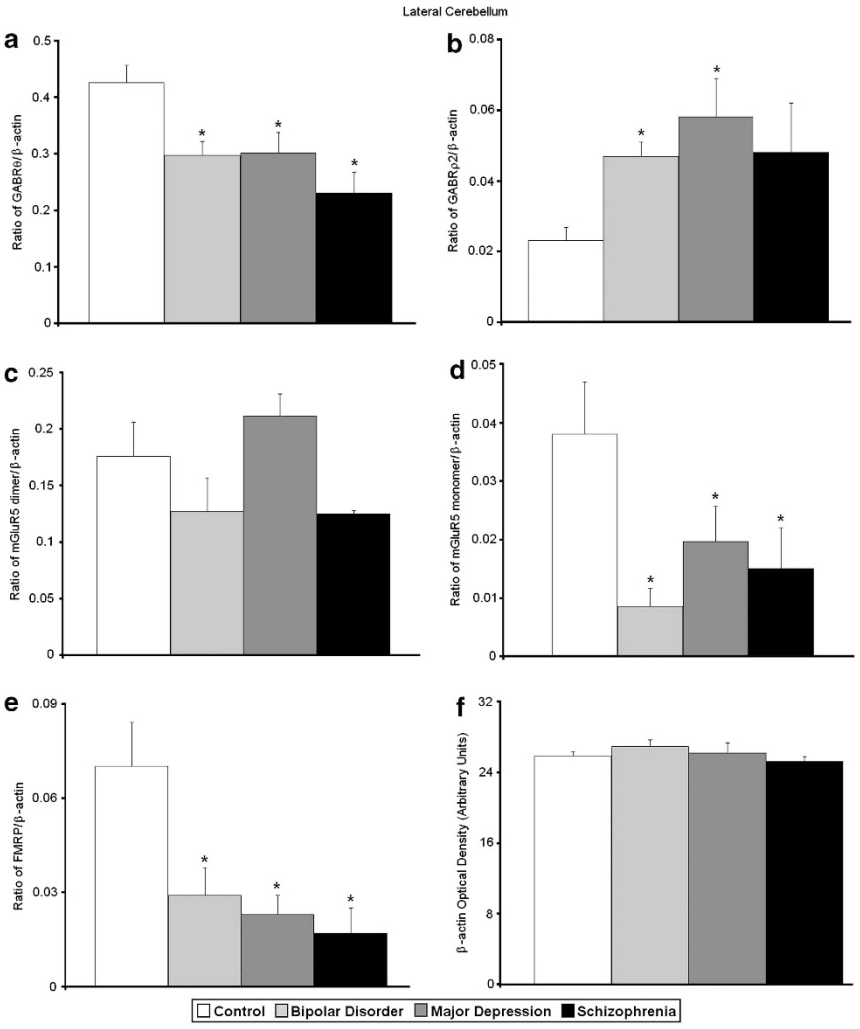

Figure 2 Expression of GABRθ/β-actin (**a**), GABRρ2/β-actin (**b**), mGluR5 dimer/β-actin (**c**), mGluR5 monomer/β-actin (**d**), FMRP/β-actin (**e**), and β-actin (**f**) in lateral cerebella of healthy controls versus subjects with bipolar disorder, major depressive disorder and schizophrenia. Histogram bars shown as mean ± s.e., $^{*}P<0.05$. FMRP and β-actin data reprinted from Fatemi *et al.*,[18] with permission from Elsevier.

mGluR5 monomer/β-actin and FMRP/β-actin ($P<0.024$, $P<0.001$ and $P<0.030$, respectively), and GABRθ/NSE, mGluR5 monomer/NSE and FMRP/NSE ($P<0.011$, $P<0.001$ and $P<0.011$, respectively) (Table 4; Figures 4 and 5). There were no significant changes in protein levels for GABRρ2 or mGluR5 dimer in BA9.

Analysis of confounds for protein data in lateral cerebellum and BA9. In the analysis of protein data from lateral cerebella, no significant differences were found between groups on hemisphere side, ethnicity, gender, history of substance abuse, severity of alcohol abuse or substance abuse, post-mortem interval, age, pH or brain weight (Table 1). We also compared the groups on family history and suicide, and found significant differences ($P<0.0001$ and $P<0.029$, respectively), but further analysis revealed that these factors had no significant impact on any of the results. We did find that subjects with schizophrenia and bipolar disorder had significantly longer duration of illness than did those with depression ($t(47)=2.47$, $P<0.018$). Age of onset was significantly later for subjects with major depression compared to subjects with schizophrenia or bipolar disorder ($t(41)=3.63$, $P<0.001$). ANOVA controlling for age of onset and duration of illness did find that subjects with depression displayed significantly higher mGluR5 dimer/NSE ($F(2,31)=4.31$, $P<0.02$) and mGluR5 dimer/β-actin ($F(2,31)=4.31$, $P<0.02$) than subjects with bipolar disorder or schizophrenia while controlling for duration. However, as mGluR5 dimer values did not change significantly between the groups, this finding is not meaningful.

For protein data from BA9, no significant differences were found between diagnostic groups on hemisphere side, history of substance abuse, severity of alcohol abuse or substance abuse, post-mortem interval, age or pH (Table 2). Nor did we find significant differences on use of barbiturates, opiates, amphetamines, cocaine or propoxyphene. We also compared subjects with schizophrenia versus subjects with bipolar disorder on disease duration, age of onset, use of antipsychotic, antidepressant and anticonvulsant medications,

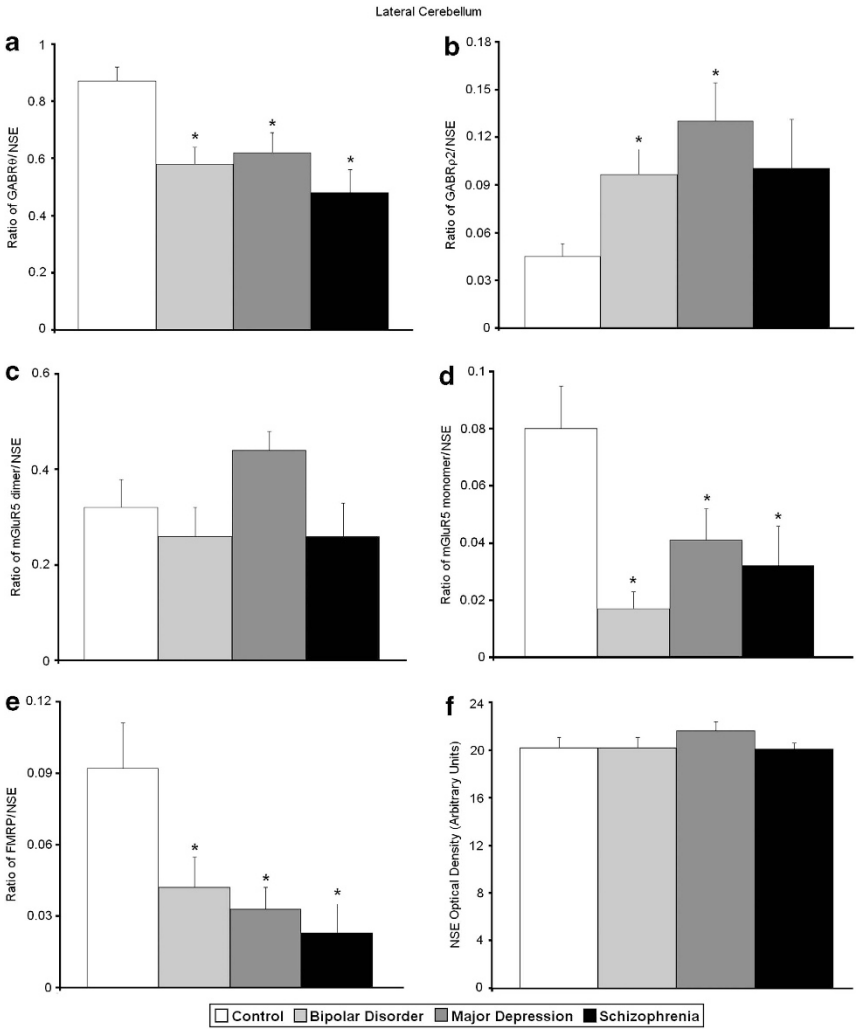

Figure 3 Expression of GABRθ/NSE (**a**), GABRρ2/NSE (**b**), mGluR5 dimer/NSE (**c**), mGluR5 monomer/NSE (**d**), FMRP/NSE (**e**), and NSE (**f**) in lateral cerebella of healthy controls versus subjects with bipolar disorder, major depressive disorder and schizophrenia. Histogram bars shown as mean ± s.e., $^*P<0.05$.

Table 4 Western blotting results for FMRP, GABRθ, mGluR5 and GABRρ2 values expressed as ratios to β-actin and neuronal-specific enolase (NSE) in BA9

	ANOVA		*Control*		*Schizophrenia*		*Bipolar disorder*	
	F	P	*Protein*	P	*Protein*	P	*Protein*	P
GABRθ/β-actin	4.04	**0.022**	1.34 ± 0.40	RG	1.06 ± 0.32	**0.017**	1.07 ± 0.44	**0.024**
GABRρ2/β-actin	0.38	NC	0.23 ± 0.17	RG	0.21 ± 0.14	NC	0.26 ± 0.23	NC
mGluR5 dimer/β-actin	1.39	NC	0.78 ± 0.38	RG	0.57 ± 0.42	NC	0.70 ± 0.49	NC
mGluR5 monomer/β-actin	10.72	**0.001**	0.19 ± 0.13	RG	0.089 ± 0.058	**0.001**	0.071 ± 0.042	**0.001**
FMRP/β-actin	3.85	**0.027**	0.81 ± 0.40	RG	0.54 ± 0.40	**0.018**	0.56 ± 0.29	**0.030**
β-actin	0.19	NC	7.06 ± 1.68	RG	7.31 ± 1.23	NC	7.29 ± 1.82	NC
GABRθ/NSE	4.54	**0.014**	1.51 ± 0.40	RG	1.23 ± 0.40	**0.019**	1.20 ± 0.36	**0.011**
GABRρ2/NSE	0.54	NC	0.26 ± 0.20	RG	0.23 ± 0.14	NC	0.30 ± 0.27	NC
mGluR5 dimer/NSE	0.66	NC	0.90 ± 0.59	RG	0.72 ± 0.67	NC	0.74 ± 0.50	NC
mGluR5 monomer/NSE	8.14	**0.001**	0.22 ± 0.17	RG	0.11 ± 0.07	**0.003**	0.086 ± 0.052	**0.001**
FMRP/NSE	4.26	**0.019**	1.05 ± 0.55	RG	0.71 ± 0.50	**0.029**	0.64 ± 0.36	**0.011**
NSE	0.01	NC	6.48 ± 1.79	RG	6.54 ± 2.27	NC	6.44 ± 2.61	NC

Abbreviations: ANOVA, analysis of variance; NC, no change; RG, reference group.
Bold values indicate significant ($P<0.05$) values.

and found no significant differences. We did find that 21.1% of bipolar patients died by suicide versus none for the other diagnostic groups ($\chi^2(2)=10.96$, $P<0.027$). We also found that there were significantly more female subjects ($\chi^2(2)=10.38$, $P<0.006$) in the bipolar group (78.9%) than in either normal controls (41.4%) or subjects with

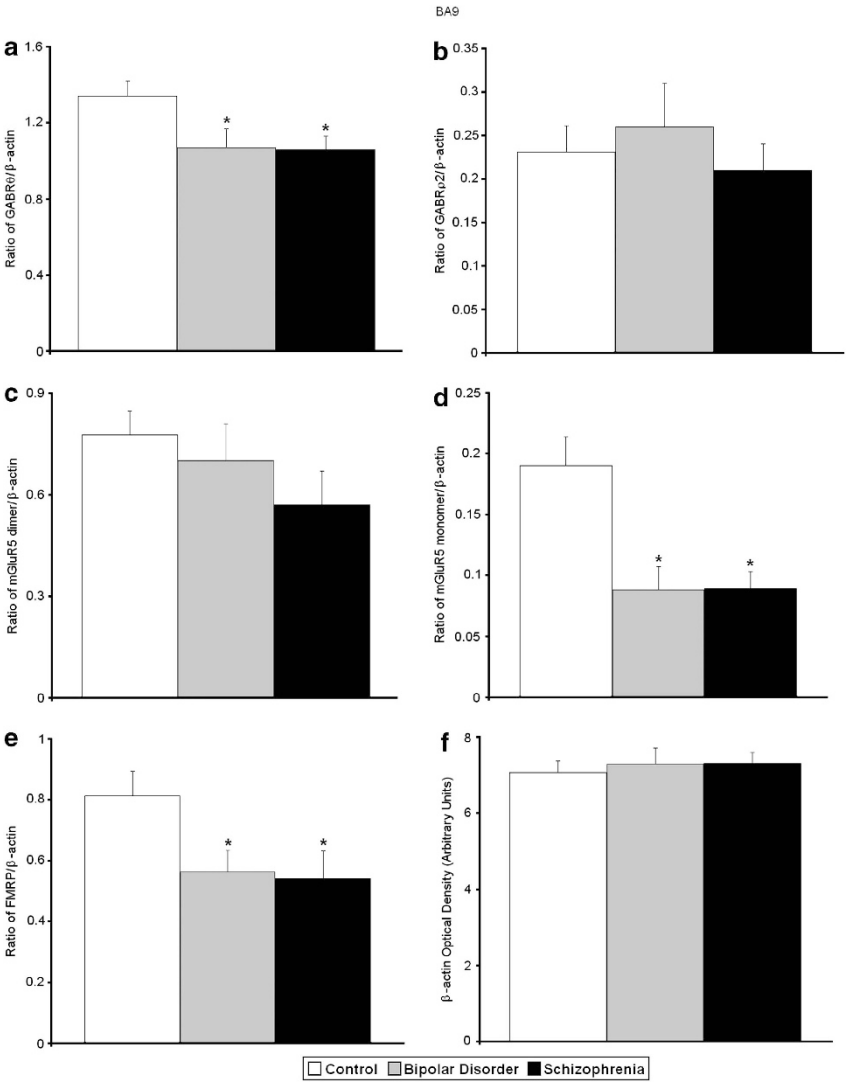

Figure 4 Expression of GABRθ/β-actin (**a**), GABRρ2/β-actin (**b**), mGluR5 dimer/β-actin (**c**), mGluR5 monomer/β-actin (**d**), FMRP/β-actin (**e**) and β-actin (**f**) in BA9 of healthy controls versus subjects with bipolar disorder and schizophrenia. Histogram bars shown as mean ± s.e., $^*P<0.05$.

schizophrenia (30%). We also found higher rates of mood stabilizer use in patients with bipolar disorder (47.4%) than in patients with schizophrenia (5%) ($\chi^2(1)=9.17$, $P<0.002$). Further analyses controlling for gender, mood stabilizer use and suicide found the initial differences on outcome measures as a function of diagnostic groups to be unchanged.

qRT-PCR results for GABRθ, GABRρ2, FMRP and mGluR5 in lateral cerebellum and BA9. For qRT-PCR experiments, all values were normalized against both β-actin and GAPDH, and these values were averaged. In the lateral cerebella, ANOVA identified group differences for *GABRQ* (GABRθ; $P<0.046$), *GABRR2* (GABRρ2; $P<0.017$) and *GRM5* (mGluR5; $P<0.034$) (Table 5). There were significantly reduced mRNA values for *GABRQ* ($P<0.016$) and *GRM5* ($P<0.039$) in the lateral cerebella of subjects with schizophrenia (Table 5), similar to protein changes in the same region. *GABRR2* mRNA was significantly increased ($P<0.019$) in the lateral cerebella in subjects with bipolar disorder, mirroring similar changes in protein levels, and *GRM5* mRNA expression was significantly reduced ($P<0.009$) in subjects with major depression (Table 5). In BA9, ANOVA identified group differences for *GABRR2* ($P<0.003$) and *GRM5* ($P<0.048$) (Table 5). In BA9 of subjects with schizophrenia, there was significantly increased mRNA for *GABRQ* ($P<0.03$). In BA9 of subjects with bipolar disorder, there was significantly increased mRNA for *GABRR2* ($P<0.0001$) and significantly reduced mRNA for *GRM5* ($P<0.04$), similar to changes in mGluR5 protein levels in the same region (Table 5). FMR1 mRNA values did not show significant changes in either of the brain areas (Table 5).

Discussion

The current studies demonstrate abnormal processing of mRNA and protein expression for two novel $GABA_A$

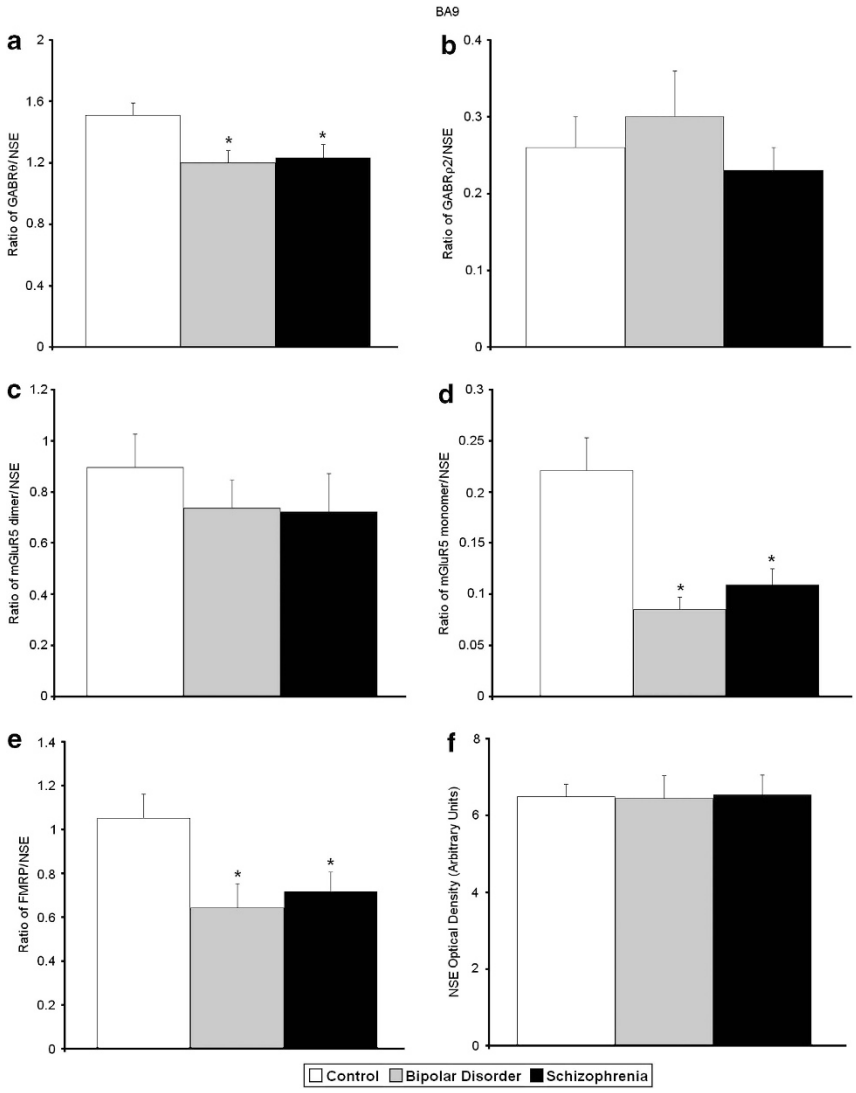

Figure 5 Expression of GABRθ/NSE (**a**), GABRρ2/NSE (**b**), mGluR5 dimer/NSE (**c**), mGluR5 monomer/NSE (**d**), FMRP/NSE (**e**) and NSE (**f**) in BA9 of healthy controls versus subjects with bipolar disorder and schizophrenia. Histogram bars shown as mean ± s.e., $^{*}P<0.05$.

receptors, θ and ρ2, as well as FMRP and mGluR5 in lateral cerebella and BA9 of subjects with schizophrenia and mood disorders. The most salient results included: (1) FMRP protein levels were significantly decreased in all the brain sites in schizophrenia, bipolar disorder and major depression; (2) mGluR5 protein levels were significantly reduced in all the brain sites in schizophrenia and bipolar disorder; (3) mRNA levels for mGluR5 were significantly reduced in lateral cerebellum of subjects with schizophrenia and major depression, and BA9 of subjects with bipolar disorder; (4) Protein levels for GABRθ were reduced significantly in all the brain sites in schizophrenia, bipolar disorder and major depression; (5) mRNA levels for GABRθ were elevated significantly in BA9 of subjects with schizophrenia, in contrast mRNA for the same receptor was decreased significantly in lateral cerebellum of subjects with schizophrenia; (6) Protein levels for GABRρ2 were increased significantly in lateral cerebellum of subjects with bipolar disorder and major depression; simultaneously, mRNA for the same receptor was also increased significantly in all the brain sites in subjects with bipolar disorder.

The GABRθ gene (*GABRQ*) is clustered with $GABA_A$ receptor epsilon (*GABRE*) and $GABA_A$ receptor alpha 3 (*GABRA3*) at Xq28.[29] In rat, GABRθ mRNA has been shown embryonically (E17/E19) to localize to the hypothalamus, tegmentum, pontine nuclei and medulla, suggesting a possible role in midbrain development.[30] GABRθ mRNA is expressed in multiple brain regions in human including amygdala, dorsal raphe, hippocampus, hypothalamus, locus coeruleus and substantia nigra.[31] The locus coeruleus is relevant to psychiatric disorders, as it is the largest noradrenergic nucleus and has important roles in the regulation of anxiety states, vigilance, attention and memory functions.[32] GABRθ forms a functional receptor when coexpressed with alpha, beta and gamma subunits.[31] The functional properties of $GABA_A$ receptors that include the θ subunit have not been well characterized.[30]

Table 5 qRT-PCR results for GABRQ, GABRR2, GRM5 and FMR1 in lateral cerebella, and BA9 of subjects with schizophrenia and mood disorders

Lateral cerebellum		*Schizophrenia*		*Bipolar disorder*		*Major depression*	
Gene	*ANOVA*	*Fold change*	P	*Fold change*	P	*Fold change*	P
GABRQ	**0.046**	0.64	**0.016**	1.25	0.47	0.730	0.11
GABRR2	**0.017**	1.33	0.11	1.58	**0.019**	1.019	0.91
GRM5	**0.034**	0.49	**0.039**	0.80	0.39	0.530	**0.009**
FMR1	0.099	0.65	0.16	1.06	0.85	0.606	0.052
BA9		*Schizophrenia*		*Bipolar disorder*		*Major depression*	
Gene	*ANOVA*	*Fold change*	P	*Fold change*	P		
GABRQ	0.075	1.43	**0.03**	1.03	0.78	NTA	
GABRR2	**0.003**	1.21	0.32	2.10	**0.0001**		
GRM5	**0.048**	1.05	0.68	0.77	**0.04**		
FMR1	0.705	0.88	0.31	0.96	0.68		

Abbreviations: ANOVA, analysis of variance; FMR1, fragile X mental retardation 1; NTA, no tissue available; qRT-PCR, quantitative real-time polymerase chain reaction.
Note: for lateral cerebella, ANOVA is based on six comparisons: C versus S, C versus B, C versus D, S versus B, S versus D and B versus D.
For BA9, ANOVA based on three comparisons: C versus S, C versus B and S versus B.
Bold values indicate significant ($P<0.05$) values.

Recent studies have investigated possible associations of the gene that codes for GABRθ (*GABRQ*) with multiple disorders.[33–36] However, single-nucleotide polymorphisms of GABRQ were not associated with susceptibility to bipolar disorder,[33–34] migraine[35] or essential tremor.[36] However, the GABRQ-478F allele showed an association with the improvement of tremor with ethanol use among men.[36]

The levels of GABRθ receptor protein are reduced significantly in both BA9 and lateral cerebellum of the subjects with schizophrenia (Figure 6). In contrast, mRNA for GABRθ receptor is downregulated in the lateral cerebella, whereas in BA9 its mRNA is upregulated (Figure 6). As both mRNA and protein are concordantly downregulated in the lateral cerebellum, a severe chronic receptor deficit may be responsible for our observed results; while in BA9, increased mRNA expression may be a compensatory response to chronic receptor downregulation, suggesting that different mechanisms may be at work (Figure 6).

In subjects with bipolar disorder, GABRθ receptor protein is reduced in the lateral cerebellum while its mRNA is upregulated (Figure 7). By the same token, protein for this receptor is downregulated in BA9, with its mRNA level unchanged (Figure 7). Here, the mechanisms for these alterations may again be different in the two brain sites. In lateral cerebellum, chronic GABRθ protein downregulation could lead to upregulation of its mRNA in a feedback loop. In BA9, normal mRNA levels with decreased protein levels indicate a defective step either in processing of protein in rough endoplasmic reticulum or subsequent cell compartments (such as Golgi or secretory granules), leading to reduced protein production (Figure 7).

In subjects with major depression, although protein levels for GABRθ are reduced significantly in lateral cerebellum, its mRNA levels remain normal (Figure 8). This scenario again indicates that the deficit lies at rough endoplasmic reticulum or a subsequent cellular compartment causing the chronic receptor protein downregulation (Figure 8). In the absence of available BA9 tissue, the fate of GABRθ in major depression will await future determinations.

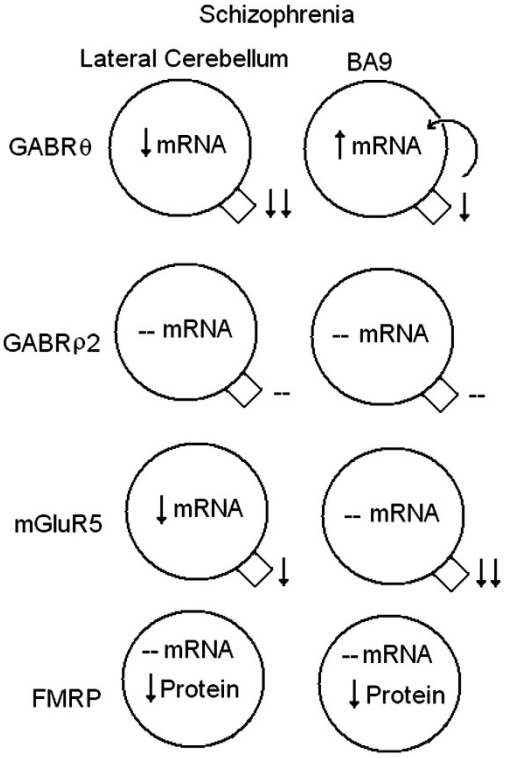

Figure 6 Summary of mRNA and protein expression for GABRθ, GABRρ2, mGluR5 and FMRP in lateral cerebella and BA9 of subjects with schizophrenia. Concordant results for mRNA and protein were obtained for GABRθ and mGluR5 in lateral cerebellum. Decreased expression of GABRθ protein in BA9 may lead to a positive feedback loop, increasing mRNA expression. Protein levels for mGluR5 and FMRP were reduced significantly in both brain sets. ↑, increased expression; ↓, reduced expression, –, no change.

The gene that codes for GABRρ2 (*GABRR2*) is localized to 6q15.[37] GABRρ2 mRNA is widely distributed in the brain, including prefrontal cortex, hippocampus and cerebellum.[38,39] In adult rat cerebellum, GABRρ2 has been localized to

Purkinje cells and basket-like cells only.[40] GABRρ2 has been shown to associate with α1 and γ2 receptor subunits.[41] In cerebellum, $GABA_A$ receptors that include the ρ2 subunit help mediate a component of phasic inhibitory GABAergic transmission at interneuron–Purkinje cell synapses.[42]

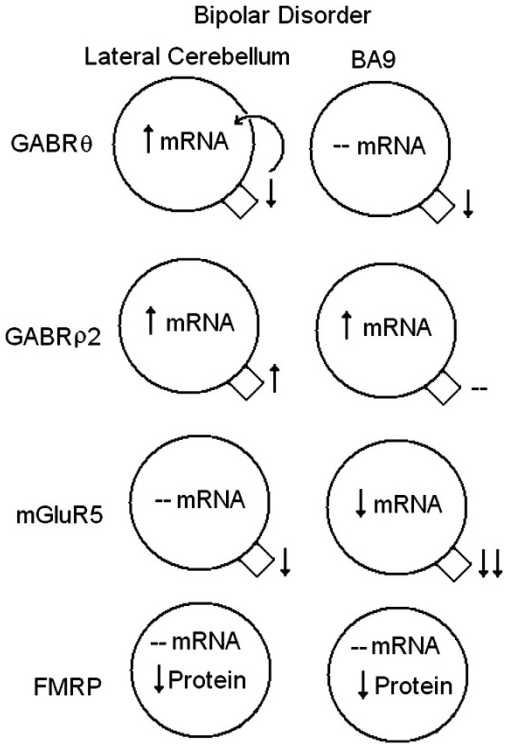

Figure 7 Summary of mRNA and protein expression for GABRθ, GABRρ2, mGluR5 and FMRP in lateral cerebella and BA9 of subjects with bipolar disorder. Concordant results for mRNA and protein were obtained for GABRρ2 in lateral cerebellum. Decreased expression of GABRθ protein in lateral cerebellum may lead to a positive feedback loop, increasing mRNA expression. Protein levels for GABRθ, mGluR5 and FMRP were decreased significantly in both brain sites. ↑, increased expression; ↓, reduced expression, –, no change.

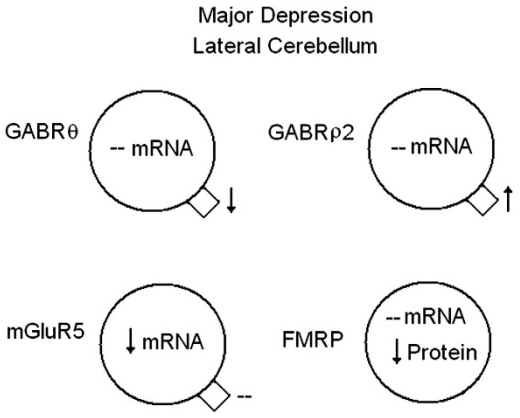

Figure 8 Summary of mRNA and protein expression for GABRθ, GABRρ2, mGluR and FMRP in lateral cerebella of subjects with major depression. There were no concordant results in subjects with major depression. However, protein levels for GABRθ and FMRP were reduced significantly, whereas it increased for GABRρ2 in major depression. ↑, increased expression; ↓, reduced expression, –, no change.

A recent study has demonstrated an association between an single-nucleotide polymorphism of *GABRR2* (GABRρ2) (rs1570932) and a component of the bipolar phenotype, namely bipolar patients with psychotic symptoms, similar to those experienced by subjects with schizophrenia.[33] A second study found an single-nucleotide polymorphism (rs12201676) associated with bipolar disorder that is flanked by *GABRR1* (15 kb away) and *GABRR2* (17 kb away) genes.[43] *GABRR2* has also been associated with alcoholism.[44]

Levels of GABRρ2 mRNA and protein did not change in lateral cerebellum or BA9 of subjects with schizophrenia (Figure 6). However, in subjects with bipolar disorder, a concordant and significant increase was observed in mRNA and protein levels of GABRρ2 in lateral cerebellum, indicating chronic upregulation in gene and protein product in this disorder (Figure 7). Interestingly, in BA9 of bipolar subjects, GABRρ2 mRNA levels were also elevated significantly, but no protein change was observed (Figure 7). In major depression, GABRρ2 protein levels were also elevated but without any change in mRNA, indicating abnormalities in processing GABRρ2 protein in rough endoplasmic reticulum compartment or subsequent cellular stations (Figure 8). Thus, GABRρ2 changes were confined to brains of subjects with mood disorders and were not seen in schizophrenia and, at least in the case of bipolar disorder, reflect upregulation of GABRρ2 mRNA/protein.

The *FMR1* gene is located at Xq27.3. FMRP has been shown to localize in multiple regions of neurons, including the soma, dendrites, synaptic spines and the axon.[11,45,46] FMRP controls multiple post-transcriptional events, including splicing, nuclear export and translation.[46,47] FMRP protein is significantly downregulated in schizophrenia, bipolar disorder and major depression in lateral cerebellum,[18] and in BA9 for subjects with schizophrenia and bipolar disorder in the absence of any mRNA abnormalities (Figures 6–8). This

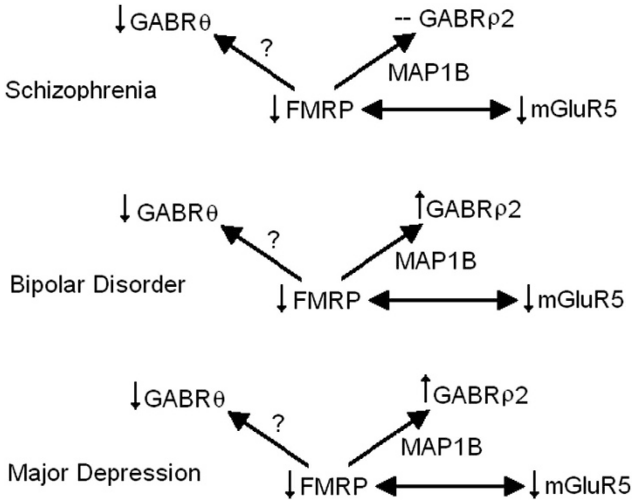

Figure 9 Summary of relationships between GABRθ, GABRρ2, mGluR5 and FMRP in three major psychiatric disorders: schizophrenia, bipolar disorder and major depression. Although there are clear biochemical connections between FMRP, mGluR5 and GABRρ2, no direct relationship can be established between GABRθ and FMRP. ↑, increased expression; ↓, reduced expression, –, no change.

picture is similar to what we have described in idiopathic cases of autism without evidence of any effects in the gene for FMRP, and thus replicative of the post-transcriptional abnormalities affecting protein synthesis (Figure 9). Changes in mRNA expression do not always correlate with similar changes in protein expression, including expression of FMRP. A recent study found that in subjects with the FMR1 premutation (expanded 5′ CGG repeat, but without full symptoms of FXS), there were both significantly increased FMR1 mRNA and significantly reduced levels of FMRP.[48] Similarly, the recent findings of Kovács *et al*.[21] demonstrated the downregulation of FMRP protein levels in the absence of any change in FMR1 mRNA or expansion of the 5′ CGG triplet repeat in peripheral blood lymphocytes of subjects with schizophrenia. As reduced FMRP expression has been identified in four major psychiatric disorders, identifying the post-transcriptional abnormalities that may be responsible for this reduction would have a major impact on the etiology and treatment of these disorders. Additionally, verification of reduced FMRP protein levels in peripheral blood lymphocytes of subjects with schizophrenia confirm our data at least in schizophrenia, and validate our additional new findings of reduced FMRP in BA9 of subjects with schizophrenia.

The gene for mGluR5 is located at 11q14.2-q14.3. mGluR5, like other metabotropic glutamate receptors contains seven membrane-spanning domains and a large extracellular N-terminus,[49] and are G-protein coupled. Metabotropic glutamate receptors are found throughout the CNS, with high concentrations in cerebral cortex, hippocampus, striatum, hypothalamus, midbrain, cerebellum, medulla and pons.[50] There were concordant and significant reductions in levels of mRNA and protein for mGluR5 in lateral cerebellum of subjects with schizophrenia (Figure 6). In BA9, despite significant reductions in protein levels, mRNA levels were normal (Figure 6). Thus, protein levels for mGluR5 were downregulated in both brain sites in schizophrenia. In a similar vein, mGluR5 protein levels were reduced significantly in both lateral cerebellum and BA9 in subjects with bipolar disorder despite normal mRNA levels, indicating post-transcriptional abnormalities in the pathway for mGluR5 protein synthesis (Figure 7). Interestingly, in lateral cerebellum of subjects with major depression, despite downregulated mRNA for mGluR5, the protein levels were normal (Figure 8). It is possible that unknown mechanisms affecting transit for protein rescue the product for release, despite low turnover for its mRNA, in major depression.

Recently, Matosin *et al*.[51] showed no significant alteration in mGluR5-binding density or mGluR5 protein levels in dorsolateral prefrontal cortex of subjects with schizophrenia. However, close inspection of their western blotting data showed highly oversaturated bands for the monomeric mGluR5 protein levels for both control and subjects with schizophrenia, potentially masking any differences between the two groups. Although several other reports did not show any change in mGluR5 protein[52,53] or mRNA[54–56] in prefrontal cortex of subjects with schizophrenia, these results could be due to the use of different analytic techniques or brain regional effects. However, other reports have reported the decreases in mGluR5 mRNA in prefrontal cortex of subjects with schizoaffective disorder[56] and in those with major depression,[57] supporting our current data showing significant decreases in mGluR5 protein levels in lateral cerebellum and BA9 of subjects with schizophrenia and bipolar disorder, and decreases in mRNA levels in lateral cerebella of subjects with schizophrenia and major depression, and BA9 of subjects with bipolar disorder. Additionally, we have previously observed increased expression of mGluR5 protein in BA9 and cerebellar vermis of children with autism (Figure 9).[22,23] However, although there is a great deal of overlap in the symptomologies of autism and FXS, there is less overlap between FXS and schizophrenia, and mood disorders.

Interactions between the aforementioned four proteins may alter GABAergic transmission. The cytoplasmic domains of GABRρ1 and GABRρ2 interact with MAP1B (Figure 9).[58] Disruption of ρ-MAP1B interactions leads to a doubling of the inward current of $GABA_C$ receptors from bipolar cells in retinal slices in the presence of low levels of GABA.[58] MAP1B mRNA is targeted by FMRP[10] (Figure 9), and in FMR1-knock out mice there is an abnormal upregulation of MAP1B.[59] With reduced expression of FMRP, one might speculate that there would be increased expression of MAP1B in subjects with schizophrenia and mood disorders. However, a preliminary study involving anterior cingulate cortex found reduced expression of MAP1B protein in subjects with bipolar disorder, with no change in subjects with schizophrenia or major depression.[60] Further experiments involving multiple brain sites would be required to see if this is a regional difference or if there is a global reduction. Altered expression of MAP1B could in turn cause changes in GABAergic neurotransmission through GABA receptors that contain ρ subunits. Currently, we do not know of a link between FMRP and GABRθ (Figure 9). No changes in GABRθ mRNA were identified among the $GABA_A$ receptor subunits that show reduced expression in animal models of FXS.[7–9]

In conclusion, FMRP is significantly downregulated in the lateral cerebella[18] and BA9 from subjects with schizophrenia, bipolar disorder and major depression, potentially causing GABA receptor changes and altered expression of mGluR5 in the three disorders in the absence of any FMR1 chromosomal abnormalities. Additionally, we have identified selective abnormalities in mRNA and protein levels of two novel $GABA_A$ receptors, namely GABRθ and GABRρ2, in subjects with schizophrenia and mood disorders. These changes could potentially explain changes in GABAergic transmission and consequent deficits associated with these disorders including anxiety, panic, and impaired learning and memory. Our results also identify potential novel targets for future pharmacologic intervention. Lastly, despite significance and novelty of these results, the study should be considered exploratory, requiring further future confirmation in other brain sites.

Conflict of interest

The authors declare no conflicts of interest. Dr Fatemi has patents related to Reelin as a marker of psychiatric disorders. He derives no income as yet from these patents.

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Disclaimer. NIMH had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report and in the decision to submit the paper for publication.

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