The Role of Reduced Expression of Fragile X Mental Retardation Protein in Neurons and Increased Expression in Astrocytes in Idiopathic and Syndromic Autism (Duplications 15q11.2-q13)

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Fragile X syndrome (FXS), caused by lack of fragile X mental retardation protein (FMRP), is associated with a high prevalence of autism. The deficit of FMRP reported in idiopathic autism suggests a mechanistic overlap between FXS and autism. The overall goal of this study is to detect neuropathological commonalities of FMRP deficits in the brains of people with idiopathic autism and with syndromic autism caused by dup15q11.2-q13 (dup15). This study tests the hypothesis based on our preliminary data that both idiopathic and syndromic autism are associated with brain region-specific deficits of neuronal FMRP and structural changes of the affected neurons. This immunocytochemical study revealed neuronal FMRP deficits and shrinkage of deficient neurons in the cerebral cortex, subcortical structures, and cerebellum in subjects with idiopathic and dup(15)/autism. Neuronal FMRP deficit coexists with surprising infiltration of the brains of autistic children and adults with FMRP-positive astrocytes known to be typical only for the fetal and short postnatal periods. In the examined autistic subjects, these astrocytes selectively infiltrate the border between white and gray matter in the cerebral and cerebellar cortex, the molecular layer of the cortex, part of the amygdala and thalamus, central cerebellar white matter, and dentate nucleus. Astrocyte pathology results in an additional local loss of FMRP in neurons and their shrinkage. Neuronal deficit of FMRP and shrinkage of affected neurons in structures free of FMRP-positive astrocytes and regions infiltrated with FMRP-expressing astrocytes appear to reflect mechanistic, neuropathological, and functional commonalities of FMRP abnormalities in FXS and autism spectrum disorder. ***Autism Res*** *2018.* © 2018 International Society for Autism Research, Wiley Periodicals, Inc.

Lay summary: Immunocytochemistry reveals a deficit of fragile X mental retardation protein (FMRP) in neurons of cortical and subcortical brain structures but increased FMRP expression in astrocytes infiltrating gray and white matter. The detected shrinkage of FMRP-deficient neurons may provide a mechanistic explanation of reported neuronal structural and functional changes in autism. This study contributes to growing evidence of mechanistic commonalities between fragile X syndrome and autism spectrum disorder.

Keywords: idiopathic autism; duplication 15q11.2-q13/autism; fragile X mental retardation protein; neuron; astrocyte

Introduction

Transcriptomic profiling reveals shared molecular neuropathology across major psychiatric disorders including autism spectrum disorder (ASD), schizophrenia, bipolar disorder, and depression [Gandal et al., 2018]. Clinical, genetic, and neuropathological studies disclose an overlap of autism, intellectual deficits (IDs), and epilepsy in different developmental disorders [Abekhoukh & Bardoni, 2014; Belmonte & Bourgeron, 2006; Casanova, 2014; Casanova & Casanowa, 2014; Darby & Clark, 1992; Wegiel et al., 2015], including fragile X syndrome (FXS) [Hodapp et al., 1990; Kidd et al., 2014]. The list of commonalities is expanded by results of meta-analysis of gene expression in ASD [Ch'ng, Kwok, Rogic, & Pavlidis, 2015] and fragile X mutations [Nishimura et al., 2007] demonstrating similarities in the affected molecular pathways.

This study focuses on the links between FXS and idiopathic and syndromic autism. Autism is associated with a high prevalence of ID: 31% of individuals diagnosed with

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autism have an IQ below 70, while 23% have an IQ in the 71–85 range [Centers for Disease Control and Prevention, 2014], as well as with epilepsy, reported in 33% of subjects with autism [Tuchman & Rapin, 2002]. ASD includes syndromic autism, caused by isodicentric or interstitial 15q duplications, collectively referred to as maternal origin duplications 15q11.2-q13, or dup(15). The reported prevalence of autism in individuals with dup(15) ranges from 69 [Rineer, Finucane, & Simon, 1998] to 81% [Depienne et al., 2009; Hogart, Wu, LaSallae, & Schanen, 2010], whereas moderate/severe ID was reported in five of five cases in clinical studies [Battaglia, Parrini, & Tancredi, 2010; Thomas et al., 2003] and in six of eight cases examined postmortem [Wegiel et al., 2015]. In dup (15), the prevalence of epilepsy ranges from 63 [Conant et al., 2014] to 87% [Wegiel et al., 2015].

Autistic behavior in 25–47% of individuals with FXS [Brown & Cohen, 2013; Hatton et al., 2006] suggests mechanistic links between FXS and autism. Studies of individuals with ASD with either maternally-derived 15q duplications or fragile X mutations reveal commonalities in the affected molecular pathways [Nishimura et al., 2007]. FXS is a chromosome X-linked disorder most commonly caused by an expansion of a CGG repeat in the 5′ untranslated region of the fragile X mental retardation-1 gene (FMR1) [Verkerk et al., 1991]. In individuals with FXS, a repeat length exceeding 200 CGGs (full mutation) and methylation of the repeat and the promoter region [Oberlé et al., 1991; Verkerk et al., 1991] results in silencing of the FMR1 gene and absence of fragile X mental retardation protein (FMRP) [Verheij et al., 1993]. FMRP is an RNA-binding protein that also interacts with numerous cytoplasmic and nuclear proteins. Any abnormality in FMRP level and function affects the translation of multiple genes and pathways [Brown et al., 2001]. FMRP is involved in neuronal development, including neuronal differentiation, migration, and development of neuronal circuits [Till, 2010], and in regulation of the translation of a subset of proteins involved in synaptic development and plasticity [Comery et al., 1997; Irwin et al., 2001], and it plays a critical role in the pruning and maturation of dendritic spines [Churchill et al., 2002; Greenough et al., 2001; Irwin et al., 2001]. Decrease or lack of FMRP in neurons results in a disruption or lack of these regulatory mechanisms and structural and functional developmental abnormalities contributing to the FXS clinical phenotype, including a high prevalence of autism, ID [Hodapp et al., 1990], and epilepsy detected in 16% of individuals dually diagnosed with autism and FXS [Kidd et al., 2014].

The link between mechanisms critical for FXS and autism is supported by biochemical studies demonstrating reduced expression of FMRP in autism. Fatemi and Folsom [2011] reported a 75% reduction in FMRP expression in the vermis and a 50% reduction in the superior frontal cortex in adults with autism. Fatemi, Folsom, Kneeland, and Liesch [2011] measured protein levels for downstream molecules and found reduced expression of FMRP in the BA9 of adults with autism, increased expression of mGluR5 in children with autism, and increased expression of glial fibrillary acidic protein (GFAP) in children and adults with autism. These alterations may contribute to the pathogenesis of FXS [Hagerman, 2002] as well as to the autistic phenotype in individuals diagnosed with FXS [Hagerman & Harris, 2008].

The aim of this study was to establish global and brain region-specific patterns of neuronal FMRP deficits in people diagnosed with ASD and to test the hypothesis that neuronal FMRP deficits are a common pathology in both idiopathic autism and syndromic autism caused by dup (15). FMRP is expressed not only in neurons but also in astrocytes, but expression in astrocytes is limited to the prenatal and a short postnatal period in the human brain [Devys, Lutz, Rouyer, Bellocq, & Mandel, 1993] and mouse brain [Pacey & Doering, 2007]. However, our study revealed an unexpected multiregional infiltration of the brains of children and adults with autism with FMRP-positive astrocytes and neuron shrinkage in affected areas. Therefore, the second aim was to establish the topographic pattern of brain infiltration with FMRP-positive astrocytes, the impact of this pathology on neurons, and the prevalence of this pathology in idiopathic and syndromic autism. Progress in understanding of abnormal brain development in FXS and in detecting similarities between FXS, idiopathic, and syndromic autism may help to elucidate mechanistic, neuropathological, and clinical commonalities and lead to development of treatments applicable to different developmental disorders.

Methods

To detect global patterns of both the neuronal deficits of FMRP and FMRP overexpression in astrocytes, the entire formalin-fixed brain hemisphere was preserved from each of nine individuals with idiopathic autism 5–39 years of age, nine with dup(15)/autism 9–39 years of age, and nine control subjects 8–47 years of age (Wegiel et al., 2015). Neither the average age nor the average postmortem interval differed significantly among the three groups. Mean group ages were 19.3 years (standard deviation [*SD*] 11.8) for idiopathic autism; 19.0 years (*SD* 9.7) for dup(15)/autism; and 27.4 years (*SD* 13.4) for controls (one-way ANOVA: $F = 1.49$, $P = 0.24$). The average postmortem intervals (PMI) were: idiopathic autism, 18.4 hr (*SD* 14.0); dup(15)/autism, 21.9 hr (*SD* 10.0); control, 15.2 hr (*SD* 7.3) (one-way ANOVA: $F = 0.84$, $P = 0.45$). In individuals with dup(15)/autism, mean brain weight was 1,179 g (*SD* 177), 15% lower than the average of 1,382 g (*SD* 76) among control subjects, and 19% lower than the average

of 1,458 g (*SD* 146) among individuals with idiopathic autism (one-way ANOVA: $F = 9.56$, $P = 0.0009$). In the dup (15) group, the ratio between males and females was the same (4:5), whereas in the idiopathic autism group, the males were overrepresented (8:1). In the idiopathic autism group, four of nine (44%) were diagnosed with epilepsy or seizures, whereas in the dup(15) group, the ratio was strikingly high—eight of nine (89%). Death was seizure-related in two cases of idiopathic autism, but in the dup(15)/autism group, sudden unexpected death of subjects with known epilepsy (SUDEP) was reported in all eight (89%) cases diagnosed with epilepsy or seizures.

The study was approved by the institutional review board for the New York State Institute for basic research in developmental disabilities (IBR). Medical records were obtained after receiving consent for release of information from the subjects' legal guardians.

In clinical studies of individuals with dup(15), blood samples and lymphoblastoid cell lines were used for molecular genetic examination including detection of chromosome 15 polymorphism, Southern blot analysis of dosage, detection of methylation state at *SNRPN exon α* [Mann et al., 2004], fluorescent in situ hybridization, and array comparative genomic hybridization [Wang, Liu, Parokonny, & Schanen, 2004]. In eight cases, tetrasomy, and in one case, hexasomy of the Prader–Willi/Angelman syndrome critical region, was found. The origin of the abnormality was maternal in eight cases of dup(15). In one subject, the origin of the duplication was not determined. In the dup(15)/autism group, seven subjects' clinical diagnoses of autism or ASD were confirmed by application of the Autism Diagnostic Interview-Revised [Lord, Rutter, & Le Couteur, 1994].

After fixation in 10% buffered formalin, the brain hemisphere was dehydrated in ascending concentrations of ethyl alcohol, embedded in polyethylene glycol (Sigma), and cut into serial coronal hemispheric 50-μm-thick sections, which were stored in 70% ethyl alcohol [Wegiel et al., 2012]. For global mapping of FMRP expression in neurons and astrocytes, mouse monoclonal antibody (mAb 6B8), which recognizes the 340–355 aa region of human FMRP [LaFauci et al., 2013], was applied. The antibody was designed and produced at IBR, whereas the commercialized antibody is listed as mouse mAb MMS-5231 (BioLegend, Dedham, MA). To retrieve antibody epitopes, free-floating sections immersed in citric buffer were microwaved. mAb 6B8 with a protein concentration of 1.6 mg/mL was diluted 1:120 in 10% fetal bovine serum in phosphate buffered saline (PBS). Sections were incubated overnight at room temperature, then washed and treated for 30 min with biotinylated sheep anti-mouse IgG antibody diluted 1:200 (Healthcare BioSciences, Marlborough, MA), and incubated for 1 hr in ExtrAvidin peroxidase (1:200) (Sigma-Aldrich, St. Louis, MO). The product of reaction was visualized with diaminobenzidine solution in PBS. Sections were lightly counterstained with hematoxylin. The immunophenotype of astrocytes was characterized by single or double immunostaining with antibodies detecting GFAP (polyclonal antibody G9269, Sigma-Aldrich) and mouse mAb 6B8 detecting FMRP.

Tissue Sampling for Standardized Semiquantitative Evaluation of FMRP Expression in Neurons and Astrocytes

Coronal hemispheric sections cut on the level of the amygdala were used to evaluate FMRP expression in the amygdala, entorhinal cortex, caudate nucleus, putamen, globus pallidus, claustrum, anterior portion of the thalamus, and neocortical ribbon as well as the wall of the third and lateral ventricles, the uncul sulcus, and the extension separating the ventral portion of the amygdala from the hippocampal formation. In coronal hemispheric sections cut at the level of the body of the hippocampus, the immunolabeling was evaluated in the hippocampus, thalamus, lateral geniculate nucleus (LGN), claustrum, the neocortical ribbon, the wall of the lateral ventricle including the temporal lateral ventricle, the hippocampal sulcus, and the extension of this sulcus between the dentate gyrus and cornu ammonis (CA). In mid-sagittal cerebellar sections cut on the level of the dentate nucleus, neuronal immunoreactivity with mAb 6B8 was evaluated, as was astrocyte infiltration in the borderline between the white matter and cortex in cerebellar lobes, dentate nucleus, and central white matter.

Semiquantitative Ranking of FMRP-Positive Astrocyte Infiltration of Brain Structures

In hemispheric cerebral sections immunostained with mAb 6B8, the topographic expansion of FMRP-positive astrocyte infiltration was concentrated in the borderline between the white matter and the cortex in the apical portion of the gyri. Stage 1 corresponded to infiltration of 1–2 gyri, stage 2 to 3–5 gyri affected, and stage 3 to more than 5 gyri or all gyri positive. In most affected cases, the entire or almost entire borderline between the white matter and cortex was massively infiltrated with mAb 6B8-positive astrocytes (stage 4). Similar criteria were applied for evaluation of the mAb 6B8-positive astrocyte infiltration in the cerebellum, with stage 1 marked by infiltration of 1–2 lobes, stage 2 with 3–5 lobes affected, and stage 3 with more than 5 or all lobes positive.

The topographic pattern of amygdala infiltration with mAb 6B8-positive astrocytes was strikingly repetitive. In stage 1, the dorsomedial region close to the wall of the uncal sulcus (including anterior amygdaloid area, anterior cortical amygdaloid nucleus, and central amygdaloid nucleus) was infiltrated. In stage 2, the infiltration expanded to the complex of basolateral nuclei (including dorsal and ventromedial parts). In stage 3, the pathology

seen in stages 1 and 2 was accentuated by infiltration of the amygdala border with white matter and septa between nuclei. Stage 4 was identified in cases with massive infiltration of nuclei and septa.

Quantitative analysis was not applied, because in many areas, FMRP-positive astrocytes form such dense conglomerates that distinguishing an astrocyte domain defined by individual astrocyte processes is questionable. These conglomerates are typical of the ventricular wall, the molecular layer of the cortex in cortical sulci, deep layers of the cortex and adjacent white matter, the extension of the hippocampal sulcus separating the molecular layer of the dentate gyrus and the CA, and the uncal sulcus separating the ventral portion of the amygdala from the hippocampal formation.

The Kruskal–Wallis test by rank with adjustment for ties [Kruskal & Wallis, 1952] was used to test the overall statistical significance of differences in semiquantitatively assessed rankings of brain infiltration with FMRP-positive astrocytes. Post hoc pairwise comparisons among the three groups were tested using Dunn's test [Dunn, 1964]. Adjustments for multiple comparisons were performed across the ten brain regions for overall tests and among groups for post hoc comparisons to preserve a false-discovery rate (FDR) of 0.05 [Benjamini & Hochberg, 1995].

Results

FMRP Reduction/Loss in Neurons in Idiopathic and Syndromic Autism

In idiopathic autism and dup(15) autism, neuron immunoreactivity with mAb 6B8 was generally reduced, and higher magnifications revealed striking differences between immunoreactivity of individual neurons ranging from strong labeling to lack of labeling. Deficit/loss of immunoreactivity in the neuron body was paralleled with a reduction in the nucleus and the almost complete loss in dendrites, resulting in reduction/loss of reaction in the neuropil (Fig. 1).

In individuals with autism, the reduction and loss of FMRP immunolabeling in the neuron body was observed, together with a reduction in neuron body size and a change of cell shape from pyramidal or multipolar to elongated, spindle like with almost invisible dendrites. This pattern was observed in the cortex, different segments of the CA, amygdala, and thalamus. Approximately 50% of pyramidal neurons in the CA1 sector revealed only weak reaction with mAb 6B8, and they were smaller than the mAb 6B8-positive neurons. A similar reduction in immunoreactivity and neuron size was observed in the CA4 sector (Fig. 1e,f). In approximately 50% of granule cells in the dentate gyrus, immunoreactivity was reduced to moderate or weak. In the amygdala of subjects with autism, 66% of neurons revealed a mild reduction of FMRP, but in the other one third of neurons very weak staining for FMRP and reduction of cell size was a common finding. A strikingly broad spectrum of differences in FMRP expression was observed in both the magnocellular and parvocellular layers of the LGN, and the deficit was associated with a prominent shrinkage of large and small neurons (Fig. 1g–i).

Approximately one third of Purkinje cells revealed only traces of immunoreactivity with mAb 6B8, another one third revealed weak reaction, and other Purkinje cells were stained moderately/strongly. A similar deficit in nuclear and cytoplasmic immunoreactivity was detected in neurons in the dentate nucleus. Weakly stained Purkinje cells and neurons in the dentate nucleus were usually smaller than moderately/strongly stained neurons.

Brain Region-Specific Patterns of Infiltration With FMRP-Positive Astrocytes

Brain region-specific infiltration with FMRP-positive astrocytes appears to be a unique form of pathology observed in children and adults diagnosed with idiopathic autism and syndromic autism caused by dup(15). Infiltration affected the subcortical white matter and deep cortical layers as well as the molecular layer, the amygdala, thalamus, cerebellar subcortical white matter and adjacent granule cell layer, cerebellar central white matter, and dentate nucleus. In a few control cases, single astrocytes or clusters of mAb 6B8-positive astrocytes were found (Fig. 2a, Table 1). Semiquantitative ranking of brain infiltration with FMRP-positive astrocytes revealed significantly more frequent and severe infiltration in all regions except the hippocampal formation and dentate nucleus in idiopathic autism compared to control subjects. Infiltration in the duplication 15 autism group was significantly more severe in all examined regions than in control subjects. Comparison of the pattern and severity of changes in the idiopathic and duplication 15 autism groups did not reveal significant differences, except enhanced infiltration in cerebellar subcortical white matter in dup(15) group. Age and PMI were tested as potential cofactors for these findings. However, application of Spearman rank correlations revealed no significant association between PMI and FMRP ratings and between age and FMRP ratings, except for a positive correlation of PMI with ratings for the ventricle wall and projections to the white matter ($P = 0.0211$).

Infiltration of the Subcortical White Matter, Deep Cortical Layers, and Neocortical Molecular Layer

The subcortical white matter and deep layers of the cortex (Fig. 2) revealed strong susceptibility to mAb 6B8-positive astrocyte infiltration, reflected in the presence of this pathology in all cases of dup(15), with an

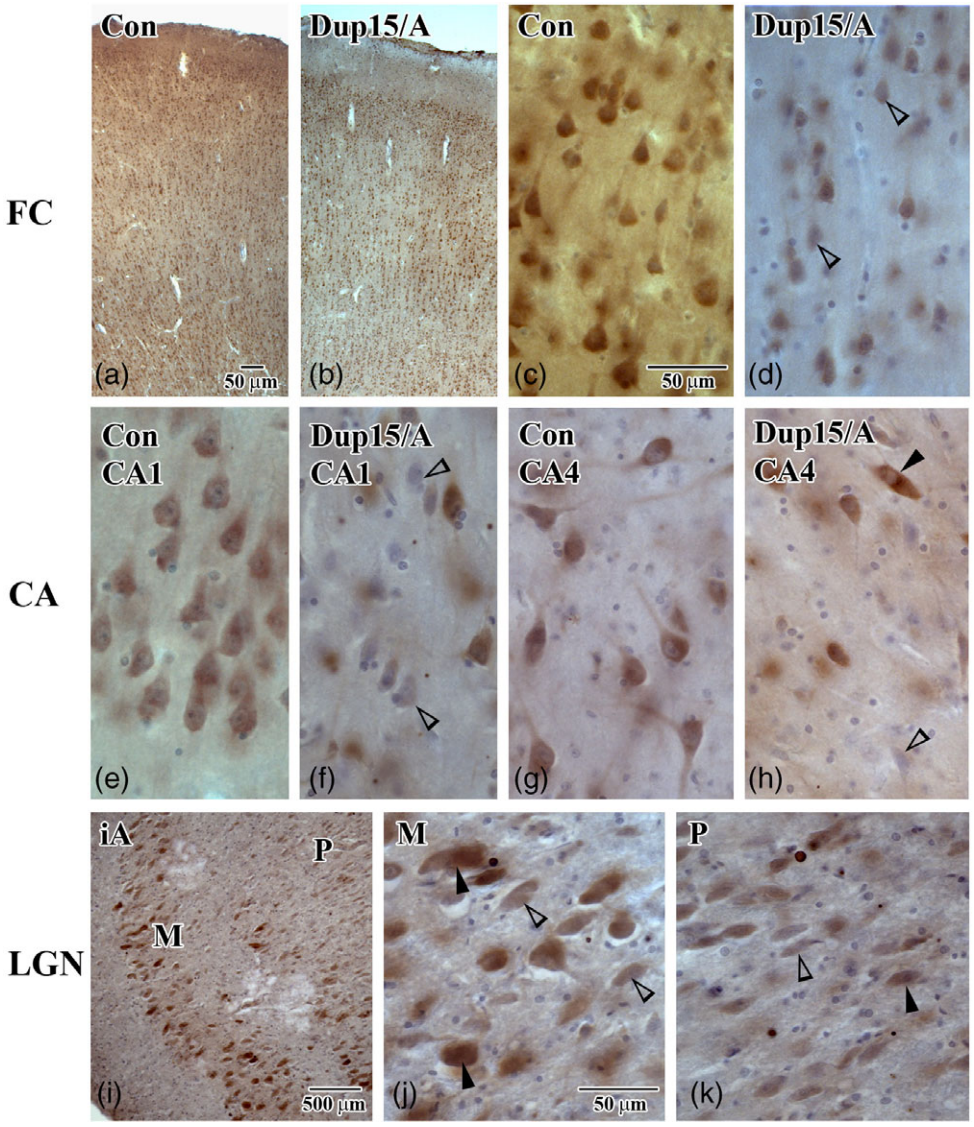

Figure 1. Frontal cortex immunostained with mAb 6B8 demonstrates FMRP expression in neurons in control subject (a) and reduced expression in dup15/autism case (b). Higher magnification of cortical neurons in the control subject showed strong FMRP immunoreactivity in neuron soma, and weaker immunoreactivity in nucleus and dendrites (c). In the cortex in dup15/A case, reduced immunoreactivity in the neuronal soma and processes, and almost no staining of neuropil reflected loss of FMRP in neuron soma, axons, dendrites, and synapses. In dup(15)/autism, the FMRP-deficiency was associated with reduced size of neuronal soma and nucleus (d). Moderate FMRP immunoreactivity in the soma and dendrites of neurons in the CA1 (e) and CA4 (g) sectors was observed in the control subject. Prominent deficit or loss of FMRP in the neuronal soma, nucleus, and dendrites was associated with reduction of the size and a distortion of neuron shape (arrowheads) in a subject with dup(15)/autism (f, h). The mosaic pattern of FMRP expression in neurons in the magnocellular (M; i, j) and parvocellular part (P; i, k) of the LGN in idiopathic autism was associated with a decrease or loss of FMRP in most of neurons, marked reduction of neuron size, and change in neuron shape from oval to spindle-like (arrowheads).

average grade of infiltration of 3.3, and in seven of nine (78%) cases with idiopathic autism (graded as 2.7). In three of nine control individuals, a few mAb 6B8-positive astrocytes were detected in the subcortical zone (average grade 0.3). In the early stages, infiltration was limited to the subcortical zone and to the adjacent deep layer of the neocortex in the top portion of a few cortical gyri. The process progresses with infiltration of more gyri and expansion from the top to the base of gyri. In some cases, almost the entire borderline between the cortex and white matter was infiltrated with mAb 6B8-positive astrocytes. A continuous thick layer of mAb 6B8-positive astrocytes was detected in the entire border between the white matter and cerebral cortex in a 39-year-old female diagnosed with dup15, autism, epilepsy, and SUDEP (Fig. 2d–g).

In the neocortex, subcortical white matter infiltration was paralleled by infiltration of the molecular layer with

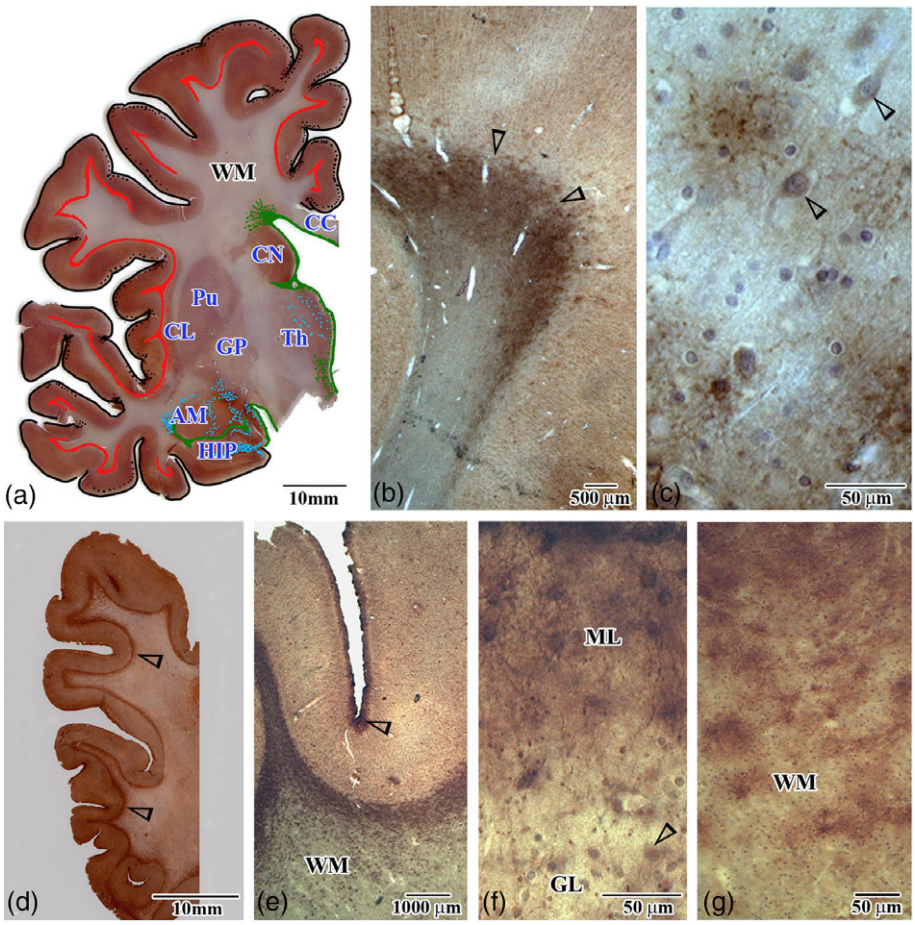

Figure 2. A hemispheric section from the brain of a subject with dup(15) autism immunostained with mAb 6B8 (a) illustrates the range of regional differences in FMRP expression with a relatively strong and uniform reaction in the cortical ribbon; slightly weaker in the amygdala (Am), hippocampus (HIP), and caudate nucleus (CN); much weaker in the putamen (Pu) and thalamus (Th); very weak in the globus pallidus (GP); and almost undetectable in the cerebral white matter (WM). This image also illustrates the pattern of brain infiltration with FMRP-positive astrocytes at the border between the white matter and the cortex (marked with red line), the cortical molecular layer (black dots), and in the amygdala, thalamus, and hippocampal sulcus (blue dots). FMRP immunolabeling in astrocytes in the ventricular subependymal zone and the adjacent white matter (green) was present not only in individuals with idiopathic and syndromic autism but also in control subjects. (b) Illustrates the most common pattern of neocortical infiltration with FMRP-positive astrocytes in the tip of the gyrus (arrowheads; b; dup (15). Infiltration of the cortex was associated with almost a total loss of FMRP in neurons in the affected deep layers of the cortex (arrowheads; c; idiopathic autism). Continuous infiltration of the entire border between the white matter and cortex with FMRP-positive astrocytes in a subject with dup(15) (arrowheads; d). Infiltration of the white matter and cortex borderline was paralleled by infiltration of the molecular layer (ML) of the cortex, especially massive in the wall and the bottom of the cortical sulci (e) associated with loss of FMRP in neurons of the granule layer (GL; f). Subcortical infiltration expands 1–2 mm into the white matter (WM; e, g).

a row of astrocytes in the middle of the molecular layer. Massive infiltration in the entire molecular layer was common in the wall and bottom of cortical sulci (Fig. 2e,f). This was found in 89% of dup(15) autism cases and in 78% of subjects with idiopathic autism cases, but was not detected in the cerebral cortex in control cases.

Infiltration of the Amygdala and Thalamus

The amygdala (Fig. 3) was the most consistently and significantly affected subcortical structure infiltrated with mAb 6B8-positive astrocytes in all subjects with dup(15) autism and in 78% of cases of idiopathic autism. The average grade of progression of changes was estimated at 2.9 and 2.5, respectively. The common component of this astrocytosis was infiltration of the amygdala with FMRP-positive astrocytes in the border between the white matter and the nuclei at the dorsomedial regions. In advanced stages, infiltration was accentuated in the borders between nuclei, including septa between basolateral and lateral nuclei. In the most advanced stages, massive FMRP-expressing astrocyte infiltration was observed in the entire anterior amygdaloid area,

Table 1. Topography and Semiquantitative Ranking of Brain Infiltration With FMRP-Positive Astrocytes in Idiopathic Autism, dup15/Autism and Control Subjects

		Neocortex		EC	Amygdala	Thalamus	Hippocampal formation	Ventricles	Cerebellum		
Group	Case	Subcortical white matter and adjacent cortex	Cortical mol. layer	Subcortical white matter	Dorso-medial nuclei and septa between nuclei	Focal	Hippocampal and uncal sulcus wall with expansions to the molecular layer in the subiculum, CA and DG	Ventricle wall and projections to the white matter	Subcortical white matter and adjacent cortex	Central white matter	Dentate nucleus
IA	1	4	4	2	3	4	4	4	2	2	0
IA	2	2	2	3	4	1	2	2	1	1	1
IA	3	3	2	2	1	0	2	4	2	0	0
IA	4	0	0	0	0	0	2	1	0	0	0
IA	5	4	4	4	4	4	2	4	3	3	1
IA	6	0	0	0	0	0	1	2	1	0	0
IA	7	3	2	3	3	1	2	2	1	1	1
IA	8	4	3	4	4	4	4	4	1	2	0
IA	9	4	4	4	4	4	4	4	2	0	0
Average		2.7	2.3	2.4	2.5	2.0	2.5	3.0	1.5	1.4	0.3
D15A	1	4	4	1	3	1	4	4	2	2	1
D15A	2	4	3	1	3	1	4	2	3	2	1
D15A	3	1	0	0	3	1	3	4	2	2	0
D15A	4	4	3	4	4	1	4	2	1	0	0
D15A	5	4	3	4	4	2	3	3	3	2	2
D15A	6	4	3	1	3	2	4	4	3	2	2
D15A	7	4	2	4	4	1	4	3	3	3	3
D15A	8	1	1	1	1	0	2	1	2	0	0
D15A	9	4	2	1	2	0	2	2	3	1	0
Average		3.3	2.3	1.9	2.9	1.3	3.3	3.0	2.6	2.2	1.8
C	1	0	0	0	0	0	1	2	0	0	0
C	2	0	0	0	0	0	1	1	0	0	0
C	3	0	0	0	0	0	3	2	0	0	0
C	4	0	0	0	0	0	1	1	0	0	0
C	5	1	0	2	1	0	3	2	0	0	0
C	6	0	0	1	0	0	1	1	0	0	0
C	7	1	0	1	1	0	1	1	1	0	0
C	8	1	0	1	0	0	1	1	0	0	0
C	9	0	0	0	0	0	1	1	1	0	0
Average		0.3	0	0.5	0.2	0	1.4	1.3	0.2	0	0
Overall χ^2		13.92	13.44	6.85	13.33	10.88	11.72	10.54	17.16	10.70	7.08
Overall *P*		0.0009	0.0012	0.0326	0.0013	0.0043	0.0028	0.0051	0.0002	0.0047	0.0290
D15A vs. C		0.0004	0.0020	0.0413	0.0011	0.0057	0.0010	0.0062	0.0001	0.0019	0.0117
IA vs. C		0.0077	0.0013	0.0186	0.0027	0.0039	ns	0.0047	0.0136	0.0250	ns
D15A vs. IA		ns	ns	ns	ns	ns	ns	ns	0.0388	ns	ns

FMRP, fragile X mental retardation protein; Case, case number; EC, entorhinal cortex; CA, cornu Ammonis; DG, dentate gyrus; IA, idiopathic autism; D15, chromosome 15q11.2-13.1 duplication syndrome; C, control.

Overall significance of differences in semiquantitatively assessed ranking of brain infiltration with FMRP-positive astrocytes is tested with the Kruskal–Wallis test by rank with adjustment for ties. (All χ^2 values have 2 degrees of freedom.) Post hoc pairwise comparisons are tested using Dunn's test. Adjustment for multiple comparisons is made across regions for overall tests and among group comparisons for post hoc comparisons to preserve a FDR of 0.05; all overall comparisons remained significant after this adjustment.

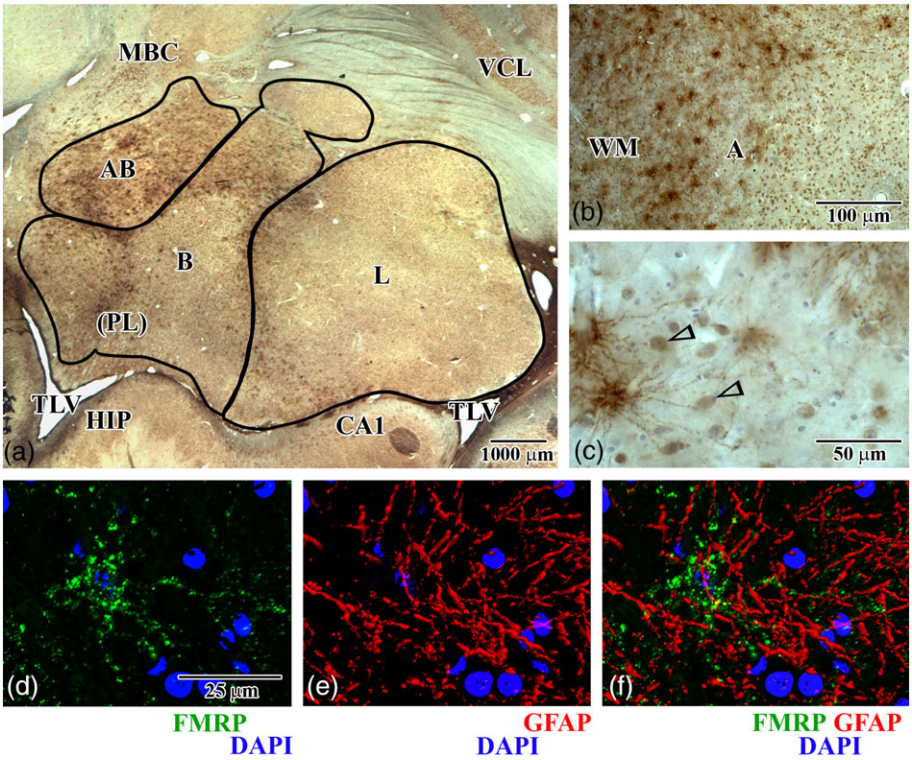

Figure 3. Infiltration with FMRP-positive astrocytes of the dorsomedial part of the amygdala including the accessory nucleus (A), different subdivisions of the basal nucleus (B) including paralaminar nucleus (PL; Avino et al., 2018), as well as the border zone between the basal (B) and lateral nucleus (L). Very strong immunoreactivity in the wall of the temporal lateral ventricle (TLV) showed expansions to the white matter but not to the amygdala. There were no signs of infiltration of the adjacent magnocellular basal complex (MBC) or ventral claustrum (VCL) (a). Selective infiltration of the amygdala was associated with loss of FMRP in neurons and reduction of the size of the neuronal soma (b, c) in a subject with dup(15)/autism. Double staining for confocal microscopy showed mAb 6B8-positive dots or clusters of dots within an FMRP-positive astrocyte (d, f), and a GFAP-positive cytoskeleton in FMRP-negative astrocytes (e, f) without signs of FMRP and GFAP colocalization. Cell nucleus stained with DAPI.

anterior cortical amygdaloid nucleus, and central nucleus, but infiltration of the lateral nucleus was significantly less severe. In infiltrated regions in the amygdala of subjects with autism, FMRP expression in neurons was reduced or absent. Weak infiltration limited to the mediodorsal region of the amygdala was found in two control cases.

Double staining with antibody 6B8 detecting FMRP and antibody detecting GFAP showed clusters of dots within FMRP-positive astrocytes soma and processes and absence of GFAP-positive cytoskeleton in these cells (Fig. 3).

Focal infiltration detected in the medial portion of the thalamus was found in 78% of cases of dup(15) autism and in 67% of cases of idiopathic autism (Table 1), with the severity of infiltration classified as 1.3 and 2.0, respectively. mAb 6B8-positive astrocytes were not detected in the thalamus of control subjects.

The topographic selectivity of FMRP-positive astrocyte infiltration in the subcortical zone and molecular layer of the neocortex, the amygdala, and thalamus was accentuated by the absence of this pathology in the caudate nucleus, putamen, globus pallidus, magnocellular basal complex and claustrum, including the ventral claustrum, despite common and significant infiltration of the adjacent amygdala and peri-amygdalar white matter.

Infiltration of the Cerebellum

In the cerebellum of subjects with autism, FMRP-positive astrocytes infiltrated white matter in the folia of the cerebellar cortex and at the borderline between white matter and granule cell layer as well as the adjacent granule cell layer (Fig. 4). Infiltration was found in all dup(15)/autism cases and in eight of nine cases with idiopathic autism. Infiltration was detected in more cortical lobes, and the number of astrocytes was higher in dup(15)/autism than in idiopathic autism (with average grade of 2.6 and 1.5, respectively). More prominent cortical pathology was associated with infiltration of the central cerebellar white matter in seven of nine cases of dup(15)/autism (grade: 2.2). In idiopathic autism, white matter infiltration was found in five of nine cases (grade: 1.4). The dentate nucleus was infiltrated in five of nine cases of dup(15)/autism and in three of nine idiopathic autism cases. Infiltration of the granule layer and dentate nucleus resulted in reduction/loss of FMRP expression in the affected

neuron soma and nucleus, cell shrinkage, and deformation. In two control subjects, very few FMRP-positive astrocytes were detected in the white matter of the folia but not in the central white matter or dentate nucleus.

Similarities of FMRP Immunoreactivity in the Hippocampal Formation and Subventricular Zone of Autistic and Control Subjects

Two features distinguished hippocampal formation and subventricular zone immunoreactivity: (a) their presence in all control subjects and individuals with idiopathic and syndromic autism, and (b) stronger immunoreactivity and larger immunoreactive area in autistic than in control subjects (Fig. 5).

In the hippocampal formation, symmetric infiltration with mAb 6B8-positive astrocytes was common on both sides of the hippocampal sulcus in the stratum moleculare of the subiculum, sectors 1 and 2 of the CA, and the stratum moleculare of the dentate gyrus. Infiltration of the granule layer and dentate nucleus resulted in reduction/loss of FMRP expression in the affected neuron soma and nucleus, cell shrinkage, and deformation. The number of immunoreactive astrocytes and area infiltrated was often two or three times greater in autistic than in control subjects.

FMRP-positive astrocytes were observed in all control and autistic subjects in the ventricular subependymal zone and adjacent white matter in the subcallosal stratum (above the caudate) and stria terminalis (below the caudate), in the wall of the lateral ventricles in the temporal lobe, and in the wall of the fourth ventricle, with astrocytes infiltrating cerebellar white matter and a portion of the flocculus and nodulus.

Impact of FMRP-Positive Astrocytes on Neurons

Vast areas of the cerebral and cerebellar cortex, amygdala, and cerebellar nuclei were infiltrated by FMRP-positive astrocytes. Regardless of brain region, the neuronal response to direct contact of a neuron with the processes of FMRP-positive astrocytes was similar: loss of FMRP in the neuron cytoplasm, nucleus, and dendrites, and shrinkage and deformation of the neuronal soma. Direct contact between FMRP-positive astrocyte processes engulfing the neuron soma and the loss of neuronal FMRP was typical for both deep layers of the cortex and neurons close to the infiltrated molecular layer (Fig. 2), the amygdala (Fig. 3), the medial part of the thalamus (not shown), and the cerebellar dentate nucleus (Fig. 4).

Discussion

Immunolabeling of brain sections of control subjects with mAb 6B8 showed high expression of FMRP in the neuronal soma, moderate expression in the nucleus and dendrites, and weak expression in white matter axons. This pattern is consistent with other studies demonstrating FMRP in the neuron soma, nucleus, dendrites, spines, axonal growth cones, and mature axons [Antar, Afroz, Dictenberg, Carroll, & Bassell, 2004; Centonze et al., 2008; De Rubeis & Bagni, 2010; Feng, Absher, et al., 1997; Feng, Gutekunst, et al., 1997]. The reported distribution reflects FMRP trafficking between the cytoplasm and nucleus, binding to the mRNA [Bassell & Warren, 2008], and formation of a messenger ribonucleoprotein complex that associates with polyribosomes and affects post-transcriptional regulation of protein synthesis [Bassell & Warren, 2008; Lu et al., 2004; Sung et al., 2003].

Mechanistic Commonalities of FMRP Deficits in Autism and Other Disorders

In FXS, the deficit of FMRP disrupts post-transcriptional regulation of many target RNAs [De Rubeis & Bagni, 2010] and leads to structural and functional anomalies. Immunocytochemistry reveals the topography and range of regional FMRP deficits in the brains of individuals diagnosed with autism. The proportion of deficient neurons varies in individual brain structures from a few to a majority. These differences may contribute to regional FMRP deficits detected in biochemical studies, including a 55% reduction of FMRP expression in the superior frontal cortex [Fatemi & Folsom, 2011] and a 75% decline in the vermis in adults with autism [Fatemi et al., 2011]. Multiregional FMRP deficits detected in individuals diagnosed with idiopathic and syndromic autism caused by dup(15) indicate that autism is associated with an FMRP deficit regardless of autism etiology. Results of a study by Fatemi, Kneeland, Liesch, and Folsom [2010] demonstrating a reduction by 78% in the amount of FMRP in the lateral part of the cerebella in subjects with schizophrenia, by 60% in bipolar disorder, and by 68% in major depression disorder suggest that FMRP deficit-related mechanistic abnormalities are not limited to ASD but instead are a component of other disorders with a broad spectrum of clinical manifestations.

Immunocytochemistry expands biochemical data by demonstration of striking differences in neuronal FMRP expression not only in different brain regions but also within cytoarchitectural subdivisions, neuronal circuits, and individual neurons. The mosaic pattern of neuronal FMRP deficits and associated structural abnormalities within functionally different brain structures and neuronal circuits appears to be an indicator of disruption of brain integrity contributing to a broad spectrum of functional deficits in autism. Commonalities detected in biochemical and immunocytochemical studies indicate that FMRP deficits and consequences of these deficits are

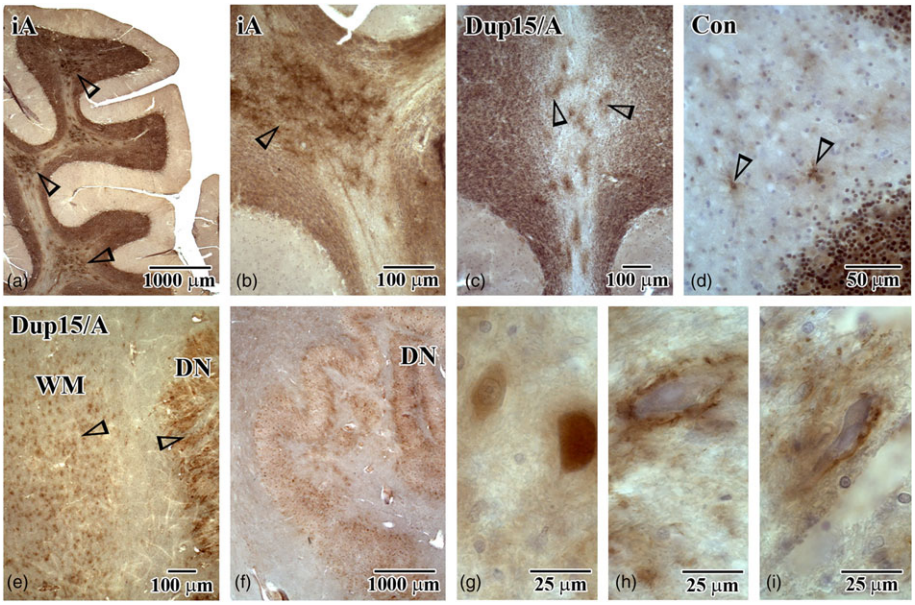

Figure 4. In the cerebellar folia, FMRP-positive astrocytes infiltrated the borderline between the cortex and white matter and the deep portion of the granule cell layer (arrows) in an individual diagnosed with idiopathic autism (a, b) and in a subject with dup15/autism (c). Sporadically, a few small FMRP-positive astrocytes were detected in white matter in the folia of a control subject (d). Massive infiltration of the cerebellar central white matter (WM; e) and the dentate nucleus (DN; e, f) with FMRP-positive astrocytes (arrowheads). Strong immunoreactivity in neurons in the dentate nucleus of a control subject (g) but loss of FMRP in shrunken neurons (arrowheads) surrounded by FMRP-positive astrocytic processes (h, i) was present in the cerebellum of a subject diagnosed with dup15/autism.

nonspecific markers of mechanistic overlaps in many disorders including ASD.

Link Between Deficit/Lack of FMRP and Reduced Neuronal Soma and Nucleus Volume

Immunocytochemistry reveals that FMRP deficit/loss is associated with a reduced size of the neuron soma and nucleus. This pathology is typical for the cerebral cortex, the amygdala, LGN, and hippocampal formation, including the subicular complex and CA. Abnormal neuronal FMRP expression appears to be a marker of the link between FMRP deficit, neuronal shrinkage, and dysfunction of affected circuits. The deficit of FMRP and the reduced size of FMRP-deficient neurons found in the LGN in individuals with idiopathic and syndromic autism are associated with clinical deficits. LGN neurons are involved in visual information processing in the dorsal and ventral stream [Felleman, Burkhalter, & Van Essen, 1997; Milner & Goodale, 1995; Ungerleider & Mishkin, 1982]. The dorsal stream is dependent mainly on afferent input from the magnocellular pathway of the LGN specialized in the analysis of object motion. The ventral stream is dependent mainly on afferent input from the parvocellular pathway involved in object recognition based on shape, size, color, and texture [Milner & Goodale, 1995]. Abnormal expression of FMRP is considered a cause of altered visual processing in FXS [Kogan et al., 2004]. The deficit of FMRP immunoreactivity and reduction of neuron size in magnocellular and parvocellular LGN in the brains of individuals with idiopathic and syndromic autism detected in this study may contribute to functional deficits, including abnormal global processing along the dorsal visual pathway and weak visuospatial coherence reported in autism [Pellicano, Ginbson, Maybery, Durkin, & Badcock, 2005]. The FMRP deficit in the LGN in idiopathic and syndromic autism, resembling the absence of FMRP in FXS, illustrates neuropathological and behavioral overlaps in autism and FXS.

There is no single neurobiological feature that uniquely distinguishes children with ASD from their typically developing peers [Müller & Amaral, 2017], but smaller than normal neuron size is one of the most consistent pathologies reported in autism. Smaller neurons found in the amygdala, the medial septal nucleus, and cerebellar nuclei were the earliest pathology identified in the brains of individuals with autism and were reported by Bauman and Kemper [1985]. Their findings were confirmed in numerous morphometric studies of the cerebral cortex, subcortical structures, and cerebellum [Casanova et al., 2006; Jacot Descombes et al., 2012; Santos et al., 2011; Schumann & Amaral, 2006; Simms, Kemper, Timbie, Bauman, & Blatt, 2009; Wegiel et al., 2014, 2015]. A multiregional, prominent brain region-specific deficit of neuronal soma volume in children with autism is documented by significant neuronal soma volume deficits varying between 5 and 34%

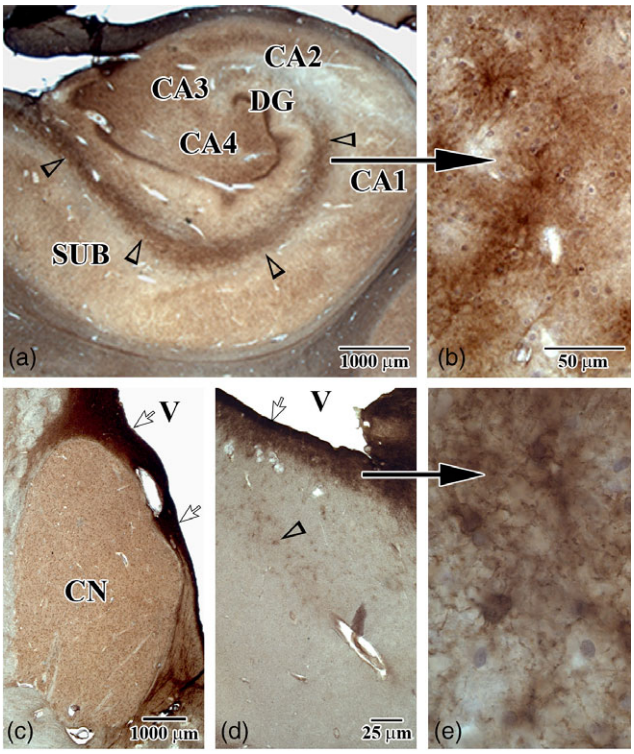

Figure 5. Pattern of distribution of FMRP-positive astrocytes in a control subject. In a control subject, the border between the dentate gyrus (DG) and the subiculum (SUB) and CA1 sector (arrowheads) was enriched with FMRP-positive astrocytes (a; high magnification, b). Neuronal immunoreactivity was strong and detectable in CA2, 3, 4, and was weak in the CA1 sector and subiculum. Subependymal and subventricular zone (arrows) showed very strong FMRP immunolabeling close to the caudate nucleus (CN) (c). A similar pattern was detected in the wall of the ventricle (arrow; V) in the cerebellum (d; high magnification, e). Only a few FMRP- positive astrocytes (arrowhead; d) migrated from the FMRP-positive periventricular zone to the central cerebellar white matter.

in 14 of 16 examined brain regions. A very severe neuronal soma volume deficit (>30%) in the largest and the smallest neurons examined in stereological studies [Wegiel et al., 2014] and a prominent deficit of FMRP in smaller neurons in the cerebral cortex, hippocampal formation, amygdala, and magno- and parvocellular part of the LGN, and other brain structures of subjects with idiopathic and syndromic autism suggest that neuronal FMRP deficit/loss and reduction in neuron size act across a broad spectrum of types of neurons, brain regions, and neuronal circuits in ASD. Deficit/lack of FMRP regulation may contribute to abnormal neuron growth and development. This hypothesis matches the molecular and cellular functions associated with FMRP mRNA targets that include cell morphology, cellular development, and cell growth [Bagni & Oostra, 2013]. The combination of neuronal FMRP deficits with distortions of neuron size and shape in unrelated brain regions with different functions suggests that this pattern of pathology contributes to the complexity of autism symptoms.

Multiregional Brain Infiltration With FMRP-Positive Astrocytes as a Second Factor Reducing Neuronal FMRP Expression in ASD

This study reveals two patterns of FMRP deficit associated with distortion of neuron size/structure/function: multiregional diffuse FMRP deficit in neurons, which is astrocyte independent, and a regional pattern linked to gray matter infiltration with FMRP-positive astrocytes. The detected pattern of FMRP expression in astrocytes in children and adults with autism is strikingly different than in a normally developing human brain with astrocyte FMRP expression limited to a prenatal and a short postnatal period. FMRP-positive astrocytes are typical for the mouse hippocampus at E17, P1, and P7, but they are absent in 2-month-old mice [Pacey & Doering, 2007]. Expression of FMRP and GFAP in astrocytes is developmentally regulated. Only negligible expression of FMRP is detected in mature glial cells in the mouse [Bakker et al., 2000] and human brain [Devys et al., 1993], whereas GFAP appears late in embryonic life and gradually increases throughout postnatal life [Rozovsky, Wei, Morgan, & Finch, 2005]. This distinction of the two types of astrocytes is reflected in the lack of or traces of GFAP in FMRP-positive astrocytes detected in this study. The protracted maturation trajectory of the amygdala beyond the perinatal period in autistic subjects discovered by Avino et al. (2018) might be related to glial developmental abnormalities postulated by authors. One may assume that the massive selective infiltration of the amygdala with FMRP-positive astrocytes extended well beyond the physiological postnatal period might be a sign of glial developmental abnormality contributing to disturbed maturation, migration, and growth of amygdala neurons and clinical consequences described by the authors of the original report.

Immunostaining with mAb 6B8 revealed a topographically selective infiltration of the cerebral cortex including the subcortical plate, adjacent layer VI, and molecular layer. In many but not all autistic subjects supernumerary neurons were detected in the subcortical white matter and in the molecular layer of the cortex [Avino & Hutsler, 2010; Bailey et al., 1998; Hutsler & Casanova, 2016]. Subcortical neurons are considered remnants of the early generated subcortical plate which plays a role in proper cortical development and in regulating interregional communication [Chun & Shatz, 1989; Hutsler & Casanova, 2016]. The detected colocalization of supernumerary neurons in the subcortical plate and molecular layer with infiltration of FMRP positive astrocytes in autistic children and adults suggests a link between persistence of developmentally defective neurons and

developmentally desynchronized FMRP-positive astrocytes. These developmental anomalies appear to be signs of permanent regional distortion of cortical structure and function in a majority of subjects diagnosed with idiopathic autism and all with dup15.

Activation of Different Types of Astrocytes in Autism

Astrocytes are a major regulator of neuron, dendrite, and synapse development and function, and neuronal pathology is inseparable from astrocyte pathology. They directly regulate migration of neurons to target areas, cortical lamination, development of dendritic arborization, spine density, synaptogenesis, synapse strength and function (tripartite synapses with astrocyte as the third party), and concentration of neurotransmitters [Jacobs, Nathwani, & Doering, 2010; Nedergaard, Ransom, & Goldman, 2003; Perea, Navarrete, & Araque, 2009]. Astrocytes are a key player in developmental disorders including FXS and autism [Jacobs et al., 2010]. Immunocytochemistry showed selective increase in GFAP immunoreactivity in the granular cell layer and white matter in the cerebellum but not in the middle frontal gyrus or anterior cingulate gyrus of subjects with autism [Vargas, Nascimbene, Krishnan, Zimmerman, & Pardo, 2005]. Western blots revealed global astrocyte activation in the brain of subjects with autism, with an increase in the level of GFAP in the superior frontal cortex (45%), parietal cortex (75%), and cerebellum (49%) [Fatemi, Folsom, Reutman, & Lee, 2008; Laurence & Fatemi, 2005]. Two other types of astrocytes were studied in autism, including aquaporin 4 (AQP4)-positive astrocytes expressing a transmembrane water channel protein typical for perivascular astrocytes [Badaut, Lasbennes, Magistretti, & Regli, 2002], and connexin 43 (Cx43)-positive astrocytes with Cx43 localized in gap junctions providing interastrocytic coupling in astrocyte networks [Giaume, Koulakoff, Roux, Holcman, & Rouach, 2010; Pannasch & Rouach, 2013]. Separation of GFAP-positive astrocytes from AQP4-positive astrocytes is reflected in insignificant increases of AQP4 in Brodmann area 40 (BA40) and the significant decrease of AQP4 in the cerebellum detected simultaneously with a significant increase in GFAP in these regions [Fatemi et al., 2008]. The increase of Cx43 expression in BA40 of subjects with autism was not significant [Fatemi et al., 2008]. Comparison of distribution and activation of GFAP-, AQP4-, Cx43-, and FMRP-positive astrocytes reveals that FMRP-positive astrocytes represent a distinct population of astrocytes typical for the early postnatal period but selectively infiltrating several brain regions in children and adults diagnosed with idiopathic autism and dup(15) autism. Absence or negligible immunoreactivity for GFAP in FMRP-positive astrocytes separates these two types of astrocytes as well as their different role in pathological conditions, including epilepsy.

Links Between Dup(15) and FMRP Abnormalities

A prominent reduction of FMRP expression in neurons but an increase in a subpopulation of astrocytes in individuals with dup(15) might be related to an extra copy of genes in the critical region of the duplicated chromosome 15. The 15q11–13 duplicated region contains at least 30 genes, including several genes considered contributors to the autism phenotype, such as the γ-aminobutyric acid type A ($GABA_A$) receptor subunit genes (α5, β, and γ3) [Buxbaum et al., 2002; Cook et al., 1998; Hogart, Nagarajan, Patzel, Yasui, & LaSalle, 2007]. Genome-wide expression profiling of lymphoblastoid cell lines identified 68 genes that were dysregulated in autism with the fragile X mutation and dup(15) [Nishimura et al., 2007]. Potentially relevant to the detected abnormal FMRP expression might be duplication of the cytoplasmic FMRP-interacting protein 1 (CYFIP1) gene [Hogart et al., 2010; Nishimura et al., 2007]. The CYFIP1 gene, located in the 15q critical region, is the best candidate to contribute to autistic behavior, ID, and hyperactivity [Abekhoukh & Bardoni, 2014; Bagni & Oostra, 2013].

Closing Remarks

The findings of this study suggest that the detected complex of neuropathological changes has a potential to contribute to a broad spectrum of diagnostic behavioral abnormalities and to a high prevalence of comorbidities including IDs and epilepsy. The clinical complexity of ASD might be an effect of several factors. The deficit of FMRP results in deregulation of binding to mRNA and loss of control of synthesis of a broad range of neuronal proteins in different brain structures and neuronal networks. The mosaic pattern of FMRP expression suggests that in autism, neuronal networks integrate altered signaling of neurons, with FMRP expression ranging from no deficit to absence of FMRP, and a broad spectrum of structural alterations, including reduction of neuron soma and nucleus size, change of neuron shape, and associated functional impairments. Multiregional but topographically selective infiltration with FMRP-positive astrocytes acts as an additional modifier of neuronal network structure and function, leading to additional disruption of functional brain integrity. The similarities in the type, topography, and severity of FMRP deficit-related abnormalities in idiopathic and syndromic autism detected in this study appear to be a sign of shared pathways within ASD and a mechanistic link to FXS pathology documented by molecular studies.

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Author Contributions

Dr. Jarek Wegiel designed the study, carried out immunostainings and tissue examination, and drafted the manuscript. Drs. W.T. Brown, G. La Fauci, Tatyana Adayev, Richard Kascsak, and Ms. Regina Kascsak designed, produced, and characterized mAb 6B8 and revised the manuscript. Dr. M. Flory performed statistical analysis. Dr. V. Martinez-Cerdeno contributed to interpretation of FMRP findings in idiopathic and syndromic autism and to manuscript revision. Drs. I. Kuchna, K. Nowicki, and W. Kaczmarski helped in selection of cases and tissue, reviewing records, and writing the manuscript. Drs. T. Wisniewski and Jerzy Wegiel contributed to research design, neuropathological and immunocytochemical evaluation, and manuscript revisions. All authors approved the final version of the submitted manuscript.

Conflict of Interest

The authors declare that they have no competing interests.

Consent

This postmortem study has been performed using anonymized, coded brain tissue samples. Selected clinical records were extracted from the anonymized, coded Autism Speaks Autism Tissue Program database by authorization by the project's principal investigator.

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