Alterations of Amino Acids and Monoamine Metabolism in Male Fmr1 Knockout Mice: A Putative Animal Model of the Human Fragile X Mental Retardation Syndrome

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ABSTRACT

The Fragile X syndrome, a common form of mental retardation in humans, is caused by silencing the fragile X mental retardation (FMR1) gene leading to the absence of the encoded fragile X mental retardation protein 1 (FMRP). We describe morphological and behavioral abnormalities for both affected humans and Fmr1 knockout mice, a putative animal model for the human Fragile X syndrome. The aim of the present study was to identify possible neurochemical abnormalities in Fmr1 knockout mice, with particular focus on neurotransmission. Significant region-specific differences of basal neurotransmitter and metabolite levels were found between wildtype and Fmr1 knockout animals, predominantly in juveniles (post-natal days 28 to 31). Adults (postnatal days 209 to 221) showed only few abnormalities as compared with the wildtype. In juvenile knockout mice, aspartate and taurine were especially increased in cortical regions, striatum, hippocampus, cerebellum, and brainstem. In addition, juveniles showed an altered balance between excitatory and inhibitory amino acids in the caudal cortex, hippocampus, and brainstem. We detected very few differences in monoamine turnover in both age stages. The results presented here provide the first evidence that lack of FMRP expression in FMRP knockout mice is accompanied by age-dependent, region-specific alterations in neurotransmission.

INTRODUCTION

The Fragile X syndrome, associated with a chromosomal abnormality, is one of the most common forms of inherited mental retardation in humans (Oostra, 1996; Jin & Warren, 2000). The Fragile X syndrome is based on a large expansion of a CGG repeat sequence (more than 200 to 230 copies in the full mutation) in the 5'- untranslated region of the fragile X mental retardation (FMR1) gene (Fu et al., 1991; Oberle et al., 1991; Verkerk et al., 1991). Caused by this repeat expansion, a hyper-methylation takes place in a CpG island, including the promotor region, resulting in an inactivation of the fragile X mental retardation protein 1 gene (FMR1 gene), loss of expression of FMR1 mRNA transcripts (Pieretti et al., 1991),

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and absence of the encoded fragile X mental retardation protein (FMRP) (Devys et al., 1993).

The syndrome, which occurs more frequently in males than in females (Oostra, 1996; Comery et al., 1997), is characterized by a mild to severe mental retardation (IQ between 20 and 60) with notable deficits in language, visuo-spatial memory, attention, and executive functions in humans (Freund & Reiss, 1991). In addition, emotional and psychosocial deficits have been described, including hyperactivity and autism (Baumgardner et al., 1995; Hagerman, 1996; Turk & Graham, 1997; Bailey et al., 1998). The brain pathology of these patients includes abnormalities in the cerebellum (Reiss et al., 1991a; 1991b), decreased volume of the superior temporal gyrus (Reiss et al., 1994), and an enlarged volume of the caudate nucleus (Reiss et al., 1995). On the cellular level, immature spine morphology and reduced synaptic size have been observed in autopsy material from patients suffering from Fragile X syndrome (Rudelli et al., 1985; Hinton et al., 1991; Irwin et al., 2000; 2001).

That finding that the FMRP gene is highly conserved among species, including mice (Verkerk et al., 1991; Ashley et al., 1993a; 1993b), and that the expression pattern at the mRNA and protein level is almost identical in different tissues of human and mice (Abitbol et al., 1993; Bachner et al., 1993; Devys et al., 1993; Hinds et al., 1993; Khandjian et al., 1995), together with the identification of a murine homologue of the FMR1, led to the generation of the Fmr1 knockout mouse, which can serve as an animal model for the experimental analysis of the Fragile X syndrome (Dutch-Belgian Fragile X Consortium, 1994). Although the nature of the mutation in the mouse model (gene interruption by insertion of a neomycin cassette into exon 5 of Fmr1) is different from that observed in most Fragile X syndrome patients (CGG repeat amplification), both changes within the genome lead to the silencing of the synthesis of functional mRNA or protein (Oostra & Hoogeveen,

1997). Previous studies characterizing the animal model drew attention to the behavioral and morphological differences between normal and muted mice that match, at least in part, those observed in affected humans (Dutch-Belgian Fragile X Consortium, 1994; D'Hooge et al., 1997; Paradee et al., 1999; Dobkin et al., 2000; van Dam et al., 2000). A study using FVB mice, the strain used in the present study, revealed significant deficits in the Morris water maze in comparison with wildtypes (Dobkin et al., 2000). The morphological abnormalities of synaptic structures in FMRP-deficient humans and mice (namely, increased number of immature dendritic spines and abnormal dendritic morphology), together with the observation that Fmr1 mRNA is strongly expressed during brain development and in regions of high synaptic plasticity, an involvement of this protein in synapse maturation and elimination and/or synthesis of proteins related to postsynaptic function was proposed (Comery et al., 1997; Feng et al., 1997; Weiler & Greenough, 1999; Irwin et al., 2000).

On the neurochemical level, neither human patients nor the mouse mutants displaying the Fragile X syndrome have been analyzed. Therefore, in the study described here, we performed a screening of a wide range of neurotransmitters and neuromodulators, including amino acids and monoamines and their metabolites, for the neurochemical characterization of the Fmr1 mouse mutants. We usied high performance liquid chromatography (HPLC) to quantify the basal tissue concentrations of amino acids, i.e. aspartate and glutamate (acting as excitatory neurotransmitters), γ-aminobutyric acid (GABA) and taurine (acting as inhibitory neurotransmitters/modulators) and glutamine and alanine, as well as monoamines (dopamine, serotonin) and their metabolites in eight distinct brain areas of juvenile (postnatal days 28 to 31) and adult (postnatal days 209 to 221) male Fmr1 knockout mice and compared with age-matched wildtype animals.

EXPERIMENTAL

Animals

Male wildtype and Fmr1 knockout mice with FVB/NJ strain background were genotyped by the polymerase chain reaction (for details see Consortium, 1994) to confirm the normality or mutation of the Fmr1 gene in wildtype and knockout mice, respectively. Mice were housed under strictly controlled conditions (light:dark = 12:12 hours) with food and water ad libitum. Litters remained with their mother until postnatal day 21 (PND 21), when they were weaned and housed as offspring-groups until used in the experiments. All experimental protocols were approved by the ethical committee of the government of the state of Saxony-Anhalt according to the German guidelines for the care and use of animals in laboratory research (§8, Abs. 1, 25.05.1998). All experiments were performed in accordance with the European Communities Council Directive of November 1986 (86/609/EEC).

Chemicals

Dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), 4-hydroxy-3-methoxyphenylacetic acid (HVA), 3-methoxytyramine (3-MT), serotonin (5-HT), 5-hydroxyindole-3-acetic acid (5-HIAA), ethylenediaminetetraacetic acid, sodium metabisulfite, ortho-phtaldialdehyde, and octanesulfonic acid were purchased from SIGMA (Germany). Perchloric acid, acetonitrile, and methanol were from MERCK (Germany). The compounds aspartate, glutamate, glutamine, taurine, alanine, and GABA were purchased from SERVA (Germany).

Tissue preparation

Neurochemical measurements were carried out in two groups of age: Juveniles (12 wildtype and 10 Fmr1 knockout mice from 8 litters aged PND 28 to 32) and adults (8 wildtype and 10 Fmr1 knockout mice from 6 litters aged PND 209 to 221).

After decapitation, the brains were quickly removed and placed on an ice-cold dissection plate. Each brain was sectioned into eight different areas: medial prefrontal cortex (mPFC), frontal cortex (includes the orbital, primary motor, and frontal part of the somatosensory cortex), caudal cortex (includes the caudal part of the somatosensory, and the visual cortex), striatum (includes striatum, nucleus accumbes, and ventral pallidum), hippocampus, thalamus (includes thalamic and hypothalamic nuclei, and substantia nigra), cerebellum, and brainstem. The tissue from both hemispheres were pooled and frozen in liquid nitrogen and stored at –80°C until assayed. For the quantification of amino acids and monoamines brain tissue wet weight was determined, and each piece was placed into a vial containing ice-cold homogenization buffer (0.1 M perchloric acid, 0.1 mM ethylenediaminetetraacetic acid, 2 mM sodium metabisulfite). After homogenization with an ultrasonic tissue disrupter (Sonoplus HD60, Bandelin, Germany) the samples were centrifuged (14,000 x g for 15 min at 4°C) and the supernatants were filtered through a syringe filter (0.2 micron pore size; Whatman, USA) before HPLC analysis.

Reversed-phase HPLC analysis

For reversed-phase high performance liquid chromatography (HPLC), the supernatants of each sample were divided, using one part for amino acid analysis and one part for measuring monoamines, respectively. Concentrations of the amino acids aspartate, glutamate, glutamine, taurine, alanine, and GABA were determined by automatic pre-column-derivatization using ortho-phtaldialdehyde and fluorescence detection (following a modified version of the method of Lindroth & Mopper, 1979). Briefly, the HPLC analysis was performed

on a C18 reversed-phase column (125 x 4 mm, packed with LiChrospher® 5 μm, MERCK, Germany), using a non-linear gradient of 5% to 75% methanol in 0.15 M phosphate buffer, pH 7.0, at a flow rate of 1.5 mL/min.

Dopamine and its metabolites DOPAC, HVA, and 3-MT, as well as 5-HT and its metabolite 5-HIAA, were measured in a single run on an HPLC column (100 x 3 mm packed with YMC ODS-AQ®, 5 μm, YMC, Germany) under isocratic conditions, with electrochemical detection. The mobile phase consisted of a 75 mM phosphate buffer containing 2 mM ethylenediaminetetraacetic acid, 0.1 mM octanesulfonic acid, and 3.5% (v/v) acetonitrile (pH 4.6). Electrochemical detection was achieved by setting a glassy carbon working electrode at +780 mV against an Ag/AgCl reference electrode of the electrochemical detector (LC4C, BAS, USA).

The final amount in the tissue sample was expressed as micrograms/g wet tissue for amino acids or ng/g wet tissue for monoamines, using an external calibration curve.

Statistical analysis

Values reported are means ± SEM. Statistical significance for the comparison of genotypes (wildtype vs. Fmr1 knockout) was performed by the Mann-Whitney U-test using SigmaStat 2.0 (Jandel, Germany) and considered significant when p < 0.05.

RESULTS

For each brain region, we calculated the tissue levels of aspartate, glutamate, glutamine, taurine, alanine, and GABA, as well as the ratio of excitatory and inhibitory amino acids [(aspartate + glutamate)/(taurine + GABA)] for both wildtype and Fmr1 knockout mice (see Fig. 1 and Table 1 for juveniles, Fig. 1 and Table 3 for adults),

allowing for some assessment of the functional state of the neuronal network. Similarly, the tissue levels of DA, DOPAC, HVA, 3-MT, 5-HT, and 5-HIAA, as well as the ratios of DOPAC/DA, HVA/DA, DOPAC+HVA/DA, and 5-HIAA/5-HT, which reflect changes in monoamine metabolism, were calculated (Table 2 for juveniles and Table 4 for adults). Because the tissue levels of 3-MT were measurable only in the caudal cortex and striatum, and the ratio of 3-MT/DA revealed no significant differences between genotypes, the ratio data are not shown.

In general, knockout of the Fmr1 gene was accompanied by age- and region-specific neurochemical alterations. Of all brain areas investigated, the frontal and caudal cortex, striatum, cerebellum, hippocampus, and the brainstem of juvenile mice displayed the most obvious neurochemical differences between wildtype and Fmr1 knockout mice. Throughout the analyzed areas in the juvenile brain, the amino acids taurine and aspartate, followed by alanine, glutamine, and GABA, showed the most prominent differences between wildtype and Fmr1 knockout mice. Glutamate was unaltered in all regions of both ages which were investigated (Table 1 and Table 3). In the caudal cortex, hippocampus, and brainstem of the juvenile Fmr1 knockout mice, the balance between excitatory and inhibitory amino acids was shifted toward increased inhibition (Table 1), whereas in adults no difference was found. Differences in the tissue levels and the metabolism of the dopaminergic system were detectable in the frontal cortex, striatum, and cerebellum of juveniles, and in the mPFC and brainstem of adults. The tissue levels and the metabolism of the serotonergic system were affected in the caudal cortex and brainstem of juveniles and in the hippocampus of adults.

In more detail, the following differences between wildtype and Fmr1 knockout mice were observed:
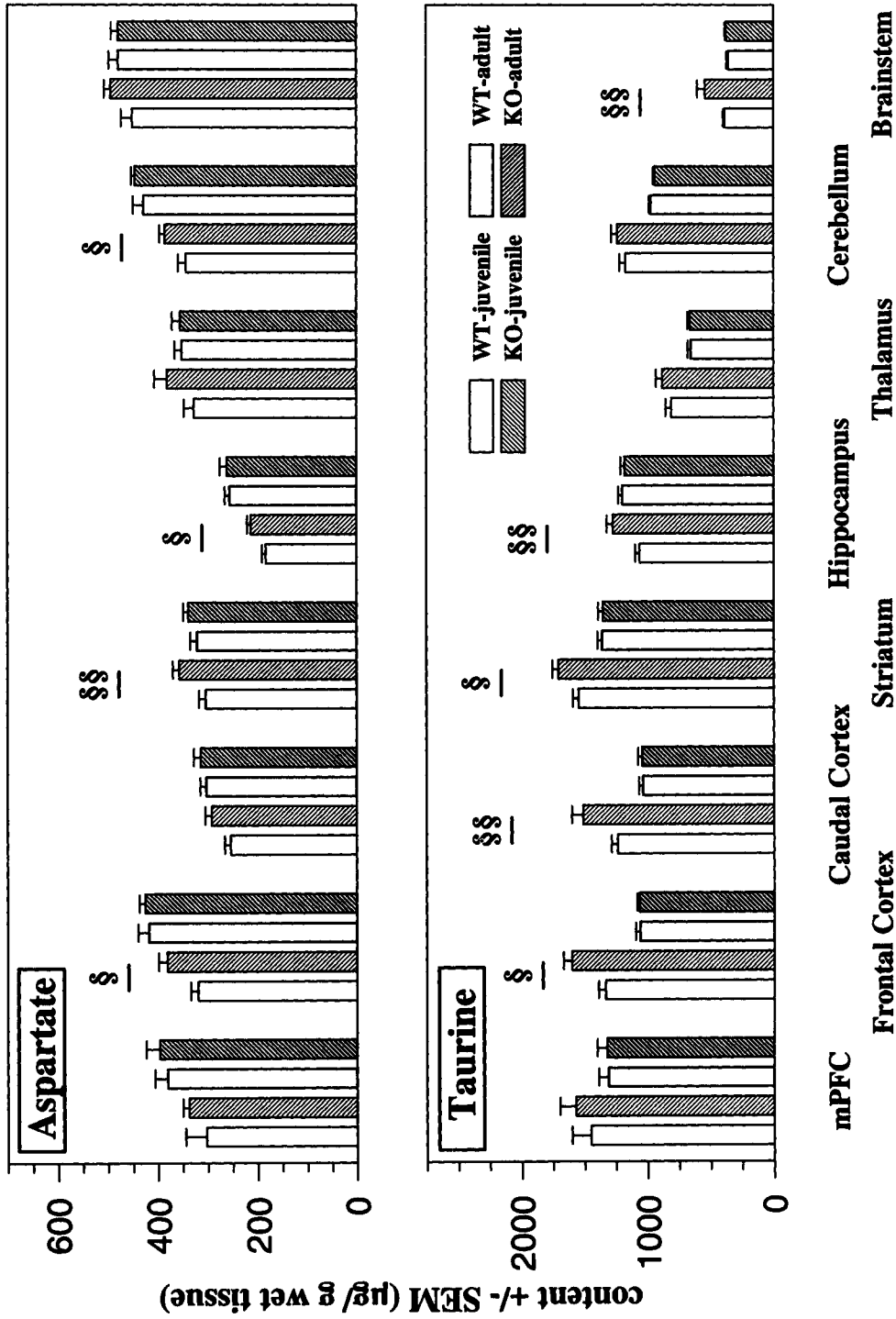
Fig. 1: Effect of Fmr1 knockout (KO) on the brain tissue levels of aspartate and taurine in juvenile and adult mice compared with wildtype mice (WT). In the juveniles, an increase of aspartate, as well as taurine, was detected in most brain regions of Fmr1 knockout mice. In contrast, in adults no brain region investigated displays a difference between genotypes. mPFC: medial prefrontal cortex: § p < 0.05. §§ p < 0.01 Fmr1 knockout vs. wildtype mice.

TABLE 1

Juveniles: Tissue levels of amino acids and excitation/inhibition quotients

	Aspartate	Glutamate	Glutamine	Taurine	Alanine	GABA	(Aspartate+Glutamate)/ (Taurine+GABA)
Medial prefrontal cortex							
Wildtype	302.9 ± 42.3	1559.2 ± 192.0	541.4 ± 62.0	1451.5 ± 151.1	61.0 ± 7.9	204.1 ± 30.0	1.13 ± 0.06
Fmr1 knockout	338.3 ± 34.5	1630.2 ± 81.7	403.3 ± 48.8	1572.4 ± 126.3	67.4 ± 5.5	129.3 ± 23.9	1.18 ± 0.05
Frontal cortex							
Wildtype	319.2 ± 14.8	1324.4 ± 62.4	709.3 ± 42.8	1333.9 ± 53.0	60.5 ± 2.8	214.1 ± 7.8	1.06 ± 0.03
Fmr1 knockout	381.0 ± 17.8	1429.8 ± 57.0	619.6 ± 28.7	1601.2 ± 67.8§	66.1 ± 4.5	221.2 ± 9.3	1.00 ± 0.02
Caudal cortex							
Wildtype	253.9 ± 11.5	1210.0 ± 54.9	766.0 ± 44.3	1239.0 ± 47.7	56.5 ± 3.5	190.6 ± 9.0	1.03 ± 0.02
Fmr1 knockout	292.1 ± 2.9†	1271.1 ± 58.6	698.4 ± 35.6	1510.7 ± 88.3‡	66.4 ± 9.3	222.5 ± 16.4	0.91 ± 0.00‡
Striatum							
Wildtype	303.6 ± 13.5	1424.8 ± 47.0	691.6 ± 34.8	1543.8 ± 47.9	68.2 ± 3.3	243.7 ± 16.4	0.97 ± 0.03
Fmr1 knockout	356.2 ± 13.4‡	1465.9 ± 49.2	619.4 ± 23.0	1765.9 ± 49.6§	72.2 ± 4.5	281.8 ± 19.7	0.92 ± 0.02
Hippocampus							
Wildtype	183.0 ± 7.5	1073.9 ± 38.2	668.9 ± 33.5	1062.4 ± 32.9	63.3 ± 2.7	179.5 ± 6.1	1.01 ± 0.03
Fmr1 knockout	213.6 ± 6.7‡	1114.2 ± 25.1	599.9 ± 19.2	1272.4 ± 48.6‡	69.3 ± 4.0	186.2 ± 7.9	0.92 ± 0.02§
Thalamus							
Wildtype	326.8 ± 19.5	1283.2 ± 55.0	714.5 ± 36.8	808.5 ± 43.4	52.2 ± 2.6	389.2 ± 25.3	1.36 ± 0.06
Fmr1 knockout	380.7 ± 25.4	1293.5 ± 43.0	618.4 ± 24.1	886.3 ± 47.8	57.3 ± 3.0	357.1 ± 15.8	1.38 ± 0.08
Cerebellum							
Wildtype	342.6 ± 16.9	1480.9 ± 59.4	649.7 ± 39.7	1169.7 ± 50.2	38.9 ± 4.1	142.9 ± 6.9	1.39 ± 0.03
Fmr1 knockout	385.3 ± 10.1§	1452.9 ± 35.4	732.9 ± 21.0§	1235.4 ± 47.1	45.3 ± 3.4	131.8 ± 4.3	1.35 ± 0.03
Brainstem							
Wildtype	451.0 ± 21.9	965.3 ± 32.2	499.8 ± 16.1	394.8 ± 42.4	172.4 ± 1.8	169.3 ± 7.8	2.25 ± 0.14
Fmr1 knockout	494.2 ± 13.3	1019.3 ± 50.1	531.2 ± 26.1	535.4 ± 62.8‡	45.1 ± 2.3‡	231.7 ± 28.4‡	2.08 ± 0.12‡

Amino acid tissue levels are given in micrograms/g wet tissue (mean ± SEM). Differences between genotypes are highlighted.
§p < 0.05; ‡p < 0.01 wildtype vs. Fmr1knockout (Mann-Whitney U-test)
†p = 0.06

TABLE 2

Juveniles: Tissue levels and metabolism of monoamines

	DA	DOPAC	HVA	3-MT	DOPAC/DA	HVA/DA	DOPAC-HVA/DA	5-HT	5-HIAA	5-HIAA/HT
Medial prefrontal cortex										
Wildtype	505.3 ± 221.6	192.4 ± 59.6	132.6 ± 21.1	n.d.	0.59 ± 0.15	1.11 ± 0.47	1.44 ± 0.58	315.0 ± 58.1	316.9 ± 37.5	1.60 ± 0.45
Fmr1 knockout	281.1 ± 163.4	100.2 ± 43.6	126.9 ± 19.5	n.d.	0.92 ± 0.26	2.98 ± 0.87	3.90 ± 1.08	173.3 ± 53.8	272.0 ± 25.6	3.06 ± 0.90
Frontal cortex										
Wildtype	80.9 ± 42.5	63.1 ± 9.2	117.4 ± 11.6	n.d.	5.30 ± 1.70	15.80 ± 6.30	21.20 ± 8.00	484.0 ± 15.2	212.4 ± 25.1	0.44 ± 0.05
Fmr1 knockout	28.5 ± 9.0	37.9 ± 4.5§	111.0 ± 9.5	n.d.	6.20 ± 1.20	20.50 ± 4.00	26.70 ± 5.20	417.7 ± 29.1	242.1 ± 26.2	0.62 ± 0.09
Caudal cortex										
Wildtype	301.7 ± 45.1	57.0 ± 6.6	49.9 ± 6.0	20.9 ± 3.6	0.20 ± 0.01	0.17 ± 0.01	0.37 ± 0.02	333.5 ± 17.3	168.9 ± 26.2	0.56 ± 0.06
Fmr1 knockout	280.3 ± 28.0	101.3 ± 19.4	62.6 ± 7.8	18.4 ± 2.4	0.39 ± 0.09	0.23 ± 0.03	0.62 ± 0.12	370.9 ± 26.3	270.1 ± 26.4§	0.80 ± 0.15
Striatum										
Wildtype	5841.2 ± 340.2	897.7 ± 55.5	708.0 ± 38.0	340.2 ± 13.3	0.16 ± 0.01	0.12 ± 0.05	0.28 ± 0.02	498.3 ± 19.9	369.7 ± 29.9	0.76 ± 0.07
Fmr1 knockout	4912.7 ± 449.1	991.4 ± 77.5	707.9 ± 52.3	277.7 ± 23.3	0.22 ± 0.03	0.15 ± 0.06†	0.37 ± 0.04†	541.0 ± 29.4	420.5 ± 17.1	0.79 ± 0.04
Hippocampus										
Wildtype	21.4 ± 2.5	12.6 ± 1.6	37.1 ± 5.3	n.d.	0.61 ± 0.07	1.92 ± 0.24	2.54 ± 0.27	322.6 ± 21.5	312.4 ± 17.2	1.00 ± 0.06
Fmr1 knockout	22.5 ± 4.9	10.7 ± 7.3	36.2 ± 3.4	n.d.	0.56 ± 0.08	1.85 ± 0.26	2.40 ± 0.30	366.2 ± 24.6	335.2 ± 13.7	0.95 ± 0.06
Thalamus										
Wildtype	585.7 ± 183.8	174.6 ± 18.7	161.2 ± 13.7	n.d.	0.57 ± 0.10	0.58 ± 0.11	1.15 ± 0.21	708.1 ± 47.2	527.4 ± 61.8	0.80 ± 0.11
Fmr1 knockout	356.0 ± 148.7	167.6 ± 22.7	134.6 ± 14.6	n.d.	0.81 ± 0.13	0.68 ± 0.12	1.49 ± 0.24	682.5 ± 51.5	512.0 ± 52.0	0.96 ± 0.12
Cerebellum										
Wildtype	7.7 ± 1.7	15.3 ± 2.9	19.1 ± 3.1	n.d.	2.43 ± 0.50	3.00 ± 0.80	5.38 ± 1.06	80.0 ± 9.2	78.4 ± 4.4	1.15 ± 0.16
Fmr1 knockout	3.9 ± 0.7§	17.3 ± 11.5	9.9 ± 1.2†	n.d.	6.27 ± 4.69	1.86 ± 0.24	8.74 ± 5.35	71.5 ± 11.0	77.7 ± 5.2	1.28 ± 0.18
Brainstem										
Wildtype	21.6 ± 1.8	26.7 ± 2.7	36.6 ± 4.9	n.d.	1.33 ± 0.18	1.75 ± 0.20	3.08 ± 0.36	668.1 ± 33.0	473.5 ± 31.3	0.75 ± 0.06
Fmr1 knockout	62.8 ± 40.2	43.8 ± 19.9	47.6 ± 18.3	n.d.	1.09 ± 0.06	1.28 ± 0.14	2.30 ± 0.20	709.2 ± 36.0§	555.7 ± 52.1	1.70 ± 0.04

Monoamine values are given in ng/g wet tissue (mean ± SEM). Differences between genotypes are highlighted.
§p < 0.05, †p < 0.01 wildtype vs. Fmr1 knockout (Mann-Whitney U-test)
n.d. = not detectable

TABLE 3

Adults: Tissue levels of amino acids and excitation/inhibition quotients

	Aspartate	Glutamate	Glutamine	Taurine	Alanine	GABA	(Aspartate+Glutamate)/ (Taurine+GABA)
Medial prefrontal cortex							
Wildtype	380.9 ± 25.5	1548.2 ± 103.3	418.9 ± 50.7	1315.7 ± 73.9	42.6 ± 3.6	208.0 ± 14.2	1.29 ± 0.02
Fmr1 knockout	396.6 ± 27.3	1578.5 ± 87.8	354.6 ± 33.2	1326.1 ± 73.9	48.7 ± 3.4	208.4 ± 14.4	1.29 ± 0.03
Frontal cortex							
Wildtype	418.3 ± 20.9	1493.0 ± 55.7	566.2 ± 29.3	1060.7 ± 35.7	43.9 ± 2.0	187.1 ± 8.8	1.53 ± 0.02
Fmr1 knockout	425.2 ± 11.5	1509.1 ± 33.0	503.9 ± 11.7§	1064.5 ± 19.0	45.4 ± 1.8	181.6 ± 2.5	1.55 ± 0.02
Caudal cortex							
Wildtype	303.3 ± 11.0	1293.5 ± 38.9	554.4 ± 31.1	1035.6 ± 33.4	39.4 ± 1.5	165.7 ± 4.1	1.33 ± 0.02
Fmr1 knockout	314.2 ± 13.2	1297.4 ± 44.9	513.5 ± 20.5	1043.2 ± 30.9	41.2 ± 1.7	171.9 ± 5.7	1.33 ± 0.02
Striatum							
Wildtype	321.1 ± 13.4	1324.2 ± 36.9	619.5 ± 32.2	1360.8 ± 33.4	51.8 ± 2.8	307.3 ± 10.3	0.99 ± 0.02
Fmr1 knockout	338.5 ± 10.2	1331.4 ± 28.5	574.0 ± 22.4	1352.9 ± 37.1	52.2 ± 2.0	315.3 ± 10.7	1.00 ± 0.01
Hippocampus							
Wildtype	255.7 ± 9.5	1360.0 ± 26.9	511.3 ± 28.3	1196.9 ± 32.6	51.9 ± 2.1	208.4 ± 5.5	1.15 ± 0.01
Fmr1 knockout	261.1 ± 13.5	1364.2 ± 50.2	467.2 ± 20.8	1179.3 ± 33.9	56.4 ± 2.4	212.0 ± 6.0	1.17 ± 0.02
Thalamus							
Wildtype	351.3 ± 14.0	1197.1 ± 26.2	531.5 ± 27.6	654.9 ± 23.4	41.6 ± 1.2	353.0 ± 9.6	1.54 ± 0.05
Fmr1 knockout	354.3 ± 15.8	1205.8 ± 29.2	493.2 ± 17.4	662.1 ± 17.2	44.1 ± 1.9	348.3 ± 14.9	1.54 ± 0.03
Cerebellum							
Wildtype	428.2 ± 20.8	1460.3 ± 23.9	651.7 ± 19.8	968.3 ± 14.8	34.9 ± 1.6	132.5 ± 2.1	1.72 ± 0.03
Fmr1 knockout	445.5 ± 7.3	1420.9 ± 23.4	572.6 ± 17.2§	939.4 ± 13.2	33.4 ± 1.0	131.9 ± 3.7	1.74 ± 0.02
Brainstem							
Wildtype	479.2 ± 18.9	852.0 ± 23.3	395.6 ± 12.5	356.0 ± 12.0	32.1 ± 0.8	159.3 ± 5.4	2.59 ± 0.04
Fmr1 knockout	479.4 ± 14.2	849.5 ± 15.1	379.4 ± 9.4	371.8 ± 9.6	33.0 ± 1.8	162.7 ± 3.8	2.49 ± 0.06

Amino acid tissue levels are given in micrograms/g wet tissue (mean ± SEM). Differences between genotypes are highlighted.
§$p < 0.05$; †$p < 0.01$ wildtype vs. Fmr1knockout (Mann-Whitney U-test)

TABLE 4

Adults: Tissue levels and metabolism of monoamines

	DA	DOPAC	HVA	3-MT	DOPAC/DA	HVA/DA	DOPAC-HVA/DA	5-HT	5-HIAA	5-HIAA/HT
Medial prefrontal cortex										
Wildtype	▓▓▓	246.3 ± 35.5	184.3 ± 9.7	n.d.	▓▓▓	0.61 ± 0.14	▓▓▓	288.9 ± 36.6	302.9 ± 14.2	1.15 ± 0.12
Fmr1 knockout	▓▓▓	174.2 ± 34.7	170.1 ± 10.0	n.d.	▓▓▓	1.13 ± 0.17	▓▓▓	240.3 ± 30.9	305.3 ± 20.2	1.36 ± 0.10
Frontal cortex										
Wildtype	40.3 ± 7.4	61.1 ± 4.4	100.0 ± 7.4	n.d.	1.67 ± 0.15	2.79 ± 0.29	4.46 ± 0.43	641.3 ± 14.9	247.4 ± 16.4	0.38 ± 0.02
Fmr1 knockout	42.5 ± 8.1	59.9 ± 3.2	98.9 ± 5.1	n.d.	1.64 ± 0.15	2.80 ± 0.33	4.44 ± 0.48	688.8 ± 22.8	237.0 ± 9.7	0.34 ± 0.01
Caudal cortex										
Wildtype	324.2 ± 37.1	60.8 ± 4.8	42.5 ± 3.8	18.6 ± 2.5	0.19 ± 0.01	0.14 ± 0.01	0.33 ± 0.02	433.9 ± 13.5	180.4 ± 10.7	0.42 ± 0.02
Fmr1 knockout	387.2 ± 27.1	64.2 ± 4.7	54.5 ± 3.9	23.6 ± 2.8	0.17 ± 0.01	0.14 ± 0.01	0.31 ± 0.01	451.1 ± 12.3	182.5 ± 8.8	0.41 ± 0.02
Striatum										
Wildtype	9442.3 ± 411.2	1138.9 ± 80.4	851.8 ± 53.8	375.9 ± 25.6	0.12 ± 0.01	0.09 ± 0.01	0.21 ± 0.01	800.2 ± 23.8	479.4 ± 20.6	0.60 ± 0.02
Fmr1 knockout	9811.1 ± 352.4	1162.8 ± 25.9	922.3 ± 33.5	422.5 ± 15.9	0.12 ± 0.01	0.10 ± 0.01	0.21 ± 0.01	804.1 ± 16.7	490.1 ± 16.9	0.61 ± 0.02
Hippocampus										
Wildtype	17.2 ± 1.8	19.7 ± 1.5	22.7 ± 2.7	n.d.	1.17 ± 0.06	1.33 ± 0.09	2.50 ± 0.13	▓▓▓	362.0 ± 24.7	0.89 ± 0.06
Fmr1 knockout	17.9 ± 0.7	22.8 ± 0.9	25.3 ± 1.3	n.d.	1.28 ± 0.03	1.44 ± 0.10	2.71 ± 0.12	▓▓▓	421.6 ± 22.2	0.92 ± 0.03
Thalamus										
Wildtype	150.8 ± 16.5	123.9 ± 10.0	134.6 ± 7.4	n.d.	0.85 ± 0.05	0.93 ± 0.05	1.77 ± 0.09	923.1 ± 36.7	625.1 ± 28.2	0.68 ± 0.04
Fmr1 knockout	179.1 ± 22.3	123.9 ± 7.4	137.9 ± 6.6	n.d.	0.76 ± 0.07	0.86 ± 0.09	1.62 ± 0.15	855.6 ± 63.0	623.5 ± 28.2	0.75 ± 0.04
Cerebellum										
Wildtype	5.3 ± 0.4	10.5 ± 0.5	5.8 ± 1.3	n.d.	2.06 ± 0.16	1.07 ± 0.20	3.13 ± 0.23	55.0 ± 4.3	73.1 ± 5.0	1.36 ± 0.11
Fmr1 knockout	6.2 ± 0.5	11.8 ± 0.7	5.8 ± 0.5	n.d.	1.96 ± 0.10	0.98 ± 0.10	2.94 ± 0.18	53.1 ± 2.7	67.3 ± 2.4	1.28 ± 0.03
Brainstem										
Wildtype	15.2 ± 0.6	26.7 ± 1.4	12.7 ± 1.3	n.d.	1.78 ± 0.11	0.84 ± 0.08	2.61 ± 0.19	593.7 ± 29.8	357.6 ± 19.0	0.60 ± 0.02
Fmr1 knockout	21.6 ± 2.9	32.3 ± 1.5	16.7 ± 1.2	n.d.	1.66 ± 0.13	0.85 ± 0.08	2.51 ± 0.20	627.9 ± 20.8	385.4 ± 14.3	0.62 ± 0.02

Monoamine values are given in ng/g wet tissue (mean ± SEM). Differences between genotypes are highlighted.
§p < 0.05 wildtype vs. Fmr1 knockout (Mann-Whitney U-test)
n.d. = not detectable

Juveniles: As shown in Fig. 1 and Table 1, when compared with wildtype mice, aspartate was significantly increased in Fmr1 knockout mice in the frontal cortex (+19%), striatum (+17%), hippocampus (+17%), and cerebellum (+12%).

Glutamine was significantly decreased in the cerebellum (−14%). Taurine was significantly increased in frontal cortex (+20%), caudal cortex (+22%), striatum (+10%), hippocampus (+20%), and brainstem (+40%). The levels of *alanine* were increased in the frontal cortex (+18%), and brainstem (+21%). GABA: an increase in the brainstem (+29%) was measured. When compared with wildtype mice, the ratio of (aspartate+ glutamate)/ (taurine+GABA) in Fmr1 knockout mice was lower in the caudal cortex (−12%), hippo-campus (−9%), and brainstem (−15%).

As indicated in Table 2, tissue levels of DA were decreased in cerebellum (−49%), and DOPAC levels were decreased in the frontal cortex (−40%). The amounts of HVA were decreased in cerebellum (−64%). Whereas the ratio of *DOPAC/DA* was unchanged in all brain regions, the *HVA/DA* ratio was increased in the striatum (+25%). The ratio of *DOPAC+HVA/DA* showed significant differences between the two genotypes in the striatum (+32%). In Fmr1 knockout mice, tissue levels of 5-HT were increased in the brainstem exclusively (+18%). The levels of 5-HIAA were increased only in the caudal cortex (+43%). When characterizing the metabolism of serotonergic neurons, we obtained no significant differences of the 5-HIAA/5-HT ratio.

Adults: In contrast to the juvenile brain, the adult animals displayed only a few significant differences between wildtype and Fmr1 knockout mice (Fig. 1, Tables 3 and 4). For amino acids, only glutamine was decreased in the frontal cortex (−11%) and cerebellum (−12%).

The tissue levels of DA were decreased in the mPFC (−41%) and increased in the brainstem (+42%) of Fmr1 knockout mice compared with wildtype mice. The amounts of DOPAC (+23%)

and HVA (+31%) were increased in the brainstem. The ratio of *DOPAC/DA* was increased in the mPFC (+34%), but the *HVA/DA* ratio showed no significant change. The ratio of *DOPAC+HVA/DA* was increased in the mPFC (+59%). Whereas the levels of 5-HT were increased in the hippocampus (+13%), the amounts of 5-HIAA showed no significant differences between genotypes throughout the brain areas investigated. Furthermore, no changes of the *5-HIAA/5-HT* ratio were observed.

DISCUSSION

The present data comparing wildtype and Fmr1 knockout mice on the neurochemical level provide the first evidence that lack of FMRP expression in male mice is paralleled by age- and region-specific abnormalities in basal tissue levels of amino acids and monoamine metabolism, as well as an altered balance between excitatory and inhibitory amino acid transmitter systems. In summary:

i) those brain regions which are known for high expression of FMRP in normal subjects, namely, cortex, striatum, hippocampus, and cerebellum (Abitbol et al., 1993; Hinds et al., 1993; Hergersberg et al., 1995; Feng et al., 1997), and in addition the brainstem, displayed the most obvious differences between both genotypes;

ii) numbers of neurochemical differences between wildtype and Fmr1 knockout mice were higher in juveniles (23 significant differences) than in adults (9 significant differences);

iii) in juvenile brains, the most affected substrates were the amino acids, especially taurine and aspartate, which showed elevated basal levels in Fmr1 knockout mice; neither juveniles nor adults showed significant alterations between genotypes in the most abundant excitatory amino acid, glutamate;

iv) the decreased ratio of excitation/inhibition, namely, (aspartate+glutamate)/(taurine+GABA), in juvenile wildtype mice implies an increased level of neuronal inhibition in caudal cortex, hippocampus, and brainstem of Fmr1 knockout mice;

v) very few differences between genotypes were found for monoamine metabolism; in juveniles, increased metabolism of dopamine was found exclusively in the striatum, whereas in adults, increased metabolism was found in the mPFC; differences in serotonin metabolism were measured in neither juveniles nor adults.

It is widely accepted, that the precise local and temporal interplay between excitatory and inhibitory neural activity is of critical importance for the formation of the final arrangement of functional neuronal connections (Singer, 1995; Katz & Shatz, 1996; Micheva & Beaulieu, 1997; Yuste & Sur, 1999). The precise causal relationship between neurochemical and the morphological, as well as behavioral, abnormalities in Fmr1 knockout mice remains to be analyzed by further studies, in particular whether altered amino acids, as well as the mismatch of excitation/inhibition, are the consequence of or the cause for the structural and behavioral abnormalities described for the Fragile X syndrome.

Taurine, which was elevated in various brain regions of juvenile Fmr1 knockout mice, is assumed to play a role as an inhibitory neurotransmitter or neuromodulator (Pasantes-Morales & Gamboa, 1980; Huxtable, 1989; 1992; Oja & Kontro, 1990), in addition to its role in osmoregulation, protein phosphorylation, calcium regulation, membrane stabilization, and as a trophic factor during neuro-development (Huxtable, 1989; 1992; Lima, 1999; Neuringer et al., 1994; Palackal et al., 1986; Sturman, 1993). Since it has been suggested that in the developing brain, taurine rather than GABA is the major inhibitor of neuronal excitation, especially during phases when GABA may still have excitatory

function (van den Pol et al., 1998; Khalilov et al., 1999; Leinekugel et al., 1999), high levels of taurine during early postnatal phases (Agrawal et al., 1968; Miller et al., 2000) might suppress electrical activity at a time when other inhibitory systems (GABA) are not yet fully matured (Huxtable, 1989). The role of aspartate, the second amino acid that showed significant alterations in Fmr1 knockout mice, in synaptic transmission is controversial. A recent study has strengthened the evidence for a transmitter role of aspartate in excitatory fiber systems, for example, in the hippocampus (Gundersen et al., 1998).

Taking together the excitatory (glutamate and aspartate) and inhibitory (taurine and GABA) transmitters, the decreased ratio of (aspartate + glutamate)/(taurine + GABA) in juvenile but not in adult Fmr1 knockout mice indicates a shift toward increased neuronal inhibition, especially in the caudal cortex, hippocampus, and brainstem of young mice. Such an altered balance of excitation and inhibition in young, but not in adult Fmr1 knockout mice could explain the electrophysio-logical findings of reduced hippocampal long-term potentiation (LTP) in juvenile Fmr1 knockout mice as compared with wildtype mice (Aggoun et al., 1999). In adult mice, no differences in basal electrical activity and induction of LTP could be found (Godfraind et al., 1996; Paradee et al., 1999). The mismatch of excitation/inhibition found in the brainstem could also underlie the enhanced audio-genic seizure susceptibility described for juvenile Fmr1 knockout mice as compared with wildtype mice (Musumeci et al., 2000). Why, in the present study, adult animals no longer showed this mis-match, despite the even more pronounced seizure susceptibility in adult Fragile X knockout animals, (Chen et al., 2001; Musumeci et al., 2000) remains an open question.

The neurochemical abnormalities measured in juvenile Fmr1 knockout mice appear to be transient because they are no longer found in the

brain of adult FMRP-deficient mice. This time window is at the end of the phase of neuronal and synaptic development in the normal brain (White et al., 1997) and matches well with a period of great vulnerability of the neuronal network toward the lack of FMRP expression, which was shown in a recent study (Nimchinsky et al., 2001). Perhaps some compensatory mechanisms that start to act in the juvenile brain (where neurochemical differences between genotypes are clear) gradually counterbalance the neurotransmission back to normal levels in adults.

To what extent the spectrum of neurochemical changes, seen in several brain regions particularly in young animals, may underlie the emotional and mental deficits observed in adult mice lacking FMRP expression requires further investigation. Furthermore, it would be interesting to assess if comparable neurochemical changes occur in human fragile X patients, and if the magnitude of neurochemical changes correlates with the severity of the mental and/or emotional impairment in humans, as well as in the animal model.

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