*Neuroscience* 301 (2015) 520–528

# BRAIN ACETYLCHOLINE AND CHOLINE CONCENTRATIONS AND DYNAMICS IN A MURINE MODEL OF THE FRAGILE X SYNDROME: AGE, SEX AND REGION-SPECIFIC CHANGES

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**Abstract—Fragile X syndrome is a learning disability caused by excess of CGG repeats in the 5′ untranslated region of the Fragile X gene (FMR1) silencing its transcription and translation. We used a murine model of this condition, *Fmr1* knock-out mice (KO) to study acetylcholine (ACh) metabolism and compared it to that of wild-type control mice (WT). Brain endogenous ACh (D0ACh), free choline (D0Ch), their deuterated variants D4ACh and D4Ch and mole ratios (AChMR and ChMR) were measured by gas chromatography–mass spectrometry in the cerebral hemisphere, cerebral cortex, hippocampus and cerebellum, following D4Ch administration. Regression analysis indicated a significant decrease with age (negative slope) of D4ACh, AChMR, D4Ch and ChMR in WT mice. Age dependence was only present for D4ACh and AChMR in KO mice. Analysis of variance with age as covariate indicated a significant greater D4Ch in the cerebral cortex of KO females when compared to WT females. Contrasts between sexes within genotypes indicated lower D0Ch in cortex and cerebellum of female KO mice but not in WT and lower D4Ch in hippocampus of female KO and WT mice. In conclusion, after adjusting for age, D0ACh concentrations and synthesis from deuterium-labeled Ch were similar in KO and control WT mice in all brain regions. In contrast, significant changes in Ch dynamics were found in hippocampus and cerebral cortex of KO mice that might contribute to the pathogenesis of FXS. Published by Elsevier Ltd. on behalf of IBRO.**

**Key words: acetylcholine turnover, mice, cholinergic function, GCMS, *fmr1*-KO mice.**

## INTRODUCTION

Fragile X syndrome (FXS) is a learning disability caused by CGG repeats in excess of 200 in the non-coding 5′ region of the Fragile X mental retardation gene (FMR1) (Bell et al., 1991; Fu et al., 1991; Verkerk et al., 1991) resulting in FMR1 protein deficiency (Pieretti et al., 1991). Subjects with FXS show developmental delays, hyperactivity, attentional problems, poor eye contact, hand flapping, hand biting, perseveration in behavior and language, and irritability or frequent tantrums (Hagerman, 1997). Subjects carrying between 55 and 200 CGG repeats do not develop FXS but have a high incidence of a tremor-ataxia syndrome (FXTAS) that develops in old age predominantly in males and primary ovarian deficiency in females (Hagerman, 2013).

Morphological dendritic abnormalities have been demonstrated during brain development and into adulthood in humans (Rudelli et al., 1985; Irwin et al., 2001) and the cerebral cortex (Nimchinsky et al., 2001) and cerebellum (Koekkoek et al., 2005; Huber, 2006) of murine models of the FXS.

The development of mice carrying an inactivated FMR1 gene so that functional FMRP is not produced (*Fmr1*-knock out) has offered outstanding opportunities to study the molecular basis of the FX syndrome (Consortium, 1994). These animals show variable deficits in spatial learning (D'Hooge et al., 1997; Van Dam et al., 2000; Baker et al., 2010) that are strain-dependent (Dobkin et al., 2000), decreased startle reactivity (Nielsen et al., 2002), increased spontaneous motor activity (Fish et al., 2013), changes in social interaction (Liu and Smith, 2009) and increased seizure susceptibility (Musumeci et al., 2000; Chen and Toth, 2001).

The well-established role of cerebral cholinergic mechanisms in learning (Berger et al., 1979; Bartus et al., 1985; Harrell et al., 1987; Chrobak et al., 1988; Luine and Hearns, 1990; Dawson et al., 1991) makes the evaluation of cholinergic function an attractive goal in the analysis of the pathophysiology of learning disabilities. Acetylcholine (ACh) modulates neuronal development and morphology (Bruel-Jungerman et al., 2011). Of particular interest in the case of FXS and animal models of this condition in which abnormal dendritic morphology has been described, is the potential of ACh to affect the maturation of dendrites. This has been demonstrated in the retina, where cholinergic transmission stabilizes certain dendrites and influences dendritic remodeling

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*Abbreviations:* ACh, acetylcholine; ANCOVA, analysis of covariance; D0ACh, endogenous ACh; D0Ch, endogenous free choline; FXS, fragile X syndrome; KO, knock-out; WT, wild type.

http://dx.doi.org/10.1016/j.neuroscience.2015.06.036
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during synaptogenesis (Lohmann et al., 2002). ACh is also known to enhance the survival of newborn neurons in the adult mouse dentate gyrus and olfactory bulb (Kaneko et al., 2006). Thus, a deficit in ACh availability or turnover could contribute to the pathogenesis of FXS.

A global influence of ACh on cerebral function could also be exerted through its control of the cerebral circulation (Scremin et al., 1973; Scremin, 1991) which could affect the ability of enhanced neuronal function to trigger adaptive adjustments of local blood flow in the case of a cholinergic deficit.

Pharmacological interventions on muscarinic ACh receptors support a role of cholinergic mechanisms in several electrophysiological and behavioral alterations observed in FXS murine models (D'Antuono et al., 2003; Volk et al., 2007; Veeraragavan et al., 2011; Fish et al., 2013). However, there is lack of a systematic analysis of cholinergic function in this animal model. In particular, ACh levels and synthesis, availability of its precursor choline and the dynamics of Ch uptake from the blood and its utilization for the synthesis of ACh have never been explored in *Fmr1*-knock-out (KO) mice.

The normal level of ACh in the brain tissue results from a dynamic steady state between synthesis and release of this transmitter. Since stores of ACh in brain tissues are small and could not sustain release in the absence of synthesis for more than a few minutes, ACh turnover is a good indication of the level of cholinergic transmission. Acetylcholine is synthesized by a reversible reaction between acetyl-CoA and Ch, catalyzed by choline acetyltransferase (ChAT) and it appears to be determined by the availability of its precursors, acetyl-CoA and Ch and the concentrations of the products (Jenden, 1979b). Choline for ACh synthesis is supplied through a high-affinity, Na-dependent choline uptake system (HACU) mediated by a protein (CHT-1) (Okuda and Haga, 2003) that transports Ch into cholinergic synaptic terminals. Net production from phospholipids, and plasma Ch are other possible sources of Ch for ACh synthesis (Jenden, 1979a).

The approach undertaken in this study has been to determine, in the entire hemisphere and key brain regions, the tissue levels and turnover rates of ACh and its precursor and degradation product Ch as affected by the genotype (FMR1KO vs wild-type (WT) mice), age and sex. This approach has been fruitful in the characterization of other syndromes with underlying cholinergic dysfunctions.

## EXPERIMENTAL PROCEDURES

### Mouse strains and genotyping

Mice of two strains were used, *Fmr1*-knock out (FVB.129P2-Fmr1tm1Cgr/J, Jackson Laboratories #004624) with females homozygous and males heterozygous for the targeted mutation, and their WT controls, (FVB.129P2-Pde6b+ Tyrc-ch/AntJ, Jackson Laboratories #004828). Animals were purchased from The Jackson Laboratories (Bar Harbor, Main, USA) and bred at the VA Greater Los Angeles Veterinary Medical Unit. Each litter was genotyped using PCR. The genotyping conditions and primer sequences were provided by the Jackson laboratory. Primers used are forward (for homozygous): 5′-CACGAGAC TAGTGAGA CGTG-3′, forward (for WT): 5′-TGTGATAGAATATGC AGCAT GTGA-3′ and reverse (for both) 5′-CTTCTGGC ACCTCCAGCTT-3′. PCR products were run in 1.5% gel and shown at 400 bp for homozygous, 131 bp for WT and together 400 bp and 131 bp for heterozygous.

### Animal procedures and sample collection

The research environment and protocol for animal experimentation were approved by the Institutional Animal Care and Use Committee (IACUC) of the West Los Angeles Veterans Affairs Healthcare System. The animal facility at this Institution is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Mice were kept in an environmentally controlled room with lights on at 07:00 h and off at 19:00 h. Endogenous free choline (D0Ch), choline labeled with 4 atoms of deuterium (D4Ch), endogenous ACh (D0ACh) and ACh labeled with 4 atoms of deuterium (D4ACh) were measured in brain tissue samples by GCMS. Animals were injected intraperitoneally with 73 μmoles/kg of the D4Ch tosylate salt solution, and 4 min later they were euthanized by microwave radiation focused to the head using a 5-kW Microwave Fixation Biostat unit (Gerling Applied Engineering, Modesto, CA, USA). Brains were removed immediately and flash frozen in powdered dry ice. The brains were bisected along their sagittal axis and in one hemisphere, selected at random, the entire cerebrum was sampled. In some animals, cerebral cortex of the frontal, parietal, temporal and occipital cortex pooled as one as well as hippocampus and cerebellum were obtained by microdissection of the other hemisphere. These tissue samples were homogenized in ice-cold 15% 1 N formic acid, 85% acetone for analysis of Ch, ACh and their deuterated variants mentioned above. Ch and ACh labeled with 9 deuterium atoms (D9Ch and D9ACh, respectively) were added to the tubes containing the homogenates in precisely known amounts to serve as internal standards. The homogenate was centrifuged and the supernatant transferred to clean centrifuge tubes and extracted with diethyl ether to remove acetone and lipids. The aqueous residue remaining after the ether extractions was used for GCMS determination of Ch and ACh as described by Jenden et al. (1973). First, these compounds are extracted in the following way: to the aqueous residue mentioned above is added an equal volume of 1 M TAPS buffer, pH 9.2 and 2 volumes of 1 mM dipicrylamine in methylene dichloride. After shaking and centrifuging, the aqueous phase is discarded; the organic phase is transferred to a clean centrifuge tube and evaporated to dryness. Then a solution of silver p-toluenesulfonate (5 mM in acetonitrile:0.5 ml) and 50 μl propionyl chloride are added, shaken, allowed to stand at room temperature for 5 min and evaporated to dryness. N-demethylation is carried out with sodium benzenethiolate in anhydrous butanone; this is followed by two liquid partition steps to separate tertiary amines from neutral and acidic

compounds, and an aliquot of the residue is injected into the GCMS system consisting of a 7890 Gas Chromatograph with a DB-5MS UI capillary column coupled to a 5975C Mass Detector (Agilent Technologies). Masses at 58, 60 and 64 are monitored corresponding to the base peaks generated by electron impact ionization of the N-demethylated D0, D4 and D9 ACh and Ch, respectively. This method is capable of detecting $10^{-13}$ -mole of Ch and ACh (Jenden et al., 1973).

In order to avoid technical difficulties and animal stress inherent to restraint and tail trauma, D4Ch was injected intraperitoneally (i.p.) instead of intravenous tail injection as performed in previous accounts for deuterium (Jenden et al., 1974) or radioactive isotope-labeled ACh precursors (Durkin et al., 1977). In order to determine the optimal time of euthanasia, the normal kinetics of D4Ch brain uptake from blood after intraperitoneal (i.p.) injection was studied. For this purpose, mice were injected i.p. with D4Ch tosylate and then euthanized by microwave irradiation of the head 1, 2, 4, 8 and 16 min after injection. D4Ch and D4ACh levels in the cerebral cortex, hippocampus, cerebellum and striatum were measured as described above. In order to obtain a global estimate of brain D4Ch uptake and D4ACh synthesis and cancel out variability among regions, values of each variable were calculated as a percent of their average value for each region, pooling all regions in the final analysis of D4Ch and D4ACh as a function of time.

### Data analysis

The data on brain D4Ch uptake were fit to a first-order absorption and elimination model (Dhillon and Gill, 2006) described as:

$$\frac{dD}{dt} = k_a D_0 - kD \quad (1)$$

where $k_a$ = absorption rate constant; $k$ = elimination rate constant; $t$ = time after D4Ch injection; $D$ = amount of D4Ch in the brain; $D_0$ = amount of D4Ch at the injection site.

Following integration:

$$D = D_0 k_a [\exp(-kt) - \exp(-k_a t)]/(k_a - k) \quad (2)$$

Eq. (2) was used to fit the data of brain D4Ch as a function of time after i.p. injection (Fig. 1).

The data on D4ACh were fit to a first-order model as;

$$D = A[1 - \exp(-kt)] \quad (3)$$

where $D$ = amount of D4ACh in the brain $k$ = rate constant; $t$ = time after D4Ch injection and $A$ = final D4ACh level.

The possible effect of age on the measured variables was tested with linear (least squares) regressions of each variable on age (days). Range of animal ages was FMR1WT = 105–359 days and FMR1KO = 107–373 days. This analysis was performed on the whole hemisphere because that region reflects the overall influence of age on the variables studied and includes the larger number of mice providing better statistical power.

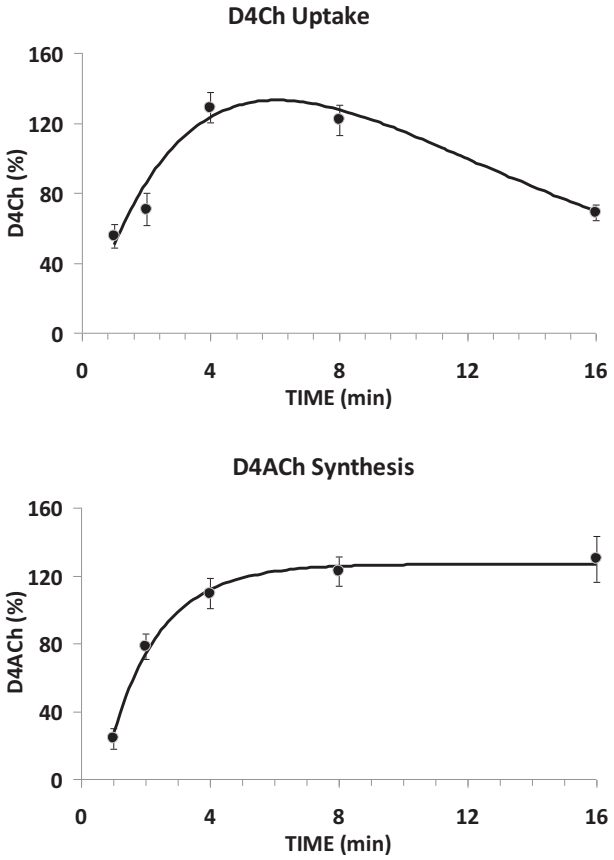


**Fig. 1.** Time course of D4Ch uptake from blood (top panel) and synthesis of D4ACh (bottom panel). The D4Ch data was fit to a first-order absorption and elimination model as described in the methods section using Eq. (2) and the data on D4ACh synthesis to a first-order growth model using Eq. (3). Number of cases are: 1 min = 7, 2 min = 8, 4 min = 17, 8 min = 4, 16 min = 8.

Analysis of covariance (ANCOVA) with age as covariate was performed for each region and variable with factors genotype (*Fmr1*-KO and WT), and sex (female, male) and followed by post hoc contrasts between levels of factors that showed statistically significant main effects in ANCOVA ($P < 0.05$). The results were displayed in graphs and tables as mean ± standard error (S.E.). Calculations were done with the statistical packages NCSS.LLC (Kaysville, Utah) and GraphPad Prism, (GraphPad Software Inc. La Jolla, California, USA). Number of animals for each analysis is shown in the tables and figure legends. Mean values of brain tissue D0ACh, D4ACh, D0Ch, and D4Ch concentrations were expressed in tables and graphs as nmoles $g^{-1}$.

## RESULTS

Uptake of D4Ch as a function of time after i.p. injection showed a steep increase with time up to 4 min after i.p. injection, followed by a lower rate of decline (Fig. 1, top panel). After 4 min D4ACh levels stabilized (Fig. 1, bottom panel). Consequently, all animals were euthanized at 4 min post-injection.

Regression analysis was performed on the entire hemisphere data to test for age dependence of each variable and the significance of possible differences in slopes between FMR1KO and FMR1WT mice. A significant decrease with age (negative slope) of D4ACh, AChMR, D4Ch and ChMR was found in FMR1WT mice. The findings in FMR1KO mice revealed instead a decrease with age only for D4ACh and AChMR (Table 1 and Figs. 8 and 9). A significant difference between slopes of FMR1KO versus FMR1WT mice existed for D4Ch (Table 1). The data shown on age-dependent trends corresponds to the whole hemisphere and males and females have been pooled.

ANCOVA with age as covariate indicated statistical significance for the factor region for all variables, sex for D0Ch and D4Ch and genotype for D4Ch. ANCOVA was then performed for each region to test for main effects of genotype and sex followed, if significant, by Tukey–Kramer post hoc contrasts. Mean values of each variable adjusted for age in each region are presented in Figs 2–7. No significant differences in the concentrations of D0ACh between FMR1KO and FMR1WT were found in any region (Fig. 2). The same was true for D4ACh (Fig. 3) and ACh mole ratio (Fig. 4). A significant increase of D4Ch (Fig. 6) was found in the cerebral cortex region of FMR1KO females when compared to WT females.

Contrasts between sexes within genotypes indicated lower D0Ch in cortex and cerebellum of female FMR1KO mice but not in WT controls (Fig. 5) and lower D4Ch in hippocampus of female KO and WT mice (Fig. 6).

## DISCUSSION

Cholinergic function has been historically evaluated *in vivo* by diverse means of estimating the turnover of ACh (Sparf, 1973; Jenden et al., 1974). An alternative approach is the measurement of ACh release into microdialysis probes inserted into the brain (Day et al., 2001). Although this approach can follow changes in cholinergic activity over time in conscious tethered animals, difficulties with this technique reside in the invasive nature of it and the fact that only a single or a few cerebral

**Table 1.** Parameters of linear regressions of all variables on Age (days) in the whole cerebral hemisphere

| Variable | *n* | Intercept | Slope × $10^{-3}$ | Slope prob. |
|---|---|---|---|---|
| *FMR1WT* | | | | |
| D0ACh | 29 | 35.66 ± 0.91 | −3.13 ± 3.80 | 0.417 |
| D4ACh | 29 | 1.7 ± 0.14 | −1.51 ± 0.60 | 0.0023* |
| AChMR | 29 | 0.049 ± 0.007 | −0.046 ± 0.027 | 0.013* |
| D0Ch | 27 | 28.51 ± 1.63 | 10.23 ± 6.99 | 0.156 |
| D4Ch | 27 | 3.33 ± 0.30 | −4.16 ± 1.29 | 0.0035* |
| ChMR | 27 | 0.11 ± 0.019 | −0.15 ± 0.047 | 0.0035* |
| *FMR1KO* | | | | |
| D0ACh | 72 | 36.49 ± 1.16 | −0.90 ± 4.30 | 0.831 |
| D4ACh | 66 | 1.76 ± 0.17 | −1.50 ± 0.50 | 0.046* |
| AChMR | 66 | 0.049 ± 0.005 | −0.041 ± 0.014 | 0.005* |
| D0Ch | 66 | 25.77 ± 3.61 | 20.1 ± 13.3 | 0.134 |
| D4Ch | 66 | 2.82 ± 0.33 | −1.50 ± 1.20 | 0.226§ |
| ChMR | 66 | 0.10 ± 0.02 | −0.089 ± 0.06 | 0.142 |

Statistical significance is indicated as: (*) Null hypothesis (Slope = 0) rejected at $P < 0.05$ probability;
(§) Null hypothesis (slopes of FMR1KO and FMR1WT are equal) rejected at $P < 0.05$ probability (noted on the FMR1KO data in the table).

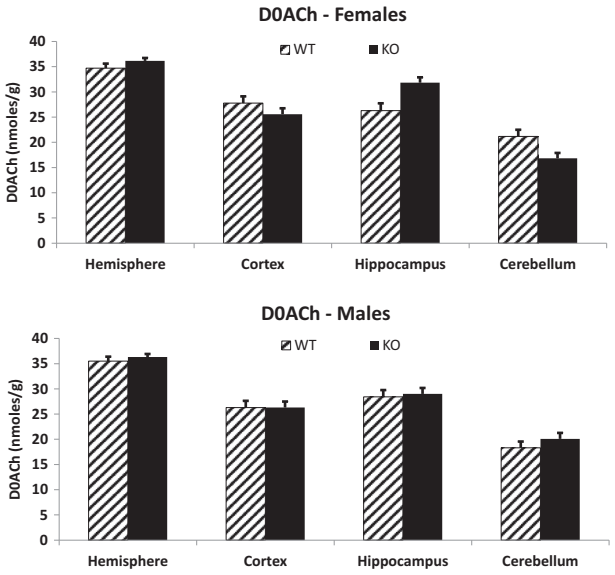


**Fig. 2.** D0ACh levels (nmoles/g, mean ± S.E.) of female (top panel), and male (bottom panel) *Fmr1*-KO and WT control mice in the four regions studied. ANOVA with age as covariate and factors genotype and sex indicated lack of statistical significance for both factors in all regions. Number of cases are for females, hemisphere = 52, cortex = 18, hippocampus = 17 and cerebellum = 18 and for males, hemisphere = 50, cortex = 14, hippocampus = 16 and cerebellum = 17.

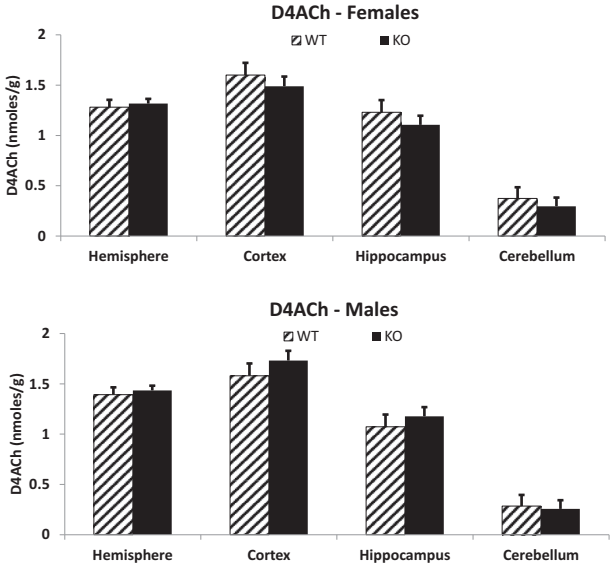


**Fig. 3.** D4ACh levels (nmoles/g, mean ± S.E.) of female (top panel) and male (bottom panel) *Fmr1*-KO and WT control mice in the four regions studied. ANOVA with age as covariate and factors genotype and sex indicated lack of statistical significance for both factors in all regions. Number of cases are for females, hemisphere = 50, cortex = 14, hippocampus = 16 and cerebellum = 13 and for males, hemisphere = 48, cortex = 12, hippocampus = 13 and cerebellum = 12.

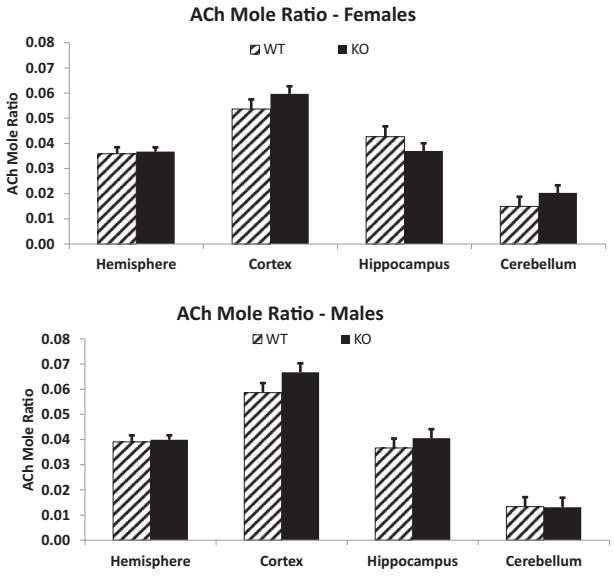


**Fig. 4.** ACh mole ratios of female (top panel) and male (bottom panel) *Fmr1*-KO and WT control mice in the four regions studied. ANOVA with age as covariate and factors genotype and sex indicated lack of statistical significance for both factors in all regions. Number of cases are for females, hemisphere = 50, cortex = 14, hippocampus = 16 and cerebellum = 13 and for males, hemisphere = 48, cortex = 11, hippocampus = 12 and cerebellum = 12.

regions can be studied at any given time. Moreover, the rapid enzymatic degradation of ACh requires the use of acetylcholinesterase inhibitors that alter ACh release due to activation of pre-synaptic cholinergic receptors by excess ACh. This requires the use of muscarinic blockers to avoid it. In contrast, measurement of ACh turnover by administration of Ch labeled with radioactive or stable isotopes can evaluate cholinergic function without anatomical restrictions or the need of pharmacological interventions. As discussed above, the rate of ACh turnover is closely coupled to the rate of ACh release and thus it reflects accurately the level of cholinergic function. Rapid microwave fixation of conscious animals allows the study of ACh, Ch and their isotopic variants without post-mortem changes.

Our results indicate a negative dependence on age of ACh synthesis from D4Ch and in ACh mole ratio, as well as in the uptake of D4Ch from blood and Ch mole ratio in FMR1WT mice, all indications of a progressive decrease in key cholinergic parameters as animals aged. This appears to be in line with numerous observations on the decline of function of the cholinergic system in humans and animals with age (Bartus et al., 1982; Dumas and Newhouse, 2011) and the decrease in Ch uptake from blood in older human subjects (Cohen et al., 1995). It is noteworthy that although a decrease in D4ACh and ACh mole ratio was also present in FMR1KO mice, no such phenomenon was demonstrated for D4Ch and Ch mole ratio, arguing for a better stability of those variables in this genotype.

After adjusting for age, D0ACh concentrations and D4ACh synthesized from D4Ch were similar for FMR1KO and their WT controls in females and males. Thus, our results do not support the hypothesis of a deficit in brain ACh as a cause of the neurophysiologic

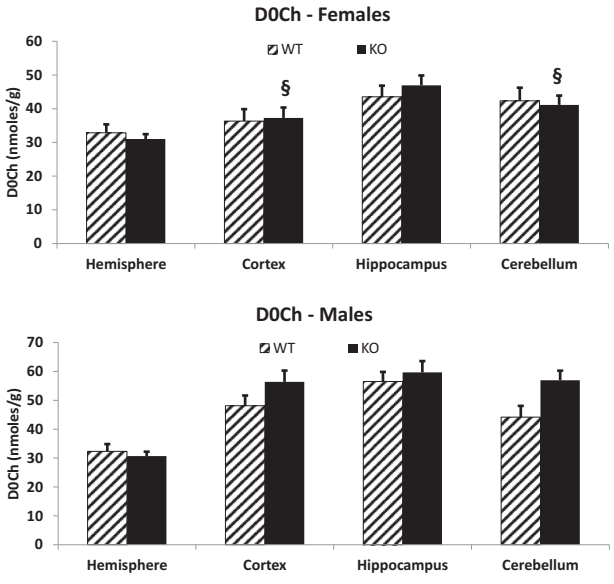


**Fig. 5.** D0Ch levels (nmoles/g, mean ± S.E.) of female (top panel) and male (bottom panel) *Fmr1*-KO and WT control mice in the four regions studied. ANOVA with age as covariate indicated significance for the factors genotype ($P = 0.036$) and sex ($P = 0.0003$) for cortex and sex ($P = 0.015$) for hippocampus. Results of post hoc contrasts between levels of significant factors are indicated in this figure as (§): Significantly different from males ($P < 0.05$). Number of cases are for females, hemisphere = 47, cortex = 13, hippocampus = 16 and cerebellum = 13, and for males, hemisphere = 47, cortex = 11, hippocampus = 12 and cerebellum = 12.

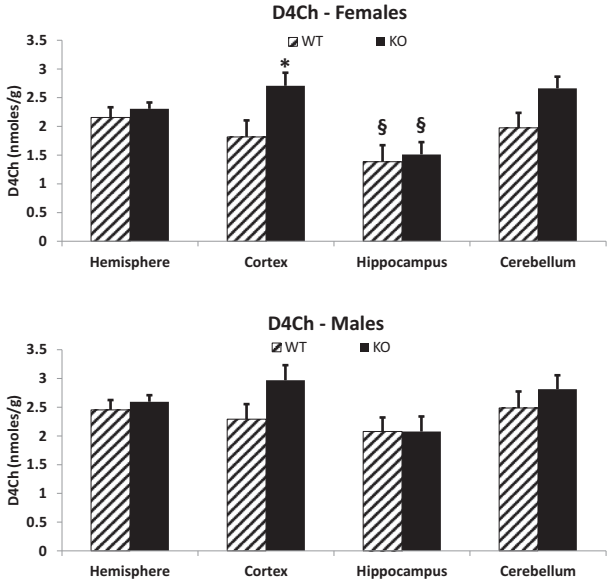


**Fig. 6.** D4Ch levels (nmoles/g, mean ± S.E.) of female (top panel) and male (bottom panel) *Fmr1*-KO and WT control mice in the four regions studied. ANOVA with age as covariate indicated significance for the factor genotype ($P = 0.006$) for cortex and sex for hippocampus ($P = 0.01$) and hemisphere ($P = 0.046$). Results of post hoc contrasts between levels of significant factors are indicated in this figure as: (*) Significantly different from WT and (§) significantly different from males. Number of cases are: for females, hemisphere = 47, cortex = 13, hippocampus = 13 and cerebellum = 16 and for males, hemisphere = 47, cortex = 12, hippocampus = 13 and cerebellum = 12.

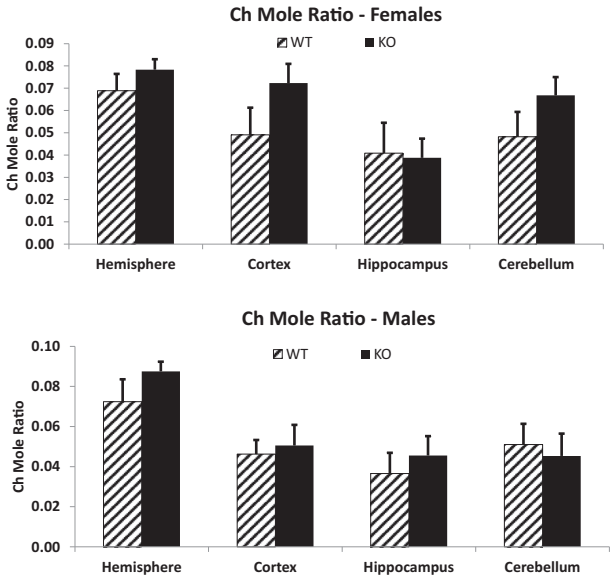


**Fig. 7.** Ch mole ratio levels of female (top panel) and male (bottom panel) *Fmr1*-KO and WT control mice in the four regions studied. ANOVA with age as covariate and factors genotype and sex indicated lack of statistical significance for both factors in all regions. Number of cases are for females, hemisphere = 47, cortex = 13, hippocampus = 13 and cerebellum = 16 and for males, hemisphere = 47, cortex = 12, hippocampus = 13 and cerebellum = 12.

and learning abnormalities reported for FMR1KO mice and by extension, of clinical characteristics of the FX syndrome of humans.

In contrast to the essentially normal ACh concentrations and dynamics in FMR1KO mice, significant alterations in Ch parameters were found in this FXS model. Although endogenous Ch concentration (D0Ch) was not different between FMR1KO and WT control mice, we found a lower D0Ch in cortex and cerebellum of female FMR1KO mice when compared to males of the same genotype, but no changes in the WT controls. The same disparity between sexes was found for D4Ch uptake from the blood in the hippocampus of FMR1KO and WT controls. In the case of the cerebral cortex, while D0Ch was lower in FMR1KO females than males, D4Ch uptake from blood was higher in female FMR1KO than in WT controls, an apparent paradox to be discussed below.

Free Ch concentration in brain cells results from the balance between Ch utilization in the synthesis of phospholipids and its hydrolysis from those molecules such as phosphatidylcholine (Freeman and Jenden, 1976). In addition, the brain blood compartment can be a source or a sink for brain Ch depending on blood Ch concentration and the rate of brain blood flow (Scremin and Jenden, 1993). Any of those factors may be invoked to explain a higher Ch uptake from the blood in the face of a lower endogenous free Ch as observed in the cerebral cortex (such as a lower Ch released from phospholipids or a higher utilization in their synthesis partially compensated by a higher Ch uptake from blood) but the information available in this study does not permit to elucidate between that and many other possibilities. Ch availability is of key significance for nervous system functions since this molecule is a precursor not only of ACh, but also of phosphatidylcholine and sphingomyelin that are constituents of all cell membranes and sources of second messenger molecules used in receptor-mediated intraneuronal signaling (Blusztajn and Wurtman, 1983) (Jenden, 1990).

The observed sexual dimorphism of Ch parameters reported here must be analyzed in the context of hormonal effects on the cholinergic system. Estrogen has been shown to modulate the release of ACh in the hippocampus (Farr et al., 2000; Gabor et al., 2003; Marriott and Korol, 2003), and cholinergic expression in basal forebrain neurons (Pongrac et al., 2004; Abraham et al., 2009). A sexual dimorphism of cholinergic function takes on different manifestations that have been extensively reviewed (Rhodes and Rubin, 1999). The present observations are consistent with a previous report showing greater Ch uptake by forebrain microvessels in female rats (Shimon et al., 1988). Age and sex dependence of variables related to ACh dynamics may also be related to the influence of age and sex on the expression of FMRP in FMR1KO mice (Singh and Prasad, 2008).

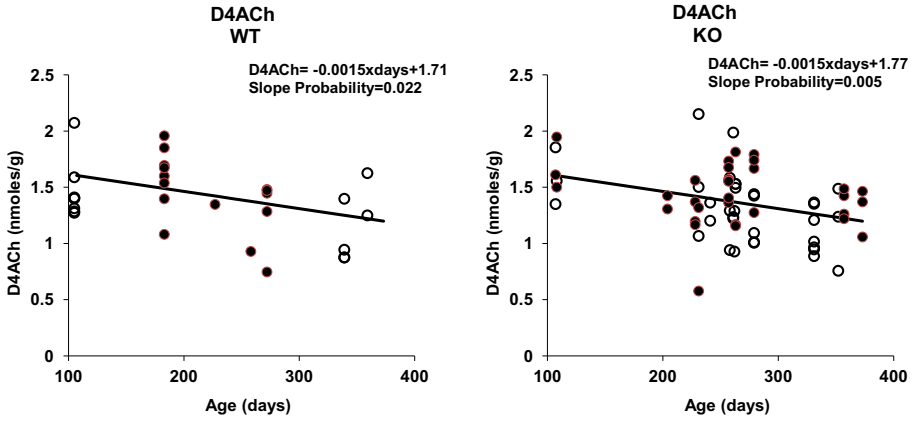


**Fig. 8.** Linear regression of D4ACh (nmoles/g) of the whole hemisphere on age (days) in wild-type mice (left panel) *Fmr1*-KO mice (right panel). Male (filled circles) and female (open circles) mice have been pooled. Regression parameters and number of mice of pooled data are shown in Table 1.

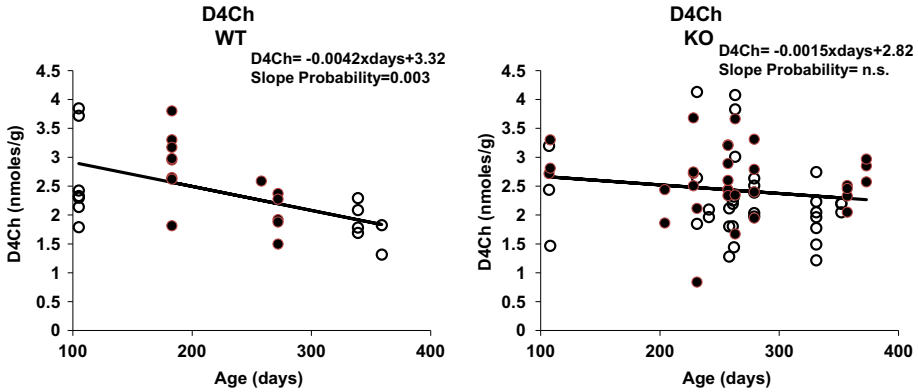


**Fig. 9.** Linear regression of D4Ch (nmoles/g) of the whole hemisphere on age (days) in wild-type mice (left panel) *Fmr1*-KO mice (right panel). Male (filled circles) and female (open circles) mice have been pooled. Regression parameters and numbers of mice of pooled data are shown in Table 1.

In terms of behavioral differences between female and male FMR1KO versus WT controls, there is a paucity of information but a significant difference between these genotypes has been reported for dark/light activity ratios with higher activity in FMR1KO females but not in males (Baker et al., 2010).

It has been reported that the MRS proton Ch peak is reduced in FXS subjects (Kesler et al., 2009; Bruno et al., 2013), an observation that appears contradictory with lack of changes in free Ch between KO and WT control mice of either sex reported here. It is important to note however that the MRS ''choline peak'' in proton MRS, centered at 3.2 ppm, includes resonance from many choline-containing compounds, such as phosphocholine, glycerol 3-phosphocholine as well as free Ch itself, in addition to other metabolites not related to Ch (Podo, 1999). In fact, the contribution of free Ch to this peak is negligible since the reported concentration of the MRS choline peak is several thousand-folds greater than that of free Ch.

In summary, the majority of variables related to ACh metabolism appear normal in FMR1KO mice. However, some region, age and sex-specific abnormalities of Ch metabolism have been found that deserve further exploration in this and other models of the FX syndrome since alteration of availability and dynamics of Ch may result in derangements of membrane composition and neuronal connectivity through second messengers derived from Ch-containing phospholipids that might contribute to the pathogenesis of FXS.

*Acknowledgments—This work was supported by a VA RR&D Senior Research Career Scientist Award to O.U. Scremin.*

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*(Accepted 21 June 2015)*
*(Available online 24 June 2015)*