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Acute ethanol produces ataxia and induces *Fmr1* expression via histone modifications in the rat cerebellum

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Abstract

Background: The cerebellum is fundamental for motor coordination and therefore crucial in ethanol-induced ataxia. Ethanol contributes to cerebellar pathophysiology. Fragile-X Mental Retardation protein (FMRP) is a complex regulator of RNA and synaptic plasticity implicated in Fragile-X Tremor and Ataxia Syndrome, a phenotype featuring increased *Fmr1* mRNA expression. Recent studies have implicated glutamatergic targets of FMRP in hereditary cerebellar ataxias including the main cerebellar excitatory amino acid (*Eaa1*) transporter and a subtype of metabotropic glutamate receptor (*Grm5*). However, ethanol-induced changes in cerebellar *Fmr1* expression and its epigenetic regulation have not been investigated. Here, we examined the effects of acute ethanol exposure on ataxic behavior, gene expression, and epigenetic regulation of the *Fmr1* gene and its glutamatergic targets in the rat cerebellum.

Methods: Male adult Sprague-Dawley rats received acute ethanol (2g/kg) intraperitoneally 1 hour prior to ataxic behavioral testing on the accelerating rotarod and were sacrificed immediately thereafter. Cerebellar tissues were analyzed for gene expression and epigenetic regulation of the *Fmr1* gene and its glutamatergic targets in the rat cerebellum using real-time quantitative PCR and chromatin immunoprecipitation.

Results: Acute ethanol exposure caused marked ataxia on the accelerating rotarod test compared with saline-treated controls. This ataxic response was associated with increases in mRNA levels of *Fmr1*, *postsynaptic density 95 (Psd95)*, *Eaa1*, and *Grm5* in the cerebellum. In addition, we found increased H3K27 acetylation at both the promoter region of *Fmr1* and at a proposed cAMP response-element binding (CREB) site downstream of the *Fmr1* transcription start site. Furthermore, acute ethanol exposure significantly increased *Creb1* and the histone acetyltransferases CREB binding protein (*Cbp*), and *p300* mRNA transcripts.

Conclusions: Overall, ethanol regulates cerebellar *Fmr1* expression most likely via HAT-mediated increase in histone acetylation. We propose that FMRP regulation of glutamatergic

transcripts plays an important role in disrupting the excitatory-inhibitory balance in the cerebellum underlying ethanol-induced ataxia.

Keywords

Epigenetics; Ataxia; Cerebellum; *Fmr1*; CREB

Introduction

The cerebellum has been shown to be important in mediating ethanol-induced motor disturbances and cerebellar ataxia is one of the most consistent physical manifestations of alcohol intoxication (Dar, 2015). Despite the significance of the cerebellum in mediating ethanol-induced ataxia, the molecular mechanisms underlying ataxia remain poorly understood (Dar, 2015). Further mechanistic comprehension will allow for better screening and reshaping treatment options for patients who may be more susceptible to developing alcohol use disorders (AUDs) due to innate resistance to ethanol-induced ataxia (Kaplan et al., 2013).

In recent years, epigenetics has become increasingly important in our understanding of how gene expression is altered during alcohol or drugs of abuse exposure (Berkel and Pandey, 2017; Renthal and Nestler, 2008). Epigenetic mechanisms can translate environmental influences into changes in gene expression with functional consequences (Kouzarides, 2007). Our lab has consistently shown that both acute and chronic ethanol exposure alters chromatin architecture via DNA methylation and histone acetylation changes such that gene expression and behavior is ultimately affected (Pandey et al., 2017). Specifically, the anxiolytic effects of acute ethanol are associated with increases in histone acetylation and CREB (cAMP response-element binding) binding protein (CBP) and p300 levels in the amygdala (Pandey et al., 2008; Teppen et al., 2016). In contrast, withdrawal from chronic ethanol exposure results in anxiety-like behaviors which is associated with decreased histone acetylation and CBP expression in the amygdala (Pandey et al., 2008). Such chromatin remodeling via CREB pathways provides a biological basis for the anxiolysis seen with acute ethanol exposure and the negative affective states seen in alcohol use disorder (Asher et al., 2002; Pandey et al., 2017). Furthermore, CREB is known to regulate ethanol sensitivity in the cerebellum (Acquaah-Mensah et al., 2006).

A limited number of studies have begun to explore the epigenetic effects of alcohol in the cerebellum, a brain region particularly sensitive to the effects of ethanol and crucial to ethanol-induced motor impairments (Dar, 2015). One of our recent studies found increased DNA methylation of the δ subunit of the GABA_A receptor associated with reduced cerebellar mRNA and protein levels in the post-mortem brain of alcohol use disorder subjects (Gatta et al., 2017). A preclinical study examining the epigenetic effects of perinatal alcohol exposure found decreased CBP expression and decreased histone acetylation in the cerebellar cortex of rats treated with perinatal ethanol vapor to model fetal alcohol spectrum disorder (Guo et al., 2011). Another developmental study found the small non-coding microRNA miR-29b decreases with ethanol exposure and induces neuronal apoptosis in cerebellar primary granule neuronal cultures (Qi et al., 2014). Despite the growing interest in

epigenetics in the context of ethanol intoxication, no studies to date have explored the effects of acute ethanol exposure in the adult cerebellum. This is important because ethanol exerts a wide swath of effects in different tissue and cell types due to its small size and high permeability and although the ataxic response to ethanol is well-characterized behaviorally, there remains a gap in our understanding of the molecular mechanisms underlying ataxia (Dar, 2015). Since we have shown that a single low dose of ethanol is sufficient for epigenetic changes in the amygdala and anxiolytic-like effects in rats (Pandey et al., 2008; Sakharkar et al., 2012; Teppen et al., 2016), we investigated the association between the ataxic effects of a single high dose of acute ethanol and associated molecular and epigenetic changes in the cerebellum.

We focused on the Fragile-X Mental Retardation (*FMR1*) gene, since it is a well-known epigenetic target which, when altered, yields functional consequences in humans (Hagerman and Hagerman, 2013). Fragile-X Mental Retardation Protein (FMRP) is a complex regulator of RNA that shapes synaptic protein expression and dendritic spine density by binding to important glutamatergic transcripts including post-synaptic density scaffolding protein, *Psds95*, and a subtype of metabotropic glutamate receptor, *Grm5* (Ascano et al., 2012; Darnell et al., 2011; Zalfa et al., 2007). While FMRP has been traditionally believed to repress translation by stalling bound target mRNAs, recent research has elucidated a role in stabilizing synaptic mRNA transcripts, including *Psds95*, which is important for synthesizing new proteins crucial in synaptic plasticity (Todd et al., 2003; Zalfa et al., 2007). Furthermore, epigenetic targets of FMRP including *Chp* and *p300* have been identified in the two most comprehensive studies characterizing FMRP mRNA targets (Ascano et al., 2012; Darnell et al., 2011). The best-known *FMR1*-related phenotype, Fragile-X Mental Retardation, results from gene silencing due to over 200 CGG trinucleotide repeats and corresponding increased methylation upstream of the gene (Hagerman and Hagerman, 2013). A less well-known phenotype called Fragile-X Tremor and Ataxia Syndrome (FXTAS) results from a pre-mutation condition with 55–200 CGG trinucleotide repeats, and is characterized by paradoxically increased mRNA expression of *FMR1* and, importantly, cerebellar ataxia and intention tremor (Hagerman, 2013; Jacquemont et al., 2003). These patients have been shown to have altered cortical and cerebellar *GRM5* expression (Lohith et al., 2013; Pretto et al., 2014) and recent studies have identified glutamatergic targets of FMRP that are implicated in hereditary cerebellar ataxia disorders including *EAAI*, the main excitatory amino acid transporter in the cerebellum (Choi et al., 2017). Moreover, alcohol abuse is known to underlie earlier onset and greater severity of FXTAS (Dorn et al., 1994; Kogan et al., 2008). Given the FXTAS phenotype and synaptic plasticity mechanisms involving mGluR-dependent synthesis of targets regulated by FMRP (Ifrim et al., 2015; Nalavadi et al., 2012), we propose a role for FMRP in shaping synaptic responses in the cerebellum during ethanol-induced ataxia.

FMRP has recently been implicated in the altered molecular landscape associated with ethanol exposure. Adolescent rats exposed to alcohol showed dendritic spine density deficits, increased *Fmr1* mRNA expression, and increased H3K27 acetylation occupancy of the *Fmr1* gene in the hippocampus (Mulholland et al., 2018). This followed previous reports of altered hippocampal FMRP expression in mice following chronic ethanol vapor treatment (Spencer et al., 2016). One other recent study has elucidated that ethanol-induced translation

of the GABA type B receptor protein in the hippocampus requires FMRP, as shown in wild type mice compared to *Fmr1*-knockout mice (Wolfe et al., 2016). This finding supports the notion that FMRP facilitates increased expression of its targets as was shown with *Psd95* (Ifrim et al., 2015; Todd et al., 2003; Zalfa et al., 2007). Overall, in the context of ethanol administration, these studies indicate FMRP has a regulatory role in controlling synaptic protein expression and shaping dendritic spines, albeit only in the hippocampus. These effects, combined with information regarding FXTAS, led us to hypothesize that ethanol-induced motor deficits may be partially mediated by epigenetic regulation of *Fmr1* expression in the cerebellum.

Materials and Methods

Animals

Male Sprague-Dawley rats (n=12) were obtained from Harlan Laboratories (Indianapolis, IN) and were used for all subsequent behavioral and molecular experiments. Rats were given *ad libitum* access to food and water while maintained on a 12:12 hour light/dark cycle. Rats arrived at Post-natal Day (PD) 80 and were allowed 10 days to acclimate to the facility. All animal procedures followed Institutional Animal Care and Use Committee Guidelines and NIH directives for the Care and Use of Laboratory Animals. Animals were pair-housed throughout the entire course of the acute ethanol rotarod experiment and these same rats were used for all biochemical studies.

Measurement of ataxia using rotarod test

Rats (PD91 or PD92) were habituated on the accelerating rotarod from IITC Life Science (Woodland Hills, CA) on day 1, followed by two days of actual training with 3 trials of 180 seconds each day without injections. The rotarod cylinder apparatus is 9.5 cm in diameter and 15 cm wide; the rotarod begins spinning at 5 rotations per minute and accelerates to 20 rotations per minute over 180 seconds. Latency to fall is recorded for every trial of training. During training, rats were placed back on the rotarod apparatus if they fell off before the full trial was completed. By the end of training, all rats were able to remain on the rod for the entire 180 second rotarod session, nearly every trial. No injections were administered during rotarod training. On the fourth day (test day), rats aged PD94 or PD95 received a single intraperitoneal (i.p.) injection of ethanol (2 g/kg, 20% w/v) or equivalent dose of normal saline. One hour following injection of ethanol or saline, pair-housed rats were tested pairwise on the rotarod for three trials. Time spent on the rod is calculated as an average latency to fall across the three trials measured on the last training day to obtain baseline performance and across the three trials on the test day to assess the effects of ethanol or saline treatment.

Tissue collection for biochemical studies

Immediately following rotarod assessment on test day, animals were decapitated under isoflurane anesthesia for tissue collection. Tissue collection occurred about 70 minutes following ethanol or saline administration. Brain tissue, specifically the whole cerebellum, was quickly dissected, frozen and kept at -80°C until used for biochemical studies. The whole cerebellum was pulverized on dry ice with a mortar and pestle and then cerebellar

tissues were used for parallel mRNA quantification and chromatin immunoprecipitation analyses as described below. Blood samples were collected at the time of brain collection for measurement of blood ethanol levels using Analox Alcohol Analyzer (Lunenburg, MA).

mRNA quantification by real-time PCR

Total RNA was isolated from rat cerebellum with Qiazol and purification using miRNeasy kit (Qiagen, Valencia, CA). RNA was then reverse transcribed in duplicate with random primers and reverse transcriptase from the cDNA Reverse Transcription Kit (ThermoFisher Scientific, Waltham, MA). Quantitative real-time PCR (qPCR) was performed using a CFX Connect qPCR system and SYBR Green master mix (BioRad, Hercules, CA). The BioRad CFX Manager software suite was used to analyze qPCR data (BioRad, Hercules, CA). Primers corresponding to mRNAs for the genes of interest (Table 1) were added to the master mix, and Hypoxanthine-guanine phosphoribosyltransferase (*Hprt1*) was utilized as a reference gene. qPCR conditions were a 2-minute hold at 95 °C, followed by 40 cycles of 95°C for 5 seconds and 60 °C for 30 seconds, before a melt step with 5 second plate-reads for every 0.5°C increase from 65°C to 95°C. Target cDNA (*Fmr1*, *Cbp*, *p300*, *Psd95*, *Grm5*, *Eaa1*) was measured alongside *Hprt1*, with every sample run in triplicate. Relative mRNA levels were determined by normalization to *Hprt1* using the Ct method (Livak & Schmittgen 2001; Kyzar et al., 2016). Results are expressed as fold change in mRNA levels compared with saline-treated controls.

Measurement of histone acetylation occupancy at *Fmr1* loci

In order to examine the histone acetylation levels specifically at several locations of the *Fmr1* gene promoter and gene body in the cerebellum of ethanol- and saline-treated rats, we performed chromatin immunoprecipitation (ChIP) assays using antibodies against specific histone residues as described previously (Kyzar et al., 2016; Zhang et al., 2018). Pulverized cerebellum tissue was fixed in methanol-free formaldehyde and DNA was sheared by sonication. The resulting DNA-chromatin complex was immunoprecipitated with an antibody directed to acetylated H3K27 (Cell Signaling, #4353). The precipitated DNA was quantified using qPCR with a SYBR Green master mix (BioRad, Hercules, CA) and primers designed for the *Fmr1* promoter (Forward: 5'-CAC GAC CTG TTA CTA CCC TTT G-3', Reverse: 5'-GGT AAC TGG GCA GAT ACT TTG-3'), two predicted CREB sites (Wang et al., 2012) at the *Fmr1* transcription start site (Forward: 5'-TCA GCT GCC CAA GAG AGA-3', Reverse: 5'-ACC GGA AGT GAA ACC GAA A -3' [site 1]), and just downstream of the transcription start site in the first exon (Forward: 5'-GGA CGA GAA GAT GGA GGA GC-3', Reverse: 5'-CGG TAG CCC GTT ACC TTG TA-3' [site 2]) as well as a distal site in the second intron (Forward: 5'-GCC ATT CTG CTT TAT TCT AGT TAT ACT C-3', Reverse: 5'-AGT CAG AAT TTG TGG AAA TCT GTT G-3' [site 3]) which we previously reported to have altered H3K27 acetylation (H3K27Ac) in the adult hippocampus following adolescent ethanol exposure (Mulholland et al., 2018). These four ChIP primer locations are shown in the Figure 3A *Fmr1* gene map. After subtracting input DNA Ct values from each respective sample, the Ct method was used to calculate fold change in the levels of H3K27Ac at the *Fmr1* gene (Livak and Schmittgen, 2001; Kyzar et al., 2016).

Statistical Analysis

All statistical analyses were conducted in SigmaStat Version 3.5 (Systat, San Jose, CA). For the rotarod behavior, two-way ANOVA was conducted to assess effects of treatment and time with Tukey tests employed for post hoc pairwise comparisons. For biochemical data, Student t-tests were conducted to compare differences between ethanol-treated and saline-treated groups when data passed tests of normality and equal variance. In one case, nonparametric Mann Whitney test was employed due to failure to pass equal variance test.

Results

Acute ethanol treatment impairs rotarod behavior

Adult male rats learned the rotarod motor behavior over three days of habituation and training and on the fourth day (test day) they were treated with acute ethanol (2 g/kg) or saline and then tested on the rotarod 1 hr later. Two-way ANOVA showed a main effect of treatment ($F_{1,20} = 33.28$, $p < 0.001$), main effect of day ($F_{1,20} = 29.40$, $p < 0.001$), and a significant interaction (treatment $\times$ day) ($F_{1,20} = 61.45$, $p < 0.001$) (Figure 1). On test day, rats treated with ethanol display significantly reduced time to fall off the rotarod apparatus one hour following injection compared to their saline-treated counterparts, indicating that acute ethanol produces marked ataxia ($p < 0.001$). Rats in the ethanol group additionally display significantly reduced time to fall off the rotarod apparatus compared to their baseline training performance ($p < 0.001$). No difference was observed during the learning of the motor behavior as indicated by similar performance between groups during rotarod training ($p = 0.159$) and saline-treated rats show no differences between test day and baseline training ($p = 0.103$). Rats treated with acute ethanol had a blood ethanol level average of 191 ± 3.8 mg/dL (mean $\pm$ SEM) at the time of brain collections about 70 minutes after acute ethanol injection.

Acute ethanol produces increased *Fmr1* mRNA expression and increased histone acetylation at the *Fmr1* promoter in the cerebellum

The motor impairment caused by acute ethanol treatment (2 g/kg) is associated with significantly increased *Fmr1* mRNA expression in the cerebellum compared to saline-treated counterparts (Figure 2A: $t_{10} = -3.78$, $p = 0.004$, t-test). Rats treated with acute ethanol also show significantly upregulated H3K27 acetylation at the *Fmr1* promoter (Figure 2B: $t_{10} = -4.28$, $p = 0.002$, t-test). The increased H3K27 acetylation at the *Fmr1* promoter may be a potential mechanism by which the epigenetic effects of alcohol increase gene transcription.

Acute ethanol produces alterations in histone acetylation at CREB sites in the *Fmr1* gene in the cerebellum

We also investigated H3K27 acetylation levels at other locations in the *Fmr1* gene where there are CREB binding sites (Mulholland et al., 2018; Wang et al., 2012). At the CREB-binding site just downstream of the *Fmr1* transcription start site, we found that acute ethanol increased H3K27 acetylation (Figure 3 [site 2]: $t_{10} = -3.28$, $p = 0.008$, t-test). At two other CREB sites in the *Fmr1* gene, we found trends toward increased H3K27 acetylation but

levels were not statistically significantly altered by acute ethanol exposure (Figure 3 [site 1]: $t_{10} = -1.83$, $p = 0.098$, t-test; Figure 3 [site 3]: $t_{10} = -1.96$, $p = 0.079$, t-test).

Acute ethanol produces increased cerebellar mRNA expression of FMRP targets

The same cohort of rats treated with acute ethanol (2 g/kg) also show increased mRNA expression of FMRP glutamatergic targets (Figure 4A) as well as increased mRNA expression of CREB-related genes that have histone acetyltransferase activity (Figure 4B). *Grm5* is significantly upregulated ($t_{10} = -2.27$, $p = 0.047$, t-test) as well as *Eaa1* ($t_{10} = -2.87$, $p = 0.017$, t-test) and *Psd95* ($U = 33$, $p = 0.015$, $n = 6$ per group Mann Whitney Test). *Creb1* is significantly upregulated ($t_{10} = -2.52$, $p = 0.030$, t-test) as are FMRP targets and histone acetyltransferases *Chp* ($t_{10} = -2.59$, $p = 0.027$, t-test) and *p300* ($t_{10} = -3.28$, $p = 0.008$, t-test). These results suggest a dysregulated glutamatergic system in the cerebellum following acute ethanol exposure associated with ataxia as measured on the rotarod thereby providing a molecular explanation for the observed increased histone acetylation mediated increase in *Fmr1* expression.

Discussion

The data shown here represent the first findings, to our knowledge, of epigenetic mediated alteration in *Fmr1* expression in the cerebellum of adult rats exposed to acute ethanol. Specifically, we provide evidence that acute ethanol stimulates the CREB pathway in the cerebellum most likely leading to increased H3K27 acetylation and downstream increased *Fmr1* gene expression. Furthermore, we show increased mRNA expression of five FMRP targets, two that play a critical role in histone acetylation: *Chp* and *p300*, and three which are associated with glutamatergic neurotransmission: *Psd95*, *Grm5*, and *Eaa1*. We hypothesize that FMRP-induced stabilization of these mRNA targets leads to increased protein expression and a hyperactive glutamatergic network in the cerebellum which may be associated with ataxia observed one hour following administration of a high dose of acute ethanol.

Ethanol produces distinct brain region-dependent transcriptional changes involved in intoxication, tolerance, and withdrawal (Contet, 2012), and this unique finding of increased cerebellar *Fmr1* expression during acute ethanol exposure suggests a role in ataxia associated with acute intoxication. Since *Fmr1* is highly-expressed in neurons (Devys et al., 1993), the protein features several RNA-binding motifs (Anderson et al., 2016), and epigenetic modifications of *Fmr1* genomic DNA lead to functional consequences (Hagerman and Hagerman, 2013; Jacquemont et al., 2003), induction of increased histone acetylation and increased gene expression by ethanol exposure is likely to significantly alter synaptic neurotransmission. Indeed, *FMR1* hyperacetylation induced by treatment with histone deacetylase (HDAC) inhibitors led to gene reactivation in a human lymphoblastoid cell line model of Fragile-X Syndrome (Chiurazzi et al., 1999), offering a potential new treatment approach for these patients (Tabolacci et al., 2016). Similarly, HDAC inhibitors have been explored as a treatment option in rodent models of alcoholism. Trichostatin A (TSA) treatment increased global histone acetylation while attenuating the anxiety-like and high alcohol-drinking behaviors of alcohol-preferring P rats (Sakharkar et al., 2014). TSA

treatment was also able to attenuate anxiety-like behavior during withdrawal after chronic ethanol exposure and normalize amygdalar deficits in dendritic spine density (Pandey et al., 2008; You et al., 2014). These findings indicate that correction of acetylation deficits with epigenetic HDAC inhibitor treatments can alter downstream gene expression and have functional consequences. Future experiments will assess the time course of epigenetic changes in the cerebellum following acute ethanol exposure as well as evaluate epigenetic changes after chronic ethanol exposure and investigate if epigenetic-targeted therapy rescues ethanol-induced gene expression alterations and mitigates motor impairments associated with ethanol exposure.

The present findings expand upon past research which has linked alcohol to alterations in the molecular and epigenetic landscape in the cerebellum of post-mortem AUD subjects (Gatta et al., 2017) and in the cerebellum of rodents exposed to perinatal ethanol (Guo et al., 2011; Qi et al., 2014) or chronic adult ethanol exposure (Auta et al., 2017). They also expand upon limited recent research illustrating a role for *Fmr1* following ethanol exposure, albeit one that was, until now, exclusive to the hippocampus (Mulholland et al., 2018; Spencer et al., 2016; Wolfe et al., 2016). *Fmr1* is a dynamic epigenetic target that can alter expression of hundreds of transcripts, but itself may also be regulated by microRNAs (Kenny et al., 2014; Liu et al., 2015; Tan et al., 2012) or its own non-coding anti-sense RNA *Fxr4* (Pastori et al., 2014; Peschansky et al., 2016). Given that acute and chronic ethanol alter microRNA and long non-coding RNA networks (Miranda et al., 2010; Osterndorff-Kahanek et al., 2018; Teppen et al., 2016), future experiments should examine other epigenetic regulations of *Fmr1* beyond the histone acetylation examined here in order to further elucidate the role of *Fmr1* and its regulatory gene network in the neural response to ethanol, particularly the ataxic effects of ethanol.

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Figure 1:
Effects of acute ethanol exposure (2 g/kg) on ataxia measured by accelerating rotarod test. Rats received no injections during rotarod training and a single injection of either ethanol or saline on test day. Data are represented as mean $\pm$ SEM, $n = 6$ per group. *** $p < 0.001$ significantly different from saline treated group

Figure 2: Effects of acute ethanol exposure on *Fmr1* mRNA expression (A) and H3K27 acetylation at *Fmr1* promoter (B) in the cerebellum of rats. Data are represented as mean $\pm$ SEM, n = 6 per group. **p<0.01 significantly different from saline treated group

A Rat *Fmr1* Gene

B H3K27Ac @ CREB binding sites in *Fmr1*

Figure 3:

Gene map of *Fmr1* ChIP primer site locations (A) and effects of acute ethanol exposure on H3K27 acetylation at CREB binding sites of the *Fmr1* gene in the cerebellum of rats (B). Data are represented as mean $\pm$ SEM, n = 6 per group. **p<0.01 significantly different from saline treated group

Figure 4:

Effects of acute ethanol exposure on cerebellar mRNA levels of selected glutamatergic targets of FMRP (A) and of cAMP response-element binding protein 1 gene (*Creb1*) and histone acetyltransferase complex CREB binding protein (*Cbp*) and *p300* (B). Data are represented as mean $\pm$ SEM, n = 6 per group. *p<0.05; **p<0.01 significantly different from saline treated group

Table 1:

The forward and reverse primer sequences utilized in qPCR reactions for amplification of cDNA corresponding with target mRNAs.

Primer Sequence	
<i>Fmr1</i> Forward	5'-AAA GTC CAG AGG GGG ATG GT-3'
<i>Fmr1</i> Reverse	5'-TCT CTC CAA ACG CAA CTG GT-3'
<i>Creb1</i> Forward	5'-AGA AGC AGC ACG AAA GAG AG-3'
<i>Creb1</i> Reverse	5'-CAC TGC CAC TCT GTT CTC TAA A-3'
<i>Chp</i> Forward	5'-TAA TGG AGG CTG CCC AGT GTG TAA-3'
<i>Chp</i> Reverse	5'-CTG GCG GAG CTT GTG TTT GAT GTT-3'
<i>p300</i> Forward	5'-AAA CAC CAG CAA CGA GAG TAC CGA-3'
<i>p300</i> Reverse	5'-TCC ATG GTG GCG TAC AGT TTC TGA-3'
<i>Psd95</i> Forward	5'-TCA TAA CTC CCC ATG CCA TT-3'
<i>Psd95</i> Reverse	5'-CTC ATG CAA ACC AGC AAA GA-3'
<i>Grm5</i> Forward	5'-ATG CAT GTA GGA GAC GGC AA-3'
<i>Grm5</i> Reverse	5'-TTT CCG TTG GAG CTT AGG GTT T-3'
<i>Eaa1</i> Forward	5'-AGT AAG TGG CGG TTT CGG AG-3'
<i>Eaa1</i> Reverse	5'-TGG TGT TAG AGA GGA CAA CTT TTC T-3'
<i>Hprt1</i> Forward	5'-TCC TCA GAC CGC TTT TCC CGC-3'
<i>Hprt1</i> Reverse	5'-TCA TCA TCA CTA ATC ACG ACG CTG G-3'