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CA1 pyramidal neuron gene expression mosaics in the Ts65Dn murine model of Down syndrome and Alzheimer's disease following maternal choline supplementation (MCS)

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Abstract

Although there are changes in gene expression and alterations in neuronal density and afferent inputs in the forebrain of trisomic mouse models of Down syndrome (DS) and Alzheimer's disease (AD), there is a lack of systematic assessments of gene expression and encoded proteins within individual vulnerable cell populations, precluding translational investigations at the molecular and cellular level. Further, no effective treatment exists to combat intellectual disability and basal forebrain cholinergic neurodegeneration seen in DS. To further our understanding of gene expression changes before and following cholinergic degeneration in a well-established mouse model of DS/AD, the Ts65Dn mouse, we assessed RNA expression levels from CA1 pyramidal neurons at two adult ages (~6 months of age and ~11 months of age) in both Ts65Dn and their normal disomic (2N) littermates. We further examined a therapeutic intervention, maternal choline supplementation (MCS), which has been previously shown to lessen dysfunction in spatial cognition and attention, and have protective effects on the survival of basal forebrain cholinergic neurons (BFCNs) in the Ts65Dn mouse model. Results indicate that MCS normalized expression of several genes in key gene ontology categories, including synaptic plasticity, calcium signaling, and AD-associated neurodegeneration related to amyloid-beta peptide (A β) clearance. Specifically, normalized expression levels were found for endothelin converting enzyme-2 (*Ece2*), insulin degrading enzyme (*Ide*), *Dyrk1a*, and calcium/calmodulin-dependent protein kinase II

(*Camk2a*), among other relevant genes. Single population expression profiling of vulnerable CA1 pyramidal neurons indicates that MCS is a viable therapeutic for long-term reprogramming of key transcripts involved in neuronal signaling that are dysregulated in the trisomic mouse brain which have translational potential for DS and AD.

Keywords

hippocampus; laser capture microdissection; choline supplementation; microarray; trisomic

Introduction

Down syndrome (DS) is caused by the triplication of human chromosome 21 (HSA21) and results in systemic deficits (Esbensen, 2010). DS is also the primary genetic cause of intellectual disability (ID), with cognitive deficits in hippocampal-dependent learning and memory, attention, and language and communication skills (Chapman and Hesketh, 2000; Esbensen, 2010; Lott, 2012; Rachidi and Lopes, 2008, 2010). Overexpression of >550 genes and putative protein-encoding transcripts, including >160 known genes termed the ‘DS critical region’ is thought to cause the neurological and peripheral phenotype associated with DS (Sturgeon and Gardiner, 2011). Further complicating the DS phenotype, by the mid-third decade of life, DS individuals develop pathological changes associated with Alzheimer’s disease (AD), including amyloid-beta peptide (A β) containing senile plaques, neurofibrillary tangles (NFTs), degeneration of cholinergic basal forebrain (CBF) neurons, and early endosomal abnormalities (Cataldo et al., 2000; Hartley et al., 2015; Hartley et al., 2014; Lai and Williams, 1989; Leverenz and Raskind, 1998; Mann et al., 1986; Senders et al., 2000; Wisniewski et al., 1985). In addition, degeneration of the CBF neuron projection system, which provides the major cholinergic innervation to the entire cortical mantle and the hippocampus (Mesulam et al., 1983; Mufson et al., 2008; Rye et al., 1984), found in DS, is similar to that observed in AD (Bierer et al., 1995; Leverenz and Raskind, 1998; Mann et al., 1986; Wisniewski et al., 1985).

Mouse models have been generated that recapitulate aspects of DS and AD for translational study. The well-established segmental trisomy Ts65Dn mouse model closely replicates human DS neuropathology (Cataldo et al., 2003; Davisson et al., 1993; Granholm et al., 2000; Holtzman et al., 1996; Salehi et al., 2006; Seo and Isacson, 2005). This model translocates a segment of mouse chromosome 16 (MMU16) and mouse chromosome 17 (MMU17) orthologous to HSA21 to <10% of the centromere of MMU17, creating a freely segregating chromosome (Akeson et al., 2001; Kahlem et al., 2004; Reeves et al., 1995) with a conservation of ~70% homology for >85 protein coding gene sequences, with over 250 genes and putative gene products triplicated (Davisson et al., 1993; Gardiner et al., 2003; Sturgeon and Gardiner, 2011). Ts65Dn mice mimic many key features of the human DS pathology, including hippocampal-dependent learning and memory deficits (Ash et al., 2014; Escorihuela et al., 1995; Hyde and Crnic, 2001; Reeves et al., 1995; Velazquez et al., 2013), attentional deficits (Moon et al., 2010; Powers et al., 2016, 2017), heightened emotionality (Driscoll et al., 2004; Moon et al., 2010), and hyperactivity (Cooper et al., 2001; Hunter et al., 2003; Reeves et al., 1995). Glutamatergic neurotransmission

dysfunction, including impaired hippocampal long-term potentiation and enhanced long-term depression, has been observed in trisomic mice (Kaur et al., 2014; Kleschevnikov et al., 2004; Rueda et al., 2010; Siarey et al., 1999). Like their human DS counterparts, the Ts65Dn mice have intact basal forebrain cholinergic neurons (BFCNs) and septohippocampal organization at birth (Granholm et al., 2000; Holtzman et al., 1996; Hunter et al., 2003), but after 6 months of age (MO), Ts65Dn mice have significant BFCN and associated CA1 hippocampal degeneration, along with astrocytic hypertrophy, and microglial activation (Cooper et al., 2001; Granholm et al., 2000; Holtzman et al., 1996; Kelley et al., 2014b; Seo and Isaacson, 2005). The septohippocampal circuit is vulnerable in both DS and AD, and Ts65Dn mice recapitulate aspects of this vulnerability through reduced hippocampal neurogenesis, synapse loss, and deficits in neuroplasticity (Belichenko et al., 2004, 2009; Granholm et al., 2003; Insausti et al., 1998; Kelley et al., 2014a, 2016; Kleschevnikov et al., 2012; Kurt et al., 2000).

Current therapeutics for DS and AD are largely ineffective and expensive, and none prevent the progression of the disease. A potential treatment modality originating from the study of mouse models of DS and AD is to supplement the maternal diet with additional choline during pregnancy and lactation (Strupp et al., 2016). Choline is an essential nutrient critical for several key developmental pathways in the brain, including the biosynthesis of acetylcholine, which regulates neuronal proliferation, differentiation, migration, plasticity and synapse formation (Strupp et al., 2016). In addition, choline is a key substrate of the phosphatidylethanolamine N-methyltransferase (PEMT) pathway, which generates phosphatidylcholine species, crucial elements in neuronal and non-neuronal lipid membranes (Lindblom and Oradd, 2009; Wang et al., 2016; Zeisel, 2010; Zeisel and Niculescu, 2006). Choline is also the primary dietary source of methyl groups, a principal driver of gene expression regulation through epigenetic programming (McGowan et al., 2008; Mehedint et al., 2010). Current dietary recommendations for choline, especially in regard to pregnant women, are likely to be inadequate for the high demand for choline during gestation (Caudill et al., 2017; Institute of Medicine, 1998; Strupp et al., 2016). Notably, maternal choline supplementation (MCS) has been demonstrated by our group to improve spatial cognition and attentional function, protect BFCNs, and normalize adult hippocampal neurogenesis in Ts65Dn offspring (Ash et al., 2014; Kelley et al., 2014a, 2016; Powers et al., 2016, 2017; Strupp et al., 2016; Velazquez et al., 2013). MCS treatment also increases choline acetyltransferase (ChAT) intensity in the hippocampus prior to BFCN degeneration in the Ts65Dn mouse model (Kelley et al., 2016). Further investigation of aging and MCS treatment shows an increase in ChAT intensity in 2N animals, but not in Ts65Dn mice when examined pre-BFCN degeneration (4-6 MO) and post-BFCN degeneration (14-18 MO), however this may be explained by the fact that unsupplemented Ts65Dn mice display septohippocampal degeneration during the aging process (Kelley et al., 2016). MCS has also been shown to protect the cholinergic system in a mouse model of amyloid-beta precursor protein (APP) overexpression (Mellott et al., 2017) and behavioral and morphologic benefits in several models of neurodevelopmental disorders (Bearer et al., 2015; Ross et al., 2016; Scremin et al., 2015; Stevens et al., 2008; Ward et al., 2008, 2009), indicating that the effects are broadly neuroprotective across several disease entities.

To understand mechanisms and signaling pathways underlying benefits of MCS at the molecular and cellular level within vulnerable neuronal populations in DS and AD, we examined gene expression changes in CA1 pyramidal neurons at two distinct timepoints, young (~6 MO; pre-BFCN degeneration) and middle-aged (~11 MO; post-BFCN degeneration) via single population expression profiling analysis. We postulate that cognitive improvement via MCS is reflected in select gene expression and septohippocampal signaling pathway changes in trisomic mice.

Materials and Methods

Mice and maternal dietary protocol

Animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Nathan Kline Institute/NYU Langone Medical Center and were in full accordance with NIH guidelines. Breeder pairs (female Ts65Dn and male C57Bl/6J Eicher × C3H/HeSnJ F1 mice) were purchased from Jackson Laboratories (Bar Harbor, ME) and mated at Nathan Kline Institute, Orangeburg, NY. Upon arrival, breeder pairs were assigned to receive one of two choline-controlled experimental diets: *i)* control rodent diet containing 1.1 g/kg choline chloride (AIN-76A; Dyets Inc., Bethlehem, PA, or *ii)* choline-supplemented diet containing 5.0 g/kg choline chloride (AIN-76A; Dyets Inc.), as described previously (Kelley et al., 2014a; Powers et al., 2016, 2017). The choline-supplemented diet provides approximately 4.5 times the concentration of choline consumed by our control group, and is within the normal physiological range (Detopoulou et al., 2008). The control diet supplies an adequate level of choline, so the offspring are not choline deficient. Breeder pairs were provided *ad libitum* access to water and their assigned diets. Standard cages contained paper bedding and several objects for enrichment (e.g., plastic igloo, t-tube, and cotton square). Mice were maintained on a 12-hour light-dark cycle under temperature- and humidity-controlled conditions.

Tissue preparation

Pups born to choline supplemented (Ts65Dn+ and 2N+) or unsupplemented maternal choline (Ts65Dn and 2N) dams were weaned on postnatal day 21 and provided *ad libitum* access to water and the control diet. Tail clips were taken and genotyped as described by Duchon et al., (Duchon et al., 2011). Mice were aged to approximately 6 MO and 11 MO and sacrificed for brain tissue accession. Mice used in this study included: 2N+ n=11 (6 MO), n= 8 (11 MO); 2N n= 12 (6 MO), n= 7 (11 MO); Ts65Dn+ n= 16 (6 MO), n= 8 (11 MO); and Ts65Dn n=19 (6 MO), n= 6 (11 MO). The study used both male and female mice. Mice were given an overdose of ketamine and xylazine and perfused transcardially with ice-cold 4% paraformaldehyde buffered in 0.15 M phosphate buffer. Tissue blocks containing the dorsal hippocampus were paraffin embedded and 6 µm-thick tissue sections were cut in the coronal plane on a rotary microtome for immunocytochemistry as described previously (Allred et al., 2012; Ginsberg, 2005a; Ginsberg, 2010). RNase-free precautions were employed, and solutions were made with 18.2 mega Ohm RNase-free water (Nanopure Diamond, Barnstead, Dubuque, IA). A second independent, mixed gender cohort of animals was perfused with 1M phosphate buffer and brains were prepared for CA1 region

microdissections at both timepoints for qPCR validation. Flash frozen samples from 2N+, 2N, Ts65Dn+, and Ts65Dn (n=9) per timepoint were accrued for RNA extraction.

Single cell microaspiration and TC RNA amplification

Laser capture microdissection (LCM) and terminal continuation (TC) RNA amplification procedures have been described in detail previously by our group (Allred et al., 2008, 2009, 2012; Che and Ginsberg, 2004; Ginsberg, 2008). Individual CA1 pyramidal neurons were microaspirated via LCM (Arcturus PixCell IIe, ThermoFisher, South San Francisco, CA). One hundred cells were captured per reaction for population cell analysis (Allred et al., 2008; Ginsberg, 2010). Microarrays (containing 100 LCM-captured CA1 neurons each) were performed per mouse brain (range 2-5 times per mouse). The full TC RNA amplification protocol is available at <http://cdr.rfmh.org/pages/ginsberglabpage.html>. This method entails synthesizing first strand cDNA complementary to the RNA template, re-annealing the primers to the cDNA, and finally *in vitro* transcription using the synthesized cDNA as a template. Briefly, microaspirated CA1 neurons were homogenized in Trizol reagent (ThermoFisher, Carlsbad, CA), chloroform extracted, and precipitated (Allred et al., 2009, 2012, 2015a, 2015b). RNAs were reverse transcribed and single-stranded cDNAs were then subjected to RNase H digestion and re-annealing of the primers to generate cDNAs with double-stranded regions at the primer interfaces. Single stranded cDNAs were digested and samples were purified by Vivaspin 500 columns (Sartorius Stedim Biotech, Goettingen, Germany). Hybridization probes were synthesized by *in vitro* transcription using ³³P and radiolabeled TC RNA probes were hybridized to custom-designed cDNA arrays without further purification.

Microarray platforms and hybridization

Array platforms consist of 1 µg of linearized cDNA purified from plasmid preparations adhered to high-density nitrocellulose (Hybond XL, GE Healthcare, Piscataway, NJ) using an arrayer robot (VersArray, Bio-Rad, Hercules, CA) (Ginsberg, 2005b, 2008). Each cDNA and/or expressed sequence-tagged cDNA (EST) was verified by sequence analysis and restriction digestion. Mouse and human clones were employed on the custom-designed array. Approximately 576 cDNAs/ESTs were utilized for the younger cohort and approximately 649 genes were utilized for the older cohort, organized into 22 gene ontology (GO) groups (Table I). The older cohort of animals had additional genes newly available and/or implicated in DS/AD pathology. Additional genes were mainly added to 3 GO categories, protein phosphatases and kinases, stress response, and sirtuins (Table I). The majority of genes are represented by one transcript on the array platform, although the neurotrophin receptors *TrkA*, *TrkB*, and *TrkC* are represented by ESTs that contain the extracellular domain (ECD) as well as the tyrosine kinase domain (TK) (Ginsberg et al., 2006, 2010).

Statistical procedures for custom-designed microarray analysis have been described in detail previously (Allred et al., 2015a, 2015b; Ginsberg, 2014; Schafer et al., 2015). Gene expression differences due to genotype or dietary condition were assessed with respect to the hybridization signal intensity ratio of the total signal of all array genes. For each gene the signal intensity ratio was modeled as a function of mouse study group, using mixed effects

models with random mouse effect to account for the correlation between repeated assays on the same mouse (McCulloch et al., 2011). Significance was judged at the level $\alpha=0.01$, two-sided; false discovery rate (FDR) based on an empirical null distribution due to strong correlation between genes (Benjamini and Hochberg, 1995; Efron, 2007) was controlled at level $\alpha=0.1$. Expression levels were graphed using a bioinformatics software package (GeneLinker Gold, Predictive Patterns, Kingston, ON).

qPCR

qPCR was performed on microdissected CA1 sections from an independent cohort of animals containing the hippocampal CA1 region from ~6 MO and ~11 MO Ts65Dn and 2N mice. Taqman qPCR primers (ThermoFisher, Waltham, MA) were utilized for qPCR (Table II). Samples were assayed on a real-time qPCR cycler (PikoReal, ThermoFisher) in 96-well optical plates with coverfilm as described previously (Alldred et al., 2008, 2012, 2015a, 2015b; Jiang et al., 2010). Standard curves and cycle threshold (Ct) were generated using standards obtained from total mouse brain RNA. The ddCT method was employed to determine relative gene level differences between study groups (ABI, 2004; Alldred et al., 2008; Ginsberg, 2010; Jiang et al., 2010). Succinate dehydrogenase A (*Sdha*) qPCR products were used as a control, as this probe demonstrated the least amount of variability of the 3 control primers tested (*Gapdh*, *Hprt1*, and *Sdha*; data not shown). Negative controls consisted of the reaction mixture without input RNA. The four study groups (Ts65Dn+, Ts65Dn, 2N+, and 2N) were compared with respect to the PCR product synthesis for each gene tested. For each gene, the PCR product synthesis was modeled as a function of mouse study group, using mixed effects models with random mouse effect to account for the correlation between repeated assays on the same mouse (Alldred et al., 2015a, 2015b; McCulloch et al., 2011). Significance was judged at the level $\alpha=0.05$, two-sided.

Results

To determine beneficial gene expression changes associated with perinatal MCS treatment, we examined CA1 pyramidal neurons at two distinct timepoints, young mice at ~6 MO (prior to BFCN degeneration) and middle-aged mice at ~11 MO (following BFCN degeneration), postulating that in addition to known cognitive benefits (Strupp et al., 2016), MCS would delay septohippocampal degeneration seen in Ts65Dn offspring (Ash et al., 2014), and approximate the gene expression levels of 2N mice. We also evaluated the impact of MCS on 2N littermates to assess the effect of early maternal choline supplementation in normal offspring.

Microarray results show marked gene expression changes at both timepoints as a function of dietary treatment and genotype in each of the 22 GO categories assayed (Table I). Interestingly, results indicate a much higher percentage of gene changes occur due to maternal dietary treatment (choline normal versus MCS) than to genotype (Ts65Dn versus 2N) (Table I). Specifically, ~6 MO mice display significant expression changes in 72.4% of genes assayed (417 of 576 genes) due to dietary intervention (e.g., Ts65Dn versus Ts65Dn+ and 2N versus 2N+), whereas 16.1% of gene expression changes are due to genotype alone (93 of 576 genes; e.g., Ts65Dn versus 2N). Similar results are seen in the ~11 MO cohort

with significant expression changes observed in 81% of the genes assayed due to maternal dietary choline intake level (526 of 649 genes), whereas 15.3% of gene expression changes are found by genotype (99 of 649 genes assayed). The remarkable alteration in expression levels by MCS treatment supports the notion that dietary methyl donor intake has a significant impact on overall gene expression patterns, likely through epigenetic programming.

Select aberrant gene expression profiles in ~6 MO Ts65Dn mice are attenuated by MCS

Results indicate gene expression changes in every GO category queried to date due to both maternal dietary treatment and genotype. For greatest mechanistic value, we sought to identify genes where genotype-driven expression level changes were impacted by MCS treatment (e.g., significant differences between Ts65Dn and 2N littermates that normalized in Ts65Dn+ mice). Accordingly, 11 genes were significantly upregulated in Ts65Dn mice that were normalized in Ts65Dn+ mice. Several of these genes are implicated in synaptic plasticity and AD-associated neurodegeneration GO categories, including the membrane-bound zinc-dependent metalloprotease endothelin converting enzyme-2 (*Ece2*), and another zinc metallopeptidase, insulin degrading enzyme (*Ide*) (Table I; Fig. 1, A–B). Interestingly, both *Ece2* and *Ide* degrade A β peptides (Eckman et al., 2003; Pacheco-Quinto and Eckman, 2013; Qiu et al., 1998; Wang et al., 2010). Additional synaptic-related genes included transcriptional regulator paired box 6 (*Pax6*), synaptotagmin 1 (*Synj1*), synaptopodin (*Synpo*), and pan-neurotrophin growth factor receptor (*p75^{NTR}*) (Table I; Fig 1C–F). Attenuated transcripts also included four genes involved in calcium-signaling regulatory pathways, adenylate cyclase 1 (*Adcy1*), calcium/calmodulin-dependent protein kinase II (*Camk2a*), mitogen-activated protein kinase 13 (*Mapk13*), and neuropeptide Y receptor Y1 (*Npy1r*), suggesting that calcium regulation is important in the process of initiating BFCN degeneration (Table I; Fig. 2A–D).

Select aberrant gene expression profiles in ~11 MO Ts65Dn mice are attenuated by MCS

In middle-aged Ts65Dn mice where BFCNs have degenerated, MCS treatment had profound effects in normalizing expression levels of several dysregulated genes in Ts65Dn+ mice. Specifically, significant normalization of 10 genes downregulated in Ts65Dn offspring occurred concomitantly with 12 genes being upregulated in Ts65Dn offspring that attained expression levels which did not differ significantly from 2N in the Ts65Dn+ offspring cohort. Attenuated genes belonged to GO-defined cellular pathways including cellular energy homeostasis, DS/AD response genes, aging and oxidative function, and excitation/inhibition of neuronal transmitters. DS/AD response genes attenuated by MCS included upregulated transcripts alpha-disintegrin and metalloproteinase domain-containing protein 10 (Adam10), dual specificity tyrosine phosphorylation regulated kinase 1A (*Dyrk1a*), which is triplicated in the Ts65Dn mouse, eukaryotic translation initiation factor 2 alpha kinase 2 (*Eif2ak2*; also termed *Prkr*), and *Mapk13*. DS/AD response genes normalized by MCS included downregulated transcripts amyloid-beta precursor protein binding family B member 1 (*Apbb1*; also known as Fe65), beta-site APP cleaving enzyme 1 (*Bace1*), caspase 8 (*Casp8*), and two truncated isoforms of tau, *Mapt2N6D* and *Mapt2N6P* (Table I; Fig 3A–I). Further, *App* is upregulated in Ts65Dn mice, and is partially attenuated by MCS treatment such that expression levels in Ts65Dn+ mice are significantly lower than Ts65Dn

offspring, but still higher than 2N littermates (data not shown). Cellular energy homeostasis genes attenuated by MCS included upregulated transcripts dual specificity tyrosine phosphorylation regulated kinase 3 (*Dyrk3*), heat-shock transcription factor 1 (*Hsf1*) and heat-shock protein family A (Hsp70) member 1A (*Hspa1a*), protein kinase AMP-activated non-catalytic subunit alpha 2 (*Prkaa2*), protein kinase AMP-activated non-catalytic subunit gamma 1 (*Prkag1*), and sestrin 2 (*Sesn2*). Adenylate cyclase 9 (*Adcy9*) was the sole downregulated cellular homeostasis gene attenuated by MCS (Table I; Fig. 4 A–G). Further, genes involved in aging and oxidative function were normalized by MCS treatment, including corticotropin-releasing hormone (*Crh*, upregulated in Ts65Dn; Fig. 5A), potassium-voltage gated channel, subfamily B member 1 (*Kcnb1*, downregulated in Ts65Dn; Fig. 5B), potassium voltage-gated channel interacting protein 3 (*Kcnip3*, downregulated in Ts65Dn; Fig. 5C) and the transcription factor mutL homolog 1 (*Mlh1*, upregulated in Ts65Dn; Fig. 5D). Downregulated transcripts within the excitation/inhibition of neuronal transmitters pathway attenuated by MCS included the death domain-associated protein (*Daxx*, Fig. 6A) and gamma-aminobutyric acid (GABA) type A receptor beta-1 subunit (*Gabrb1*, Fig. 6B).

Assessment of select gene expression by qPCR

qPCR was performed on an independent cohort of ~6 MO trisomic and 2N mice using RNA extracted from CA1 hippocampal microdissections. Results indicated that *Synj1* is significantly downregulated in Ts65Dn mice by dietary intervention (e.g., Ts65Dn+ versus Ts65Dn), although there was no genotype difference (e.g. Ts65Dn vs. 2N; Fig 7). *Mapk13*, *Camk2a*, and *p75^{NTR}* did not show any significant differences in the admixed microdissected tissue (Fig 7).

Using an independent ~11 MO cohort, qPCR revealed that *Apbb1*, *App*, and *Casp8* showed partial or full normalization of genotype-related changes by MCS. However, expression differences for *Apbb1* and *Casp8* in the admixed tissues assayed by qPCR were in the opposite direction of the microarray results (Fig. 8A), illustrating one of the caveats of comparing single-population expression profiles with admixed cell population transcript analyses. In contrast, *Bace1* did not show a significant change in Ts65Dn mice nor attenuation due to MCS (Fig. 8A). Genes involved in stress response and cell homeostasis also showed either partial or fully normalized gene expression to 2N levels in Ts65Dn+ mice, including *Dyrk1a*, *Hsf1*, *Mapk13*, and *Prkaa2*, consistent with microarray results (Fig. 8B). In contrast, *Sesn2* did not show significant differences by maternal diet or genotype (Fig. 8B).

Discussion

We report significant expression level changes in young and middle-aged Ts65Dn mice that were normalized by MCS, including select genes in GO categories that represent AD, cellular stress, synaptic plasticity, and cell death pathways, along with other changes that would indicate MCS treatment beneficially alters gene expression within vulnerable CA1 pyramidal neurons both prior to and following BFCN degeneration. While many additional genes showed expression level changes due to either genotype or MCS treatment, we

focused on those gene expression changes in the DS model that were attenuated or reversed by MCS treatment to fully determine the mechanistic effects of the MCS treatment in an individual cell type vulnerable to degeneration, as seen in AD and DS individuals. There is greater attenuation of aberrant Ts65Dn gene expression by MCS in the ~11 MO cohort than in the ~6 MO cohort, indicating the long-term benefits of this early dietary intervention. Interestingly, young mice did not display normalization of downregulated genes, indicating that the gene expression changes that are upregulated in Ts65Dn mice may be the critical first response at the onset of BFCN degeneration. We highlight some of the key findings in relevant pathways.

Calcium signaling and metabolic dysregulation

In ~6 MO trisomic mice, MCS rescued aberrant expression involved in Ca²⁺ signaling and metabolic dysregulation. Consistent with our findings, *Camk2a* expression is upregulated in postmortem AD hippocampus and correlates with amyloid and NFT load (McKee et al., 1990; Wang et al., 2005). Downregulation of pathological *Camk2a* expression may be beneficial to homeostatic calcium signaling. Further support for normalization of calcium signaling comes from modification of the *Camk2a* target *Npy1r* (Lin et al., 2014), which independently has been shown to be regulated by BDNF as well as products of the choline PEMT pathway (Agrawal et al., 2014). Additionally, *Adcy1* is a calcium-sensitive apoptosis gene, which catalyzes the formation of cyclic AMP, and upon activation, releases glutamate (Cheng and Yakel, 2015; Conti et al., 2009). *Adcy1* is also required for maintenance of hippocampal-dependent remote contextual fear memory (Shan et al., 2008), indicating a need for proper gene expression regulation. Another gene involved in metabolic regulation is *Mapk13*, which when knocked out results in positive metabolic regulation by increasing glucose tolerance and enhancing insulin secretion (Sumara et al., 2009). Conversely, upregulation of *Mapk13* results in increased immune system activation and tau phosphorylation (Cavallini et al., 2013; Feijoo et al., 2005; Risco et al., 2012). *Mapk13* is the only gene we found that was aberrantly upregulated in both ~6 MO and ~11 MO Ts65Dn offspring of dams fed a standard choline diet, which may result in aberrant tau phosphorylation. These results indicate a need for homeostatic regulation of calcium signaling and metabolic regulation in the DS brain, especially at the onset of BFCN degeneration.

Synaptic plasticity and AD-like neurodegeneration

Synj1 is triplicated in both the Ts65Dn mouse and human DS (Martin et al., 2014). We found that *Synj1* expression levels are normalized by MCS. Upregulation of *Synj1* has been linked to increases in A β and endosomal abnormalities in DS (Cossec et al., 2012; Martin et al., 2014; Zhu et al., 2013). Notably, genetic reduction of *Synj1* leads to synaptic and behavioral rescue in an AD mouse model (McIntire et al., 2012). In contrast, *Synpo* is decreased in postmortem CA1 pyramidal neurons during the progression of dementia from mild cognitive impairment to AD, and this downregulation correlates with cognitive decline (Counts et al., 2014). Loss of *Synpo* has also been reported to coincide with loss of dendritic spines and spine apparatus, with concomitant deficits in learning and memory and synaptic plasticity (Deller et al., 2007). Therefore, a balance of *Synpo* expression is likely needed for proper synaptic activity and plasticity. Co-expression of p75^{NTR} and App has been shown to

result in apoptosis, and decrease of this pan-neurotrophin receptor has been linked to both prevention of the cognitive decline and reduction of amyloid burden (Fombonne et al., 2009; Yao et al., 2015). Further, p75^{NTR} when bound to the precursor of NGF (proNGF) or the precursor to BDNF (proBDNF), can induce apoptosis (Mufson et al., 2012; Pedraza et al., 2005; Teng et al., 2005) indicating that upregulation of this receptor is potentially detrimental to neuronal health and survival. *Ece2* has been identified as a regulator of A β clearance (Eckman et al., 2003; Pacheco-Quinto and Eckman, 2013). However, upregulation of *Ece2* is found in postmortem AD patients with vascular pathology, indicating a need for balanced expression (Miners et al., 2010, 2014). Likewise, *Ide* is a thiol metallo-endopeptidase that acts as a protease regulating cerebral A β levels (Qiu et al., 1998; Wang et al., 2010). However, *Ide*-driven breakdown of A β competes with insulin as a substrate (Haque and Nazir, 2014), and the higher insulin levels seen in DS (Anwar et al., 1998; Bergholdt et al., 2006) would likely inhibit *Ide*-mediated clearance of A β . Collectively, these findings indicate that normalization of expression changes in CA1 pyramidal neurons in Ts65Dn offspring by MCS may be the result of decreased cellular stress and metabolic homeostasis. Furthermore, attenuated expression of genes involved in synaptic plasticity and AD-like neurodegeneration may underlie the amelioration of cognitive deficits, notably memory and attentional impairments seen in Ts65Dn+ offspring as previously shown by our group (Ash et al., 2014; Kelley et al., 2014a, 2016; Powers et al., 2016, 2017; Strupp et al., 2016; Velazquez et al., 2013).

Cellular homeostasis regulation

We hypothesize that maternal choline supplementation is neuroprotective to the offspring, especially as they age. Specifically, MCS treatment partially restores cellular homeostasis, resulting in Ts65Dn mice that do not aberrantly express genes involved in cellular energy regulation, effectively attenuating aberrant phenotypic effects on DS/AD response genes, aging and oxidative function and excitation and inhibition of neuronal transmitters. For example, we observed normalized expression of two catalytic subunits in the 5' adenosine monophosphate-activated protein kinase (AMPK) complex signaling cascade that interact with each other, *Prkaa2* and *Prkag1* (Wong and Lodish, 2006). Consistent with this observation, *Prkab2*, the beta catalytic subunit of the AMPK complex, was upregulated in the postmortem human AD CA1 region by microarray assessment (Blalock et al., 2011). Related genes attenuated by MCS associated with the AMPK or downstream pathways include *Hsf1*, *Dyrk3*, and *Sesn2*. *Hsf1* has been previously shown to be involved in protein homeostasis (Su and Dai, 2016), and is activated by synaptic depolarization (Hooper et al., 2016). Further, *Hsf1* nuclear translocation and activation upregulates multiple synaptic genes including *Bdnf* and *Psd95* (Hooper et al., 2016; Wang et al., 2017). *Dyrk3* regulates stability of the mammalian target of rapamycin complex 1 (mTORC1) pathway during cellular stress, which is a downstream target of AMPK (Wippich et al., 2013). *Dyrk3* has also been shown to be involved with dendritic branching (Slepek et al., 2012), and in conjunction with *Dyrk1a*, promotes cell survival through phosphorylation of sirtuin 1 (*Sirt1*) (Guo et al., 2010), indicating dysregulation may negatively impact multiple signaling pathways, including the AMPK cascade. *Sesn2* is a critical factor mediating reactive oxygen species formation, in part by inhibiting AMPK pathway member mTORC1 (Ebnoether et al., 2017). *Sesn2* is also induced by A β exposure *in vitro* and upregulated in serum of AD patients

(Chen et al., 2014; Rai et al., 2016). Therefore, normalization of the AMPK cascade and/or downstream signaling pathway members, as seen with MCS, may be considered therapeutic targets in DS and AD.

AD-related genes

We demonstrate several key genes implicated in AD and DS pathobiology are MCS-responsive in Ts65Dn mice. For example, *Adam10* is upregulated in Ts65Dn mice and attenuated by MCS. Studies have shown increased *Adam10* levels in AD hippocampus, and two rare *Adam10* mutations attenuate α -secretase activity (Gatta et al., 2002; Kim et al., 2009). Adam10 is present and active in synaptic vesicles, including those enriched in APP C-terminal fragments (CTFs), and colocalizes with Bace1 and synaptophysin, which may result in APP processing within synaptic vesicles (Lundgren et al., 2015). *Dyrk1a* is upregulated in DS and AD (Ferrer et al., 2005; Kimura et al., 2007; Lockstone et al., 2007), may dysregulate the 3-repeat tau to 4-repeat tau ratio (Wegiel et al., 2011), and is part of the triplicated 'DS critical region' that has been implicated in cognitive impairment in DS. Importantly, normalizing *Dyrk1a* gene dosage in trisomic mice improves spatial and reference memory and related AD-like phenotypes (Altafaj et al., 2013; Garcia-Cerro et al., 2014, 2017), highlighting the importance of attenuating *Dyrk1a* expression levels in DS as a potential therapeutic approach.

There are also several downregulated genes involved in AD/DS pathology normalized in Ts65Dn+ mice, including *Bace1*. Although associated with β -cleavage of APP, leading to greater A β and β CTF expression, attenuation of Bace1 levels by MCS may indicate a counterintuitive normalization of APP processing in vulnerable neurons that reflects the importance of steady-state A β and β CTF levels, underscoring the importance of evaluating β CTF expression in DS/AD models (Jiang et al., 2010, 2016). Alternatively, Bace1 deficiency may be considered detrimental, as a loss of Bace1 expression impairs myelination (Hu et al., 2015) and causes hippocampal synaptic deficits (Petrus and Lee, 2014; Wang et al., 2014; Zhu et al., 2016), illustrating the need for normative Bace1 levels. MCS also restores the normal expression of two truncated forms of tau (*Mapt2N6D* and *Mapt2N6P*). Little is known about the normal expression of these truncated forms of tau, especially in relation to the DS phenotype, but this may reflect a compensatory change by MCS to permit optimized tau processing, including truncation of the N- and C-terminal domains. Further, the App binding protein Fe65 (*Apbb1*) is upregulated to 2N levels by MCS in trisomic mice, which may be neuroprotective. Moreover, in addition to its role in processing APP (Chow et al., 2015), Apbb1 is a key protein in the cellular response to genotoxic stress (Ryu et al., 2015). Taken together, these varied expression levels changes reveal the need for greater understanding of A β processing and tau metabolism within vulnerable neurons in DS and AD models.

Limitations of the study include a caveat that expression-profiling studies, even those that employ single-population analysis, do not infer causality, but provide highly relevant targets for the study of mechanistic interactions for *in vitro* and *in vivo* experiments as well as identify potential targets for clinical trials. We recognize that the Ts65Dn mouse model does not recapitulate the full pathobiology of AD or DS, and is one of many models that could

have been chosen for these studies. Discrepancies between human AD, DS transcriptomic profiles, and the trisomic model could be envisioned, as the Ts65Dn mouse has >90 triplicated genes that are not located on HSA21 (Sturgeon and Gardiner, 2011). Despite lacking total genetic overlap with DS and not displaying NFTs or A β deposition like AD, Ts65Dn mice are one of the few models available that mimics both behavioral and cognitive decline of AD and DS, including age-related cognitive deficits such as spatial learning, working memory, reference memory, and attention (Ash et al., 2014; Escorihuela et al., 1995; Hyde and Crnic, 2001; Reeves et al., 1995; Velazquez et al., 2013). Future studies may include studying the use of MCS in aged Ts65Dn mice as well as employing Dp16 DS models, APP overexpressing models, and tauopathy models in conjunction with the MCS paradigm. Additional studies comparing the expression profile of trisomic septohippocampal neurons with septohippocampal neurons following cholinotoxic delivery, such as either by ethylcholine mustard aziridinium ion (AF64A) (Fan and Hanin, 1999) or 192 IgG-saporin (Wiley et al., 1991) may also be considered.

In conclusion, results showed significant normalization of gene expression in Ts65Dn mice within several key GO categories as a result of MCS treatment. At the younger age point, critical upregulated genes were normalized by MCS, including those involved in calcium regulation and synaptic plasticity, both pathways critical for the normal homeostatic function at the onset of BFCN degeneration. Similarly, middle-aged trisomic mice saw significant improvement due to early MCS treatment that had lifelong beneficial consequences. These included positive regulation of genes involved in AD pathology and transcripts associated with ID. Additionally, normalization of genes involved in cellular metabolism and homeostasis may prove to be critical for normal function in the aging DS brain. In sum, MCS treatment may help prolong normal cellular function as well as reduce cognitive decline brought on by BFCN degeneration within the septohippocampal circuit in Ts65Dn mice.

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Figure 1.

~6 MO MCS-treated trisomic offspring show upregulation and reversal of select genes in synaptic plasticity and neurodegeneration GO categories. **A.** *Ece2* is upregulated 1.24 fold (2N versus Ts) in Ts65Dn mice and downregulated in Ts+ mice (Ts+ versus Ts) to 2N levels by MCS. **B.** *Ide* is upregulated 1.22 fold (2N versus Ts) in Ts65Dn mice and downregulated in Ts+ mice (Ts+ versus Ts) to 2N levels by MCS. **C.** *p75^{NTR}* is upregulated 1.26 fold (2N versus Ts) in Ts65Dn mice and downregulated in Ts+ mice (Ts+ versus Ts) to 2N levels by MCS. **D.** *Pax6* is upregulated 1.23 fold (2N versus Ts) in Ts65Dn mice and downregulated in Ts+ mice relative to 2N and Ts levels by MCS treatment, while *Pax6* levels in Ts+ are not significantly different from 2N+ levels. **E.** *Synj1* is upregulated 1.22 fold (2N versus Ts) in Ts65Dn mice and downregulated in Ts+ mice (Ts+ versus Ts) to 2N levels by MCS. **F.** *Synpo* is upregulated 1.24 fold (2N versus Ts) in Ts65Dn mice and is downregulated in Ts+ mice (Ts+ versus Ts) to 2N levels by MCS. Note that 2N mice show no significant differences due to MCS treatment in the presented genes except *Pax6*. Key: *, $p < 0.01$; **, $p < 0.001$; ***, $p < 0.0001$.

$p < 0.005$; ***, $p < 0.001$. Upward arrowhead, significant upregulation; downward arrowhead, significant downregulation. For figure clarity, we used the abbreviation Ts (for Ts65Dn mice) and Ts+ (for Ts65Dn MCS treated mice).

Figure 2.

Select calcium signaling genes were normalized by MCS treatment in Ts65Dn mice at ~6 MO. **A.** *Adcy1* is upregulated 1.21 fold (2N versus Ts) in Ts65Dn mice and downregulated in Ts+ mice relative to 2N and Ts levels by MCS treatment, while *Adcy1* levels in Ts+ are not significantly different from 2N+ levels. **B.** *Camk2a* is upregulated 1.23 fold (2N versus Ts) in Ts65Dn mice and downregulated in Ts+ mice relative to 2N and Ts levels by MCS treatment, while *Camk2a* levels in Ts+ are not significantly different from 2N+ levels. **C.** *Mapk13* is upregulated 1.28 fold (2N versus Ts) in Ts65Dn mice and downregulated in Ts+

mice (Ts+ versus Ts) to 2N levels by MCS. **D.** *Npy1r* is upregulated 1.23 fold (2N versus Ts) in Ts65Dn mice and downregulated in Ts+ mice (Ts+ versus Ts) to 2N levels by MCS. Significant differences due to MCS treatment in 2N mice were observed for *Adcy1* and *Camk2a*. Key: *, p<0.01; **, p<0.005; ***, p<0.001. Upward arrowhead, significant upregulation; downward arrowhead, significant downregulation.

Figure 3.

Expression levels impacted by perinatal MCS in ~11 MO MCS-treated offspring included select AD and DS response genes. **A.** *Adam10* is upregulated 1.52 fold (2N versus Ts) in Ts65Dn mice and downregulated in Ts+ mice (Ts+ versus Ts) to 2N levels by MCS. **B.** *Dyrk1a* is upregulated 1.30 fold (2N versus Ts) in Ts65Dn mice and downregulated in Ts+ mice relative to 2N and Ts levels by MCS treatment, while *Dyrk1a* levels in Ts+ are not significantly different from 2N+ levels. **C.** *Eif2ak2* is upregulated 1.35 fold (2N versus Ts) in Ts65Dn mice and downregulated in Ts+ mice (Ts+ versus Ts) to 2N levels by MCS. **D.**

Mapk13 is upregulated 1.22 fold (2N versus Ts) in Ts65Dn mice and downregulated in Ts+ mice relative to 2N and Ts levels by MCS treatment, while *Mapk13* levels in Ts+ are not significantly different from 2N+ levels. **E.** *Apbb1* is downregulated 1.64 fold (2N versus Ts) in Ts65Dn mice and upregulated in Ts+ mice (Ts+ versus Ts) to 2N levels by MCS. **F.** *Bace1* is downregulated 1.92 fold (2N versus Ts) in Ts65Dn mice and upregulated in Ts+ mice (Ts+ versus Ts) to 2N levels by MCS. **G.** *Casp8* is downregulated 1.58 fold (2N versus Ts) in Ts65Dn mice and upregulated in Ts+ mice (Ts+ versus Ts) to 2N levels by MCS. **H.** *Mapt2N6D* is downregulated 1.87 fold (2N versus Ts) in Ts65Dn mice and upregulated in Ts+ mice (Ts+ versus Ts) to 2N levels by MCS. **I.** *Mapt2N6P* is downregulated 2.12 fold (2N versus Ts) in Ts65Dn mice and upregulated in Ts+ mice (Ts+ versus Ts) to significantly higher levels than *Mapt2N6P* expression in 2N and 2N+ mice. Key: *, $p < 0.01$; **, $p < 0.005$; ***, $p < 0.001$. Upward arrowhead, significant upregulation; downward arrowhead, significant downregulation.

Figure 4.

Expression levels impacted by perinatal MCS in ~11 MO MCS-treated offspring included select energy homeostasis genes. **A.** *Dyrk3* is upregulated 1.32 fold (2N versus Ts) in Ts65Dn mice and downregulated in Ts+ mice relative to 2N and Ts levels by MCS treatment, while *Dyrk3* levels in Ts+ are not significantly different from 2N+ levels. **B.** *Hsf1* is upregulated 1.24 fold (2N versus Ts) in Ts65Dn mice and downregulated in Ts+ mice relative to 2N and Ts levels by MCS treatment, while *Hsf1* levels in Ts+ are not significantly different from 2N+ levels. **C.** *Hspa1a* is upregulated 7.72 fold (2N versus Ts) in Ts65Dn

mice and downregulated in Ts+ mice relative to 2N and Ts levels by MCS treatment, while *Hspa1a* levels in Ts+ are trend-level ($p<0.02$) lower than 2N+ levels but higher than 2N levels. **D.** *Prkaa2* is upregulated 1.17 fold (2N versus Ts) in Ts65Dn mice and downregulated in Ts+ mice relative to 2N and Ts levels by MCS treatment, while *Prkaa2* levels in Ts+ are not significantly different from 2N+ levels. **E.** *Prkag1* is upregulated 1.83 fold (2N versus Ts) in Ts65Dn mice and downregulated in Ts+ mice relative to 2N and Ts levels by MCS treatment, while *Prkag1* levels in Ts+ are not significantly different from 2N + levels. **F.** *Sesn2* is upregulated 1.32 fold (2N versus Ts) in Ts65Dn mice and downregulated in Ts+ mice relative to 2N and Ts levels by MCS treatment, while *Sesn2* levels in Ts+ are not significantly different from 2N+ levels. **G.** *Adcy9* is the sole downregulated gene in this GO category. *Adcy9* is downregulated 1.73 fold in Ts65Dn mice and upregulated in Ts+ mice (Ts+ versus Ts) to 2N levels by MCS. Key: *, $p<0.01$; **, $p<0.005$; ***, $p<0.001$. Upward arrowhead, significant upregulation; downward arrowhead, significant downregulation.

Figure 5.

Select age-related and oxidative function genes were reversed by MCS in ~11 MO trisomic mice. **A.** *Crh* is upregulated 1.74 fold (2N versus Ts) in Ts65Dn mice and downregulated in Ts+ mice (Ts+ versus Ts) to 2N levels by MCS. **B.** *Kcnb1* is downregulated 2.19 fold (2N versus Ts) in Ts65Dn mice and upregulated in Ts+ mice (Ts+ versus Ts) to 2N levels by MCS. **C.** *Kcnip3* is downregulated 2.62 fold (2N versus Ts) in Ts65Dn mice and upregulated in Ts+ mice (Ts+ versus Ts) to 2N levels by MCS. **D.** *Mlh1* is upregulated 1.32 fold (2N versus Ts) in Ts65Dn mice and downregulated in Ts+ mice (Ts+ versus Ts) to 2N

levels by MCS. Key: **, $p < 0.005$; ***, $p < 0.001$. Upward arrowhead, significant upregulation; downward arrowhead, significant downregulation.

Figure 6. Downregulated neurotransmitter-related genes that were attenuated by MCS treatment included **A** *Daxx* is downregulated 5.17 fold (2N versus Ts) in Ts65Dn mice and upregulated in Ts+ mice (Ts+ versus Ts) to 2N+ levels by MCS, but not to 2N levels. **B** *Gabrb1* is downregulated 1.43 fold (2N versus Ts) in Ts65Dn mice and upregulated in Ts+ mice (Ts+ versus Ts) to 2N levels by MCS. Key: **, $p < 0.005$; ***, $p < 0.001$. Upward arrowhead, significant upregulation; downward arrowhead, significant downregulation.

Figure 7. qPCR for select genes using subregional CA1 dissections of admixed cells in ~6 MO mice. *Synj1* displays significant downregulation in MCS-treated Ts65Dn offspring, consistent with microarray studies. No genes had differential expression between genotypes, in contrast to microarray data from microdissected CA1 pyramidal neurons. Key: *, $p < 0.05$.

Figure 8. qPCR for select genes using subregional CA1 dissections of admixed cells in ~11 MO mice. **A.** *Apbb1* and *Casp8* were upregulated in Ts65Dn mice and displayed significant reduction in MCS treated trisomic offspring. *App* is upregulated in Ts65Dn mice, and partially recovers to 2N levels by MCS treatment. *Bace1* showed no significant differences based on diet or genotype. **B.** *Dyrk1a* and *Prkaa2* were upregulated in trisomic mice, and displayed normalization (*Dyrk1a* trend-level $p < 0.067$) with MCS treatment in Ts65Dn offspring. *Hsf1* and *Mapk13* were downregulated due to MCS treatment in trisomic mice (*Mapk13* trend-level $p < 0.11$). *Sesn2* showed no significant differences based on diet or genotype. Key: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Upward arrowhead, significant upregulation; downward arrowhead, significant downregulation. Diamond, qPCR results validate microarray findings.

Table I

Gene expression alterations by genotype and dietary treatment

Gene Ontology Category	% altered* by Genotype**		% altered* by MCS***	
	~6 MO	~11 MO	~6 MO	~11 MO
Age of mice				
Autophagy and endosomal-lysosomal	12.0	16.0	68.0	82.0
Alzheimer's disease	8.6	26.8	51.4	80.5
Cell death	12.1	17.6	78.8	82.4
Channels	20.8	16.7	95.8	79.2
Cytoskeletal elements	14.3	5.6	68.6	75.0
GABAergic neurotransmission	4.5	9.1	72.7	72.7
Glial-enriched markers	27.3	9.1	63.6	72.7
Glucose utilization	7.7	15.4	84.6	69.2
Glutamatergic neurotransmission	11.8	23.5	61.8	85.3
G-proteins	18.2	24.2	93.9	75.8
Immediate-early genes	14.3	0.0	85.7	85.7
Monoaminergic neurotransmission	22.2	8.9	75.6	93.3
Neurodevelopment	18.2	26.1	81.8	78.3
Neurotrophins	21.1	5.0	52.6	75.0
Peptides	18.8	16.7	68.8	88.9
Proteases	23.5	14.7	70.6	58.8
Protein phosphatases/kinases	22.9	16.4	79.2	78.7
Sirtuins	n.a.	12.5	n.a.	100.0
Steroid-related markers	3.8	7.7	57.7	84.6
Stress response genes	14.3	18.2	42.9	90.9
Synaptic-related markers	31.3	9.4	81.3	78.1
Transcription factors	6.9	16.7	72.4	86.7
Total # of Genes Changed	93	99	417	526
Total # of Genes Assayed	576	649	576	649
Total % Changed	16.1	15.3	72.4	81.0

*
%(Genes Changed/Genes Assayed)**
2N vs. Ts65Dn;***
Choline Normal vs. MCS; n.a. = not assayed

Table II

qPCR TaqMan gene probes utilized for CA1 region gene expression profiling

Gene name	ID
<i>App</i>	Mm01344172_m1
<i>Apbb1</i>	Mm01274044_g1
<i>Bace1</i>	Mm00478671_m1
<i>Camk2a</i>	Mm01258148_m1
<i>Casp8</i>	Mm00802247_m1
<i>Dyrk1a</i>	Mm00432934_m1
<i>Gapdh</i>	Mm99999915_g1
<i>Hsf1</i>	Mm01201402_m1
<i>Hprt1</i>	Mm01318747_g1
<i>Mapk13</i>	Mm00442493_m1
p75NTR	Mm01309638_m1
<i>Prkaa2</i>	Mm01264790_m1
<i>Sdha</i>	Mm01352360_m1
<i>Sesn2</i>	Mm004060686_m1
<i>Synj1</i>	Mm01215509_m1