The effects of aging on the BTBR mouse model of autism spectrum disorder

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INTRODUCTION

Autism spectrum disorder (ASD) is a complex heterogeneous neurodevelopmental disorder characterized by alterations in social functioning, communicative abilities, and engagement in repetitive or restrictive behaviors. The process of aging in individuals with autism and related neurodevelopmental disorders is not well understood, despite the fact that the number of individuals with ASD aged 65 and older is projected to increase by over half a million individuals in the next 20 years. To elucidate the effects of aging in the context of a modified central nervous system, we investigated the effects of age on the BTBR T + tf/j mouse, a well characterized and widely used mouse model that displays an ASD-like phenotype. We found that a reduction in social behavior persists into old age in male BTBR T + tf/j mice. We employed quantitative proteomics to discover potential alterations in signaling systems that could regulate aging in the BTBR mice. Unbiased proteomic analysis of hippocampal and cortical tissue of BTBR mice compared to age-matched wild-type controls revealed a significant decrease in brain derived neurotrophic factor and significant increases in multiple synaptic markers (spinophilin, Synapsin I, PSD 95, NeuN), as well as distinct changes in functional pathways related to these proteins, including "Neural synaptic plasticity regulation" and "Neurotransmitter secretion regulation." Taken together, these results contribute to our understanding of the effects of aging on an ASD-like mouse model in regards to both behavior and protein alterations, though additional studies are needed to fully understand the complex interplay underlying aging in mouse models displaying an ASD-like phenotype.

Keywords: ASD, autism, BDNF, aging, synaptic marker, neuroprotection, neurodevelopmental disorder, BTBR

Despite the population statistics indicating a significant number of aged individuals with ASD, studies that investigate the effects of the neurodevelopmental disabilities including ASD, in an aged physiological context are lacking. Due to the paucity and limitations of studies and the expected increase in the aged individuals presenting ASD a multidisciplinary conference was held in March 2010 to identify gaps in knowledge regarding older adults with ASD. The panel concluded that descriptive studies of aging in humans and rodent models were needed (Piven and Rabins, 2011). A limited number of human studies have investigated the persistence of the ASD core deficits into adulthood (Ballaban-Gil et al., 1996; Seltzer et al., 2003; Shattuck et al., 2007), childhood factors that are associated with prognosis (Rumsey et al., 1985), and outcome studies (Rutter et al., 1967; Howlin et al., 2004; Renty and Roeyers, 2006). Major findings from these studies suggest that language development (Kanner et al., 1972) and Intelligence Quotient (IQ) predicts outcome (Lotter, 1974; Rumsey et al., 1985; Szatmari et al., 1989). The ASD diagnosis persists with inconsistent findings about which, if any, core deficits change (Ballaban-Gil et al., 1996; Piven et al.,...
1996; Seltzer et al., 2003), and that the adults with ASD remain dependent on others (Rutter et al., 1967) even if they have a normal IQ (Howlin et al., 2004). Despite persistent dependence some studies found that individuals with ASD had improved symptoms over time (Sztamari et al., 1995; Piven et al., 1996; Seltzer et al., 2003). However, a few limitations hinder these early studies of aging in ASD, including study individuals being only 20–30 years old, small sample sizes, and an inability to completely generalize the study findings given the heterogeneity of the population examined (Ramsey et al., 1985; Gillberg and Wing, 1999; Fombonne et al., 2001; Howlin and Moss, 2012).

Given the heterogeneous nature of ASD, mouse models have proven to be particularly useful and reliable in elucidating the mechanism of these disorders (Crawley, 2007; Moy and Nadler, 2008). There are several approaches to ASD mouse models: targeted gene mutations, defects in neurotransmitters or brain regions, inbred strains, and models based on the comorbidity of other human diseases with autism (Crawley, 2007). One strain in particular, BTBR T+tf/J, has been shown to be an especially relevant animal model of ASD (Moy et al., 2007). Extensive behavioral characterization of the BTBR (black and tan, brachyuric) mouse model has revealed low sociability compared to wild-type strains (Bolivar et al., 2007; Moy et al., 2007), resistance to change (Moy et al., 2007; Moy and Nadler, 2008), increased display of self-grooming behavior (Pobbe et al., 2010), display of repetitive behaviors (Pearson et al., 2011), and reduced display of territorial scent marking (Wohr et al., 2011). Furthermore, unusual vocalizations have also been extensively characterized in BTBR mice (Scattoni et al., 2008, 2011), in addition to instances of social avoidance and gaze aversion (Defensor et al., 2011). While the autistic-like behavioral phenotype of the young male BTBR mouse has been studied extensively, the effects of age on this ASD-like mouse model have not been elucidated. As aged human autistic tissue samples are limited and the BTBR mouse model is well characterized and widely accepted, we selected this mouse model to study the nature of the ASD phenotype in an aged context. To enhance our understanding of the aged BTBR mouse and to gain further insight into the effects of aging of ASD, we followed our extensive behavioral characterization of the aged BTBR mouse with quantitative proteomic analyses on cortical and hippocampal tissues as these two brain regions have been strongly associated with ASD (Mundy, 2003; Nadler et al., 2006). We hypothesized that both the behavioral phenotype and proteomic profile would be different between the aged ASD-like mice (or mice with an abnormal central nervous system (CNS)) and the aged wildtype mice (or mice with a presumed normal CNS). Our results contribute to our understanding of the effects of aging on an ASD-like mouse model, and it is our hope that further research will enhance our understanding of aging in an abnormal CNS.

**MATERIALS AND METHODS**

**EXPERIMENTAL ANIMALS**

All experimental animal procedures were approved by the Animal Care and Use Committee of the National Institute on Aging, National Institutes of Health. Male BTBR T+tf/J mice (n = 8, 15 months of age) and male control wildtype (WT) C57BL6J (n = 5, 15 months of age) were housed in the NIA animal facility on a 12 h light and dark cycle from 6 am to 6 pm and received ad libitum access to food and water throughout the duration of the study. All behavioral testing described in detail below was performed between the hours of 9 AM and 5 PM. Prior to behavioral testing, animals were moved from the vivarium to an experimental room and given a minimum of 30 min to habituate to the new environment. Between each experimental animal, all apparatuses were wiped down with 70% ethanol followed by D.I. water to prevent any scent retention that could possibly bias the performances of later mice.

**SOCIAL PREFERENCE ASSESSMENT**

Social behavior was assessed using a three-chambered sociability apparatus with a protocol modeled after previously established methods (Crawley, 2007). In brief, animals were first habituated for 10 min to the three-chambered sociability apparatus (60 × 40 × 22 cm, Stoelting, Wood Dale, IL) free of any stimuli and returned to their home cage for a minimum of 30 min. Prior to the return of the experimental mouse to the chamber for testing, another mouse of the same strain, age, and gender and an inanimate object both housed in identical wire mesh cylinders (15 cm tall, 7 cm in diameter) were placed in opposing compartments of the apparatus. Experimental animals were then returned to the center chamber of the apparatus and were free to choose to interact with either the mouse or object enclosed in the respective wire mesh containers. Interactions were recorded for 10 min and analyzed with ANY-Maze Video Tracking Software (Stoelting). A discrimination index (DI) was calculated that takes into account the relative exploration time spent with either the contained mouse or object: (animal time/total time—object time/total time) × 100. As socially-normal mice show a robust preference for another mouse over an inanimate object, a high DI is reflective of socially-normal behavior while a low DI is indicative of deficits in social functioning.

**NOVEL OBJECT PREFERENCE TEST**

The novel object preference test was performed as described previously (Dere et al., 2007; Park et al., 2010). In brief, mice were given 10 min to explore a 25 cm² opaque walled box with two identical objects placed equidistant from each other in the center of the box. After 10 min of habituation to the two identical objects was completed, animals were returned to their home cages for a minimum of 30 min. After the habituation task, trained experimenters replaced one of the identical objects with a novel object that had never been seen by the experimental mouse. Experimental animals were then returned to the opaque box and given 10 min in which they were free to interact with either the familiar or novel object. Both the habituation session and testing session were videotaped and the animal’s behavior automatically tracked by ANY-Maze Video Tracking Software (Stoelting). Discrimination indices were again calculated: (novel time/total time-same time/total time) × 100. A high discrimination index (DI) is indicative of an enhanced ability to preferentially recognize the novel object, suggesting intact memory functioning.
FIGURE 1 | Social function, cognitive ability, general behavior, anxiety and motor coordination in an aged ASD mouse model. Social preference was assessed using a three-chambered sociability apparatus in aged male BTBR T + tf/j mice and age-sex-matched control mice (A, B). Percentage of time spent exploring both the contained inanimate object and the contained mouse was measured for male BTBR (n = 8) and control mice (n = 5) (A). A discrimination index (DI) was calculated (B) which represents the degree to which experimental or control animals displayed a preference for exploring the contained mouse over the contained inanimate object. Cognitive functioning was assessed via the novel object preference task (NOP) (C, D). Percentage of time spent exploring either a familiar or a novel object was again measured for male BTBR (n = 8) and control mice (n = 5) (C) and a DI was calculated (D) with a higher DI indicating an increased ability to discern a novel object from a familiar object. Exploration of an open field apparatus was assessed in male BTBR and control mice was measured with regard to the following parameters: time spent exploring the peripheral or central areas of the apparatus relative to the total amount of time spent exploring (E), total distance traveled (F), number of ambulatory episodes (G), and number of jumping episodes (H). Exploration of the open field apparatus was repeated with a Light/Dark insert add-on and relative time spent in either the light half or the dark half of the chamber was recorded (I). General anxiety was assessed using the elevated plus maze task. For both BTBR T + tf/j mice and age-and sex-matched controls, relative spent in the closed vs. the open arm was recorded as a percentage of total time (J). Finally, motor coordination was assessed using a rotarod apparatus latency to fall was recorded in both BTBR T + tf/j and control mice (K, n = 5). Data are expressed as means ± s.e.m. Asterisks represent p-values as shown: ∗p < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.001. Statistical significance was measured using a Student’s t-test.

OPEN FIELD TEST
A generalized view of the behavioral characteristics of both experimental and control mice was obtained via an open field test performed as described previously (Holmes et al., 2002). In brief, each mouse was placed in a square chamber approximately 0.14 m² in size (Med Associates Inc, St. Albans, VT) and given 10 min to freely explore. Activity Monitor software (Med Associates Inc) automatically recorded multiple parameters over the duration of the 10 min test. Individual data points were then compiled and subsequently analyzed to produce the following measurements: distance traveled, time spent in the peripheral areas of the chamber, time spent in the center area of the chamber, number of ambulatory episodes, and number of jumping episodes. Relative time spent in the periphery vs. the center was calculated for each mouse by dividing the time spent in either the periphery or center by the total exploration time (600 s for all animals).

LIGHT/DARK EXPLORATION TEST
The light-dark exploration test was performed as previously described (Chadwick et al., 2011) as a means to assess general anxiety behavior. The same square chamber used in the Open field test was again used to complete the Light/Dark exploration test, with the additional insertion of a dark chamber taking up 50%
of the area of the entire apparatus (Dark box insert for mouse open field activity, Med Associates Inc). The dark chamber contains a small arched entrance to allow mice to access the dark environment; this entryway was placed toward the center of the apparatus so mice could easily access it. Time spent in either the light or dark compartment was automatically recorded by Activity Monitor software (Med Associates Inc). Relative time spent in the light chamber vs. the dark chamber was calculated for each mouse by dividing the time spent in either the light or the dark by the total exploration time (600 s for all animals).

**ELEVATED PLUS MAZE**

General anxiety behavior was also evaluated using an elevated plus maze test as described previously (Chadwick et al., 2011). In brief, animals were given 5 min to explore a plus shaped maze raised approximately 36 cm off of the ground. Two of the arms of the maze were enclosed on all sides; two arms remained open for the duration of the test. ANY-Maze Video Tracking Software (Stoelting) was used to record time spent in either the closed or open arms of the apparatus and the relative time spent in the closed or open arms was calculated by dividing the time spent in either arm by the total exploration time (300 s for all animals).

**ROTA-ROD TEST**

Motor coordination was evaluated using an accelerating Rota-Rod treadmill (Med. Associates, Inc) as described previously (Bohlen et al., 2009). Briefly, mice were given two training sessions on the spinning Rota-Rod apparatus on the day prior to the testing day (the first trial was administered in the morning and the second trial was administered in the afternoon). Each habituation training trial lasted 2 min [4 revolutions per minute (rpm)]. On the test day, the mice were placed on the Rota-Rod, which gradually accelerated from 4 to 40 rpm over a 5 min time interval. The test was performed twice per day and the latency to fall was measured and averaged.

**ANIMAL EUTHANIZATION AND TISSUE COLLECTION**

Following completion of behavioral analyses, animals were euthanized via isoflurane overdose (Butler Animal Health Supply, Dublin, OH) as described previously and in accord with approved animal procedures (Chadwick et al., 2011). Bodyweight data was collected immediately prior to euthanization for each animal. Hippocampal and cortical brain tissues were snap frozen on dry ice and stored at −80°C until used for further analyses.

**PROTEIN DIGESTION, iTRAQ LABELING AND WESTERN BLOTTING**

Protein digestion and iTRAQ labeling were processed according to the iTRAQ protocol (iTRAQ Reagents—8plex: amine-modifying Labeling Reagents for Multiplexed Relative and Absolute Protein Quantitation, ABSciex). All tissue samples (Hippocampus and Cortex; WT and BTBR) were prepared in parallel throughout the labeling procedures. Briefly, 100μg...
of protein in each condition was acetone precipitated and resuspended in 20 μL of iTRAQ dissolution buffer (0.5 M triethylammonium bicarbonate (TEAB), ABSciex) containing 0.1% ProteaseMAX detergent (Promega) to denature the proteins. The sample was then reduced by adding iTRAQ Reducing Reagent [Tris(2-carboxyethyl)phosphine (TCEP), ABSciex] to a final concentration of 5 mM and incubated at 37°C for 1 h. Subsequently, the sample was alkylated with iTRAQ Cysteine-Blocking Reagent (10 mM methyl methanethiosulfonate (MMTS), ABSciex) for 10 min at room temperature. The protein sample was then digested with 5 μg sequencing-grade trypsin (Promega) per 100 μg protein at 37°C overnight. Labeling of the samples with iTRAQ 8-plex labels was performed at room temperature for 2 h. After labeling, the samples were mixed with 0.1% formic acid, then stored at −20°C until LC/MS/MS analysis. Western blotting procedures as described previously (Maudsley et al., 2007; Martin et al., 2009a), were performed using the same protein lysates employed for iTRAQ labeling. Antibodies used were as follows: rabbit anti-BDNF [(brain-derived neurotrophic factor) Santa Cruz Biotechnology, Santa Cruz CA], rabbit antiphosphorylated TrkB and TrkB, rabbit anti-phosphorylated Akt and Akt, rabbit antiphosphorylated synapsin 1 and synapsin 1, rabbit anti-spinophilin, rabbit anti-PSD95 and rabbit anti-synaptophysin (Cell signaling technology, Inc., Danvers, MA), mouse anti-NeuN (EMD Millipore Corporation, Billerica, MA) and mouse anti-beta actin (Sigma-Aldrich, St. Louis, MO). Results were quantified and statistical analysis was performed using an unpaired student t-test. p < 0.05 was considered statistically significant.

**LC/MS/MS ANALYSIS**
Samples were analyzed using an Eksigent NanoLC Ultra 2D (Dublin, CA) and Thermo Fisher Scientific LTQ Orbitrap XL (San Jose, CA). In brief, peptides were first loaded onto a trap cartridge (Agilent), then eluted onto a reversed phase PicoFrit column (New Objective, Woburn, MA) using a linear 120 min gradient of acetonitrile (2–62%) containing 0.1% formic acid at 250 nL/min flowrate. The eluted peptides were sprayed into the LTQ Orbitrap XL. The data-dependent acquisition mode was enabled, and each FTMS MS1 scan (60,000 resolution) was followed by 6 MS2 scans (alternating CID at unit resolution and HCD at 7500 resolution on 3 precursor ions). The spray voltage and ion transfer tube temperature were set at 1.8 kV and 180°C, respectively.

**DATABASE SEARCH AND iTRAQ QUANTIFICATION**
Proteome Discoverer 1.2 (Thermo Fisher Scientific) was used for protein identification and iTRAQ quantification using
Sequest algorithms. The following criteria were followed: SwissProt mouse database; enzyme: trypsin; miscleavages: 2; static modifications: methylthio (+45.988 Da on C), iTRAQ8plex (+304.205 Da on N-terminus and K); dynamic modifications: oxidation (+15.995 Da on M), deamidation (+0.984 Da on N and Q); peptide tolerance as 25 ppm; MS2 tolerance as 0.8 Da. Peptides reported via all search engines were accepted only if they met the false discovery rate of 5%. For iTRAQ quantification, the reporter ion intensities in MS2 spectra (m/z 113–121, integration width tolerance 50 mmu) were used to calculate the expression ratios among the different conditions (Hippocampus and Cortex; WT and BTBR).

**BIOINFORMATICS ANALYSIS**

Functional annotational clustering of our proteomic data, i.e., gene ontology (GO), KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis was performed using WebGestalt (http://bioinfo.vanderbilt.edu/webgestalt/, 3/2014) as previously described (Chadwick et al., 2010a, 2011). In addition Ingenuity Pathway Analysis (http://Ingenuity.com, 3/2014) was employed for Canonical Signaling Pathway enrichment investigations. In brief, GO analysis allows for broad clustering of genes into functional groups, while KEGG pathways analysis allows for more specific clustering into signaling pathways (Maudsley et al., 2011). Inclusion criteria were set as follows: pathway groups needed to meet a minimum population of two genes from the input experimental set, and also needed to possess a probability significance of enrichment compared to a control background dataset of less than $p < 0.05$ (hypergeometric test of significance). In addition, Venn diagrams were also constructed to identify common and uniquely altered genes between hippocampus and cortex, using VennPlex (Cai et al., 2013), as described previously (Martin et al., 2009b).

**TEXTROUS! LATENT SEMANTIC ANALYSIS-BASED INVESTIGATION**

Textrous! latent semantic analysis was performed as described previously (Chen et al., 2013). In brief, corresponding gene symbols were uploaded into Textrous! (http://textrous.irp.nih.gov/, 3/2014) and then the relevant output indices, i.e., Cosine Similarity, Z-score and $p$-value were extracted. In addition to the direct list-based semantic output both the Collective processing mode (generating hierarchical word-clouds), as well the Individual processing mode (generating symbol-word heatmaps) were applied to provide more nuanced informatic appreciation of the specific input datasets.

**STATISTICAL ANALYSIS**

All statistical analyses were conducted using a Student’s $t$-test (two-tailed with equal variances: GraphPad Prism, version 5.02). $p < 0.05$ was considered statistically significant throughout the study. Error bars represent 95% confidence interval. All data represent means ± s.e.m. (standard error of mean). $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. 
Table 1 | GO term analysis for cortical protein alterations observed in aged BTBR compared to WT control.

| GO group                   | GO term                                                          | GO ID  | C     | O     | E     | R     | P     | H     |
|----------------------------|------------------------------------------------------------------|--------|-------|-------|-------|-------|-------|-------|
| Biological process         | Endoplasmic reticulum tubular network organization               | 71786  | 4     | 2     | 0.02  | 126.12| 0.0047| 293.595|
| Biological process         | Early endosome to late endosome transport                       | 45022  | 21    | 3     | 0.08  | 36.03| 0.0041| 86.0114|
| Biological process         | Vesicle-mediated transport                                       | 16192  | 909   | 14    | 3.6   | 3.88 | 0.0018| 10.64954|
| Biological process         | Secretion by cell                                                | 32940  | 693   | 11    | 2.75  | 4    | 0.0041| 9.548865|
| Biological process         | Secretion                                                       | 46903  | 795   | 12    | 3.15  | 3.81 | 0.0037| 9.265151|
| Biological process         | Transmission of nerve impulse                                    | 19226  | 735   | 11    | 2.91  | 3.78 | 0.0047| 8.79947|
| Biological process         | Neurological system process                                      | 50877  | 1237  | 16    | 4.9   | 3.26 | 0.002| 8.798642|
| Biological process         | System process                                                  | 3008   | 1695  | 19    | 6.72  | 2.83 | 0.002| 7.638085|
| Biological process         | Multicellular organismal signaling                               | 35637  | 751   | 11    | 2.98  | 3.69 | 0.0087| 7.603174|
| Biological process         | Transport                                                       | 6810   | 3338  | 30    | 13.23 | 2.27 | 0.0006| 7.313597|
| Biological process         | Establishment of localization                                    | 51234  | 3392  | 30    | 13.45 | 2.23 | 0.0006| 7.184723|
| Biological process         | Establishment of localization in cell                           | 51649  | 1756  | 19    | 6.96  | 2.73 | 0.0027| 7.012377|
| Biological process         | Cellular localization                                           | 51641  | 1977  | 20    | 7.84  | 2.55 | 0.0035| 6.262626|
| Biological process         | Localization                                                   | 51179  | 4167  | 31    | 16.52 | 1.88 | 0.0037| 4.571781|
| Cellular component         | Chaperonin-containing T-complex                                  | 5832   | 7     | 2     | 0.03  | 78.59 | 0.0039| 169.3182|
| Cellular component         | Pericentriolar material                                         | 242    | 12    | 2     | 0.04  | 45.84 | 0.0076| 97.14357|
| Cellular component         | Clathrin-sculpted vesicle                                       | 60198  | 12    | 2     | 0.04  | 45.84 | 0.0076| 97.14357|
| Cellular component         | Clathrin-coated vesicle membrane                                | 30665  | 110   | 5     | 0.4   | 12.5  | 0.0011| 36.98259|
| Cellular component         | Filopodium                                                     | 30175  | 55    | 3     | 0.2   | 15   | 0.0076| 31.7878|
| Cellular component         | Coated pit                                                     | 5905   | 57    | 3     | 0.21  | 14.48 | 0.0082| 30.20798|
| Cellular component         | Coated vesicle membrane                                         | 30662  | 150   | 5     | 0.55  | 9.17  | 0.0032| 22.87777|
| Cellular component         | Lamellipodium                                                  | 30027  | 121   | 4     | 0.44  | 9.09  | 0.0076| 19.2634|
| Cellular component         | Ruffle                                                          | 1726   | 123   | 4     | 0.45  | 8.94  | 0.0076| 18.94553|
| Cellular component         | Neuron projection                                               | 43005  | 651   | 12    | 2.37  | 5.07  | 0.0002| 18.75378|
| Cellular component         | Cell leading edge                                               | 31252  | 257   | 6     | 0.93  | 6.42  | 0.0039| 15.4537|
| Cellular component         | Clathrin-coated vesicle                                         | 30136  | 207   | 5     | 0.75  | 6.64  | 0.0076| 14.0714|
| Cellular component         | Cell projection                                                | 42995  | 1230  | 16    | 4.47  | 3.58  | 0.0002| 13.24231|
| Cellular component         | Mitochondrion                                                  | 5739   | 1525  | 18    | 5.54  | 3.25  | 0.0002| 12.02165|
| Cellular component         | Cytoplasmic part                                               | 44444  | 6772  | 44    | 24.62 | 1.79  | 7.70E-05| 7.363182|
| Cellular component         | Microtubule cytoskeleton                                       | 15630  | 863   | 10    | 3.14  | 3.19  | 0.0076| 6.760205|
| Cellular component         | Cytoskeleton                                                   | 5856   | 1790  | 15    | 6.51  | 2.3   | 0.0093| 4.672489|
| Cellular component         | Cytoplasm                                                      | 5737   | 9130  | 49    | 33.19 | 1.48  | 0.0007| 4.696255|
| Cellular component         | Cytosol                                                        | 5829   | 2372  | 18    | 8.62  | 2.09  | 0.0093| 4.245871|
| Cellular component         | Intracellular                                                  | 5622   | 12564 | 58    | 45.68 | 1.27  | 0.0012| 3.70944|
| Cellular component         | Intracellular part                                             | 44424  | 12237 | 55    | 44.49 | 1.24  | 0.0082| 2.586871|

GO term annotation of the cortical proteins possessing BTBR:WT iTRAQ expression ratios < 1.2 or > 0.8 were created using WebGestalt. Each GO term group significantly populated (p < 0.05) by at least two independent proteins are depicted. For each GO Term group the specific GO term ID, number of total proteins in the curated GO term cluster (C), the number of observed experimental proteins in the GO term group (O), the expected number of proteins in the group based on whole-genome background frequency (E), the GO term group enrichment factor (R), the GO term enrichment probability (P) and the hybrid score (H) are indicated (H).

RESULTS

ASD-LIKE BEHAVIORAL PHENOTYPE PERSISTS IN AGED BTBR MICE

Commensurate with an extant ASD phenotype aged BTBR mice demonstrated no sociability preference between an object or another mouse, while WT controls spent significantly more time with another mouse compared to an object (Figure 1A). Relative percentage of time spent with either the mouse or the object was used to calculate a social discrimination index [DI: (novel time/total time-same time/total time) × 100] (Figure 2B). WT and BTBR mice performed similarly in the novel object preference test of learning and memory, with both groups spending significantly more time with a novel object compared to a familiar object (Figures 1C,D). Both WT and BTBR mice significantly preferred to explore the peripheral areas of the chamber over the center of the chamber, though the BTBR mice did show a significantly higher preference for the periphery over the center (Figure 1E). No significant differences in total distance traveled (Figure 1F) or number of ambulatory episodes (Figure 1G) were found. BTBR mice however demonstrated significantly less jumping activity compared to WT (Figure 1H). In light-dark exploration tests BTBR mice spent significantly more time in the dark chamber compared to the lighted chamber, while WT control mice show no statistical difference in preference for either chamber (Figure 1I). In the elevated plus maze test of anxiety,
Table 2 | GO term analysis for hippocampal protein alterations observed in aged BTBR compared to WT controls.

| GO Class                  | GO Term                                                | GO ID  | C   | E   | O   | R   | P             | H             |
|---------------------------|--------------------------------------------------------|--------|-----|-----|-----|-----|----------------|----------------|
| Biological process        | Neurotransmitter secretion                             | 7269   | 99  | 15  | 1.25| 11.98| 4.4E–10        | 112.0914       |
| Biological process        | Synaptic vesicle endocytosis                           | 48488  | 22  | 6   | 0.28| 21.57| 8.46E–06       | 109.4166       |
| Biological process        | Regulation of neuronal synaptic plasticity             | 48168  | 47  | 9   | 0.59| 15.14| 4.14E–07       | 96.63861       |
| Biological process        | Synaptic vesicle transport                             | 48489  | 68  | 11  | 0.86| 12.79| 5.71E–08       | 92.64262       |
| Biological process        | Regulation of synaptic plasticity                     | 48167  | 96  | 13  | 1.21| 10.71| 1.68E–08       | 83.26964       |
| Biological process        | Neurotransmitter transport                             | 6836   | 133 | 15  | 1.68| 8.92 | 1.21E–08       | 70.62155       |
| Biological process        | Synaptic transmission                                  | 7268   | 651 | 41  | 8.23| 4.98 | 8.14E–15       | 70.16509       |
| Biological process        | Regulation of neurotransmitter levels                 | 1505   | 137 | 15  | 1.73| 8.66 | 1.54E–08       | 6765607       |
| Biological process        | Regulation of synaptic transmission                   | 50804  | 186 | 18  | 2.35| 7.65 | 3.76E–09       | 64.44981       |
| Biological process        | Regulation of neurological system process              | 31644  | 217 | 19  | 2.74| 6.92 | 5.27E–09       | 57.28507       |
| Biological process        | Regulation of transmission of nerve impulse           | 51969  | 204 | 18  | 2.58| 6.98 | 1.08E–08       | 55.6007       |
| Biological process        | Transmission of nerve impulse                          | 19226  | 735 | 41  | 9.29| 4.41 | 2.94E–13       | 55.26459       |
| Biological process        | Multicellular organismal signaling                     | 35637  | 751 | 41  | 9.5 | 4.32 | 4.13E–13       | 53.4991       |
| Biological process        | Respiratory electron transport chain                   | 22904  | 110 | 12  | 1.39| 8.63 | 8.22E–07       | 52.51466       |
| Biological process        | Signal release                                        | 23061  | 344 | 23  | 4.35| 5.29 | 8.37E–09       | 42.72878       |
| Biological process        | Generation of a signal involved in cell-cell signaling| 3001   | 344 | 23  | 4.35| 5.29 | 8.37E–09       | 42.72878       |
| Biological process        | Cell respiration                                       | 45333  | 159 | 14  | 2.01| 6.96 | 8.22E–07       | 42.35249       |
| Biological process        | Energy derivation by oxidation of organic compounds    | 15980  | 329 | 22  | 4.16| 5.29 | 1.54E–08       | 41.32802       |
| Biological process        | Cell-cell signaling                                   | 7267   | 1099| 48  | 13.9| 3.45 | 3.98E–12       | 39.3304       |
| Biological process        | Generation of precursor metabolites and energy         | 6091   | 451 | 26  | 5.7 | 4.56 | 1.07E–08       | 36.34601       |
| Biological process        | Neurological system process                            | 50877  | 1237| 44  | 15.64| 2.81 | 1.54E–08       | 21.95307       |
| Biological process        | Small molecule metabolic process                      | 44281  | 2515| 71  | 31.8| 2.23 | 1.02E–09       | 20.05082       |
| Biological process        | Cell morphogenesis involved in neuron differentiation  | 48667  | 575 | 25  | 7.27| 3.44 | 3.07E–06       | 18.96424       |
| Biological process        | Regulation of biological quality                      | 65008  | 2511| 70  | 31.75| 2.2  | 2.49E–09       | 18.92836       |
| Biological process        | Neuron projection morphogenesis                       | 48812  | 583 | 25  | 7.37| 3.39 | 3.70E–06       | 18.4138       |
| Biological process        | Neuron development                                    | 48666  | 798 | 31  | 10.09| 3.07 | 1.11E–06       | 18.28086       |
| Biological process        | Regulation of system process                          | 44057  | 455 | 21  | 5.75| 3.65 | 1.05E–05       | 18.17266       |
| Biological process        | Secretion by cell                                    | 32940  | 693 | 28  | 8.76| 3.2  | 2.39E–06       | 1799813       |
| Biological process        | Axonogenesis                                           | 7409   | 526 | 23  | 6.65| 3.46 | 7.81E–06       | 1767143       |
| Biological process        | Neuron projection development                         | 31175  | 704 | 28  | 8.9 | 3.15 | 3.07E–06       | 1736551       |
| Biological process        | Secretion                                             | 46903  | 796 | 30  | 10.05| 2.98 | 3.07E–06       | 16.42833       |
| Biological process        | Neuron differentiation                                | 30182  | 990 | 34  | 12.52| 2.72 | 3.46E–06       | 14.85371       |
| Biological process        | Nervous system development                            | 7399   | 1724| 50  | 21.8 | 2.29 | 6.25E–07       | 14.20743       |
| Biological process        | Establishment of localization in cell                | 51649  | 1756| 50  | 22.21| 2.25 | 1.01E–06       | 13.49028       |
| Biological process        | Generation of neurons                                | 48699  | 1073| 35  | 13.57| 2.58 | 6.71E–06       | 13.34706       |
| Biological process        | Cellular localization                                | 51641  | 1977| 53  | 25  | 2.12 | 2.31E–06       | 11.94914       |
| Biological process        | Establishment of localization                        | 51234  | 3392| 74  | 42.89| 1.73 | 7.81E–06       | 8.835714       |
| Biological process        | Transport                                              | 6810   | 3338| 73  | 42.21| 1.73 | 8.56E–06       | 8.765067       |
| Biological process        | SinglE–multicellular organism process                 | 44707  | 5612| 106 | 70.97| 1.49 | 4.87E–06       | 7915582       |
| Biological process        | Multicellular organism process                         | 32501  | 5644| 106 | 71.37| 1.49 | 6.63E–06       | 7715945       |
| Molecular function        | Malate dehydrogenase (oxaloacetate-decarboxylating)   | 4473   | 2   | 2   | 0.02| 81.3 | 0.0023         | 214.4915       |
| Molecular function        | (NADP+) activity                                       |        |     |     |     |     |                |                |
| Molecular function        | Malate dehydrogenase (oxaloacetate-decarboxylating)   | 16619  | 3   | 2   | 0.04| 54.2 | 0.0039         | 130.5643       |

(Continued)
Table 2 | Continued

| GO Class                  | GO Term                                                                 | GO ID  | C   | E   | O  | R   | P   | H  |
|---------------------------|-------------------------------------------------------------------------|--------|-----|-----|----|-----|-----|----|
| Molecular function        | Threonine peroxidase activity                                           | 8379   | 3   | 2   | 0.04 | 54.2 | 0.0039 | 130.5643 |
| Molecular function        | Malic enzyme activity                                                   | 4470   | 4   | 2   | 0.05 | 40.65 | 0.0063  | 89.45681  |
| Molecular function        | Hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in cyclic amides | 16812 | 10  | 3   | 0.12 | 24.39 | 0.0023  | 64.34746  |
| Molecular function        | NADH binding                                                           | 51287  | 49  | 7   | 0.6 | 11.61 | 0.0001  | 46.44    |
| Molecular function        | Calmodulin-dependent protein kinase activity                            | 4683   | 21  | 4   | 0.26 | 15.49 | 0.0017  | 42.90035  |
| Molecular function        | Proline-rich region binding                                             | 70064  | 15  | 3   | 0.18 | 16.26 | 0.0062  | 35.89571  |
| Molecular function        | Syntaxin binding                                                       | 19905  | 38  | 5   | 0.47 | 10.7  | 0.0017  | 29.6342   |
| Molecular function        | NADH dehydrogenase (ubiquinone) activity                               | 8137   | 44  | 5   | 0.54 | 9.24  | 0.0023  | 24.37763  |
| Molecular function        | NADH dehydrogenase (quinone) activity                                 | 50136  | 44  | 5   | 0.54 | 9.24  | 0.0023  | 24.37763  |
| Molecular function        | NADH dehydrogenase activity                                             | 3954   | 44  | 5   | 0.54 | 9.24  | 0.0023  | 24.37763  |
| Molecular function        | SNARE binding                                                          | 149    | 45  | 5   | 0.55 | 9.03  | 0.0023  | 23.8236   |
| Molecular function        | Cofactor binding                                                       | 48037  | 262 | 15  | 3.22 | 4.65  | 7.65E–05 | 19.14097  |
| Molecular function        | Actin filament binding                                                  | 51015  | 72  | 6   | 0.89 | 6.77  | 0.0033  | 16.79966  |
| Molecular function        | Coenzyme binding                                                       | 50062  | 186 | 11  | 2.29 | 4.81  | 0.0005  | 15.87795  |
| Molecular function        | Oxidoreductase activity, acting on NADH or NADPH, quinone or similar compound as acceptor | 16655 | 58  | 5   | 0.71 | 7.01  | 0.0062  | 15.47533  |
| Molecular function        | Calmodulin binding                                                     | 5516   | 164 | 10  | 2.02 | 4.96  | 0.0008  | 15.36067  |
| Molecular function        | Hydrogen ion transmembrane transporter activity                         | 15078  | 101 | 7   | 1.24 | 5.63  | 0.0023  | 14.85347  |
| Molecular function        | Cytoskeletal protein binding                                            | 8092   | 638 | 25  | 7.85 | 3.19  | 3.90E–05 | 14.0645   |
| Molecular function        | Oxidoreductase activity                                                 | 16491  | 711 | 24  | 8.75 | 2.74  | 0.0003  | 9.652688  |
| Molecular function        | Actin binding                                                          | 3779   | 356 | 14  | 4.38 | 3.2   | 0.0017  | 8.862563  |
| Molecular function        | GTPase activity                                                        | 42802  | 863 | 25  | 10.62 | 2.36 | 0.0012  | 6.893132  |
| Molecular function        | Anion binding                                                          | 43168  | 2402| 54  | 29.55 | 1.83  | 0.0002  | 6.769115  |
| Molecular function        | Small molecule binding                                                 | 36094  | 2630| 58  | 32.35 | 1.79  | 0.0002  | 6.621156  |
| Molecular function        | Protein binding                                                        | 5515   | 7337| 126 | 90.25 | 1.4  | 2.47E–05 | 6.450224  |
| Molecular function        | Nucleoside phosphate binding                                            | 1901265| 2437| 53  | 29.98 | 1.77  | 0.0005  | 5.842823  |
| Molecular function        | Nucleotide binding                                                     | 166    | 2436| 52  | 29.96 | 1.74  | 0.0007  | 5.489529  |
| Molecular function        | Nucleoside-triphosphatase activity                                      | 17111  | 760 | 21  | 9.35  | 2.25  | 0.0039  | 5.420105  |
| Molecular function        | Pyrophosphatase activity                                                | 16462  | 794 | 21  | 9.77  | 2.15  | 0.0063  | 4.731418  |
| Molecular function        | Hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides | 16818 | 797 | 21  | 9.8  | 2.14  | 0.0086  | 4.709411  |
| Molecular function        | Catalytic activity                                                     | 3824   | 5371| 94  | 66.07 | 1.42  | 0.0005  | 4.687463  |
| Molecular function        | Hydrolase activity, acting on acid anhydrides                           | 16817  | 802 | 21  | 9.86  | 2.13  | 0.0063  | 4.687405  |
| Molecular function        | Purine ribonucleoside triphosphate binding                             | 35639  | 1829| 38  | 22.5  | 1.69  | 0.0063  | 3.719114  |
| Molecular function        | Purine nucleoside binding                                               | 1883   | 1841| 38  | 22.65 | 1.68  | 0.0063  | 3.697108  |
| Molecular function        | Purine ribonucleoside binding                                           | 32550  | 1838| 38  | 22.61 | 1.68  | 0.0063  | 3.697108  |
| Molecular function        | Ribonucleoside binding                                                 | 32549  | 1842| 38  | 22.66 | 1.68  | 0.0063  | 3.697108  |
| Molecular function        | Nucleoside binding                                                     | 1882   | 1852| 38  | 22.78 | 1.67  | 0.0068  | 3.619719  |
| Molecular function        | Purine ribonucleotide binding                                           | 32555  | 1864| 38  | 22.93 | 1.66  | 0.0073  | 3.546884  |
| Cellular component        | Synapse                                                                | 45202  | 489 | 28  | 5.45  | 5.14  | 3.35E–11 | 53.84127  |
| Cellular component        | Mitochondrial respiratory chain                                        | 5746   | 69  | 8   | 0.77  | 10.41 | 6.79E–06 | 53.80024  |

(Continued)
## Table 2 | Continued

| GO Class                        | GO Term                              | GO ID   | C     | E     | O     | R     | P     | H     |
|---------------------------------|--------------------------------------|---------|-------|-------|-------|-------|-------|-------|
| Cellular component              | Neuron projection                     | 43005   | 651   | 33    | 7.25  | 4.55  | 1.02E–11 | 50.01087 |
| Cellular component              | Synaptic vesicle                      | 8021    | 105   | 10    | 1.17  | 8.55  | 2.14E–06 | 48.47496 |
| Cellular component              | Axon                                 | 30424   | 286   | 19    | 3.19  | 5.96  | 8.47E–09 | 48.10981 |
| Cellular component              | Respiratory chain                     | 70469   | 76    | 8     | 0.85  | 9.45  | 1.32E–05 | 46.11058 |
| Cellular component              | Clathrin-coated vesicle membrane      | 30665   | 110   | 10    | 1.23  | 8.16  | 3.21E–06 | 44.82692 |
| Cellular component              | Vesicle membrane                      | 12506   | 355   | 21    | 3.96  | 5.31  | 8.47E–09 | 42.86294 |
| Cellular component              | Cytoplasm                            | 30659   | 342   | 20    | 3.81  | 5.25  | 2.52E–08 | 39.89265 |
| Cellular component              | Cytoplasm                            | 5737    | 9130  | 170   | 101.72| 1.67  | 1.52E–24 | 39.77632 |
| Cellular component              | Cytoplasmic part                      | 44444   | 6772  | 143   | 75.45 | 1.9   | 1.86E–21 | 39.38793 |
| Cellular component              | Clathrin-coated vesicle               | 30136   | 207   | 14    | 2.31  | 6.07  | 7.60E–07 | 37.14346 |
| Cellular component              | Cell projection                       | 42995   | 1230  | 45    | 13.7  | 3.28  | 2.51E–11 | 34.76907 |
| Cellular component              | Coated vesicle membrane               | 30662   | 150   | 11    | 1.67  | 6.58  | 6.79E–06 | 34.0063  |
| Cellular component              | Cytoplasmic vesicle part              | 44433   | 401   | 21    | 4.47  | 4.7   | 6.16E–08 | 33.88979 |
| Cellular component              | Cytosol                              | 5829    | 2372  | 69    | 26.43 | 2.61  | 4.11E–13 | 32.37287 |
| Cellular component              | Coated vesicle                       | 30135   | 254   | 15    | 2.83  | 5.3   | 1.44E–06 | 30.96068 |
| Cellular component              | Mitochondrion                        | 5739    | 1525  | 48    | 16.99 | 2.83  | 5.17E–10 | 26.28082 |
| Cellular component              | Synapse part                         | 44456   | 370   | 18    | 4.12  | 4.37  | 1.46E–06 | 25.50178 |
| Cellular component              | Membrane-bounded vesicle             | 31988   | 883   | 32    | 9.84  | 3.25  | 4.82E–08 | 23.7801  |
| Cellular component              | Mitochondrial inner membrane         | 5743    | 354   | 17    | 3.94  | 4.31  | 3.58E–06 | 23.47276 |
| Cellular component              | Cytoplasmic membrane-bounded vesicle | 16023   | 862   | 31    | 9.6   | 3.23  | 8.13E–08 | 22.90041 |
| Cellular component              | Mitochondrial part                   | 44429   | 744   | 27    | 8.29  | 3.26  | 5.82E–07 | 20.32635 |
| Cellular component              | Organelle inner membrane             | 19866   | 384   | 17    | 4.28  | 3.97  | 1.01E–05 | 19.83284 |
| Cellular component              | Vesicle                              | 31982   | 970   | 32    | 10.81 | 2.96  | 3.20E–07 | 19.22476 |
| Cellular component              | Cytoplasmic vesicle                  | 31410   | 928   | 31    | 10.34 | 3     | 3.39E–07 | 19.21682 |
| Cellular component              | Intracellular part                   | 44424   | 12237 | 179   | 138.34| 1.31  | 2.42E–13 | 16.5272  |
| Cellular component              | Intracellular                        | 5622    | 12564 | 180   | 139.98| 1.29  | 1.32E–12 | 15.32446 |
| Cellular component              | Organelle membrane                   | 31090   | 2373  | 57    | 26.44 | 2.16  | 8.13E–08 | 15.3142  |
| Cellular component              | Intracellular organelle part         | 44446   | 6725  | 120   | 74.93 | 1.6   | 5.80E–10 | 14.77852 |
| Cellular component              | Organelle part                       | 44422   | 6812  | 120   | 75.9  | 1.58  | 1.42E–09 | 13.97938 |
| Cellular component              | Cytoskeleton                         | 5856    | 1790  | 45    | 19.94 | 2.26  | 1.06E–06 | 13.50281 |
| Cellular component              | Protein complex                      | 43234   | 3278  | 70    | 36.52 | 1.92  | 9.89E–08 | 13.44922 |
| Cellular component              | Cell part                            | 44464   | 14643 | 189   | 163.15| 1.16  | 1.90E–10 | 11.27665 |
| Cellular component              | Cell                                 | 5623    | 14644 | 189   | 163.16| 1.16  | 1.90E–10 | 11.27665 |
| Cellular component              | Macromolecular complex               | 32991   | 3864  | 76    | 43.05 | 1.77  | 5.44E–07 | 11.08799 |
| Cellular component              | Intracellular organelle              | 43229   | 10636 | 155   | 118.5 | 1.31  | 8.08E–08 | 9.290587 |
| Cellular component              | Organelle                            | 43226   | 10651 | 155   | 118.67| 1.31  | 8.13E–08 | 9.287781 |
| Cellular component              | Intracellular membrane-bounded organelle | 43231   | 9587  | 138   | 106.82| 1.29  | 1.32E–05 | 6.29446  |
| Cellular component              | Membrane-bounded organelle           | 43227   | 9598  | 138   | 106.94| 1.29  | 1.36E–05 | 6.277735 |

GO term annotation of the cortical proteins possessing BTBR:WT iTRAQ expression ratios <1.2 or >0.8 were created using WebGestalt. Each GO term group significantly populated (p<0.05) by at least two independent proteins are depicted. For each GO Term group the specific GO term ID, number of total proteins in the curated GO term cluster (C), the number of observed experimental proteins in the GO term group (O), the expected number of proteins in the group based on whole-genome background frequency (E), the GO term group enrichment factor (R), the GO term enrichment probability (P) and the hybrid score (R* – log10 P) are indicated (H).

Both WT and BTBR mice significantly preferred closed arms to open arms (Figure 1J). Motor coordination, assessed using a Rotarod test was similar in both groups (Figure 1K).

**ALTERED PROTEIN EXPRESSION IN CNS OF AGED BTBR MICE**

Cortical and hippocampal western blotting of proteins, selected for their ability to indicate alterations of neurosynaptic activity, was employed to detect potential expression differences between aged WT and BTBR mice. Our panel of neurosynaptic factors analyzed in cortical tissue is indicated in Figure 2A. No significant difference between WT and BTBR pro-BDNF (brain-derived neurotrophic factor) levels was detected (Figure 2B), whereas a significant decreased in BTBR mature BDNF was observed compared to WT (Figure 2C). Levels of phosphorylated TrkB receptor...
FIGURE 5 | Signaling pathway analysis of cortical and hippocampal proteins differentially regulated BTBR and WT.

Cortical and hippocampal proteins exhibiting differential regulation between BTBR and control mice were analyzed for potential functional pathway interactions using KEGG (A-cortex, B-hippocampus) and Ingenuity Canonical Signaling pathway analysis (C-cortex, D-hippocampus). For (A,B), the top 10 most significantly-populated (calculated with Hybrid scores: enrichment factor (R) \( \times -\log_{10} \) enrichment probability, P) neuronally-specific KEGG or Canonical signaling pathways are shown.

were similar in WT and BTBR cortex (Figure 2D) while the total TrkB expression was significantly lower in BTBR mice compared to WT (Figure 2E). No significant differences in Akt1 expression was noted in the BTBR model compared to WT (Figure 2F). Levels of phosphorylated synapsin 1 (Figure 2G), but not total synapsin 1 (Figure 2H) were significantly elevated in BTBR mice compared to WT. Expression of both synaptophysin (Figure 2I) and PSD95 (Figure 2J) was not affected in BTBR mice, while both spinophilin (Figure 2K) and NeuN (Figure 2L) levels were significantly elevated in BTBR mice compared to WT.

In the hippocampus we again found no significant differences in the levels of pro-BDNF between BTBR and WT (Figure 3B) that was coincident with a significant reduction in extant BDNF levels (Figure 3C). Phosphorylated TrkB expression was unchanged in BTBR mice compared to WT (Figure 3D), while there was a trend, similar to that in the cortex, to reductions of total TrkB receptor expression in the BTBR mice (Figure 3E). As with the cortex no significant alterations in Akt1 were observed (Figure 3F). Significant potentiation of both phosphorylated (Figure 3G) and non-phosphorylated synapsin 1 (Figure 3H) expression levels was seen in the BTBR hippocampus. In accordance with the cortical data we found no significant BTBR-induced changes in the hippocampal expression of synaptophysin (Figure 3I) or PSD95 (Figure 3J). Levels of both spinophilin (Figure 3K) and NeuN (Figure 3L) were again significantly potentiated in the BTBR mice, mirroring our previous cortical data.

SYSTEMIC ANALYSIS OF BTBR CENTRAL NERVOUS PROTEIN EXPRESSION

to gain an unbiased appreciation of the multiple tissue protein expression pattern changes generated in the BTBR mouse compared to WT we applied quantitative isobaric mass-tag labeling (iTRAQ) to cortical and hippocampal tissue extracts. In the cortex we identified and generated relative expression profiles for 674 proteins (Figure 4A: Table S1), while in the hippocampus 656 proteins were identified and quantified in BTBR mice (Figure 4B: Table S2). Log2-transformed iTRAQ expression ratios were employed to generate snake plot graphs of individual proteins. To initially validate some of the iTRAQ data we chose three random proteins, Rab3A, CaMKIIID and Cplx1 and found that at the western level of detection our results were comparable to our iTRAQ expression ratio data (Figures 4C,D).

GENE ONTOLOGY BIOINFORMATIC ANALYSIS OF BTBR-SPECIFIC ALTERED PROTEINS

Using a standard arbitrary cut-off of \( >1.2 \) (elevated in BTBR vs. WT) or \( <0.8 \) (reduced in BTBR vs. WT) for the iTRAQ
Table 3 | KEGG Pathway analysis for cortical proteins altered in BTBR mice compared to WT controls.

| KEGG pathway                        | C   | E   | O   | R   | P   | H   |
|-------------------------------------|-----|-----|-----|-----|-----|-----|
| Glutathione metabolism              | 50  | 2   | 0.07| 2738| 0.0188| 47.25356 |
| MTOR signaling pathway              | 52  | 2   | 0.08| 26.33| 0.0188| 45.44142 |
| Arginine and proline metabolism     | 54  | 2   | 0.08| 25.35| 0.0188| 43.7501  |
| Shigellosis                         | 61  | 2   | 0.09| 22.44| 0.0188| 38.7279  |
| Renal cell carcinoma                | 70  | 2   | 0.1 | 19.56| 0.0188| 33.75747 |
| Chronic myeloid leukemia            | 73  | 2   | 0.11| 18.75| 0.0188| 32.39564 |
| Progesterone-mediated oocyte maturation | 86  | 2   | 0.13| 15.92| 0.0188| 27.47541 |
| Gap junction                        | 90  | 2   | 0.13| 15.21| 0.0188| 26.25006 |
| Melanogenesis                       | 101 | 2   | 0.15| 13.56| 0.0192| 23.27844 |
| GnRH signaling pathway              | 101 | 2   | 0.15| 13.56| 0.0192| 23.27844 |
| Oocyte meiosis                      | 112 | 2   | 0.16| 12.22| 0.0192| 20.97806 |
| Leukocyte transendothelial migration | 116 | 2   | 0.17| 11.8 | 0.0192| 20.25705 |
| Vascular smooth muscle contraction  | 116 | 2   | 0.17| 11.8 | 0.0192| 20.25705 |
| Neurotrophin signaling pathway      | 127 | 2   | 0.19| 10.78| 0.0192| 18.50601 |
| Spliceosome                         | 127 | 2   | 0.19| 10.78| 0.0192| 18.50601 |
| Tight junction                      | 132 | 2   | 0.19| 10.37| 0.0192| 17.80217 |
| Hepatitis C                         | 134 | 2   | 0.2 | 10.22| 0.0192| 17.54466 |
| Natural killer cell mediated cytotoxicity | 136 | 2   | 0.2 | 10.07| 0.0192| 17.28716 |
| Insulin signaling pathway           | 138 | 2   | 0.2 | 9.92 | 0.0192| 17.02965 |
| Alzheimer’s disease                 | 167 | 2   | 0.24| 8.2  | 0.0261| 12.98355 |
| Chemokine signaling pathway         | 189 | 2   | 0.28| 7.24 | 0.0313| 10.89226 |
| Metabolic pathways                  | 1130| 6   | 1.65| 3.63 | 0.0188| 6.264807 |

REMARKS: KEGG signaling pathway annotation of the cortical proteins possessing BTBR:WT iTRAQ expression ratios < 1.2 or > 0.8 was created using WebGestalt. Each KEGG pathway significantly populated (p < 0.05) by at least two independent proteins are depicted. For each populated KEGG pathway the number of total proteins in the curated KEGG pathway (C), the number of observed experimental proteins in the KEGG pathway (O), the expected number of proteins in the pathway based on whole-genome background frequency (E), the KEGG pathway enrichment factor (R), the KEGG pathway enrichment probability (P) and the hybrid score (R* = log10 P) are indicated (H).

While GO annotation indicates some degree of functional commonality between groups of proteins, associating multiple proteins with classically-identified molecular signaling paradigms provides additional functional information regarding the potential physiological ramifications of specific group of differentially-regulated factors. We therefore performed both KEGG and Ingenuity-base pathway analysis upon our cortical and hippocampal BTBR protein sets. Inspecting the top ten highest scoring KEGG pathways from cortical proteins (Figure 5A: Table 3) a strong population of metabolic (Glutathione metabolism, Insulin signaling pathway, Metabolic pathways), neurotransmission/degenerative (Neurotrophic signaling pathway, Alzheimer’s disease) and ultrastructural (Gap junction, Tight junction, Spliceosome) signaling pathways was evident. Applying the same analysis to the hippocampus an intense population of signaling systems linked to oxidative metabolism [Oxidative phosphorylation, Pyruvate metabolism, Citrate cycle (TCA cycle)] and central neurodegeneration (Alzheimer’s disease, Huntington’s disease, Parkinson’s disease, Long-term potentiation) was clear (Figure 5B: Table 4). Extraction of potential physiological meaning from complex data sets is best performed using multiple informatics tools. Therefore, we also sought interpretation of the potential...
| KEGG pathway                                           | C   | E   | O   | R   | P         | H            |
|-------------------------------------------------------|-----|-----|-----|-----|-----------|--------------|
| Oxidative phosphorylation                             | 132 | 15  | 0.61 | 24.63 | 1.42E−15  | 365.6991     |
| Alzheimer’s disease                                   | 167 | 17  | 0.77 | 22.06 | 1.03E−16  | 352.6768     |
| Huntington’s disease                                  | 183 | 18  | 0.84 | 21.32 | 2.92E−17  | 352.518      |
| Parkinson’s disease                                   | 130 | 12  | 0.6  | 20   | 1.88E−11  | 214.5168     |
| Long-term potentiation                                 | 70  | 8   | 0.32 | 24.77 | 1.74E−08  | 192.2016     |
| Gastric acid secretion                                | 74  | 8   | 0.34 | 23.43 | 2.34E−08  | 178.7893     |
| Collecting duct acid secretion                        | 27  | 4   | 0.12 | 32.11 | 4.01E−05  | 141.183      |
| Pyruvate metabolism                                  | 40  | 5   | 0.18 | 27.09 | 7.57E−06  | 138.7253     |
| Metabolic pathways                                   | 1130| 37  | 5.21 | 7.1  | 5.38E−19  | 129.7114     |
| GnRH signaling pathway                                | 101 | 8   | 0.47 | 17.17 | 2.19E−07  | 114.3446     |
| Oocyte meiosis                                        | 112 | 8   | 0.52 | 15.48 | 4.04E−07  | 98.97322     |
| Calcium signaling pathway                             | 177 | 10  | 0.82 | 12.24 | 1.14E−07  | 84.98348     |
| Vasopressin-regulated water reabsorption              | 44  | 4   | 0.2  | 19.7  | 0.0002    | 72.86971     |
| Endocytosis                                           | 201 | 10  | 0.93 | 10.76 | 3.03E−07  | 70.27005     |
| Citrate cycle (TCA cycle)                             | 30  | 3   | 0.14 | 21.67 | 0.001     | 65.01        |
| Butanoate metabolism                                  | 30  | 3   | 0.14 | 21.67 | 0.001     | 65.01        |
| Propanoate metabolism                                 | 32  | 3   | 0.15 | 20.32 | 0.001     | 60.96        |
| Alanine, aspartate and glutamate metabolism           | 32  | 3   | 0.15 | 20.32 | 0.001     | 60.96        |
| Melanogenesis                                         | 101 | 6   | 0.47 | 12.87 | 4.15E−05  | 56.39572     |
| Vibrio cholerae infection                             | 54  | 4   | 0.25 | 16.05 | 0.0004    | 54.53694     |
| Shigellosis                                           | 61  | 4   | 0.28 | 14.21 | 0.0007    | 44.83116     |
| Wnt signaling pathway                                 | 150 | 7   | 0.69 | 10.11 | 4.01E−05  | 44.45221     |
| Salivary secretion                                    | 89  | 5   | 0.41 | 12.18 | 0.0003    | 42.90866     |
| Gap junction                                          | 90  | 5   | 0.42 | 12.04 | 0.0003    | 42.41546     |
| Beta-Alanine metabolism                               | 22  | 2   | 0.1  | 19.7  | 0.0071    | 42.33021     |
| Gloma                                                 | 65  | 4   | 0.3  | 13.34 | 0.0007    | 42.08639     |
| Neurotrophin signaling pathway                        | 127 | 6   | 0.59 | 10.24 | 0.0001    | 40.96        |
| Valine, leucine and isoleucine degradation            | 44  | 3   | 0.2  | 14.78 | 0.0023    | 38.99366     |
| Proteasome                                            | 44  | 3   | 0.2  | 14.78 | 0.0023    | 38.99366     |
| Epithelial cell signaling in Helicobacter pylori infection | 68 | 4   | 0.31 | 12.75 | 0.0009    | 38.83341     |
| PPAR signaling pathway                                | 70  | 4   | 0.32 | 12.38 | 0.0009    | 37.70648     |
| Long-term depression                                  | 70  | 4   | 0.32 | 12.38 | 0.0009    | 37.70648     |
| Bile secretion                                        | 71  | 4   | 0.33 | 12.21 | 0.0009    | 37.1887      |
| Cardiac muscle contraction                            | 77  | 4   | 0.36 | 11.26 | 0.0012    | 32.88842     |
| Phosphatidylinositol signaling system                 | 78  | 4   | 0.36 | 11.1  | 0.0012    | 32.4503      |
| Pentose phosphate pathway                             | 27  | 2   | 0.12 | 16.05 | 0.0103    | 31.89396     |
| Amyotrophic lateral sclerosis (ALS)                   | 53  | 3   | 0.24 | 12.27 | 0.0036    | 29.98417     |
| ErbB signaling pathway                                | 87  | 4   | 0.4  | 9.96  | 0.0016    | 2784696      |
| Inositol phosphate metabolism                         | 57  | 3   | 0.26 | 11.41 | 0.0043    | 2700212      |
| Regulation of actin cytoskeleton                      | 213 | 7   | 0.98 | 7.12  | 0.0003    | 25.0829      |
| Glycolysis/Gluconeogenesis                            | 65  | 3   | 0.3  | 10    | 0.0057    | 22.44125     |
| Prion diseases                                        | 35  | 2   | 0.16 | 12.38 | 0.0158    | 22.30063     |
| African trypanosomiasial                              | 35  | 2   | 0.16 | 12.38 | 0.0158    | 22.30063     |
| Pancreatic secretion                                  | 101 | 4   | 0.47 | 8.58  | 0.0026    | 22.17953     |
| Adipocytokine signaling pathway                       | 68  | 3   | 0.31 | 9.56  | 0.0064    | 20.97292     |
| Bacterial invasion of epithelial cells                | 70  | 3   | 0.32 | 9.29  | 0.0066    | 20.25644     |
| Renal cell carcinoma                                  | 70  | 3   | 0.32 | 9.29  | 0.0066    | 20.25644     |
| Vascular smooth muscle contraction                    | 116 | 4   | 0.54 | 7.47  | 0.0039    | 1799475      |
| Antigen processing and presentation                  | 76  | 3   | 0.35 | 8.55  | 0.0081    | 1788245      |
| Purine metabolism                                    | 162 | 5   | 0.75 | 6.69  | 0.0023    | 1765004      |
| Protein processing in endoplasmic reticulum           | 165 | 5   | 0.76 | 6.57  | 0.0023    | 1733345      |
| Lysosome                                              | 121 | 4   | 0.56 | 7.16  | 0.0044    | 16.87288     |

(Continued)
signaling pathways enriched in the BTBR tissues using canonical signaling pathway analysis (Ingenuity). Canonical signaling analysis of the cortex revealed a strong bias toward cytokine signaling (Leptin signaling in obesity, JAK family kinases in IL-6-type signaling), neurodegenerative disease related activity (Amyloid processing, CDK5 signaling, CNTF signaling) and energy metabolism (IGF-1 signaling) (Figure 5C; Table 5). Similar pathway processing of hippocampal data again revealed a strong prediction of metabolism-related activity (Oxidative phosphorylation, Mitochondrial dysfunction, G protein signaling mediated by T1bb), neurodegenerative activity (Huntington’s disease signaling) and interestingly, given the strong presentation of, Melanocyte development and pigmentation signaling in the cortex (Figure 5C), melatonin-related activity (Melatonin signaling) (Figure 5D; Table 6).

### BTBR-REGULATED COHERENT PROTEIN REGULATION PATTERNS

Along with our global analysis of protein set data from the two tissues we also investigated the predicted functional nature of the divergent expression polarity subsets of BTBR-specific proteins uniquely regulated in either the cortex or hippocampus. Using VennPlex (Figure 6A; Table 7) (Cai et al., 2013) we were able to identify multiple groups of coherently-controlled proteins across multiple CNS tissues. Using our previously developed informatics application, Textrous!, we generated physiological predictions extracted from multiple databases using latent semantic analysis (Chen et al., 2013). Using the collective processing capacity of Textrous!, which generates interactive hierarchical word-clouds, we found that the tissue-unique up or down regulated sets of proteins generated quite distinct functional signatures in the cortex or hippocampus. For example, the specifically upregulated proteins in the cortex were strongly focused into activity controlling excitatory amino acid synaptic activity (Figure S1A; Table S3), while the downregulated cortical only proteins demonstrated a strong link to a reduction of protein kinase activity (Figure S1B: Table S4). With respect to the hippocampus, the upregulated protein set appeared to be linked to increases in heat-shock factors and synaptic pathophysiology (Figure S2A: Table S5), while the downregulated hippocampal protein set suggested a reduction in oligodendrocyte generation and CNS myelination (Figure S2B: Table S6).

In addition to our global data set informatics interpretation we also sought to investigate the function of coherently-regulated groups of BTBR-associated proteins observed across multiple tissues. We therefore focused on the subset of proteins (12 upregulated; 4 downregulated: Table 7) coherently altered in a BTBR-specific manner in both the cortex and hippocampus (Figure 6B). To explore the potential functional connection between these select proteins we applied our novel informatic platform Textrous! (Chen et al., 2013). Textrous! allows the extraction of functional data, using latent semantic analysis (LSA), from even the smallest of datasets. Textrous! is a unique LSA tool as it possesses two forms of functional data output, i.e., collective and individual processing. Collective processing of the coherent BTBR protein set results in the creation of a hierarchical word-cloud revealing a strong and novel link between the BTBR ASD phenotype and N-WASP (neuronal Wiskott–Aldrich Syndrome protein) mediated activity (Figure 6C; Table 8). In addition there was a second, less strong demonstration of the importance of inherited genomic activity in ASD phenotypes. The individual processing mode in Textrous! also investigates the strongest individual protein-term relationships within a specific protein subset (Figure 6D).

![Figure 5C](image_url) Melanocyte development and pigmentation signaling

![Figure 6A](image_url) BTBR-regulated coherent protein regulation patterns

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Table 4 | Continued

| KEGG pathway                          | C | E | O | R   | P       | H       |
|---------------------------------------|---|---|---|-----|---------|---------|
| Spliceosome                           | 127| 4 | 0.59 | 6.83 | 0.005    | 15.71603|
| Chemokine signaling pathway           | 189| 5 | 0.87 | 5.73 | 0.0036   | 14.00239|
| Rheumatoid arthritis                  | 91 | 3 | 0.42 | 7.14 | 0.0127   | 13.53884|
| Fc gamma R-mediated phagocytosis      | 94 | 3 | 0.43 | 6.92 | 0.0136   | 12.91591|
| Taste transduction                    | 52 | 2 | 0.24 | 8.34 | 0.0313   | 12.54716|
| MAPK signaling pathway                | 268| 6 | 1.24 | 4.85 | 0.0032   | 12.10002|
| Arginine and proline metabolism      | 54 | 2 | 0.25 | 8.03 | 0.0325   | 11.94958|
| Pathogenic Escherichia coli infection | 56 | 2 | 0.26 | 7.74 | 0.0342   | 11.34664|
| Chagas disease (American trypanosomiasis) | 104| 3 | 0.48 | 6.25 | 0.017    | 11.05969|
| Amoebiasis                            | 106| 3 | 0.49 | 6.13 | 0.0177   | 10.73992|
| Toxoplasmosis                         | 132| 3 | 0.61 | 4.93 | 0.0309   | 7.444505 |
| Natural killer cell mediated cytotoxicity | 136| 3 | 0.63 | 4.78 | 0.0324   | 7.119595 |
| Phagosome                             | 153| 3 | 0.71 | 4.25 | 0.0416   | 5.868853 |

KEGG signaling pathway annotation of the hippocampal proteins possessing BTBR:WT iTRAQ expression ratios < 1.2 or > 0.8 was created using WebGestalt. Each KEGG pathway significantly populated (p < 0.05) by at least two independent proteins are depicted. For each populated KEGG pathway the number of total proteins in the curated KEGG pathway (C), the number of observed experimental proteins in the KEGG pathway (O), the expected number of proteins in the pathway based on whole-genome background frequency (E), the KEGG pathway enrichment factor (R), the KEGG pathway enrichment probability (P) and the hybrid score (R * −log10 P) are indicated (H).
Table 5 | Ingenuity Canonical Signaling Pathway analysis of proteins differentially regulated in the cortex of aged BTBR compared to WT controls.

| Canonical pathways | log(p-value) | Ratio | 10 x Hybrid |
|--------------------|-------------|-------|-------------|
| Leptin Signaling in Obesity | 3.97E-00 | 4.71E-02 | 1.86987 |
| Role of JAK family kinases in IL-6-type Cytokine Signaling | 2.52E-00 | 7.14E-02 | 1.79928 |
| Amyloid Processing | 3.20E-00 | 4.92E-02 | 1.5744 |
| Melanocyte Development and Pigmentation Signaling | 3.73E-00 | 4.21E-02 | 1.57033 |
| Gai Signaling | 3.16E-00 | 2.96E-02 | 0.93536 |
| Dopamine Receptor Signaling | 2.70E-00 | 3.12E-02 | 0.8424 |
| CDK5 Signaling | 2.52E-00 | 3.09E-02 | 0.77868 |
| α-Adrenergic Signaling | 2.52E-00 | 2.75E-02 | 0.693 |
| IGF-1 Signaling | 2.40E-00 | 2.80E-02 | 0.672 |
| CNTF Signaling | 1.90E-00 | 3.51E-02 | 0.6669 |
| Neurotrophic Pain Signaling In Dorsal Horn Neurons | 2.36E-00 | 2.75E-02 | 0.649 |
| G Beta Gamma Signaling | 2.50E-00 | 2.48E-02 | 0.62 |
| IL2 Signaling | 1.88E-00 | 3.28E-02 | 0.61664 |
| Thrombopoietin Signaling | 1.85E-00 | 3.12E-02 | 0.5772 |
| Renin-Angiotensin Signaling | 2.26E-00 | 2.38E-02 | 0.53788 |
| Gas Signaling | 2.22E-00 | 2.40E-02 | 0.5328 |
| Role of JAK1 and JAK3 in yc Cytokine Signaling | 1.75E-00 | 2.94E-02 | 0.5145 |
| GM-CSF Signaling | 1.74E-00 | 2.94E-02 | 0.51156 |
| Clathrin-mediated Endocytosis Signaling | 2.49E-00 | 2.02E-02 | 0.50298 |
| Regulation of Cellular Mechanics by Calpain Protease | 1.82E-00 | 2.74E-02 | 0.49868 |
| CREB Signaling in Neurons | 2.58E-00 | 1.93E-02 | 0.49794 |
| Glutamate Receptor Signaling | 1.79E-00 | 2.78E-02 | 0.49762 |
| Synaptic Long Term Potentiation | 2.14E-00 | 2.31E-02 | 0.49434 |
| Antiproliferative Role of Somatostatin Receptor 2 Interactions | 1.73E+00 | 2.78E-02 | 0.48094 |
| Agrin Interactions at Neuromuscular Junction | 1.68E+00 | 2.86E-02 | 0.48048 |
| JAK/Stat Signaling | 1.69E+00 | 2.82E-02 | 0.47658 |
| Corticotropic Releasing Hormone Signaling | 2.21E+00 | 2.07E-02 | 0.45747 |
| Neurotrophin/TRK Signaling | 1.68E+00 | 2.63E-02 | 0.44184 |
| P2Y Purinergic Receptor Signaling Pathway | 2.12E+00 | 2.08E-02 | 0.44096 |
| Renal Cell Carcinoma Signaling | 1.65E+00 | 2.53E-02 | 0.41745 |
| Insulin Receptor Signaling | 2.03E+00 | 2.01E-02 | 0.40803 |
| Melatonin Signaling | 1.65E+00 | 2.67E-02 | 0.40755 |
| FLT3 Signaling in Hematopoietic Progenitor Cells | 1.60E+00 | 2.53E-02 | 0.4048 |
| GNRH Signaling | 2.05E+00 | 1.96E-02 | 0.4018 |
| cAMP-mediated signaling | 2.23E+00 | 1.77E-02 | 0.39471 |
| Ephin B Signaling | 1.61E+00 | 2.44E-02 | 0.39284 |
| Prolactin Signaling | 1.62E+00 | 2.38E-02 | 0.38556 |
| BMP signaling pathway | 1.59E+00 | 2.33E-02 | 0.37047 |

(Continued)
### Table 6 | Ingenuity Canonical Signaling Pathway analysis of proteins differentially regulated in the hippocampus of aged BTBR compared to WT controls.

| Canonical pathways | −log(p-value) | Ratio | 10 x Hybrid |
|--------------------|---------------|-------|-------------|
| Oxidative phosphorylation | 1.27E + 01  | 1.25E–01 | 15.875 |
| Mitochondrial dysfunction | 1.33E + 01  | 8.37E–02  | 13.121 |
| GABA receptor signaling | 5.19E + 00  | 1.07E–01  | 5.5533 |
| Glutamate degradation III (via 4-aminobutyrate) | 3.02E + 00  | 1.67E–01  | 5.0434 |
| GM-CSF signaling | 4.44E + 00  | 8.82E–02  | 3.91608 |
| Huntington's disease signaling | 6.46E + 00  | 5.16E–02  | 3.3336 |
| G protein signaling mediated by Tubby | 3.47E + 00  | 9.09E–02  | 3.15423 |
| Melatonin signaling | 4.18E + 00  | 7.41E–02  | 3.09738 |
| RhoA signaling | 4.52E + 00  | 6.25E–02  | 2.82 |
| Lipid antigen presentation by CD1 | 2.68E + 00  | 1.00E–01  | 2.68 |
| Regulation of actin-based motility by rho | 3.77E + 00  | 6.52E–02  | 2.45804 |
| Role of NFAT in cardiac hypertrophy | 4.87E + 00  | 4.78E–02  | 2.32786 |
| CTAA4 Signaling in cytotoxic T lymphocytes | 3.63E + 00  | 6.25E–02  | 2.26875 |
| TCA Cycle II (Eukaryotic) | 2.90E + 00  | 7.50E–02  | 2.175 |
| Breast cancer regulation by statin | 4.65E + 00  | 4.67E–02  | 2.17155 |
| Chemokine signaling | 3.22E + 00  | 6.67E–02  | 2.14774 |
| Synaptic long term potentiation | 3.72E + 00  | 5.38E–02  | 2.0036 |
| Guanine and guanosine salvage I | 1.71E + 00  | 1.11E–01  | 1.8981 |
| CREB signaling in neurons | 4.21E + 00  | 4.35E–02  | 1.83135 |
| ß-alanine degradation II | 1.71E + 00  | 1.00E–01  | 1.71 |
| Axonal guidance signaling | 5.19E + 00  | 3.29E–02  | 1.70751 |
| GNRH signaling | 3.51E + 00  | 4.58E–02  | 1.60758 |
| Actin nucleation by ARP-WASP complex | 2.65E + 00  | 5.97E–02  | 1.58205 |
| 4-Aminobutyrate degradation I | 1.53E + 00  | 1.00E–01  | 1.53 |
| Superpathway of inositol phosphate compounds | 3.94E + 00  | 3.85E–02  | 1.5169 |
| Creatine-phosphate biosynthesis | 1.32E + 00  | 1.11E–01  | 1.4652 |
| Pyruvate fermentation to lactate | 1.32E + 00  | 1.11E–01  | 1.4652 |
| D-myo-inositol-5-phosphate metabolism | 3.32E + 00  | 4.32E–02  | 1.43424 |
| fMLP signaling in neutrophils | 3.13E + 00  | 4.55E–02  | 1.42415 |
| Methylglyoxal degradation I | 1.53E + 00  | 9.09E–02  | 1.39077 |
| Clathrin-mediated endocytosis signaling | 3.29E + 00  | 4.04E–02  | 1.32916 |
| Ephrin receptor signaling | 3.45E + 00  | 3.81E–02  | 1.31445 |
| Protein kinase A signaling | 3.95E + 00  | 3.18E–02  | 1.2561 |
| Thrombin signaling | 3.20E + 00  | 3.79E–02  | 1.2128 |
| 3-phosphoinositide biosynthesis | 3.10E + 00  | 3.87E–02  | 1.1997 |

(Continued)
DISCUSSION

In the present study we evaluated the behavior, synaptic protein alterations and targeted proteomic signatures in the hippocampus and cortex of 15 month old BTBR and WT mice to determine whether the ASD-like phenotype of BTBR mice was resistant to neuronal aging. Thus, we assessed both the behavioral phenotype and proteomic profiles between aged ASD-like mice (abnormal CNS) and aged wild type mice (with a relatively normal CNS). We found that several neurosynaptic proteins were significantly increased in both the hippocampus and cortex of the aged ASD-like mice in comparison to the aged wild type. In particular, the phosphorylated form of the pre-synaptic marker synapsin 1, and the post-synaptic marker spinophilin were increased in both the aged BTBR hippocampus and cortex; PSD95 was significantly elevated in aged BTBR hippocampus. Aged BTBR mice still presented a behavioral phenotype typically observed in young autistic mice, suggesting that the physiological actions underlying the ASD-like condition of the BTBR mice is relatively resistant to aging.

The maintenance of the ASD-phenotype into advanced age in BTBR mice is intriguing because in the normal developing CNS synapses are overproduced but then become pruned in response to neuronal activity during post-natal development (Petit, 1988; Cohen and Greenberg, 2008; Ebert and Greenberg, 2013). It is proposed that autism is caused by dysregulation of the machinery controlling synaptic messenger RNA translation leading to increased synthesis of synaptic proteins and lack of synaptic pruning, in turn inducing a hyperconnectivity and over-reinforcement of neuronal circuits. In fact it is one of the hallmark characteristics of ASD observed in the brains of both mouse models and humans with ASD (Kelleher and Bear, 2008; Gkogkas et al., 2013). Fragile X syndrome, for example which is the leading cause of inherited intellectual disability and ASD (Bhakar et al., 2012), is caused by mutations in FMR1 gene resulting in decreased expression of fragile X mental retardation protein (FMRP). This protein modulates the rate of translational elongation of mRNAs that encode synaptic proteins (Darnell et al., 2011). A decrease in FMRP impairs regulation of synaptic protein expression (Bhakar et al., 2012) and post-natal synapse elimination (Harlow et al., 2010).

As increased synaptic marker expression is associated with the autistic-like mouse phenotype it is not surprising that we found that the BTBR T-<sup>tf/j</sup> mice maintained their reduced social behavior into old age (Figure 1). This is consistent with the limited human literature that found that social impairments in individuals with ASD persist into adulthood (Ballaban-Gil et al., 1996; Szatmari, 1998; Howlin and Moss, 2012). Although increased synaptic markers have been found to be deleterious for the developing CNS, it is possible that the increased synapses and hyperconnected neuronal circuits be an effective advantage in the aging process where typically nervous system connectivity is degraded through accumulated damage. BDNF and TrkB mRNAs are localized in dendritic spines, thus age-related spine density decreases in older individuals suggest a link between BDNF and TrkB mRNA expression and impaired synaptic connections with aging (Tapia-Arancibia et al., 2008). Indeed decreases in both BDNF and TrkB expression have been documented in the hippocampus and in the neocortex during physiological aging (Phillips et al., 1991; Murray et al., 1994; Connor et al., 1997; Ferrer et al., 1999; Quartu et al., 1999; Hock et al., 2000; Holsinger et al., 2000; Fahnestock et al., 2002; Romanczyk et al., 2002; Tapia-Arancibia et al., 2008; Zhao et al., 2010).

We found that levels of mature BDNF were significantly decreased in the aged BTBR hippocampus and cortex compared with the aged WT (Figures 2A, 3A), suggesting an accelerated

Table 6 | Continued

| Canonical pathways | −log(p-value) | Ratio | 10 × Hybrid |
|--------------------|--------------|-------|-------------|
| Glutamate receptor signaling | 1.69E + 00 | 4.17E–02 | 0.70473 |
| G-Protein coupled receptor signaling | 2.38E + 00 | 2.90E–02 | 0.6902 |
| IL-1 signaling | 1.87E + 00 | 3.67E–02 | 0.68629 |
| Antiproliferative role of somatostatin receptor 2 | 1.60E + 00 | 4.17E–02 | 0.6672 |
| Remodeling of epithelial adherens junctions | 1.53E + 00 | 4.29E–02 | 0.65637 |
| JAK/stat signaling | 1.54E + 00 | 4.23E–02 | 0.65142 |
| Gloma signaling | 1.84E + 00 | 3.54E–02 | 0.65136 |
| Role of NFAT in regulation of the immune response | 2.15E + 00 | 3.00E–02 | 0.645 |
| Fatty acid β-oxidation I | 1.45E + 00 | 4.44E–02 | 0.6438 |
| RhoGDI signaling | 2.11E + 00 | 2.97E–02 | 0.62667 |
| Cardiac β-adrenergic signaling | 1.96E + 00 | 3.16E–02 | 0.61936 |
| Cardiac hypertrophy signaling | 2.15E + 00 | 2.80E–02 | 0.602 |
| Relaxin signaling | 1.93E + 00 | 3.05E–02 | 0.58865 |
| Renal cell carcinoma signaling | 1.50E + 00 | 3.80E–02 | 0.57 |
| Calcium signaling | 2.05E + 00 | 2.76E–02 | 0.5668 |
| Phospholipase C signaling | 2.06E + 00 | 2.64E–02 | 0.54384 |
| Gaq signaling | 1.80E + 00 | 2.92E–02 | 0.5256 |
| Gas signaling | 1.60E + 00 | 3.20E–02 | 0.512 |
| Molecular mechanisms of cancer | 2.15E + 00 | 2.32E–02 | 0.4988 |
| Role of tissue factor in cancer | 1.61E + 00 | 3.08E–02 | 0.49588 |
| Gap junction signaling | 1.71E + 00 | 2.76E–02 | 0.47196 |
| p70S6K signaling | 1.51E + 00 | 3.03E–02 | 0.45753 |
| Androgen signaling | 1.60E + 00 | 2.76E–02 | 0.4416 |
| Gai signaling | 1.48E + 00 | 2.96E–02 | 0.43808 |
| cAMP-mediated signaling | 1.65E + 00 | 2.65E–02 | 0.43725 |
| PPARα/RXα activation | 1.51E + 00 | 2.50E–02 | 0.3775 |
| Insulin receptor signaling | 1.38E + 00 | 2.68E–02 | 0.36984 |
| eNOS signaling | 1.39E + 00 | 2.58E–02 | 0.35862 |
| Glucocorticoid receptor signaling | 1.35E + 00 | 2.01E–02 | 0.27135 |

Canonical signaling pathway annotation of the hippocampal proteins possessing BTBR:WT iTRAQ expression ratios < 1.2 or > 0.8 was created using Ingenuity Pathway Analysis. Each signaling pathway significantly populated (p < 0.05) by at least two independent proteins are depicted. For each populated signaling pathway the −log<sub>10</sub> of the enrichment probability (p-value), the enrichment ratio and the calculated hybrid score [10 × (enrichment ratio − −log<sub>10</sub>(p-value))] are depicted.

transmembrane protein 2) and Cep290 (centrosomal protein 290) (Figure 6D).
aging phenomena, but did not find a significant change in the overall level of active TrkB receptor (Figures 2D, 3D), in the BTBR mice. In addition, we also found that there was a non-significant trend for the increase in the expression in both cortical and hippocampal levels of Akt1, one of the most important downstream targets of TrkB signaling. Thus, via a potentially complex compensatory mechanism, the BTBR mice may have increased their BDNF signaling efficiency and downstream signal transduction capacity compared to WT mice.

Our findings of reduced BDNF in both the hippocampus and cortex in old BTBR mice compared to old WT mice despite seemingly intact memory functioning may be related to this efficiency potentiation of the BDNF-TrkB system in the BTBR mice. These effects may be related to changes in endocytic proteins such as Itsn1, Golga2 and Ap3b2 (Table 7) that may affect the signaling dynamics of the TrkB receptor (Mattson et al., 2004). In addition, it is possible that the elevated levels of various synaptic markers served to protect the aging BTBR brain against age-related declines in cognition often associated with decreased levels of BDNF.

Given the complexity of the ASD, it is unlikely that BDNF signaling and various neuronal cell markers are the only protein products altered in the presence of ASD; thus we performed global quantitative iTRAQ proteomics, coupled to advanced bioinformatics in order to gain a comprehensive, systems-level understanding of potential changes in aged BTBR mice compared to WT counterparts. With respect to current relevant literature we discovered that many of these proteins differentially regulated in the brain of the old BTBR mice demonstrated strong correlations with those altered in humans with neurodevelopmental disabilities (Table S7). Nearly twenty percent of the coherently-regulated proteins across the hippocampus and cortex of aged BTBR mice

FIGURE 6 | Identification of coherent regulatory protein signatures in BTBR CNS tissues. Vennplex diagram analysis of the upregulated (BTBR:WT iTRAQ ratio > 1.2) or downregulated (iTRAQ ratio < 0.8) proteins in both the hippocampus and cortex are depicted (A). A small coherently-regulated protein subset across both tissues was evident and comprised a focused protein set strongly linked to human ASD-like conditions (B). Textrousy-based collective processing interrogation of the coherently-regulated BTBR protein set revealed, in the resultant hierarchical word-cloud, a strong potential role of skeletal modeling activity in this protein set (C). This skeletal signaling dependence of ASD was confirmed using the individual processing module of Textrousy! (D). In this heatmap output correlation strength between protein and functional term is indicated by the intensity of the teal-colored blocks.
also are strongly linked to human ASD phenotypes, suggesting that future investigation of the remaining factors may prove extremely fruitful for autism-related research (Figure 6B). With respect to the highly-focused, coherently-regulated protein set (Figure 6B: Table 7) Prrt2 was coherently altered in both the hippocampus and cortex of aged BTBR mice compared to aged WT (Table 7). Alterations in activity of Prrt2 have been found in the frontal cortex of individuals with autism (Ji et al., 2012) and have also been genetically-associated with ASD (Weber et al., 2013). Vat1l was up-regulated in both the hippocampus and cortex of the old BTBR mice.

We performed functional informatics data clustering to gain a wider appreciation of the signaling phenotype present in aged BTBR mice. Using GO term annotation it was evident in the cortex that considerable alterations in ER (endoplasmic reticulum), vesicular transport, cytoskeletal remodeling, and energy generation were apparent (Figures 4E,F). The functional GO term clustering of differentially expressed proteins in the BTBR hippocampus also indicated a role of energy generation, neurotransmitter release and synaptic activity but did not display a robust skeletal remodeling component (Table 2). We also employed KEGG and Ingenuity Canonical Signaling pathway analysis for the differentially-regulated cortex and hippocampal protein sets. At this higher level of functional interrogation (compared to GO term clustering) we also observed a subtle divergence in tissue behavior, i.e., cortical KEGG pathway analysis demonstrated a strong bias toward energy metabolic and neurotrophic/neuroprotective mechanisms (Figure 5A) while the hippocampus demonstrated a more degenerative phenotype associated with metabolic instabilities and calcium management factors (Figure 5B). Interestingly and to highlight the need for multidimensional informatics analyses for physiological interpretation, the canonical signaling pathway analysis of both tissue datasets revealed additional processes that could affect the ASD phenotype, i.e., the prominence of cytokine signaling activity in the cortex and the identification of the potential role of melatonin function in both CNS tissues (Figures 5C,D).

To further reinforce the importance of applying unbiased informatics for the analysis of complex data associated with a highly nuanced phenotype such as ASD we found that analysis of different subgroups of the data yielded interesting, tissue-specific insights into the effects of aging on ASD. Using our novel Textrous! platform we were able to identify differential tissue activity, i.e., enhanced glumatameric cortical activity coincident with attenuated kinase signaling (Figure S1). These cortical changes occurred contemporaneously with an elevation of hippocampal protein misfolding/aggregation coincident with an attenuated CNS myelination and oligodendrocyte functionality (Figure S2). Both of these hippocampal processes have been recently associated with dysfunctional brain development (Mitew et al., 2013).

When approaching complex issues such as ASD with a systems-biology approach it is important to maintain this concept of multiple levels of protein interaction, function and hierarchical architecture, i.e., functions can be tissue specific, expression polarity specific while also existing contemporaneously at a level above tissue- or polarity-dependence. For example we found that

### Table 7 | VennPlex Venn diagram analysis of up and downregulated proteins across the cortex and hippocampus of BTBR mice compared to WT controls.

| Protein | Cortex up | Cortex down | Hippo up | Hippo down |
|---------|-----------|-------------|----------|------------|
| PRRT2   | 1.951168338 | 2.381953722 |          |            |
| DYNLL1  | 1.672920918 | 1.737545789 |          |            |
| DBNL    | 1.626693344 | 1.24582423  |          |            |
| AP3B2   | 1.581785753 | 1.789312776 |          |            |
| VAT1L   | 1.391942674 | 1.401775589 |          |            |
| WDR33   | 1.379833997 | 2.510567518 |          |            |
| GOLGA2  | 1.292500223 | 1.60040989 |          |            |
| LANCL1  | 1.279121599 | 1.320074872 |          |            |
| ME3     | 1.270939623 | 1.62730661 |          |            |
| ITSN1   | 1.269638936 | 1.774926636 |          |            |
| CEP290  | 1.238621608 | 1.454207016 |          |            |
| PCYD1L1 | 1.209317219 | 1.860882083 |          |            |
| ERMN    | −1.243267187 | 1.500264003 |          |            |
| CSRP1   | −1.365689412 | 1.509867503 |          |            |
| DBI     | −1.496288514 | −2.056916039 |          |            |
| NUCB1   | −1.631205729 | −3.561025937 |          |            |
| EIF5A   | 1.517756095 | −1.65673365 |          |            |
| MM4A    | 1.391228352 | −1.887879989 |          |            |
| PTPN11  | 1.24745108  | −3.09376449 |          |            |
| ZKDB    | 1.214674609 | −1.393599577 |          |            |
| SLC17A7 | 1.204082729 | 1.315805498 |          |            |
| DPYSL4  | 1.195193712 | −1.314442062 |          |            |
| OAT     | −1.307905257 | 1.362629072 |          |            |
| Ptdc    | 1.828557315 |          |          |            |
| PCBP2   | 1.792502849 |          |          |            |
| QDPR    | 1.7625157   |          |          |            |
| SRSF4   | 1.533013648 |          |          |            |
| ADCY2   | 1.511246891 |          |          |            |
| NDUFS4  | 1.47998035  |          |          |            |
| SLC9A3R1| 1.473396618 |          |          |            |
| LETM1   | 1.455678341 |          |          |            |
| PRDX3   | 1.44243043  |          |          |            |
| IDH2    | 1.441010435 |          |          |            |
| EXOC5   | 1.41342868  |          |          |            |
| HNRRNPUL2| 1.405691244 |          |          |            |
| PTC1    | 1.37175507 |          |          |            |
| DDS5    | 1.365841661 |          |          |            |
| PPP1R9B | 1.346708939 |          |          |            |
| DPP6    | 1.340020376 |          |          |            |
| LAP3    | 1.303333413 |          |          |            |
| KCNAB2  | 1.296085587 |          |          |            |
| SLC12A5 | 1.252190507 |          |          |            |
| HOOK3   | 1.25003514  |          |          |            |
| COX5A   | 1.247002966 |          |          |            |
| WDR1    | 1.244912709 |          |          |            |
| GRM2    | 1.243617897 |          |          |            |
| CAB39   | 1.23402405 |          |          |            |
| RTN3    | 1.233971862 |          |          |            |
| TF      | 1.233243832 |          |          |            |
| FBXO41  | 1.231290747 |          |          |            |
| RAB7A   | 1.226744118 |          |          |            |
| RTN4    | 1.216538369 |          |          |            |
| TCP1    | 1.215498024 |          |          |            |

(Continued)
| Protein       | Cortex up | Cortex down | Hippo up | Hippo down |
|--------------|-----------|-------------|----------|------------|
| PRKAR2B      | 1.215235782 |             |          |            |
| CCT3         | 1.20748828 |             |          |            |
| PRDM5        | 1.198502382 |             |          |            |
| COQ9         |           | −1.24961385 |          |            |
| CLDN11       |           | −1.260614004 |         |            |
| NCALD        |           | −1.266374541 |         |            |
| F5           |           | −1.269955666 |         |            |
| EXOC6B       |           | −1.275380087 |         |            |
| CTN1         |           | −1.287911813 |         |            |
| VB1          |           | −1.361766666 |         |            |
| PICALM       |           | −1.466374966 |         |            |
| CAPNS1       |           | −1.756445493 |         |            |
| TOMM20       | −1.819905488 |           |          |            |
| MAPK3        | −1.918613356 |           |          |            |
| NUDC         |           | 1.732009523 |          |            |
| RAP2B        |           | 1.726355193 |          |            |
| KIF2A        |           | 1.663032474 |          |            |
| SETDB1       |           | 1.648568725 |          |            |
| ATP6V1D      |           | 1.640964877 |          |            |
| SYNR1        |           | 1.58532711 |          |            |
| DPYS3        |           | 1.582802831 |          |            |
| NCAN         |           | 1.571586568 |          |            |
| CTNND2       |           | 1.562506309 |          |            |
| CNRIP1       |           | 1.554254382 |          |            |
| UTP20        |           | 1.544855143 |          |            |
| ME1          |           | 1.478800627 |          |            |
| PFN1         |           | 1.477145399 |          |            |
| PPME1        |           | 1.45576369 |          |            |
| APP          |           | 1.455466641 |          |            |
| LANCL2       |           | 1.455132031 |          |            |
| GNAQ         |           | 1.453050806 |          |            |
| ARPC5L       |           | 1.451224208 |          |            |
| PRDX2        |           | 1.448929334 |          |            |
| CYFIP1       |           | 1.432347642 |          |            |
| HSPA2        |           | 1.431503207 |          |            |
| SFPQ         |           | 1.427372534 |          |            |
| TXLNG        |           | 1.42119969 |          |            |
| GRB2         |           | 1.4157442 |          |            |
| CPNE6        |           | 1.414165286 |          |            |
| PDXP         |           | 1.410098436 |          |            |
| GLOD4        |           | 1.391614359 |          |            |
| OX1          |           | 1.380069445 |          |            |
| VAMP2        |           | 1.373709575 |          |            |
| HSPA1A       |           | 1.373028035 |          |            |
| HSPA1B       |           | 1.373028035 |          |            |
| PSD3         |           | 1.371411227 |          |            |
| BSG          |           | 1.369172074 |          |            |
| PFN2         |           | 1.368469072 |          |            |
| ATP5J2       |           | 1.368321641 |          |            |
| GN1B         |           | 1.366417736 |          |            |
| DLG4         |           | 1.363981591 |          |            |
| SH3GLB2      |           | 1.36363306 |          |            |
| KTN1         |           | 1.362629072 |          |            |
| RAB3A        |           | 1.362173026 |          |            |

(Continued)
inspection of the coherently-regulated multi-tissue protein set revealed an exciting and potentially novel functional association of neuronal Wasp (Wiskott-Aldrich syndrome protein) activity and ASD (Figures 6C,D). To our current knowledge this is the first example of such a functional correlation, however it is relatively simple to predict that the molecular activities of this skeletal remodeling factor could impact a disorder such as ASD in which a less synaptically plastic neurological system is present.

In conclusion, we found that both the social behavioral phenotype and the CNS proteomic profile of this ASD model are relatively age-resistant. Aged BTBR mice possessed elevations in hippocampal and cortical synaptic markers that likely play a role in the etiology of the autistic-like phenotype. Low levels of BDNF have been found inconsistently in the ASD literature but more consistently in the aging and Alzheimer Disease literature (Chadwick et al., 2010b). Diminished mature BDNF in the aged BTBR hippocampus/cortex were seen along with complementary alterations in the downstream signaling pathways compared to WT mice. These findings in-part reinforce the potential long-term neuroprotective capacity of the ASD phenotype with respect to cognitive aging. It is therefore interesting to posit that despite lower levels of neurosynaptic plasticity that the more robust and reinforced neuronal network in ASD phenotypes may indeed act prophylactically over long periods of time compared to normal CNS networks that degrade due to unrepaired damage and synaptic loss. The complex proteotype of the

### Table 7 | Continued

| Protein | Cortex up | Cortex down | Hippo up | Hippo down |
|---------|-----------|-------------|----------|------------|
| DCLK1   | 1.207992461 |             |          |            |
| GPD1L   | 1.207286149 |             |          |            |
| HNRNPL  | 1.205847041 |             |          |            |
| NAPB    | 1.202833155 |             |          |            |
| RTN4    | 1.201798491 |             |          |            |
| PCSK1N  | 1.20147191  | 1.248474191 |          |            |
| WDR7    | 1.248967263 | 1.248958157 |          |            |
| MAG     | 1.25013637  | 1.256321905 |          |            |
| DDB1    | 1.26014124  | 1.265874043 |          |            |
| MOBP    |            | 1.268669041 |          |            |
| GAD2    | 1.2614124  | 1.270175029 |          |            |
| INA     | 1.27283353  | 1.274095426 |          |            |
| TPT1    | 1.280488507 | 1.281124693 |          |            |
| ID3G    | 1.283265073 | 1.284285221 |          |            |
| PCCB    | 1.28359839  | 1.284463003 |          |            |
| MA8RE2  | 1.295178624 | 1.297701354 |          |            |
| MRPS36  | 1.300775393 | 1.300938943 |          |            |
| MARCKS  | 1.30939843  | 1.30959086  |          |            |
| PSMD6   | 1.311237761 | 1.311237761 |          |            |
| ABAT    | 1.312784192 | 1.31355144  |          |            |
| NTM     | 1.316410553 | 1.316410553 |          |            |
| CA2     | 1.318123142 | 1.318123142 |          |            |
| HPCAL1  | 1.324474794 | 1.324474794 |          |            |
| MOG     | 1.330302869 | 1.330302869 |          |            |
| PSMA6   | 1.330302869 | 1.330302869 |          |            |
| SDHA    | 1.330302869 | 1.330302869 |          |            |
| COPB1   | 1.33093843  | 1.33093843  |          |            |
| NONO    | 1.33093843  | 1.33093843  |          |            |
| TUFM    | 1.33093843  | 1.33093843  |          |            |
| ADSS    | 1.33093843  | 1.33093843  |          |            |
| IGSF8   | 1.33093843  | 1.33093843  |          |            |
| COX5A   | 1.33093843  | 1.33093843  |          |            |
| UQCRFS1 | 1.33093843  | 1.33093843  |          |            |
| CKB     | 1.33093843  | 1.33093843  |          |            |
| PIP4K2B | 1.33093843  | 1.33093843  |          |            |
| NEFM    | 1.33093843  | 1.33093843  |          |            |
| PSMD6   | 1.33093843  | 1.33093843  |          |            |
| NEFL    | 1.33093843  | 1.33093843  |          |            |
| NDUFA8  | 1.33093843  | 1.33093843  |          |            |
| BIN1    | 1.33093843  | 1.33093843  |          |            |
| PTMS    | 1.33093843  | 1.33093843  |          |            |
| PHB2    | 1.33093843  | 1.33093843  |          |            |
| NDUFS2  | 1.33093843  | 1.33093843  |          |            |
| DSTN    | 1.33093843  | 1.33093843  |          |            |
| CCT5    | 1.33093843  | 1.33093843  |          |            |
| S100B   | 1.33093843  | 1.33093843  |          |            |
| CLASP2  | 1.33093843  | 1.33093843  |          |            |
| EPB41L1 | 1.33093843  | 1.33093843  |          |            |
| PFKL    | 1.33093843  | 1.33093843  |          |            |
| PSAP    | 1.33093843  | 1.33093843  |          |            |
| CPLX1   | 1.33093843  | 1.33093843  |          |            |
| SERPIN3K| 1.33093843  | 1.33093843  |          |            |
| AHSA1   | 1.33093843  | 1.33093843  |          |            |
| SET     | 1.33093843  | 1.33093843  |          |            |
| ATL1    | 1.33093843  | 1.33093843  |          |            |

For each specific protein studied fold change values, indicated in the table below, were generated from the iTRAQ expression ratios of (> 1.2 or < 0.8) proteins differentially regulated in the BTBR tissues [Cortex, Hippocampus (Hippo)] compared to WT.
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BTBR mice revealed molecular and functional signatures highly reminiscent of those found in humans with neurodevelopmental disabilities including ASD. Many of these identified processes play a role in neurodevelopment based on studies in murine models, and give more evidence for proteins that are playing a role

| Word                  | Cosine similarity | Z-score | p-Value |
|-----------------------|------------------|---------|---------|
| Motor                 | 0.52476314       | 1.869598279 | 0.030741909 |
| Nonsense              | 0.478414897      | 1.69975296 | 0.045132642 |
| ATPases               | 0.4700895        | 1.63196501 | 0.062853657 |
| Gtases                | 0.4626056051     | 1.56457791 | 0.064922494 |
| Exons                 | 0.466740356      | 1.649194014 | 0.049758798 |
| Growth-associated     | 0.455691506      | 1.608035438 | 0.059157898 |
| Inherited             | 0.455845768      | 1.607592194 | 0.059157898 |
| Gtase                 | 0.455708715      | 1.607066915 | 0.059157898 |
| Autosomal             | 0.453990019      | 1.600163824 | 0.056799292 |
| Polymerization        | 0.453672232      | 1.599294435 | 0.056799292 |
| Optic                 | 0.444664336      | 1.564905613 | 0.058791455 |
| EXN                   | 0.437675556      | 1.538225004 | 0.062024307 |
| GTP-binding           | 0.435679912      | 1.5306066 | 0.062846966 |
| Maps                  | 0.427384101      | 1.498930567 | 0.069930816 |
| Bundles               | 0.426675365      | 1.496230358 | 0.067328828 |
| Disabilities          | 0.423470554      | 1.483995579 | 0.06599044 |
| Gdi                   | 0.419776795      | 1.469703976 | 0.070780877 |
| Brothers              | 0.419347757      | 1.46980723 | 0.070916359 |
| Movement              | 0.417519897      | 1.461729797 | 0.072007721 |
| Acuity                | 0.417814185      | 1.45999644 | 0.072145037 |
| Cytoskeleton          | 0.414147875      | 1.448405032 | 0.073808252 |
| n-wasp                | 0.412888356      | 1.443634832 | 0.074369848 |
| Framework             | 0.411123648      | 1.436859653 | 0.075358997 |
| Recressive            | 0.409375594      | 1.430168227 | 0.076385361 |
| Wiskott-aldrich       | 0.407779814      | 1.424091426 | 0.077223236 |
| Actin                 | 0.406464694      | 1.419760659 | 0.077803841 |
| Wasp                  | 0.405802073      | 1.416543842 | 0.078214164 |
| Gtpase-activating     | 0.402282931      | 1.403109057 | 0.080308419 |
| Recycling             | 0.395770067      | 1.378245348 | 0.08141064 |
| Reorganization        | 0.391918169      | 1.363821581 | 0.082837336 |
| Prenylation           | 0.389963302      | 1.356096352 | 0.087549584 |
| Splice                | 0.388452273      | 1.350312529 | 0.08507991 |
| Missense              | 0.388195935      | 1.349330109 | 0.08668483 |
| Streaming             | 0.387060369      | 1.347842888 | 0.086829191 |
| Apparatus             | 0.3874916        | 1.346461219 | 0.08909916 |
| Pericentriolar        | 0.387010855      | 1.344805911 | 0.09312617 |
| Families              | 0.386042261      | 1.341106175 | 0.098960226 |
| Plexiform             | 0.385654365      | 1.339223953 | 0.098253363 |
| Polysomes             | 0.383798601      | 1.332524712 | 0.091265902 |
| Guanosine             | 0.383107191      | 1.329903161 | 0.097159136 |
| Splicing              | 0.382666221      | 1.328219703 | 0.092080525 |
| Cytoskeletal          | 0.381930528      | 1.325661608 | 0.092419849 |
| Mutational            | 0.381930511      | 1.325269803 | 0.092585576 |
| Coiled-coil gaps      | 0.381500118      | 1.323968383 | 0.092751522 |
| Depolymerizing        | 0.380444571      | 1.319738261 | 0.093417509 |
| Intellectual          | 0.378479462      | 1.312236202 | 0.094760067 |
| Mutations             | 0.378323187      | 1.311636902 | 0.094760067 |
| Protrusions           | 0.377198886      | 1.307351254 | 0.095603656 |
| Neurofibromatosis     | 0.376699229      | 1.305439931 | 0.095946424 |
| Actin-binding         | 0.375967678      | 1.302681498 | 0.096287381 |
| Atrophy               | 0.375377963      | 1.3003959 | 0.096800485 |
| Dynamics              | 0.373862886      | 1.29461182 | 0.097660115 |
| Pigmentary            | 0.373853033      | 1.294574204 | 0.097660115 |

Cosine similarity scores, Z-scores and probability values (p-Value) were calculated using collective processing of the 16 coherently-regulated BTBR-specific proteins in the cortex and hippocampal datasets.
in neuroprotection allowing for maintenance of cognition in the aged BTBR mice. Therefore, it is possible that the elevated levels of various synaptic markers and proteins identified proteomically such as Picalm, Itsn1, Mobp, Ina, as well as signaling actions involving Wasp functionality served to protect the aging BTBR brain against age-related declines in cognition often associated with decreased BDNF. In the current study we observed a robust maintenance of cognitive ability despite advanced age that may be due to unique neurosynaptic architecture and activity in the ASD phenotype. The role of the associated proteins and synaptic markers provide a foundation for further investigation into this intriguing mechanism.

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SUPPLEMENTARY MATERIAL
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