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Nuclear receptor binding factor 2 (NRBF2) is required for learning and memory

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Abstract

The mechanisms which underlie defects in learning and memory are a major area of focus with the increasing incidence of Alzheimer's disease in the aging population. The complex genetically-controlled, age-, and environmentally-dependent onset and progression of the cognitive deficits and neuronal pathology call for better understanding of the fundamental biology of the nervous system function. In this study, we focus on nuclear receptor binding factor-2 (NRBF2) which modulates the transcriptional activities of retinoic acid receptor α and retinoid X receptor α , and the autophagic activities of the BECN1–VPS34 complex. Since both transcriptional regulation and autophagic function are important in supporting neuronal function, we hypothesized that NRBF2 deficiency may lead to cognitive deficits. To test this, we developed a new mouse model with nervous system results in profound learning and memory deficits. Interestingly, we did not find deficits in autophagic flux in primary neurons and the autophagy deficits were minimal in the brain. In contrast, RNAseq analyses have identified altered expression of genes that have been shown to impact neuronal function. The observation that NRBF2 is involved in learning and memory suggests a new mechanism regulating cognition involving the role of this protein in regulating networks related to the function of retinoic acid receptors, protein folding, and quality control.

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Introduction

Memory loss marks the onset of mild cognitive impairment before the occurrence of dementia in Alzheimer's disease. Understanding the mechanism of learning and memory loss is important both in the neurobiology of cognition and in the biology of neurodegenerative disease. It is then critical to identify potential novel regulators of learning memory and in this study, the focus was the nuclear receptor binding factor-2 (NRBF2) which we hypothesized could be an important modulator of protein quality control. In support of a role for NRBF2 in neuronal quality control it has been shown to bind to and modulate the autophagic activities of the BECN1–VPS34 complex [1-4]. Autophagy is a highly regulated cellular process, integrating signals ranging from nutrient availability to cellular stress and as we have recently proposed is a critical component of the antioxidant defenses of the cell [5]. Regulation of autophagy is especially important for post-mitotic neurons since these cells survive lengthy periods of time without cell division which prevents the dilution of accumulated toxic materials in

proliferating cells. Furthermore, protein and organelle turnover is important for the quality control of intracellular functional components which can be damaged during normal electrophysiological and metabolic processes or environmental toxins and oxidative stress. It is then important to understand the mechanisms through which autophagy, as the key regulator of protein quality control, is controlled in neuronal cells. In addition, NRBF2 has been shown to modulate the transcriptional activities of retinoic acid receptor α (RAR α) and retinoid X receptor α (RXR α) [6, 7]. It is then possible that NRBF2 could impact learning and memory through several mechanisms.

An important site for control of autophagic regulation is the autophagosomal membranes which contain phosphatidylinositol 3-phosphate (PI3P). This signaling molecule is produced by the kinase activity of class III PI3K, VPS34, through phosphorylating phosphatidylinositol. The association of BECN1 with VPS34 plays an important role in the nucleation of autophagosomes and can be regulated by transcription, post-translational modification, as well as interactions with regulatory proteins [8-10]. Beclin has been shown to interact with a number of proteins and plays a role in autophagy and cell survival [8–11]. Furthermore, both BECN1 and VPS34 knockout mice exhibit postnatal lethality or severe neuron loss in conditional knockouts, with mechanisms involving both autophagy deficits and endosomal trafficking [11-13]. We and others have previously demonstrated an essential role for VPS34 in PI3P production, endocytic and autophagic degradation, in diverse organs [12–20]. Recent studies using an autophagy interaction network based on proteomic analyses, confirmed by either Beclin or VPS34 pulldown assays, identified a novel BECN1/VPS34 regulator, NRBF2 [1–4]. The role the BECN1-VPS34 complex and potentially NRBF2 plays in regulating learning and memory is unclear but is plausible since BECN1 is decreased in Alzheimer's disease brains and in particular microglia [21]. Furthermore, BECN1 heterozygous mice exhibited an exacerbated Alzheimer's disease pathology in mouse models [22]. Further connecting the VPS34-BECN1 complex to cognition, it has been shown that knockout of the WDR45/WIPI4 protein, an effector of VPS34, in the nervous system, resulted in greatly impaired learning and memory [23].

In addition to interacting with the BECN–VPS34 complex, NRBF2 can also interact with RAR α and RXR α [6, 7]. This is also an important alternative pathway regulating learning and memory, as evidenced by the observation that RAR/RXR deficiency causes learning and memory deficits in mouse models, and that retinoids or receptor agonists alleviate age-or disease-related memory deficits [24–30]. RAR/RXR receptors are present in mammalian nervous systems [31, 32], with levels decreasing with age [33, 34]. The blood levels of vitamin A and its derivatives in humans are also age and gender-dependent [35], and their deficiency in adult rodents results in learning and memory impairment [36–38].

These findings led us to the interesting hypothesis that NRBF2 plays a role in regulating learning and memory either through its effects of the autophagy process or the RAR/RXR system. NRBF2 whole body knockout mice can survive up to 12 months of age, while exhibiting smaller body weight at 4 months of age, and liver necrosis at 10 months [4]. These phenotypes potentially complicate a learning/memory study, especially at the age of 10 months or above. To overcome these challenges, we used a Nestincre:Nrbf2^{loxP/loxP} strategy to engineer a knockout NRBF2 in the nervous system. These mice were viable with normal body weight and without obvious pathology. With young mice (6 months of age) we observed normal learning ability of the Nestin-cre:Nrbf2^{loxP/loxP} mice in the Morris water maze hidden platform test, but impaired performance in probe trials. The learning deficits became strikingly profound in the hidden platform test at 12 months of age, consistent with an age-dependent effect, while appearance, body weight, and performances in open field and zero maze tests remain normal. The involvement of NRBF2 in learning and memory supports an important regulatory role for this protein with possible contributory mechanisms from autophagy and the RAR/RXR system.

Materials and methods

Generation of brain-specific NRBF2-deficient mice

The mouse Nrbf2 gene is located on chromosome 10qB5.1 and contains four exons. The conditional deletion strategy for Nrbf2 gene was based on the deletion of 1924 bp sequence in exon 1. The Nrbf2 recombined locus was inserted with two loxP sites that flank exon 1 along with neomycin-resistant positive selection cassette flanked by flippase recognition target (FRT) sites. The diphtheria toxin A (DT-A) gene was used as a negative selection against random integration of the vector and inserted into downstream of the short arm. Homologous recombinant embryonic stem cells of C57BL/6 mouse origin were injected into C57BL/6J-Tyrc-2J/J blastocysts, and chimeric male mice were crossed with C57BL/6 Flp deleter female mice to excise the neomycin selection cassette. The resulting excised F1 mice were mated with C57BL/6 to produce *Nrbf*2^{f/+} mice. The progeny was again mated with Nestin-Cre transgenic mice expressing Cre recombinase that conditionally delete exon 1 of Nrbf2 gene in Nestin positive cells and produce Nrbf2^{f/f}:Nestin-Cre mice (Fig. 1a). The following primers were used to detect wild-type Nrbf2 (a) and $Nrbf2^{f}$ (c) alleles:

Fig. 1 Generation of conditional Nrbf2 KO mice. a Genome structures of Nrbf2 endogenous locus and Nrbf2^{f/f}: Nestin-Cre mice. (a) Nrbf2 endogenous locus. (b) Nrbf2 recombined locus with the insertion of neomycin selection cassette flanked by FRT and loxP sites. (c) Flp-mediated excision by breeding between recombined animals and ubiquitous Flp-recombinase expressing deleter mice enabled the deletion of FRT-flanked region. (d) Cre-mediated excision by breeding between recombined animals and Nestin-Cre transgenic mice, which enables the deletion of loxPflanked region in Nestin-Cre expressing tissues. b Nrbf2 deficient mice (KO) exhibit diminished NRBF2 protein by western blot analyses compared to wildtype mice (WT) (postnatal day 25, n = 4-5 mice per genotype). Ctx: cortex, Cb: cerebellum, Str: striatum, MB: midbrain, HC: hippocampus. c NRBF2 protein levels appear normal in liver and heart in Nrbf2 deficient mice (KO) by western blot analyses compared to wildtype mice (WT) (postnatal day 25, n = 3 mice per genotype). Western blots of β-actin or GAPDH were used as loading controls.



nrbf1: 5'-CTCTCAATCCCTCCGCATCATCG-3'; nrbf2: 5'-GGTGCCTTTGCTTTAAGGCTCACG-3'.

cre1: 5'-TCGCGATTATCTTCTATATCTTCAG-3'; cre2: 5'-GCTCGACCAGTTTAGTTACCC-3'.

We have used n = 6-15 for behavioral studies from wildtype and NRBF2 deficient mice for each age group. All mouse experiments were done in compliance with the University of Alabama at Birmingham Institutional Animal Care and Use Committee guidelines.

Western blot analysis

Different regions of the mouse brain including cortex, cerebellum, striatum, midbrain, and hippocampus, as well as heart and liver, were collected for western blot analysis. Briefly, brains were dissected and homogenized in 1.5 ml centrifuge tubes in cell lysis buffer (50 mM Tris, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 1% Triton X-100, pH 7.8) containing protease cocktail inhibitors (Roche). After 15 min on ice, samples were centrifuged at 1.3×10^4 rpm for 15 min. The supernatant was transferred into a fresh tube and pellet (Triton-X-100 insoluble fractions) were resuspended in 2% sodium dodecyl sulfate (SDS) in phosphate-buffered saline. Both supernatant and pellet fractions were used for bicinchoninic acid assay to determine protein quantification and 10-20 µg protein per lane were separated on a 12% SDS-polyacrylamide gel electrophoresis, then transferred to polyvinylidene difluoride membranes (Fisher Scientific, PI88520). Antibodies used were anti-NRBF2 (ProteinTech, 24858), anti-Synaptophysin (Cell Signaling, 5461), anti-PSD95 (Cell Signaling, 3450), anti-LC3 (Sigma, L8918), anti-p62 (Abnova, H00008878), anti-LAMP1 (Abcam, ab25245), anti-p-mTOR (Cell Signaling, 2971s), anti-mTOR (Santa Cruz, SC8319), anti-p-AKT (Cell Signaling, 9271), anti-AKT (Cell Signaling, 9272), anti-HSC70 (Abcam, Ab19136), anti-VPS34 (Sigma, V9764), and anti-BECN1 (Santa Cruz, sc11427). Anti- β -actin (Sigma, A5441) was used as a loading control. ECL reagent (Fisher Scientific, PIA34075), and ImageQuantTL (GE Healthcare) software were used to analyze western blot results.

Immunohistochemistry

Brains were placed in 10% buffered formalin (Fisher Scientific) overnight at 4 °C followed by paraffin embedding. Five-micrometer-thick sections were used for hematoxylin and eosin (H&E) and immunostaining. Both chromogenic and fluorescence immunohistochemistry were performed. Sections were treated with citrate buffer 0.01 M for 30 min at 90 °C. After antigen retrieval sections were blocked with 10% fetal bovine serum and 1% bovine serum albumin. The following primary antibodies were used: anti-GFAP (Invitrogen, 131-17719, 13-0300), anti-IBA1 (Abcam, ab5076), anti-NPAS4 (Invitrogen, MA5-27592), anti-STRA6 (Proteintech, 22001-1-AP), anti-STEAP2 (Invitrogen, PA5-20405), anti-HSPA5 (Invitrogen, PA5-34941), anti-SLC24A5 (Proteintech, 27747-1-AP), anti-EPS8L2 (Proteintech, 20461-1-AP), anti-LC3 (Sigma, L8918), anti-p62 (Abnova, H00008878), anti-VPS34 (Sigma, V9764), anti-Synaptophysin (Cell Signaling, 5461), and anti-BECN1 (Santa Cruz, SC11427), anti-PSD95 (Cell Signaling, 3450), and anti-Transferrin (Abcam, ab84036). The second antibodies for chromogenic DAB staining were: OneStep polymer HRP anti-rabbit (GeneTex GTX83399) or Immpress-HRP Horse Anti-Mouse TgG polymer Reagent (30027: Lot: ZF0604). The second antibodies for immunofluorescence staining were Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Cyanine3 (Invitrogen Cat # A10520) or Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 568 (Invitrogen, A11004). DAB (3,3'-diaminobenzidine) kit (Vector SK-4100) was used in chromogenic stains. Nuclear counterstain was done by hematoxylin (Vector LS-J1042) and slides were mounted in Permount (Fisher, P36971). All immunofluorescence slides were nuclear counterstained and mounted using Invitrogen Prolong Diamond Antifade Mountant (Fisher, P36971). Keyence BZ-X microscope was used for capturing the immunostaining images.

TUNEL assay

Apoptotic cells were detected by TUNEL assay using an *in situ* cell death detection kit (Roche Diagnostic, 1176291910). Briefly, sections were subjected to antigen retrieval and then incubated with TUNEL assay mixture for 60 min at 37 $^{\circ}$ C in a humidified atmosphere in the dark.

The method was tested in pathological sections where prior studies have detected TUNEL positive cells. TdT enzyme was omitted for negative controls.

Primary neuron cultures

Primary cortical neurons were cultured from P0 mouse pups. The mice were decapitated and brain was rapidly removed and dissections were performed immediately in ice-cold Hanks' balanced sodium salts (without Ca2+ and Mg^{2+}). After dissection cerebral cortices were chopped into small pieces and collected in a 15 ml Falcon tube containing HBSS. The tissues were further incubated for 30 min at 37 °C with papain (Worthington), then mechanically dissociated with a fire-polished Pasteur pipette. Cells were pelleted by centrifugation at 25 °C for 5 min at 1000 $\times g$ and resuspended in Neurobasal medium containing 2% B27 supplement (Invitrogen), 1% Pen-Strep (10,000 U/ml, 10,000 µg/ml) and 0.5 mM L-glutamine. Cells were then plated in 24-well or 6-well plates coated with 0.1 mg/ml poly-L-lysine (Sigma). The cultures were kept in a humid incubator (5% CO₂, 37 °C).

Autophagic flux assay

For autophagic flux analysis, primary neurons were grown from $Nrbf2^{t/f}$ Cre- and $Nrbf2^{t/f}$ Cre+ pups, and at day 7 in vitro (DIV7) treated with and without 40 µM chloroquine (CQ) for 4 h, which blocks autophagy completion. Lysate was prepared from cells with and without CQ treatment, and western blot analysis for LC3 and p62 was performed.

Synaptosomal bioenergetics

Synaptosomes were prepared using the Percoll gradient method [39, 40]. The band between 10 and 23% Percoll represents synaptosomes. Fifteen micrograms of synaptosomes were aliquoted into glass-bottomed 96-well plates coated with polyethyleneimine and centrifuged at 3400g for 1 h at 4 °C, incubated with 100 nM MitoTracker for 30 min, and imaged. At this resolution, there was no difference in synaptosomal mitochondria amount between the two genotypes. Then 10 µg of synaptosomes were aliquoted into XF96 plates coated with polyethyleneimine and centrifuged at 3400g for 1 h at 4 °C. After resuspension with XF medium, the mitochondrial stress test was performed. Oxygen consumption rate (OCR) was measured at basal level and after sequential injections of Oligomycin (4 µg/ml), FCCP (2 µM), and antimycin A (10 µM). Basal (before oligomycin minus after antimycin), ATP-linked (before oligomycin minus after oligomycin), proton leak (after oligomycin minus after antimycin), maximal (after FCCP minus after antimycin), reserve capacity (maximal minus basal), and non-mitochondria (after antimycin) OCR were calculated as described previously [41–47].

In addition to mitochondrial stress test with endogenous nutrients, we also measured the function of individual mitochondrial complexes after permeabilization of the plasma membrane with PMP (Seahorse XF) bioscience proprietary reagents and providing specific substrates for each complex [48]. Oxidation of complex I linked substrates was measured by concurrent injection of PMP, pyruvate, malate, and uncoupler FCCP. This was followed by an injection of rotenone to inhibit complex I, and the addition of succinate (complex II substrate) to measure complex II substrate linked respiratory activities.

Complex IV substrate linked activities were determined in similar fashion by the addition of PMP, ascorbate, and tetramethyl-p-phenylenediamine (TMPD) with ADP. Rotenone was added to inhibit the possible reverse electron flow through complex I. Then the addition of azide was used to inhibit cytochrome c oxidase.

Behavioral tests

For the open field test [49, 50], data were recorded and analyzed using the Ethovision software. The mouse's movements were tracked with regard to total distance traveled (cm), position inside the field (time in center versus against the wall in seconds), and mean velocity (cm/s).

For Zero Maze test, we used a circular maze (70 cm diameter) that was raised 40 cm above the table. The maze was divided into four equal parts, including two open areas and two closed areas. Mice were put at a starting point, and monitored for 4 min with an Ethovision camera-driven tracker system. The system recorded the position of the animal in the arena at 5 frames/s, and the time spent in each area was analyzed. Shorter time in the open area indicates increased anxiety.

A 3-day rotarod test [49] was performed in which the mice were placed on a rotating rod (San Diego Instruments) that gradually accelerated from 4 to 40 rpm over a 5 min period [51]. On each day mice were tested for three rounds at 1 h apart. The latency to fall was recorded in seconds for each trial and the average latency to fall was calculated for each mouse on each day. A decrease of latency suggests a decrease of motor coordination.

A water maze test was performed as described in detail before [49, 52, 53]. Briefly, we used a blue plastic pool with 120 cm in diameter, and a see-through round platform which is 10 cm in diameter and located 0.5 cm below the water surface. During day 1 through day 5 of the testing period, the mice were trained to find a hidden platform that was kept in a constant position throughout these 5 days. Four trials a day were run, and all starting positions in the four quadrants were equally used (in random order). The mice were given 60 s to find the platform and 10 s to stay on the platform. The inter-trial interval was approximately 2 min. Learning of the task was evaluated by recording the swimming speed, latency to find the platform, path length, and percentage of trials with which each animal found the platform. After the end of the four trials on day 5, the mice were tested in a 60 s probe trial (i.e., trial 21), i.e., with no escape platform present. Mice that had learned the platform position would predominantly search in the "correct" quadrant of the pool in the probe trial.

For a novel placement recognition test, mice were placed in a $40 \times 40 \times 60$ cm³ ($l \times w \times h$) black Plexiglas box for 10 min to acclimate to the environment. The next day animals were placed in the same box with two identical objects for 3 min then returned to their cages. After a 2 h interval, the animals were returned to the box for a 3 min exposure to the same two objects from training but one object was placed in a new location. Exploratory behaviors consisted of the mouse's nose coming in direct contact or within 1 cm of the object. Animals were excluded if they did not have at least 5 s of exploratory behavior or 1 s total exploration of each object.

For fear conditioning test, prior to the procedure, each animal was put in the conditioning chamber with a set of environmental cues for 2 min for habituation. Then three electrical foot shocks with paired cue were delivered. They remained in the chamber for an additional 30 s. Contextual memory was assessed 24 h later by re-exposing each animal to the same chamber for 5 min for evaluating freezing behavior. Cued test was assessed after a contextual memory test with a changed environment but with cues to determine amygdala dependent learning and memory. Percent freezing was calculated using Video Freeze Version 2.1.0 (Med Associates Inc.).

For examination of food intake, physical activity, and energy expenditure, over the course of the day, we used a comprehensive laboratory animal monitoring system (CLAMS). Mice were housed singly in CLAMS cages, for 1 week before measurements were made over 3 consecutive days in a 12:12 light:dark cycle; temperature was also maintained within the CLAMS. The system simultaneously measured food consumption, physical activity (movement through infra-red beams), and energy expenditure (through indirect calorimetry). The plastic flooring of the CLAMS cage has holes, allowing separation of feces and urine from the mouse (thereby ensuring accurate food intake assessments) [54–56].

Statistical analyses

All protein and mRNA results were normalized to internal control and then summarized as mean \pm SEM; and the differences of means between wildtype and knockout mice

Fig. 2 Nrbf2 deficient mice (KO) exhibit a modest but significant increase of P62. BECN, and VPS34 by western blot analyses compared to wildtype mice (WT). Mice were all at 6 months of age, protein extractions were from the dissected hippocampus (n = 3)mice per genotype). a Western blots and quantification of NRBF2 and p62. b Western blots and quantification of BECN and VPS34. All from n = 3 mice each genotype. $Data = mean \pm SEM$ normalized to control, Student *t*-test, *p <0.05.



were evaluated with Student *t*-test or Wilcoxon rank-sum test. All the outcomes from behavior studies were summarized as mean \pm SEM. The *t*-tests were carried out to compare the genotype difference of the outcomes at each time point. For repeated measures, a linear mixed-effect regression model was conducted to evaluate the genotype difference over time. Results with a *p*-value of less than 0.05 were considered statistically significant. Experiments had a minimum of n = 3 mice for each group except a higher number of mice (n = 5-7 for open field, zero maze, rotarod, watermaze, and novel place recognition tests, n =15 for fear condition) were used for behavioral studies.

RNAseq and data analyses

Hippocampi were dissected from five WT and five knockout (KO) male mice of 6 months of age. Preparation of total RNA and polyA sequencing library, RNAseq, and initial analyses were performed at the HudsonAlpha Institute for Biotechnology. Quality control of sequencing library included size verification and bioanalysis and quantitation by real-time PCR. NovaSeq S1 5-PE per lane yields 750 M reads, 75 Gb will yield ~50 M reads per sample. Transcriptome alignment used Edico's Dragen System and the latest version of the mouse reference genome (mm10). For each sample, FeatureCounts was performed to get separate lists of counts for known genes and transcripts. To search for new transcripts, StringTie was performed and FPKM and TPM counts obtained. Further analysis was done using Linear Models for Microarray Analysis, and conserved transcription factor binding sites. Functional enrichment was done using the ToppFun application of the ToppGene Suite [57]. The *p*-value (FDR Benjamini–Hochberg corrected) was set at 0.05.

Quantitative real-time PCR analyses

RNA was prepared from the hippocampus using Trizol (Invitrogen). $0.5-2 \mu g$ of RNA was used to synthesize cDNA using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Quantitative real-time PCR was performed with SYBR Green Mastermix (Thermo Fisher Scientific) with the following conditions: 95 °C, 5 min; (95 °C, 10 s; 60 °C, 10 s; 72 °C, 15 s) × 40 cycles. Results were normalized against an internal control (β -actin). Primers used are included in Supplementary Table 1.

Fig. 3 Nrbf2 KO mice do not exhibit impairment of motor coordination on the rotarod. All behaviors were performed with male mice. a, c 12 months of age (n = 5-7 each genotype). **b**, **d** 6 months of age (n = 5-7)each genotype). We found that the latency to fall, on the Rotarod test (49) with 4-40 rpm acceleration over 5 min, was not changed in 12 months old KO mice on all 3 days (p > 0.05)compared to WT (a), while decreased on the first day in 6 months old KO mice compared to WT mice, *p < 0.05(b). c, d Total time on rod over the 3 testing days for 12 months old and 6 months old WT and KO mice also was of no statical difference.



Results

Generation of brain-specific conditional *Nrbf2* deficient mice

We used the LoxP/Cre approach to generate conditional Nrbf2 deficient mice. We inserted LoxP sites by homologous recombination to flank exon 1 of mouse Nrbf2 gene in C57BL/6 embryonic stem cells (Fig. 1a) and injected into C57BL/6J-Tyrc-2J/J blastocysts. Breeding of chimeric mice with C57BL/6 Flp deleter mice resulted in an excised neomycin selection cassette. Germline transmitted Nrbf2^{f/+} mice were bred with Nestin-Cre transgenic mice to generate *Nrbf2* nervous system-specific knockout (*Nrbf2^{f/f}*:Nestin-Cre+) mice as evidenced by significantly decreased NRBF2 protein in wide areas of the brain (Fig. 1b). Western blot analyses of NRBF2 in the off-target tissues of liver and heart demonstrated that the levels of these proteins were not changed (Fig. 1c). The Nrbf2^{f/f}:Nestin-Cre+ mice breed normally and have 5-8 pups per litter, continuously till 1.5 years of age, without apparent early mortality. The overall gross anatomy of the brain of the NRBF2 knockout mice was normal as assessed by H&E stained sagittal sections (Supplementary Fig. 1A-D).

We assessed LC3 and P62 levels in the hippocampus of 6 months old wildtype (WT) and NRBF2 knockout (KO) mice by western blot analyses. Results show that the levels of p62, BECN, and VPS34 were slightly elevated (Fig. 2), while the levels of LC3II, p-mTOR, total mTOR, p-AKT, total AKT, LAMP1, and HSP-70 were not significantly changed in KO mice (Supplementary Fig. 2A–E). There was a slight increase of LC3II/I (Supplementary Fig. 2A). Levels of the post-synaptic protein PSD95 was unchanged, while there was a modest increase in the pre-synaptic protein synaptophysin as determined by western blot analyses (Supplementary Fig. 3). Semi-quantitative immunohistochemistry analyses did not reveal remarkable changes in the levels of the post-synaptic protein PSD95, pre-synaptic protein synaptophysin, or LC3 in the hippocampus (Supplementary Fig. 4A–C). Autophagic flux was unchanged in primary cortical neurons in KO cells compared to control (Supplementary Fig. 5). Synaptosomal bioenergetics are normal at postnatal day 25 (Supplementary Fig. 6).

Neurological deficits in NRBF2 deficient mouse brain

To assess the neurological consequence of NRBF2 knockout (KO) in the nervous system, we performed behavior studies with open field and zero maze tests to assess spontaneous movement and anxiety (Supplementary Fig. 7A–D), mouse feeding, physical activity, and energy expenditures to assess whole body metabolism (Supplementary Fig. 7F), rotarod tests to assess motor coordination, Morris water maze, novel place recognition, and fear conditioning tests to assess learning and memory (Figs. 3 and 4). No significant alterations were observed in open field (Supplementary Fig. 7A, B) or zero maze (Supplementary Fig. 7C, D), both at 12 months and at 6 months of age between WT and knockout (KO) mice. Overall body weight (Supplementary Fig. 7E), mouse feeding, physical activity, and energy expenditures were unchanged at 6 months of age (Supplementary Fig. 7F).



Fig. 4 *Nrbf2* KO mice exhibit learning and memory deficits. All behaviors were performed with male mice. \mathbf{a} - \mathbf{c} , \mathbf{g} 12 months of age (n = 5-7 each genotype). \mathbf{d} - \mathbf{f} 6 months of age (n = 5-7 each genotype). \mathbf{h} , \mathbf{i} , 2 months of age (n = 15 each genotype). \mathbf{a} , \mathbf{d} Morris Water Maze escape latency is plotted. KO mice exhibited longer escape latencies in multiple testing days both at 12 (\mathbf{a}) and 6 months (\mathbf{d}) of age compared to age-matched controls while the difference between the WT and the KO curve is bigger at 12 months of age. \mathbf{b} , \mathbf{c} , \mathbf{e} , \mathbf{f} KO mice exhibited less time spent in SE quadrant (\mathbf{b} , \mathbf{e}) and less SE location crossing (\mathbf{c} , \mathbf{f}) compared to WT mice in probe trial test, at both 12 (\mathbf{b} , \mathbf{c}) and 6 (\mathbf{e} , \mathbf{f}) months of age, while the differences between WT

In the rotarod test, WT and knockout (KO) mice performed similarly at age of 12 months (Fig. 3a), while at age of 6 months, at day 1 there was a difference in performance between WT and KO mice (Fig. 3b). The mixed-effect regression model suggested that the overall time on rod combining all 3 days tended to be lower in KO mice at both 12 and 6 months of age, but did not reach statistical significance (p = 0.180 and p = 0.064, respectively) (Fig. 3c, d).

Significant deficits were observed in the Morris Water Maze at 12 months of age in knockout (KO) mice compared to WT mice as evidenced by escape latency in the hidden platform test (Fig. 4a) and less time spent in the SE quadrant (p = 0.0004 by two-tailed *t*-test and p = 0.009 by Wilcoxon rank-sum test) and fewer SE location crossing (p = 0.0002by two-tailed *t*-test and p = 0.0081 by the Wilcoxon ranksum test) during the probe trial (Fig. 4b, c).

Deficits were also observed in KO mice at 6 months of age, although the differences between WT and knockout

and KO mice are bigger at 12 months of age. The difference in time spent in the SE quadrant between WT and KO mice at 6 months of age (e) is not significant in Student *t*-test (p = 0.069). g KO mice are impaired in memory retention in the novel place recognition test at 12 months of age. During training both WT and KO mice explore objects in both locations equally. Testing was performed 2 h after training, during which KO mice exhibited no preference to the object placed at the novel place. h, i KO mice are impaired in memory retention in both the contextual (h) and the cued (i) fear conditioning test (2 months of age, n = 15 each genotype). Data = mean ± SEM, Student *t*-test, *p < 0.05 compared to control mice.

(KO) mice were less pronounced (Fig. 4d–f). A linear mixed model suggested that on average the escape latency for KO mice was 12.4 s (95% CI 4.8–20.0, p = 0.0018) longer than WT mice; and the time decreased 5.0 s (95% CI 3.2–6.8, p < 0.0001) per day for both WT and KO mice. There was no significant difference in the trend between genotypes. The SE duration (p = 0.069 by two-tailed *t*-test, and p = 0.062 by Wilcoxon test) and the SE crossing frequency (p = 0.030 by two-tailed *t*-test, and p = 0.068 by Wilcoxon test) tended to be lower in KO mice.

To further characterize the learning and memory phenotype, we performed a novel place recognition test and found that the KO mice exhibit deficits in this test at 12 months of age compared to WT mice (Fig. 4g). In a larger cohort with n = 15 mice each, we also found that the KO mice exhibit deficits in fear conditioning at 2 months of age (Fig. 4h, i).

Significant decrease of expression of mRNA of SLC24A5 and EPS8L2, with significant accumulation of their respective proteins in the hippocampus of the NRBF2 knockout (KO) mice

Because of our observation that NRBF2 knockout (KO) primary neurons exhibit normal autophagic flux and NRBF2 knockout hippocampus only had subtle changes in autophagy protein levels, we further examined whether NRBF2 knockout has a significant impact in the hippocampal transcriptome. Hippocampi were dissected from 6 months old mice and subjected to RNAseq analyses. We confirmed that Nrbf2 gene expression was significantly decreased in the KO mice (to <1/36). From 24,306 genes analyzed, we found 47 whose expression levels had a >1.5fold change (Supplementary Table 2). Pathway analyses using ToppFun application of the ToppGene Suite [57, 58] show that these genes are enriched for protein folding and quality control, as well as membrane vesicular proteins (downregulated), and immediate early genes/transcription factors (upregulated) (Fig. 5a). Real-time RT-PCR confirmed many of the genes identified as regulated by NRBF2, including decreases of EPS812 (epidermal growth factor receptor kinase substrate 8-like protein, implicated in cytoskeletal remodeling), SLC24a5 (solute carrier family 24, member 5, linked to ER and Golgi), HSPA5 (heat shock protein 5, linked to Alzheimer's disease), PDIA4 (protein disulfide isomerase associated 4, linked to ER and Golgi), Steap2 (six-transmembrane protein of prostate 1, linked to ion transport and protein folding), and Stra6 (stimulated by retinoic acid 6); and an increase of TFRC (transferrin receptor) in NRBF2 knockout mice (Fig. 5b). LC3B, ATG14, and LAMP1 mRNAs did not change in NRBF2 knockout hippocampus. BECN mRNA is upregulated by ~20%. LAMP2a mRNA is trending down, whereas p62 mRNA is trending up.

Immunohistochemistry studies with TFRC, Steap2, HSPA5, and VPS34 did not show significant changes in the overall levels of these proteins (Supplementary Fig. 8). However, upon closer examination, we found that SLC24A5, STRA6, NPAS4d, and EPS812, as well as BECN1 and P62 proteins, exhibit an aggregate-like accumulation in the CA1 pyramidal/Radiatum/Lacunosum Moleculare layers, consistent with cell-specific disruption of protein quality control (Fig. 6).

Discussion

Since NRBF2 has been shown to interact with RXR/RAR transcription factors as well as the VPS34/BECN complex, we hypothesized that NRBF2 is important for learning/ memory and tested this in a series of experiments using a



Fig. 5 Changed gene expression in the hippocampus of the Nrbf2 KO mice. a Upregulated genes (red/lighter circle nodes) enriched in transcription factors and downregulated genes (green/darker circle nodes) enriched in membrane proteins and protein folding are down. Enriched terms (p-value < 0.05 FDR B&H) are represented as blue rectangles. b Real-time RT-PCR (primers as listed in "Supplemental Table 1") quantification of autophagy-lysosomal pathway genes and of NRBF2 target genes as revealed by RNAseq analyses (n = 3, 1 year of age). Upregulated in Nrbf2 KO include tfrc (transferrin receptor). Downregulated in Nrbf2 KO include eps8l2 (epidermal growth factor receptor kinase substrate 8-like protein, implicated in cytoskeletal remodeling), hspa5 (heat shock protein 5, linked to Alzheimer's disease), pdia4 (protein disulfide isomerase associated 4, linked to ER and Golgi), slc24a5 (solute carrier family 24, member 5, linked to ER and Golgi), steap2 (linked to ion transport and protein folding), and stra6 (stimulated by retinoic acid 6).

novel mouse model with nervous system-specific NRBF2 knockout. We found that NRBF2 knockout mice exhibited normal physical activity, food intake, whole body energy expenditure, or metabolic reliance on the energy source (Supplementary Fig. 7). No change of synaptosomal bioenergetics was observed (Supplementary Fig. 6). By H&E staining, no evidence of neurodegeneration was observed at



Fig. 6 Accumulation of proteins in the hippocampus in the NRBF2 knockout mice. Immunohistochemistry staining of brains in wildtype (WT) and Nrbf2 knockout (KO) mice by antibodies against SLC24A5, EPS8L2, NPAS4, STRA6, P62, and BECN1, n = 3 each at 6 months. Arrows point to aggregates.

6 months or 1 year of age (Supplementary Fig. 1). Strikingly, we observed significant learning and memory deficits in NRBF2 knockout mice, revealed by the Morris Water Maze, Novel Place Recognition, and fear conditioning test (Fig. 4). Interestingly we observed a decrease of freezing in both the contextual and cued fear conditioning test (Fig. 4). Importantly, normal behaviors in the open field and zero maze tests, were consistent with a lack of overt defects in movement or increased anxiety (Supplementary Fig. 7). However, there was a decrease in time spent on the rotarod in the first day of testing in 6-month-old knockout mice, and a trend for an overall decrease of motor coordination on the rotarod, consistent with potentially perturbed neuronal function (Fig. 3). Taken together these data as consistent with a specific deficit in both hippocampal mediated and amygdala mediated learning.

With regard to the potential involvement of altered autophagy and transcriptional function of NRBF2, we performed studies to compare autophagic homeostasis and transcriptomics in the hippocampus of the control and NRBF2 knockout mice. We found that in response to NRBF2 knockout, there was a modest elevation of P62, LC3II/I, BECN, and VPS34 proteins, with BECN mRNA also modestly elevated. LC3B and ATG14 mRNAs were unchanged. Thus the change of levels of BECN and possibly p62 may be due to both transcriptional activation and decreased turnover.

Despite the fact that prior studies indicating the role of NRBF2 in autophagic flux, we have not detected any changes in autophagic flux in primary neurons, with and without rapamycin. Neither have we detected any changes in p-mTOR or p-AKT levels in the brain. Autophagic flux in primary neurons is similar in WT and the knockout mice. This potentially can be due to the fact that the primary neurons are from postnatal day 0 mouse brains and that the western blot analyses are from adult brains. A proteotoxicity/quality control response to NRBF2 knockout will likely progress with age and not evident in the younger cells. Further, a change in levels of autophagy-related proteins does not always result in a change in activities, whether they have become rate-limiting steps or not or whether other

compensatory changes in the autophagy pathway have occurred may be important.

We have not detected any overt pathological changes in the knockout brain based on TUNEL staining and H&E staining. Furthermore, western blot analyses of PSD95 and synaptophysin proteins did not detect any changes in the knockout mice, suggesting a normal synaptic structure. This is not surprising as learning and memory can be altered with epigenetic, transcriptional, or other signaling changes in the brain [59-61]. Since NRBF2 has also been shown to interact with peroxisome proliferator-activated receptor alpha (PPAR- α), thyroid hormone receptor β (TR β), retinoic acid receptor α (RAR α), and retinoid X receptor α (RXR α) with the c-terminal 33 amino acids essential for the interaction in a yeast 2 hybrid system [6]. These interactions may influence NRBF2 function in the brain through an alternative mechanism to autophagy. Using RNAseq analyses, we found that the NRBF2 knockout altered the transcriptome in the mouse hippocampus. TFRC is upregulated by NRBF2 knockout, this may lead to increased cellular iron uptake and toxicity [62]. In human brains, TFRC is highly expressed in the hippocampus [63]. In mice, TFRC whole-body knockout is embryonic lethal due to severe anemia [58]. The aging model SAMP8 mice which also exhibit learning and memory deficits have a decreased Tfrc gene expression [64]. At present we do not know whether the alteration of its gene expression is a direct consequence of NRBF2 regulation of their transcription or indirect consequence downstream of NRBF2 deficiency. Additionally, it is unknown whether these changes contribute to the learning and memory deficits observed in NRBF2 knockout mice and whether they may be targeted to improve learning/memory.

Among the genes downregulated in the hippocampi of the NRBF2 knockout mice, Eps8l2 is involved in actin remodeling and its knockout leads to hearing loss [65, 66]. HSPA5 is a chaperone protein potentially implicated in Alzheimer's disease [67]. HSPA5 transcription can be regulated by RXR::RAR and NRF2 [68, 69]. In addition to decreased TRFC and learning and memory deficits, the aging model SAMP8 mice also have increased HSPA8 and HSPA1A gene expression [64]. A decrease of Hspa5 mRNA has been shown in the hippocampus in aged cognitively impaired rats compared to young or aged cognitively unimpaired rats [70], and may result in misfolded proteins and decreased neuronal function. We do not know whether the change of its gene expression occurs in neurons versus populations of glia. Nonetheless, a recent single-cell transcriptomics study demonstrated that early changes in gene expression are cell type-specific while later changes in gene expression are shared among cell types and primarily involved in global stress response [71]. STRA6 is also a retinoic acid-responsive gene and may mediate cellular uptake of vitamin A and mediate RXR and RAR function [72]. Steap2 protein is a metalloreductase capable of spanning membranes, including the plasma membrane, vesicular tubular structures, and Golgi, and may be important for copper and iron homeostasis and protein trafficking [73, 74].

Considering that the NRBF2 knockout mice exhibit a profound deficit in learning and memory, it is important to understand how the downstream regulated genes are involved in the process. The upregulation of genes potentially can be compensatory to pathology or mediating pathology. In this regard, paradoxically, TFRC is upregulated in NRBF2 knockout brains and downregulated in AD brains [75], while Eps812 expression is downregulated in NRBF2 knockout brains while upregulated in APOE4/4 AD compared with APOE3/3 AD [76]. PDIA4 is a protein disulfide isomerase family protein important for protein quality control, and its expression is downregulated in NRBF2 knockout brains in this study while increased in AD brains [77]. Interestingly, and consistent with a protein quality control defect, we found aggregates of SLC24A5, EPS8L2, HPAS4, BECN1, and P62 in the CA1 Pyramidal/Radiatum/Lacunosum Moleculare layers. These may be due to a failure of quality control of these proteins, and may in turn contribute to altered synaptic physiology and cognitive deficits. SLC24A5 is a Na⁺/Ca²⁺-K⁺ exchanger previously shown to regulate pigmentation and localized to the trans-Golgi, while mitochondrial localization has also been noted recently [78-80]. EPS8L2 is an EPS8-like protein and is predicted to play a role in actin remodeling, extracellular vesicle production, and lysosome function [65, 66, 81, 82]. How these aggregates contribute to the learning and memory deficits will need to be further investigated. Recently, it has been reported that NRBF2 mRNA is downregulated in parahippocampal gyrus in human postmortem brains with Alzheimer's disease compared to healthy controls, and that whole body NRBF2 knockout mice exhibit impaired memory, LTP, autophagy, and AB accumulation, further suggesting that a deficiency in NRBF2 may contribute to Aß accumulation, dementia and cognitive deficits in Alzheimer's diseases [83]. Our study provides insight into NRBF2 function in the nervous system and pathologies associated with its dysfunction. Taken together, we have shown that NRBF2 is important for learning and memory, and this may involve multiple interactive mechanisms. Future studies will determine whether these changes contribute to, or serve as an adaptive response that attenuates, the learning and memory deficits observed in NRBF2 knockout mice, and determine utility in dissecting neuronal versus glial function in cognition and dementia in the context of Alzheimer's disease.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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