Investigation of Riluzole’s Synaptic Protection Mechanism Through HSF1-BDNF Axis

Yi Zhang
University of Tennessee Health Science Center

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Abstract
The FDA-approved amyotrophic lateral sclerosis (ALS) drug Riluzole has great potential in treating Alzheimer’s disease (AD) based on promising animal data as well as its known action on modulating synaptic transmission. However, its detailed mechanism of action is not fully understood. Here, we proposed work aiming to address this aspect via focusing on the Heat Shock Factor 1 (HSF1)-dependent mechanisms. We found that Riluzole could increase HSF1 and BDNF (Brain-Derived Neurotrophic Factor) expression both at transcriptional and translational levels. CA1 (Cornu Ammonia, the first region in the hippocampal circuit), is its main target. We also demonstrated a protective role of Riluzole on rat primary neuronal culture which was abolished by a HSF1 inhibitor. Current data together suggest that Riluzole’s synaptic-protective mechanism is highly possible through a HSF1-BDNF axis. The positive outcome from this study will facilitate filling our knowledge gap and interpretation of the ongoing clinical trials of Riluzole in AD in which the final data will be released later this year.

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Investigation of Riluzole’s Synaptic Protective Mechanism Through HSF1-BDNF Axis

Author: Yi Zhang

Advisor: Francesca-Fang Liao, Ph.D.

A Thesis Presented for The Graduate Studies Council of The University of Tennessee Health Science Center in Partial Fulfillment of Requirements for the Master of Science degree from The University of Tennessee
in Biomedical Sciences: Neuroscience
College of Graduate Health Sciences

August 2019
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Abstract

Yi Zhang

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The FDA-approved amyotrophic lateral sclerosis (ALS) drug Riluzole has great potential in treating Alzheimer’s disease (AD) based on promising animal data as well as its known action on modulating synaptic transmission. However, its detailed mechanism of action is not fully understood. Here, we proposed work aiming to address this aspect via focusing on the Heat Shock Factor 1 (HSF1)-dependent mechanisms. We found that Riluzole could increase HSF1 and BDNF (Brain-Derived Neurotrophic Factor) expression both at transcriptional and translational levels. CA1 (Cornu Ammonia, the first region in the hippocampal circuit), is its main target. We also demonstrated a protective role of Riluzole on rat primary neuronal culture which was abolished by a HSF1 inhibitor. Current data together suggest that Riluzole’s synaptic-protective mechanism is highly possible through a HSF1-BDNF axis. The positive outcome from this study will facilitate filling our knowledge gap and interpretation of the ongoing clinical trials of Riluzole in AD in which the final data will be released later this year.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Aβ</td>
<td>Soluble oligomeric abeta</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>CAMKII</td>
<td>Ca²⁺/calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>HSF1</td>
<td>Heat Shock Factor 1</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>MAP2</td>
<td>Microtubule Associated Protein 2</td>
</tr>
<tr>
<td>PSD95</td>
<td>Postsynaptic Density Protein 95</td>
</tr>
<tr>
<td>Syn I</td>
<td>Synapsin I</td>
</tr>
<tr>
<td>UTHSC</td>
<td>University of Tennessee Health Science Center</td>
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</table>
Chapter 1

Introduction

1.1 Background

1.1.1 Alzheimer’s disease

Alzheimer’s disease (AD) is one most devastating neurodegenerative disease that affects more than 35 million people in the world (5.5 million in U.S) (Querfurth and LaFerla, 2010). Researchers have found synaptic dysfunction in AD patients’ brain (Selkoe, 2002). Overarching theme is that the soluble oligomeric Abeta(Aβ) is neurotoxic and could directly induce synaptic dysfunction (Selkoe, 2002, Oddo et al., 2003). In other word, AD is indeed a synaptic disease. However, clinical trials of promising drugs targeted to Aβ all failed (Doody et al., 2014), which makes researchers re-consider the Aβ mechanism of AD: may not be the right target because it may be too late to overturn the disease when Aβ has been built. Up to now, there are only two kinds of synaptic protective drugs approved by FDA to relieve AD symptoms at early stage: cholinesterase inhibitors (Aricept, Exelon, Razadyne) and memantine. Under this circumstance, exploring the synaptic protection mechanism and developing new drugs for AD becomes central tasks for scientists in the neuroscience and pharmacology society.

1.1.2 Riluzole

Riluzole is a FDA approved drug for amyotrophic lateral sclerosis (ALS, also called Lou Gehrig’s disease). It inhibits the release of glutamic acid from cultured neurons, from brain slices, and from corticostriatal neurons in vivo. It also blocks some of the postsynaptic effects of glutamic acid by noncompetitive blockade of N-methyl-D-aspartate (NMDA) receptors (Doble, 1996). Riluzole could increase nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) in cultured mouse astrocytes (Mizuta et al., 2001), and this finding shows possible neuron protection of Riluzole on AD (Appleby et al., 2013). Recent studies have shown that in classic Alzheimer’s disease model (5XFAD transgenic mice), Riluzole could reduce Aβ plaques in the brain and partly improve memory after
consistently feeding mice Riluzole in drinking water for five months (Okamoto et al., 2018). The drug is also being tested currently in mild AD patients by Rockefeller University and the end-point results are anticipated to be released in a year.

1.1.3 HSF1-BDNF axis

Heat shock factor 1 (HSF1) is an evolutionarily highly conserved transcription factor that coordinates stress-induced transcription and directs versatile physiological processes in eukaryotes. The rapidly induced gene activation in stress has facilitated key findings on transcriptional processes and elucidated survival mechanisms in protein-damaging conditions. Although the main focus has been on HSF1-induced expression of chaperone genes, HSF1 controls a wide set of target loci in stressed cells and directs versatile physiological processes also in non-stressed circumstances, including development, metabolism and aging. Its central role in core physiological pathways and the capacity to orchestrate genome-wide transcription has revealed HSF1 as a fundamental director of cellular processes, and advanced our understanding on how gene expression is coordinated in cells, organs and entire animals (Vihervaara and Sistonen, 2014).

In neuronal cells, HSF1 could up-regulate expression of several important downstream synaptic proteins such as PSD95, Syn I and BDNF (Chen et al., 2014, Wang et al., 2017) (Figure 1.1).

1.2 Hypothesis

Up to now, Riluzole’s mechanism of action is not fully understood, but it has been shown repeatedly to modulate glutamate neurotransmission. In vitro experiment shows that Riluzole could increase HSF1 (heat shock factor 1) for neuronal survival under glutamate excitotoxic stress (Liu et al., 2011). Under this circumstance, we hypothesis that Riluzole could have synaptic protection through the founded HSF1-BDNF axis.
Figure 1.1: HSF1-BDNF axis

Through Hsp90 inhibitor, stress-response transcription factor HSF1 could be activated to up-regulate important downstream synaptic protein expression, like PSD95, Syn I and BDNF.

Chapter 2

Materials and Methods

2.1 Experimental Models

2.1.1 Mice

All animal procedures were performed in accordance with the Animal Scientific Procedures Act and with the approval of the Institutional Animal Care and Use Committee (IACUC) at University of Tennessee Health Science Center (UTHSC). WT B6 mice were purchased from the Jackson Lab (Stock #018582).

2.1.2 Primary neuron culture

Cortical and hippocampal neurons were isolated and purified from E17 embryos of Sprague Dawley (SD) rats as previously described (Wang et al., 2017).

For staining, primary neuron culture was fixed with 4% PFA after washing with PBS for 10-15 minutes. Then was washed with PBS for three times, 5min each time. After being washed with 0.3% PBST for 15 min, we blocked it with 10% goat serum for 30 minutes. And incubated with mouse anti-MAP2 (1:800; Sigma) for 60 minutes at room temperature. After washing with PBST three times (5 minutes each time), incubated with anti-mouse fluorescent antibody for 60 minutes. Nuclei were stained with DAPI.

2.1.3 Chemical compounds/reagents

KRRBB11 was purchased from Tocris; Riluzole was purchased from EMD Millipore.
2.2 Methods

2.2.1 Compounds administration

The solvent used for Riluzole administration is 1% Tween-80 in sterile saline. Riluzole was injected intraperitoneally (IP) at 10-20 mg/Kg once a day for one or two days.

2.2.2 RNA extraction and analysis (qRT-PCR)

Total RNA was extracted from fresh brain tissue (hippocampus, cerebellum, midbrain and cortex) by using Trizol reagent (Life Technology). For RNA, fresh mouse brain tissue was put into 1 mL Trizol and was incubated for 5 min. Then 0.2 mL chloroform was added in before 15 second-vortex. Then let the solution incubate for 2-3 min. Let the solution centrifuge for 15 min at 12,000 g at 4°C. Then transfer the aqueous phase containing RNA to a new tube and add 0.5 mL of isopropanol, then incubate for another 10 min. Centrifuge for 10 min at 12,000 g at 4°C. Re-suspend the pellet in 1 mL 75% ethanol, vortex briefly and centrifuge for 10 min at 7,500 g at 4°C. Vacuum the RNA pellet for 5-10 min and add 20-50 µL DEPC-H2O to dissolve it.

The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used to synthesize the first strand cDNA from the samples with an equal amount of RNA. Synthesized cDNAs were then amplified and analyzed on Real-Time PCR Systems (Invitrogen) using SYBR Green Master Mix (5 Prime). Primers used were listed in Table 2.1.

2.2.3 Histology and immunohistochemistry

Mouse transcardial perfusion was applied by saline and 4% PFA, post-fixed in 4% PFA overnight. Mouse brains were paraffin-embedded and 16µm sections were deparaffinized by passing through 100% xylene and rehydrated through serial dilutions of ethanol (100%, 95%, and 75%). Hematoxylin and Eosin (H&E) staining was performed and images were captured under a light microscope. For OCT embedding, perfused brains were cryoprotected by 30% sucrose infiltration and then sectioned at 16µ using a cryostat (Leica). Anti-HSF1 (1:500; cell signaling), anti-MAP2 (1:800; Sigma), anti-BDNF (1:500; Santa Cruz Biotechnology) antibodies were used and nuclei were stained with DAPI.

2.2.4 Protein analysis by Western blots

Western blots were performed as previously described (Wang et al., 2017). Antibodies (Abs) were used as follows: rabbit anti-HSF1, PSD95, p-CREB,CREB, Syn I and
Table 2.1: Primers used for qRT-PCR of the genes of HSF1 and BDNF exons

<table>
<thead>
<tr>
<th>Gene</th>
<th>Strand</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Hsf1</td>
<td>Forward</td>
<td>CACTCTGTGCCCAAGTATGG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCTGTAGGCTGGAGATGGAG</td>
</tr>
<tr>
<td>Bdnf mExon I</td>
<td>Forward</td>
<td>AGTCTCCAGGACAGCAAAGC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TGCAACCGAAATGAAAATAACC</td>
</tr>
<tr>
<td>mExon IIA</td>
<td>Forward</td>
<td>GGATTTGTCCAGGTGGTAGT</td>
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<tr>
<td>mExon IIB</td>
<td>Forward</td>
<td>GCGGTGAGGCTGGAAATAGA</td>
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<tr>
<td>mExon IIC</td>
<td>Forward</td>
<td>GTGGTGAAGCCCAAAAGA</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>AACCATAGTAAGAAAGGATGGTC</td>
</tr>
<tr>
<td>mExon III</td>
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<td>GAGACTGCCTCCACTCC</td>
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<tr>
<td>mExon IV</td>
<td>Forward</td>
<td>GCTGCCTTGATTTTTTCTGGA</td>
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<tr>
<td>mExon V</td>
<td>Forward</td>
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<td>mExon VI</td>
<td>Forward</td>
<td>AGCGTGACAACAAATGTGACT</td>
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<td>mExon VIIA</td>
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<td>CTCCCTGAAAGTGAAGTTTTTGT</td>
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<td>mExon VIIIB</td>
<td>Forward</td>
<td>CTGTATCCCGACCTCTGTCC</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>CACACACCAGCCTTCTC</td>
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<td>mExon VIII</td>
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<tr>
<td>mExon IXA</td>
<td>Forward</td>
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<tr>
<td>mExon IX coding</td>
<td>Forward</td>
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</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GACCTTTTTACAGACTGTGACC</td>
</tr>
<tr>
<td>mExon</td>
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<td>AAGGATGGTCATCATTCTCTA</td>
</tr>
<tr>
<td>IIA,IIIB,III,IV,VI, VIII,IXA</td>
<td>Forward</td>
<td>GCAAATTCAACCGGCACAG</td>
</tr>
<tr>
<td>Gapdh</td>
<td>Reverse</td>
<td>CTCGCTCCTGGAAAGATGG</td>
</tr>
</tbody>
</table>
Chapter 2. Materials and Methods

p-CAMKII (1:1000; Cell Signaling); mouse anti-Akt (1:1000; Cell Signaling), rabbit anti-BDNF (1:500; Santa Cruz Biotechnology); mouse anti-β-actin (1:10000; Cell Signaling), and anti-mouse IgG and anti-rabbit IgG horseradish peroxidase-conjugated Abs (1:5000; Sigma-Aldrich).

2.2.5 In situ hybridization

Fresh brain slides (16µm) were stored at -80°C freezer. Remove brain slides from freezer and thaw for 5 min at 55°C. Fix them for 10 min in 4% paraformaldehyde, 4°C. Then wash 5 min. in 0.5x SSC at room temperature. Immerse slides in proteinase K solution, 1-5µg/ml in RNase Buffer for 10 min at room temperature. The amount of proteinase K needs to be optimized with each new preparation. Once optimized aliquots can be frozen down and used for some time. Wash for 10 min. in 0.5xSSC at room temperature. Dry around sections with Kimwipe, lay slides flat in an air tight box with a piece of filter paper which has been saturated with Box Buffer (4xSSC, 50% formamide) on the bottom. Cover each section with 100µl of rHB2 without probe. Prime sequence of HSF1 and BDNF probe can be found in Table 2.2. Then incubate at 42°C for 1-3 hours. Add 2.0µl probe per slide (stock solution 300,000 cpm/µl in 1XTE) 1.0µl tRNA per slide (50mg/ml stock) Heat 3min, 95°C immediately add 17.0µl ice cold rHB2 per slide, vortex, place on ice. Add 20µl of above hybridization mix to each 100µl of prehybridization solution directly into the bubble covering the section. Incubate overnight at 55°C. Wash 2 times 10 min, each in 2x SSC with beta Mercapto EtOH-EDTA at room temperature. Immerse in RNase A solution (20µg/ml in RNase buffer) 30 min at room temperature. Wash 2x 10 min each in 2x SSC with beta Mercapto EtOH-EDTA at room temperature again. Wash 2 hours in 4 liters of 0.1x SSC with beta Mercapto EtOH-EDTA, 55°C. Wash 2 times 10 min. each in 0.5x SSC without beta Mercapto EtOH or EDTA at room temperature. Dehydrate 2 min. each in 50%, 70%, and 90% ethanol containing 0.3M NH4Ac. Dry in vacuum desiccator (3-4 hrs.), store with desiccant until autoradiography. Dip in Kodak NTB2 nuclear emulsion diluted 1:1 with water at 42°C, dry for 2 hours in the dark, expose in the dark at 4°C with desiccant for 2-8 weeks. Then develop at 15°C follow the steps:a) 3 min. Kodak D19 developer, diluted 1:1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer ARMS</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsf1 S2</td>
<td>GCCTTCAGCCACTCCTTCTAAA</td>
<td></td>
</tr>
<tr>
<td>Hsf1 AS2T7</td>
<td>TAATACGACTCACTATAGGGTGGACAGCATCAGAGGAGTAAAG</td>
<td></td>
</tr>
<tr>
<td>Bdnf 715S</td>
<td>GGCGCCCATGAAAGAAGTAAAC</td>
<td></td>
</tr>
<tr>
<td>Bdnf 1634AS</td>
<td>CGGCAACAAACCACACATTAT</td>
<td></td>
</tr>
</tbody>
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with water; b) 20 seconds in water stop rinse; c) 3 min. Kodak Fixer, full strength; d) Wash 3 times 5 min. each in water; e) Counterstain with Hematoxylin and Eosin.

2.3 Statistical Analysis

Quantity One and Image J software were used for data analysis. Data are shown as mean ± SEM. Statistical significance was determined by t tests (two-tailed) for two groups or ANOVA (with Dunnett’s multiple comparisons test, Dunn’s multiple comparisons test, or Tukey’s multiple comparisons test) for three or more groups. P values less than 0.05 were considered to be statistically significant.
Chapter 3

Results

3.1 Comparisons of Riluzole Delivery Routes Between i.p and p.o

Intraperitoneal injection (i.p) and oral gavage (p.o) of Riluzole are the two main methods shown in previously published research papers (Okamoto et al., 2018). Researchers dissolved Riluzole in drinking water and fed the mice. However, this method may make it hard to calculate the volume mice drank. We used five mice (2.5 months old) and set them into three groups, treat them with saline and with Riluzole, using i.p and p.o (10mg/Kg and 20mg/Kg each mouse) respectively. Then isolated the hippocampus and extracted proteins to detect HSF1 and BDNF level. We didn’t find significant differences between mice with different injection concentrations, but found i.p group showed stronger signal than p.o group (Figure 3.1A) after quantification (Figure 3.1B), indicates that delivery method through i.p is more effective than p.o in our Riluzole treatment system. So, we used i.p as the drug delivery method in the following experiments.

3.2 Uneven Expression and Distribution of HSF1 and BDNF in Different Brain Regions after Riluzole Injection

Then we used two mice and inject them with Rilzole (20mg/Kg) and divided the whole brain into two halfs to run WB and qPCR respectively. We isolated four different parts of the brain (hippocampus, cerebellum, midbrain and cortex) to detect HSF1 and BDNF levels. Through WB, we found that both HSF1 and BDNF increase mainly in hippocampus after rilzule injection (Figure 3.2A). This is consistent with our qPCR data with the other half parts of samples, showing both HSF1 and BDNF in hippocampus have nearly two-fold increase at transcriptional level (Figure 3.2B and C). Meanwhile, hippocampus is the main affected area in AD brain, and would be highly possible where the promising drug protective area. Based on our data, we would focus on studying hippocampus in the next experiments.
Figure 3.1: Delivery of Riluzole through i.p is more effective than through p.o.

Five 2.5 month-old B6 mice were subjected into saline injection (vehicle), i.p with Riluzole (10mg/Kg and 20mg/Kg) and p.o with Riluzole (10mg/Kg and 20mg/Kg). Hippocampus was isolated for protein extraction and run for Western-blot. (A) i.p group showed more detectable protein expression compared with p.o group. (B) Quantified data through image J. to analyze Western-blot results.
Chapter 3. Results

Figure 3.2: HSF1 and BDNF increase mainly in the hippocampal area of the brain.

Two 2.5 month-old B6 mice were injected with saline and Riluzole (20mg/Kg) respectively and isolated hippocampus, cerebrum, midbrain and cortex to extract protein and mRNA for analysis of gene expression. (A) Western-blot showed both HSF1 and BDNF increase mainly happens in hippocampus compared with other parts in brain; qPCR showed mRNA increase of (B) HSF1 and (C) BDNF mainly happens in hippocampus area compared with other parts in brain.
3.3 HSF1 and BDNF Protein Expression Increase in Hippocampus after Riluzole Injection

Next, we focused on protein expression in hippocampus after Riluzole injection. We used three mice, one injected with saline and the other two inject with 20mg/Kg Riluzole, one and two doses respectively. Then isolated the hippocampus and extracted proteins to run WB. We found both HSF1 and BDNF protein expression increase in hippocampus after two doses of Riluzole injection (Figure 3.3A). This observation was confirmed through quantification (Figure 3.3C). Interestingly, BDNF increase is mainly on the pro-BDNF form; we did not observe difference between the treatment groups on the mature m-BDNF levels. We also detected proteins related to the HSF1-BDNF axis and found that p-CREB increased after Riluzole treatment (Figure 3.3B). However, no change in PSD95, Syn I, p-AKT and p-CAMKII was detected after Riluzole injection (Figure 3.3B).

3.4 HSF1 Mainly Increases in the CA1 Region of Hippocampus

We then wanted to figure out the specific region in hippocampus area where HSF1 level increase. We used four mice, one was injected with saline and other three were injected with Riluzole (20mg/Kg) at two doses. After perfused and fixed with 4% PFA as described, we isolated brains and prepared slides and used immunohistochemistry (IHC) to stain the slides with HSF1 antibody. We found HSF1 mainly increase in CA1 region (Figure 3.4B, C and D) and verified the positive signal is neuron area through co-staining with neuron specific antibody, MAP2 (microtubule associate protein 2) and nuclear antibody DAPI (Figure 3.5). However, in CA3, dentate gyrus and cortex, there was no difference after Riluzole injection compared with vehicle (Figure 3.4B, C and D).

3.5 BDNF Mainly Increases in the CA1 and CA3 Region of Hippocampus

We also wanted to figure out the specific regions in hippocampus area where BDNF level increase. We used four mice: one was injected with saline and other three were injected with Riluzole (20 mg/Kg) at two doses. After perfused and fixed with 4% PFA as described, we isolated brains and prepared slides and used immunohistochemistry (IHC) to stain the slides with BDNF antibody. We found that BDNF was mainly increased in CA1 and CA3 region (Figure 3.6A and B) and verified the positive signal is from the neuron-rich area through co-staining with a neuron specific antibody, MAP2 (microtubule associate protein 2) and nuclear antibody DAPI (Figure 3.7). In dentate gyrus and cortex, there was no difference after Riluzole injection compared with vehicle (Figure 3.6C and D).
Three 2.5 month-old B6 mice were injected with saline (vehicle), Riluzole (20 mg/Kg) one dose (Rx1) and two doses (Rx2) respectively. Hippocampus was isolated to extract protein for protein analysis. (A) HSF1 and BDNF (pro-BDNF) show increase after Riluzole treatment. (B) CREB shows increase but there is no increase detected on p-AKT, p-CAMKII, PSD 95 and Syn I. (C) Quantified data through image J. to analyze HSF1 and pro-BDNF Western-blot results.
Figure 3.4: CA1 is the main region in which HSF1 is increased in hippocampus

Three 2.5 month-old B6 mice were injected with Riluzole (20 mg/Kg) and one was injected with saline as vehicle. After perfusion and brain-fix, brain slides were made and immunohistochemistry (IHC) on HSF1 was performed. HSF1 increase after Riluzole injection mainly shows in CA (A), but no difference was found in CA3 (B), dentate gyrus (C) and cortex (D). Image J was used for data quantify. Data represent mean ± SEM; *P<0.05; n.s, no significance.
Figure 3.5: Co-staining of HSF1 with neuronal makers in CA1 region in hippocampus

To verify the HSF1 positive signal locates in neuron area, we co-stained brain sections with (A) HSF1, (B) MAP2 and (C) DAPI. Image J was used for data quantify. Data represent mean ± SEM; *P<0.05; n.s, no significance.
Figure 3.6: CA1 and CA3 are the main regions in which BDNF is increased in hippocampus.

Three 2.5 month-old B6 mice were injected with Riluzole (20 mg/Kg) and one was injected with saline as vehicle. After perfusion and brain-fix, brain slides were made and immunohistochemistry (IHC) on BDNF was performed. BDNF increase after Riluzole injection mainly shows in (A) CA1 and (B) CA3, but no difference was found in (C) dentate gyrus and (D) cortex. Image J was used for data quantify. Data represent mean ± SEM; *P<0.05; n.s, no significance.
Figure 3.7: Co-staining in hippocampus

To verify the BDNF positive signal locates in neuron area, we co-stain hippocampus with (A) BDNF, (B) MAP2 and (C) DAPI. Image J was used for data quantify. Data represent mean ± SEM; *P<0.05; n.s, no significance.
3.6 HSF1 and BDNF mRNA Expression Is Increased in Hippocampus after Riluzole Injection

We have found that both HSF1 and BDNF protein expression increase in hippocampus after Riluzole injection. We speculated the mRNA levels of these two molecules were up-regulated after Riluzole treatment. We used six mice, three were injected with saline and three were injected with Riluzole (20 mg/Kg) at two doses. Then we isolated fresh brain tissue (hippocampus) and extracted RNA as described. RNA analysis was performed through qPCR detection and we found that both HSF1 and BDNF displayed nearly two-fold increase at transcriptional levels with statistically significance (Figure 3.8A and B). This result is consistent with the initial data we get in detecting the spread of HSF1 and BDNF in different parts of brain (Figure 3.2B and C).

There are nine promoters before the BDNF coding region in the sequence structure (Maynard et al., 2016) (Figure 3.9).

We then further detected which promoter(s) induce the transcriptional activity after Riluzole treatment. With the help of Dr. Sakata, we designed the specific exons primers of the nine promoters and use the same six samples to run qPCR. After data analysis, we found that promoter II, III, VI, VIII and IX contributed to the BDNF mRNA increase with statistical significance (Figure 3.10).

Figure 3.8: HS1 and BDNF mRNA level increases after Riluzole treatment
Three 2.5 month-old B6 mice were injected with Riluzole (20 mg/Kg) for two doses and three were injected with saline as vehicle. Fresh brain tissue was isolated and mRNA was extracted from hippocampus for qPCR. Both (A) HSF1’s and (B)BDNF’s mRNA level showed two-fold increase after Riluzole injection. Data represent mean ± SEM; *P<0.05, **P<0.01; n.s, no significance.
**Figure 3.9:** Schematic of transcript production from BDNF gene

Transcription is initiated from promoters upstream of individual 5’-untranslated regions (UTRs) and spliced to the common coding exon IX. Each transcript uses one of two polyadenylation sites.

Figure 3.10: Changes of different BDNF mRNA exons after Riluzole treatment

Three 2.5 month-old B6 mice were injected with Riluzole (20 mg/Kg) for two doses and three were injected with saline as vehicle. Fresh brain tissue was isolated and mRNA was extracted from hippocampus for qPCR. Both (A) HSF1’s and (B) BNDF’s mRNA levels showed two-fold increase after Riluzole injection. Data represent mean ± SEM; *P<0.05, **P<0.01, ***P<0.001; n.s, no significance.
3.7 Riluzole Could Protect Neuron Against Aβ Toxicity Through HSF1 Pathway

Finally, we wanted to determine Riluzole’s neuronal protection in primary neuron culture. We used Sprague Dawley (SD) rats and hippocampal neurons were isolated from E17 embryos as previously described (Liu et al., 2011, Swanger et al., 2015). The overall cell density was around 30,000-40,000 cells per unit. Then we developed the primary neuron culture for around 14 days (14 DIV) to allow its full maturation. We used soluble Aβ (7PA2 condition media, 1:5 dilution) (Walsh et al., 2002) to treat it and found significant neurons fraction plus greatly decreased dendritic neuron, which showed Aβ’s direct toxicity towards neurons (Figure 3.11A and B). 0.5 µM Riluzole was added into the neuron and there was no toxicity detected (Figure 3.11C). And Riluzole could protect neuron from Aβ toxicity after adding into the 7PA2 treatment group.

To link Riluzole’s neuronal protection to HSF1-BDNF axis, we used HSF1 inhibitor, KRIBB11(Chen et al., 2014,Yoon et al., 2011) to block HSF1-BDNF pathway. We found that when we added 0.25µM KRIBB11 into the cell culture with 0.5µM Riluzole had already been added, when treated with soluble Aβ(7PA2), Riluzole did not display neuronal protection anymore (Figure 3.12A and B). Both neuron number/density and average dendritic lengthen showed great decrease with statistically significant when the same concentration KRIBB11 was added in (Figure 3.13B and Figure 3.14B). Much less neuronal “degradation” with decreased dendritic spines was observed (Figure 3.11D) and the neuronal protective effect showed statistically significant after quantification at both 0.25µM and 0.5µM Riluzole based on neuron number/density and average dendritic length (Figure 3.13A and Figure 3.14A).
Riluzole could have neuronal protective role against Aβ toxicity

Hippocampal neurons were isolated and purified from E17 rat embryos. Then (A) without treatment, treated (B) with soluble Aβ (7PA2), (C) with only 0.5μM Riluzole and (D) with 0.5μM Riluzole plus soluble Aβ(7PA2). After treatment, cell culture was stained with MAP2 antibody and nuclei were stained with DAPI as described.
Figure 3.12: HSF1 inhibitor KRIBB11 could block Riluzole’s neuronal protection

Hippocampus neurons could be protected from Aβ toxicity (A) with only 0.5µM Riluzole but (B) blocked by 0.25µM KRIBB11. Cell culture was stained with MAP2 antibody and nuclei were stained with DAPI as described.

Figure 3.13: Neuron number/density change after Riluzole and KRIBB11 treatment

Neuron number/density was counted when (A) Riluzole was added into soluble Aβ (7PA2) treatment primary neuron culture at 0.25µM and 0.5µM concentration (B) KRIBB11 was added at the same concentration with Riluzole. Data represent mean ± SEM; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; n.s, no significance.
Figure 3.14: Dendritic length change after Riluzole and KRIBB11 treatment

Dendritic length of neuron was calculated with Image J software when (A) Riluzole was added into soluble Aβ (7PA2) treatment primary neuron culture at 0.25μM and 0.5μM concentration (B) KRIBB11 was added at the same concentration with Riluzole. Data represent mean ± SEM; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; n.s, no significance.
Chapter 4

Discussion

The findings of this study offer evidence that the promising drug towards AD, Riluzole, would play neuron protective role through HSF1-BDNF axis. We demonstrate that both HSF1 and BDNF would increase at mRNA and protein levels in hippocampus after Riluzole treatment in experimental mice (young adult). *In vitro* experiments on primary rat neuronal culture also demonstrate Riluzole’s protective role and it could be blocked through a HSF1 inhibitor.

We first dissected four different parts of the brain to see the change of HSF1 and BDNF expression after Riluzole treatment and found that hippocampus is the main area showing the significant changes. The Western Blot analysis has shown that HSF1, BDNF and p-CREB increase after Riluzole treatment in hippocampus. It should be noticed that even though some literatures present data with separate bands on pro-BDNF and m-BDNF (Chhibber and Zhao, 2017) using the same antibody as we used here for Western Blot analysis, they didn’t show the full membrane image; we observed additional bands between pro-BDNF and m-BDNF with unclear identity (*Figure 3.3A*). Interestingly, we did not observe increase on some important synaptic related proteins like PSD95 and Syn I, and CAMKII after Riluzole treatment, unlike in the case of using Hsp90 inhibitors to activate HSF1. This issue will require confirmation and further investigation in the future.

Our immunohistochemistry (IHC) identifies that hippocampal CA1 is the main region where HSF1 expression is increased after Riluzole treatment compared with other parts in the hippocampus. But why does CA1 selectively respond to Riluzole? Considering that CA1 constitutes the primary output of the hippocampus and, along with subiculum, are the first hippocampus areas affected in Alzheimer’s disease (Masurkar, 2018), CA1 principle/Pyramydal neurons are arguably the most vulnerable cells. Latest single cell sequencing and next gene sequencing (NGS) technology have been applied to further study distinct molecular gene markers in different neuronal subtypes in hippocampus area. Through latest gene analysis, researchers found that cell type-specific profiling of mouse neurons clustered primarily by tissue location, with a clear separation between the ECII, hippocampus regions (CA1, CA2, CA3, DG) and neocortical regions (S1 and V1). Moreover, many AD-relevant pathways were significantly enriched among vulnerable neurons in CA1 (Roussarie et al., 2018). This may partly explain our finding but further
analysis of gene data is required to study specific gene changes in different neuron cell subtypes in response to Riluzole.

We also use immunohistochemistry (IHC) to identify the specific regions in hippocampus with marked changes in BDNF expression and found that both CA1 and CA3 indicate significant increase (Figure 3.6A and B). Compared with the HSF1 staining results (Figure 3.4A and B), CA1 is the area show signal increase both in HSF1 and BDNF, supporting our hypothesis and Western Blot/qPCR data. It remains to be explained why we see BDNF but not HSF1 increase in CA3. Selective degeneration of pyramidal neurons in regions CA1 and CA3 of the hippocampus is a common structural correlate of several neurodegenerative conditions including Alzheimer’s disease, epilepsy and stroke (Mattson, Guthrie, and Kater, 1989), this finding also supports our IHC data. However, the signal background is relatively high and due to critics on the antibody’s increasing ‘false positive’, we turned to in situ hybridization (Obernosterer, Martinez, and Alenius, 2007) to study the interplay between HSF1 and BDNF. We didn’t detect significant increase based on our initial in situ hybridization data (Appendix Figures A.1 and A.2) and we speculate that it was due to the limited number of mice and not-optimized experiment conditions.

The qPCR analysis identified promoter II, III, VI, VIII and IX would contribute to induce BDNF transcriptional activity after Riluzole treatment. Other researchers once reported that BDNF production from promoters I or II, but not IV or VI, mediates the effects of BDNF on aggressive behavior (Maynard et al., 2016). This may partly explain our finding that promoter II’s activity. Even though we didn’t observe significant increase on promoter I activity, we still see it average over two-fold increase. Considering this is only one-time experiment with three mice, more mice and repeated experiments would be further required to validate these promoters activity in the future.

Although we demonstrate that Riluzole could protect neurons against Aβ toxicity in vitro and HSF1 inhibitor KR1BB11 could make Riluzole invalid, more solid evidence should be provided through HSF1 knockout cell. We tried to isolate primary neurons from HSF1 knockout mouse through time-mating but the experiments did not progress well due to technical issue. Stable HSF1 knockout (KO) mice colony (including forebrain -specific conditional KO) and primary cell culture are two necessary systems for future study on HSF1-BDNF axis. Additionally, thus far, we only used young B6 wild type mice for the initial studies of Riluzole treatment. In future, we will use AD mice models such as J20 or 5XFAD to determine their response to Riluzole. In fact, we have ongoing studies of using B6 as well as HSF1+/- mice at various ages (young, middle and old). Furthermore, the optimal concentration and process of riluzole towards Aβ requires further investigation. Real-time observation could be set up in the future to see Riluzole’s neuronal protection in details.

In conclusion, we have conducted a series of pilot studies to determine Riluzole’s beneficial effects and neuroprotective mechanisms in mice and in neuronal cultures. The preliminary data indicate that Riluzole could induce HSF1 and BDNF increase in the CA1 region and could attenuate Aβ-induced neuronal toxicity through HSF1-BDNF axis.
Appendix A

Supplementary Data

*Figure A.1: HSF1 in situ hybridization in hippocampus*

To detect BDNF mRNA expression in brain of Riluzole-treatment mice, we perform *in situ* hybridization in hippocampus slice. With two (A) saline treatment (vehicle) mouse and three (B) Riluzole treatment mouse.
To detect BDNF mRNA expression in brain of rRluzole-treatment mice, we perform *in situ* hybridization in hippocampus slice. With two (A) saline treatment (vehicle) mouse and three (B)riluzole treatment mouse.

**Figure A.2:** BDNF *in situ* hybridization in hippocampus
List of References


Yoon, Young Ju et al. (2011). “KRIBB11 inhibits HSP70 synthesis through inhibition of heat shock factor 1 function by impairing the recruitment of positive transcription elongation factor b to the hsp70 promoter”. In: *The Journal of Biological Chemistry* 286.3, pp. 1737–1747. ISSN: 1083-351X. DOI: 10.1074/jbc.M110.179440.
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