Faculty Excellence Award Application Form

Name: Ashok N. Hegde, Ph.D.

Rank: Professor (William Harvey Professor of Biomedical Science)

Department: Biological and Environmental Sciences

Award applying for:
(Check one)

	Excellence in Teaching Award*
	Excellence in Online Teaching Award*
X	Excellence in Scholarship & Creative Endeavors Award*
	Excellence in University Service*
	Excellence in Scholarship of Teaching & Learning Award*
	Department/Program Excellence Award^
	Irene Rose Community Service Award^
	Laurie Hendrickson McMillian Faculty Award^

*college selection required before being forwarded to university
^university awards
College nominees' final applications received by Center for Teaching and Learning (ctl@gcsu.edu) by March 1, 2019.

Please insert the required documentation in the pages below for the award category you have noted above. Detailed information associated with each award is available online at the Center for Teaching and Learning website.

GEORGIA COLLEGE
GEORGIA'S PUBLIC LIBERAL ARTS UNIVERSITY

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19 February 2020

Selection Committee

Excellence in Scholarship and Creative Endeavors Award

Dear selection committee:

LETTER OF NOMINATION AND SUPPORT: DR. ASHOK HEGDE

I wish to express my strong support for Dr. Ashok Hegde's application for the Georgia College Excellence in Scholarship and Creative Endeavors Award for 2019/2020 as both his nominator *and* as Chair of the Department of Biological & Environmental Sciences.

Dr. Hegde has been a very active researcher, mentoring both graduate and undergraduate students, since coming to Georgia College. In the last four years, Dr. Hegde has had nine publications. He has published research articles with a post-doctoral fellow at GC as a co-author. On his review articles, two graduate students and two undergraduate students from GCSU are co-authors. Thus, his scholarship allows active participation by both graduate and importantly, undergraduate students. His rigorous research program is all the more remarkable considering this is in addition to his teaching and leadership of our unique Pre-Med Mentoring program.

Dr. Hegde's publications from Georgia College have appeared in highly regarded journals with significant impact factors. The **impact factor** (**IF**) of an academic journal is a measure of the frequency with which the "average article" in a journal has been cited in a particular year or period. It is frequently used to indicate relative importance of a journal within its field with journals with higher impact factors deemed to be more important than those with lower ones. For example, *Frontiers Molecular Neuroscience* (IF: 5.076), *Frontiers in Aging Neuroscience* (IF: 4.504), *Biomolecular Concepts* (IF: 4.46), and *Neurobiology of Learning and Memory* (IF: 3.244) are all highly regarded journals in the field. Dr. Hegde's papers published from Georgia College have been cited well. For example, his peer-reviewed article "Proteolysis, synaptic plasticity and memory," has 40 citations since its publication in 2017. His scholarly work has been widely disseminated as well. This is evident by the number of views of his papers published in open-access journals. His paper published in *Frontiers in Psychiatry* has over 4100 views. A recent peer-reviewed article he published with Georgia College undergraduates as authors has had over 1700 views within two months of its publication.

Dr. Hegde's scholarship is recognized nationally and internationally. He regularly receives invitations to write review articles and book chapters. Recently he was invited to be a Guest Associate Editor for a Research Topic in 'Learning and Memory' for the *Frontiers* group of journals. Under this Research Topic, he has served an editor for articles submitted to *Frontiers in Systems Neuroscience, Frontiers in Aging Neuroscience, Frontiers in Molecular Neuroscience* and *Frontiers in Human Neuroscience.*

Prior to this, he organized a Research Topic called "Ubiquitin and the Brain" for *Frontiers in Molecular Neuroscience*, the top journal in neuroscience in terms of citations, and wrote an editorial for the Research Topic from Georgia College. To date, this Research Topic has received over 167,000 views.

Dr. Hegde's research is on how the synapses in the brain change in response to learning which is technically termed "synaptic plasticity." This ability of synapses to change forms the basis of learning and memory. Specifically, Dr. Hegde investigates how targeted protein degradation regulates molecular signaling pathways that are responsible for changing synapses in the brain.

Dr. Hegde has continued his research on synaptic plasticity and has adapted his research project to allow undergraduate participation. He not only brought neuroscience research to our institution but also has developed a highly sought-after Medical Neuroscience course. Since arriving at Georgia College, Dr. Hegde established a neuroscience research program comparable to that seen at research universities. He has enhanced undergraduate research at Georgia College by obtaining a *highly competitive R15 grant* from National Institutes of Health. With this grant, he was able to provide summer stipends for his research students, further enhancing their immersive experiences. His efforts in persuading the university administration to acquire a new Confocal Laser Scanning Microscope has added significantly to the research infrastructure in the Department of Biological and Environmental Sciences and has provided impetus for fellow faculty members to use Confocal Microscopy in their mentored undergraduate research.

In fostering research scholarship in undergraduate students, Dr. Hegde believes in full immersion in projects as a way to better understand the subject at hand. In the lab, he starts the students out on simple projects to learn the experimental techniques, before giving them their own project. When the students participate in preparation of review articles, he gives them the tasks of literature search and collection of relevant papers. He also has the students participate in preparing the response to peer review. Thus, the students actively engage in each step of the research process, and each student takes ownership of the project.

To enhance research experiences of undergraduate students in his laboratory, Dr. Hegde has instituted a two-tiered mentoring system. Dr. Hegde mentors the students on devising experimental strategies and interpreting the results as well giving the students the "big picture." The second tier is peer-mentoring. The students whom he has trained are now training other undergraduate students in the lab. The trained student with experience in the lab mentors the novice on technical aspects of experiment at the laboratory bench. His mentoring style is one that encourages independence.

In summary, Dr. Ashok Hegde's research scholarship is of an exceptionally high caliber and aligns with our mission of mentored undergraduate research experiences. It is of a high quality, it is viable, and definitely productive. It is therefore with great pleasure that I nominate Dr. Hegde and I whole-heartedly support his application for the Georgia College Excellence in Scholarship and Creative Endeavors Award.

Sincerely,

Indiren Pillay, PhD

David Geffen School of Medicine

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February 14, 2020

Dear Faculty Excellence Award Review Committee:

It is my pleasure to write a letter to support Dr. Ashok N. Hegde, as a highly deserving candidate for the Excellence in Scholarship and Creative Endeavors Award at Georgia College and State University (GCSU). Dr. Hegde's research focuses on synaptic plasticity that underlies learning and memory. His work specifically focuses on the role of protein degradation by the ubiquitin-proteasome pathway. In many human diseases such as Alzheimer's, memory loss is the first symptom to be observed. In addition, in Alzheimer's and other neurodegenerative diseases, the proteolysis by the ubiquitin-proteasome pathway is impaired. Therefore, Dr. Hegde's research is important not only from the point of view of basic research but also has significance for translational research that impacts society. I am a Professor in the Department of Physiology, David Geffen School of Medicine at UCLA. I am also a member of Integrative Center for Learning and Memory, Brain Research Institute at UCLA. I have many years of experience in the field of synaptic plasticity and learning and I have published many peer-reviewed papers in this research area. Moreover, I have served as a member and Chair of the Learning and Memory Study Section at NIH's Center for Scientific Review. Therefore, I believe I am qualified to assess the quality and significance of Dr. Hegde's scholarly work.

At GCSU, Dr. Hegde has continued to make important contributions to the field of synaptic plasticity. For example, his research has discovered a new role for the proteasome in regulating signaling to the nucleus. The process of sending signals from the periphery of nerve cells to the nucleus is important for changing the strength of the synapses for the long-term, which forms the basis of long-term memory. The observations made by Dr. Hegde and his students at GCSU has the potential to devise new ways to improve memory. The kind of research he is undertaking at GCSU is comparable to that carried out in research-intensive (R1) institutions.

Dr. Hegde's scholarly/research activity at GCSU has resulted in publications in high impact journals. For example, Frontiers in Neuroscience journals rank at the top in the field of neuroscience based on the number of citations. Neurobiology of Learning and Memory and Learning and Memory are well known journals in our field. His scholarly work is well respected and recognized. Some of the articles he has published from GCSU have already been cited many times. For example, his article "Proteolysis, synaptic plasticity and memory" which was published in 2017 has already been cited 41 times.

Dr. Hegde is training undergraduates to carry out high quality research. He is implementing the best practices in neuroscience undergraduate research training in his laboratory at GCSU. He not only teaches the undergraduate students how to do

experiments but also how to design experiments, and analyze and interpret data. In addition, he trains his students to collect, review and assess scientific literature. He engages his students in all stages of the publication process such as preparation of the manuscript, responding to the comments by the reviewers and so on. Thus, the students under his mentorship gain a sophisticated and realistic understanding of scientific research.

The high quality of Dr. Hegde's research is also evident in the fact that at GCSU, he was able to obtain an R15 grant from the National Institutes of Health. The competition for these grants is severe and even the best applications typically undergo multiple rounds of revision and review before securing funding. Remarkably, Dr. Hegde's R15 proposal was funded on the first attempt.

Dr. Hegde's work on the role of proteolysis in synaptic plasticity is nationally and internationally known. He has taken intellectual leadership in his area of research. For example, he and Dr. Fred van Leeuwen of Maastricht University in Netherlands organized a Research Topic for Frontiers in Molecular Neuroscience on the role of proteolysis in the normal and abnormal nervous system which culminated in the publication of 19 articles which were then published together as an eBook. Dr. Hegde did the work for the publication of this eBook after joining GCSU and wrote the editorial with Dr. Leeuwen.

Dr. Hegde has also contributed to other areas of neuroscience research. After joining his current position at GCSU, he has collaborated and contributed to studies of a gene implicated in schizophrenia (called RGS4) and he provided his expertise in electrophysiology to the research on the effects of dehydration on synaptic plasticity which has implications for understanding cognitive impairment that occurs with aging.

In summary, Dr. Ashok Hegde's research/scholarship at GCSU is outstanding and I recommend him with the greatest enthusiasm for the Excellence in Scholarship and Creative Endeavors Award.

Sincerely,

Thomas J. O'Dell, Ph.D.

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Todd C. Sacktor, M.D.
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The Robert F. Furchgott Center for Neural and Behavioral Science

February 24, 2020

Re: Letter of support for Dr. Ashok Hegde
Excellence in Scholarship and Creative Endeavors Award Committee
Georgia College

To the Committee,

This letter is to endorse, in the strongest possible terms, the nomination of Dr. Ashok Hegde for the Georgia College Excellence in Scholarship and Creative Endeavors Award. Ashok has made significant contributions to the field of synaptic plasticity, focusing on how protein degradation changes the strength of synapses in the nervous system. The ability of the brain to change synaptic strength allows it to form memories. Therefore, elucidating the processes that govern alteration of synaptic strength is of great importance for understanding normal memory, as well as the mechanisms of age-related memory loss and memory impairment that occurs in many brain disorders. Ashok's work centers on how molecules important for memory are selectively tagged with a small protein called ubiquitin, and how these ubiquitin-tagged molecules are degraded by a protein-complex named the proteasome. Thus, his work has made important contributions towards our knowledge of how proteolysis regulates synaptic plasticity.

When Ashok moved to Georgia College in August 2015, to continue a unique Pre-Med Mentoring Program, it was a change in direction for his career. The new position offered him possibilities as well as challenges. He had the opportunity to think about research questions and pursue science as a scholarly pursuit while training students to carry out laboratory research. He has not only adapted well to his new environment, but he is enriching it.

On arriving at Georgia College, Ashok applied for an R15 grant from the National Institutes of Health (NIH). Based on my many years of experience as a physician-scientist with NIH grants (in applying for such grants as well as in reviewing them), I know that grants from the NIH are not easy to obtain — usually only about the top 10% of the grant applications are funded. These grants are awarded based on merit as assessed in a peer-review process by a committee whose members are drawn from various universities and research institutions around the country. In addition, for an R15 grant, an applicant has to compete with undergraduate institutions with a long history of NIH-funded research, including

Bowdoin College in Maine, Oberlin in Ohio, and Davidson in North Carolina. In order to rise to the top, the science in a grant application has to be exceptional/outstanding. Ashok's R15 grant application from Georgia College was funded on the first submission. Because Ashok's effort has made research by his undergraduate students nationally competitive, increased recognition has been brought to Georgia College.

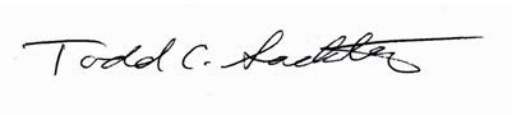
In addition, since joining Georgia College, Ashok has published several peer-reviewed articles in high-impact journals. For example, *Frontiers in Neuroscience* journals are cited more than any other journals in Neuroscience. The journal *Frontiers in Aging Neuroscience* ranks 5th among all open access neuroscience journals and 4th among the Geriatrics and Gerontology journals based on citations. In addition to citations, a currently used metric to assess the efficacy of dissemination is to look at the views an article gets when published in an open-access journal. Ashok's articles consistently register numerous views. For example, his paper in *Frontiers in Psychiatry* has over 4100 views. His latest peer-reviewed article with Georgia College undergraduates as co-authors is published in *Frontiers in Aging Neuroscience.* This article is noteworthy in terms of its reach. Within two months of publication, it has racked up more than 1700 views.

Ashok's approach to science is deep and well-thought. This is reflected in his style of research. He has devoted his scientific career to understanding the role of ubiquitin-proteasome-mediated protein degradation. Since joining Georgia College, he has published his initial findings on a new role for the protein degradation in the regulation of signals sent to the nucleus for gene transcription, which eventually change the strength of synapses. The new roles for the proteasome that he is discovering will enhance our understanding of the role of protein degradation and how it interacts with protein synthesis. This work has significance for understanding how the threshold for long-term synaptic plasticity and memory is determined, which in turn has potential practical applications for improving memory.

Apart from his research contributions, Ashok has taken leadership in terms of dissemination of ideas and findings in his field of research. He organized a Research Topic called "Ubiquitin and the Brain: Roles of Proteolysis in the Normal and Abnormal Nervous System." Several papers from scientists all over the world were published under this Research Topic. Ashok, along with his fellow Editor, Fred van Leeuwen, oversaw the peer-review of the articles. He and Fred then wrote an editorial to give an overview of the Research Topic, which was then published as an eBook. This Research Topic has over 167,000 views thus far.

In conclusion, Ashok's scholarly approach to neuroscience research is highly impressive. He is a candidate of great merit for the Excellence Award in Scholarship and Creative Endeavors, and I recommend him without hesitation for the Award.

Sincerely yours,



Todd Charlton Sacktor, M.D.
SUNY Distinguished Professor of Physiology & Pharmacology
Anesthesiology, and Neurology

PUBLICATIONS (evidence of each is included)

1. Hegde, A.N., Smith, S.G., Duke, L., Pourquoi, A, and Vaz, S. (2019) Perturbations of Ubiquitin-Proteasome-Mediated Proteolysis in Aging and Alzheimer's Disease. *Front. Aging Neurosci.* 11: 324

- Review Process: Anonymous peer-review; reviewers' names revealed at the time of publication.
- Selectivity and Competitiveness: Impact Factor (2017): 4.504

  Frontiers in Aging Neuroscience is the 5th most cited open access journal in Neurosciences. It is also the 4th most cited Geriatrics & Gerontology journal in the world.
- Dissemination: This article was published on December 6, 2019. It has had 1,815 views (as of February 24, 2020).
- Scope: *Frontiers in Aging Neuroscience* is a leading journal in its field, publishing rigorously peer-reviewed research that advances our understanding of the mechanisms of Central Nervous System aging and age-related neural diseases.

2. Hegde, A. N. and Smith, S. G. (2019). Recent developments in transcriptional and translational regulation underlying long-term synaptic plasticity and memory. *Learn. Mem.* 26:307-317.

- Review Process: Anonymous peer-review.
- Selectivity and Competitiveness: Impact Factor (2016): 2.894
- Scope: *Learning & Memory* welcomes high-quality original work covering all areas of neuronal plasticity, learning, memory, and their models, conducted in humans and other vertebrate and invertebrate species with the following approaches: behavior, cognition, computation, neuroanatomy, neurophysiology, neuropharmacology, biochemistry, genetics, and cell and molecular biology. The journal publishes Research Papers and Brief Communications, as well as commissioned Reviews and Commentaries.

3. Vashisht A., Bach S.V., Fetterhoff D., Morgan J.W., McGee M. and **Hegde A.N.** (2018). Proteasome limits plasticity-related signaling to the nucleus in the hippocampus. *Neurosci Lett*. 687:31-36.

- Review Process: Anonymous peer-review.
- Selectivity and Competitiveness: Impact Factor (2018): 2.173
- Scope: *Neuroscience Letters* is devoted to the rapid publication of short, high-quality papers of interest to the broad community of neuroscientists. Papers in all areas of neuroscience - molecular, cellular, developmental, systems, behavioral, cognitive, and computational - will be considered for publication.

4. Vashisht, A., Morykwas, M., **Hegde, A.N.,** Argenta L. and McGee, M.P. (2018). Age-dependent changes in brain hydration and synaptic plasticity. *Brain Res.* 1680:46-53.

- Review Process: Anonymous peer-review.
- Selectivity and Competitiveness: Impact Factor (2017): 3.125; *Brain Research* is Ranked 44th among the 149 Neuroscience journals which puts it in the top 30%.
- Scope: *Brain Research* publishes papers reporting interdisciplinary investigations of nervous system structure and function that are of general interest to the international community of neuroscientists.

5. Hegde, A.N. and van Leeuwen, F.W. (2017) Editorial: Ubiquitin and the Brain: Roles of Proteolysis in the Normal and Abnormal Nervous System. *Front. Mol. Neurosci*. 10:220.

- Review Process: Review by the Editors of the journal.
- Selectivity and Competitiveness: Impact Factor (2017): 5.076. Based on the Impact Factor, the journal ranks among the top 17% of the journals. Frontiers in Neuroscience journal series is the most cited in the Neurosciences.
- Dissemination: This editorial was written to introduce a Research Topic which has had 168,294 views (as of February 24, 2020). It was then published as an eBook.

  https://www.frontiersin.org/research-topics/1934/ubiquitin-and-the-brain-roles-of-proteolysis-in-the-normal-and-abnormal-nervous-system
- Scope: *Frontiers in Molecular Neuroscience* publishes rigorously peer-reviewed research that aims to identify key molecules underlying the structure, design and function of the brain across all levels.

6. Hegde, A. N. (2017). Proteolysis, synaptic plasticity and memory. *Neurobiol. Learn. Mem.* 138:98-110.

- Review Process: Anonymous peer-review.
- Selectivity and Competitiveness: Impact Factor (2017): 3.244
- Dissemination: This article has been cited 41 times.
- Scope: *Neurobiology of Learning and Memory* publishes articles examining the neurobiological mechanisms underlying learning and memory at all levels of analysis ranging from molecular biology to synaptic and neural plasticity and behavior. The journal is especially interested in manuscripts that examine the neural circuits and molecular mechanisms underlying learning, memory and plasticity in both experimental animals and human subjects.

Continued on next page

7. Hegde A. N. (2017) Proteolysis and Synaptic Plasticity. In: *Molecular Mechanisms of Memory* (Sara S., ed.), *Learning and Memory: A Comprehensive Reference*, 2nd Edition (Byrne, J., ed. [4 vols.]), Oxford: Academic Press. vol 4, pp 257-277.

- Review Process: Reviewed by the Section Editor and then by Editor-in-Chief.
- Selectivity and Competitiveness: Impact Factor: Not applicable. However, according to Google Scholar, this book has 70 citations. Academic Press is an imprint of Elsevier, a leading Science Publisher.

  "*Learning and Memory: A Comprehensive Reference, Second Edition* is the authoritative resource for scientists and students interested in all facets of learning and memory. This updated edition includes chapters that reflect the state-of-the-art of research in this area."

  Link to Publisher's website:

  https://www.elsevier.com/books/learning-and-memory-a-comprehensive-reference/byrne/978-0-12-805159-7

8. Bach, V. and **Hegde, A. N.** (2016). The Proteasome and Epigenetics: Zooming in on Histone Modifications. *Biomol. Concepts* 7: 215-227.

- Review Process: Anonymous peer-review.
- Selectivity and Competitiveness: Impact Factor (2018): 4.46
- Dissemination: This article has had 13 citations.
- Scope: *BioMolecular Concepts* is a peer-reviewed open access journal fostering the integration of different fields of biomolecular research. The journal aims to provide expert summaries from prominent researchers, and conclusive extensions of research data leading to new and original, testable hypotheses.

9. Ding, L., Styblo, M., Drobná Z. and **Hegde, A.N.** (2016). Expression of the Longest RGS4 Splice Variant in the Prefrontal Cortex Is Associated with Single Nucleotide Polymorphisms in Schizophrenia Patients. *Front.Psychiatry* 7:26.

- Review Process: Anonymous peer-review; reviewers' names revealed at the time of publication.
- Selectivity and Competitiveness: Impact Factor (2016): 3.532
- Dissemination: This article has had 4,110 views (as of February 24, 2020).
- Scope: *Frontiers in Psychiatry* publishes rigorously peer-reviewed research across a wide spectrum of translational, basic and clinical research.

Note: (1) The Impact Factor (IF) of an academic journal is a metric that is related to the average number of citations that articles published in that journal receive. The higher the IF, the greater the selectivity and bigger the impact of the journal. There are usually slight variations from year-to-year and hence the year is shown next to the IF in the above list. To put things in perspective, an IF of 5.076 (#5 in the list) put the journal in the top17%. An IF of 3.125 (#4 in the list) puts the journal in the top 30%; **(2)** The scope of a journal is from the journal's website; **(3)** Where available, the number of views or citations for a paper is provided under 'Dissemination.'

When an undergraduate researcher shows me the results of her latest experiment, I smile in pure delight. Not just because the nerve cells decked in red and green fluorescent colors are a sight to behold and our hypothesis is correct, but mainly because of the gleam in the student's eye. I let her savor the moment because it is hard earned through painstaking and systematic experimentation. At Georgia College, I not only experience the joy of science but also the joy of teaching science. There is something deeply satisfying about mentoring students to ask precise scientific questions, to design and execute experiments and to analyze data. I train and prepare students to conduct rigorous neuroscience research as well as to become peer-mentors to the new students who join my lab. My scholarship is not only integrated with **Research Mentoring** but also with my **Teaching in the Classroom**.

Research Mentoring

Our research addresses the question of how the brain stores information for a long time. Memory for things, places and events can last for weeks, years, and even for a life-time. The basic process is that the sensory input changes the strength of the connection between nerve cells called the synapses. The ability of the brain to change the strength of synapses is called "synaptic plasticity" which is what allows the brain to form memory. My students study what happens in the brain at the molecular and cellular level when it stores information for the long-term.

The specific focus of our research is how regulated protein degradation contributes to the formation of long-term memory. My research students at Georgia College and I have discovered a new mechanism by which some proteins belonging to the protein degradation machinery can help turn on genes that are important for making synapses stronger. Our findings have broad implications for understanding normal memory as well as memory impairment that occurs in neurodegenerative diseases such as Alzheimer's. By manipulating protein degradation selectively near synapses, we can enhance synaptic strength. This can potentially yield methods to improve memory. Our initial studies have revealed that these methods can also rescue synaptic malfunction that occurs in Alzheimer's disease. A future goal is to develop reagents to rescue memory loss in Alzheimer's disease model mice which will have the potential to translate to therapy for human patients.

At Georgia College, I have had the pleasure of recruiting highly motivated and bright students for research through my classes and the Pre-Med Mentoring Program that I run. I have striven to give them the best possible research experience and to raise the profile of our undergraduate research. For example, I received an *extremely competitive* R15 grant ($381,367) from the National Institutes of Health, the first of its kind for Georgia College. A key aspect of R15 is supporting participation of undergraduates in high quality research. Through my efforts, our Department has obtained a Confocal Laser Scanning Microscope which allows students to do cutting-edge research. My approach in engaging undergraduates has been quality rather than quantity. Even so, I have trained 10 undergraduates during the last four years in laboratory research techniques. Some of the promising ones have stayed in the lab and have pursued their own research projects and are authors on a recent publication.

My research students share my fascination for neuroscience which is said to the be the final frontier in biology. While we are a small part of this vast field, my students appreciate that we are addressing important and interesting research questions. I have my students actively participating in gathering research data as well as in bringing the manuscripts to publication. When their names appear in international publications with Georgia College affiliation, they are contributing in their

own way toward pre-eminence of our institution. I take great pride in the accomplishments of my students who are my collaborators in the pursuit of exciting research problems in neuroscience.

Teaching in the Classroom

It was heartening when a pre-med student told me that she was inspired to pursue a career in neurology because of my *Medical Neuroscience (BIOL 4950)* course. I think the students like my courses because I bring my background as a scientist/researcher into all aspects of my teaching to enable them to be better learners and to help them succeed. My research specialty is learning and memory which I use to help students to study properly and retain the subject. At the beginning of the course, I explain to them how attention works and how the brain absorbs and retains information. Neuroscience research has shown that spaced learning is better than massed learning. I make sure my students understand how to implement this principle in their plan of study.

Because of my research background in learning and memory, I am naturally enthused to adopt techniques and technologies that help the students to be active learners. For example, I frequently ask questions in my classes using Poll Everywhere which allows students to answer anonymously using their cell phones. I also assign tasks of "thought experiments." For example, another course that I teach, *Medical Physiology (BIOL 4950)* also has a good bit of neuroscience in it. In this class, I divided the students into two groups and each group had to redesign a molecule important for communication between nerve cells. In the brain, communication occurs by one nerve cell sending an Action Potential which is a jolt of tiny current flowing through its cable-like axon that connects it to another nerve cell. The Action Potential is an all-or-none phenomenon, meaning either it is "on" or "off." The "on" switch is the opening of the sodium channel (which is a protein). The "off" switch is triggered by opening of the potassium channel (which is also a protein). The first group had to theoretically design a sodium channel molecule to alter the properties of the Action Potential, for example its duration. The second group had to do a similar exercise to tweak the potassium channel molecule. Each group had to brainstorm ideas and figure out how to do the assigned task. Then they talked to the whole class about it. The students loved this exercise. In the Student Rating of Instruction Results, among other laudatory comments was the following: *"I really like the in−class practice questions. Great class."*

My expertise as a scientist helps me enhance other courses that I teach as well. Prior to switching to neuroscience at the post-doctoral level, I did research in molecular biology/cell biology. I bring this experience to bear when I teach *Cellular and Molecular Physiology (BIOl 3200)* and explain to students "how we know what we know." I have added histopathology to my *Histology (BIOL 4140)* class which came from my research interest in neurodegenerative diseases.

As part of the Pre-Med Mentoring Program, I teach *Seminar in Medical Culture (BIOL 3750)* which has an "active-learning" format. As part of this course, I do Clinical Case Studies. The students are given clinical information bit by bit as if a patient is coming to an emergency room or an outpatient visit. The students have to come up with *plausible hypotheses* as to the patient's condition. The goal here is to scientifically understand a disease and its treatment. The students love this approach because they get to play "medical detectives."

I use my scientific and research experience in my course design and other aspects of pedagogy. In all my courses, I strive to teach students information literacy through assignments such as writing a scientific review paper. In my classes, I encourage my students to understand the subject matter by asking questions which is a hallmark of scientific inquiry. Thus, my research scholarship plays an integral role in my classroom teaching.

frontiers in Aging Neuroscience

MINI REVIEW
published: 06 December 2019
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Perturbations of Ubiquitin-Proteasome-Mediated Proteolysis in Aging and Alzheimer's Disease

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The ubiquitin-proteasome pathway (UPP) has multiple roles in the normal nervous system, including the development of synaptic connections and synaptic plasticity. Research over the past several years has indicated a role for the UPP in aging without any overt pathology in the brain. In addition, malfunction of the UPP is implicated in Alzheimer's disease (AD) and dementia associated with it. In this mini review article, we assess the literature on the role of protein degradation by the UPP in aging and in AD with special emphasis on dysregulation of the UPP and its contribution to cognitive decline and impairment.

Keywords: memory, learning, tau, amyloid beta, protein degradation, cognitive impairment, synaptic plasticity

OPEN ACCESS

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INTRODUCTION

Ubiquitin has long been known to be associated with pathologies of the brain, including that of Alzheimer's disease (AD; Ciechanover and Brundin, 2003). Our understanding of the link between ubiquitin-mediated proteolysis and neurodegenerative diseases such as AD, however, has only begun to improve with the elucidation of the mechanistic details of protein degradation. Although proteolysis by the ubiquitin-proteasome pathway (UPP) was originally assumed to operate only on abnormal proteins, research over many years has shown physiological roles for the UPP in various cells, including neurons. In the UPP, ubiquitin attachment to lysine residues in substrate proteins occurs through a series of enzymatic reactions in a highly regulated manner. Additional ubiquitin molecules are attached to the first ubiquitin to form a polyubiquitin chain. The polyubiquitinated protein is degraded by the proteasome (Glickman and Ciechanover, 2002).

In this mini review article, we provide a brief overview of the research on the UPP in the context of normal aging as well as AD and examine the effect on cognition wherever possible.

UPP AND THE AGING BRAIN

Several cellular functions are altered with aging. It is reasonable to hypothesize that ubiquitin-proteasome-mediated proteolysis is also impaired with aging. Investigations, however, have not yielded consistent results. For example, proteolysis by the UPP was tested in mice of different ages using GFP-reporter mice (Cook et al., 2009) that expressed GFP along with a short amino acid sequence (ACKNWFSSLSHFVIHL), originally identified in yeast, that serves as a signal for degradation by the UPP (Gilon et al., 1998). The authors used organotypic hippocampal culture and

whole-brain sections and found no significant changes in the amount of GFP from 6 to 18 months of age. It is not clear whether the degradation of the GFP-reporter protein mimics the degradation of endogenous substrates of the UPP. In addition, this study used MG-132, which is not a highly specific proteasome inhibitor (Cook et al., 2009).

Another study tested the capacity of the 26S proteasome to degrade polyubiquitinated substrates in the cortex, hippocampus, and cerebellum of 3-week-old (young) and 24-month (old) rats and found no impairment in degradative capacity with aging. Additionally, despite age-dependent augmentation in overall cellular protein content, there were no increases in the quantity of the proteasome as measured by rocket immunoelectrophoresis (Walker, 1984). Therefore relative to total cellular protein, the percentage of the proteasome was lower in brain regions of the old rats (19% less in cortex, 31% less in the cerebellum, and 37% less in the hippocampus) compared to corresponding regions in the brains of young rats (Giannini et al., 2013). When considered in relation to human aging, the rat model at 24 months is slightly younger compared to the mouse model at 18 months. This is because the average lifespan of rats is 36 months, and that of mice is 24 months, and thus an 18-month-old mouse is equivalent to a 60-year-old human whose lifespan is 80 years (Sengupta, 2013; Ackert-Bicknell et al., 2015).

Genetic experiments using *Drosophila*, however, found a decrease in 26S proteasome function with increasing age as measured in whole flies or fly heads. This study was based on the identification of Rpn11 (a subunit of the proteasome) as a suppressor of age-related neurodegeneration. The amount of Rpn11 was decreased at an age where memory impairment is seen in flies (*Drosophila* lifespan is 60–70 days; 20–30 days post eclosion is considered "old age"; Tonoki et al., 2009).

Other investigations tested specific questions with respect to aging, such as susceptibility to intermittent hypoxia. One study tested proteasome activity and CREB phosphorylation along with making behavioral assessments. With intermittent hypoxia, CREB phosphorylation and proteasome activity decreased, and spatial learning was impaired. All these effects were more pronounced in aged rats compared to young (Gozal et al., 2003).

The relationship between the UPP and aging in the nervous system has also been investigated with respect to ubiquitin-conjugating enzymes. For example, a ubiquitin ligase called mahogunin (Mgrn1) is mostly cytoplasmic in hippocampal neurons. With aging, much of Mgrn1 is found in the nucleus, where it associates with transcriptionally active regions to induce expression of genes critical for coping with a reduction in proteolytic activity (Benvegnù et al., 2017).

A molecule critical for synaptic plasticity and thus cognition, Arc, has also been investigated with respect to aging. Arc is controlled at multiple levels, including transcription and ubiquitin-mediated degradation. In aged rats (24 months), the basal level of Arc is increased, and this was thought to be the result of decreased degradation. Consistent with this idea, levels of Ube3a, the ubiquitin ligase that targets Arc for degradation, is decreased in the hippocampus of aged rats (Fletcher et al., 2014).

THE UPP AND AD

AD usually affects people 65 years or older, although early-onset familial forms do occur. Much of the patient population falls under the "sporadic" category, in which the exact cause of the disease remains uncertain. AD begins with mild cognitive impairment and, as the disease progresses, patients suffer from severe cognitive defects. At later stages, brain pathologies with plaques and tangles are observed. It is generally accepted that two main types of pathological phenomena occur in the AD brain. One is the accumulation of amyloid β (Aβ), the clumps of which lead to the development of plaques. The second is the accumulation of phosphorylated microtubule-associated protein tau, which ultimately forms tangles. The UPP is linked to both of these pathways of AD pathogenesis (**Figure 1**).

Inhibition of the Proteasome in AD

In AD, ubiquitinated proteins accumulate, and it is believed that the proteolytic system in neurons is overwhelmed by aggregating proteins. Based on this logic, investigations were made of the proteasome in both postmortem human AD brains and in the brains of AD model mice. To help understand the results of these experiments, a brief description of the structure of the proteasome is necessary.

The main proteolytic complex that degrades the polyubiquitinated proteins is the 26S proteasome, comprising a cylindrical 20S catalytic core and a 19S regulatory particle (RP) attached on either side of the 20S cylinder. The function of the 19S RP is to recognize and bind the polyubiquitinated substrate proteins, unfold them, and channel them into the narrow opening (13 Å) of the catalytic core for degradation. The catalytic core consists of seven α subunits in two outer rings and seven β subunits in the two inner rings of the cylindrical shape. The 20S core has trypsin-like, chymotrypsin-like, and postglutamyl peptidase activities (Hegde, 2004).

Some of the studies of postmortem AD brains measured the catalytic activities of the 20S proteasome. One study that obtained postmortem brains within ∼2.5 h of autopsy found a decrease in chymotrypsin-like and postglutamyl peptidase activities in the parahippocampal gyrus, superior and middle temporal gyri, and inferior parietal lobule. This study found no change in the overall quantities of the α and the β subunits of the 20S proteasome (Keller et al., 2000).

The proteasome is also inhibited in the brains of AD model mice. For example, in mice carrying mutant amyloid precursor protein (APP) transgene (Tg2576), $A\beta_{(1-42)}$ accumulate in neurons, which adversely affects the sorting of membrane receptors [such as the epidermal growth factor receptor (EGFR) and TrkB receptor] through the multivesicular body (MVB). The MVB pathway is critical for the degradation of the endocytosed plasma membrane receptors, which can occur through the lysosome or the proteasome. Investigation of the EGFR, using cortical and hippocampal neuronal cultures from Tg2576 mice and wildtype littermates, found that both ubiquitination and proteasome-mediated degradation of the receptor are impaired in neurons of APP mutant

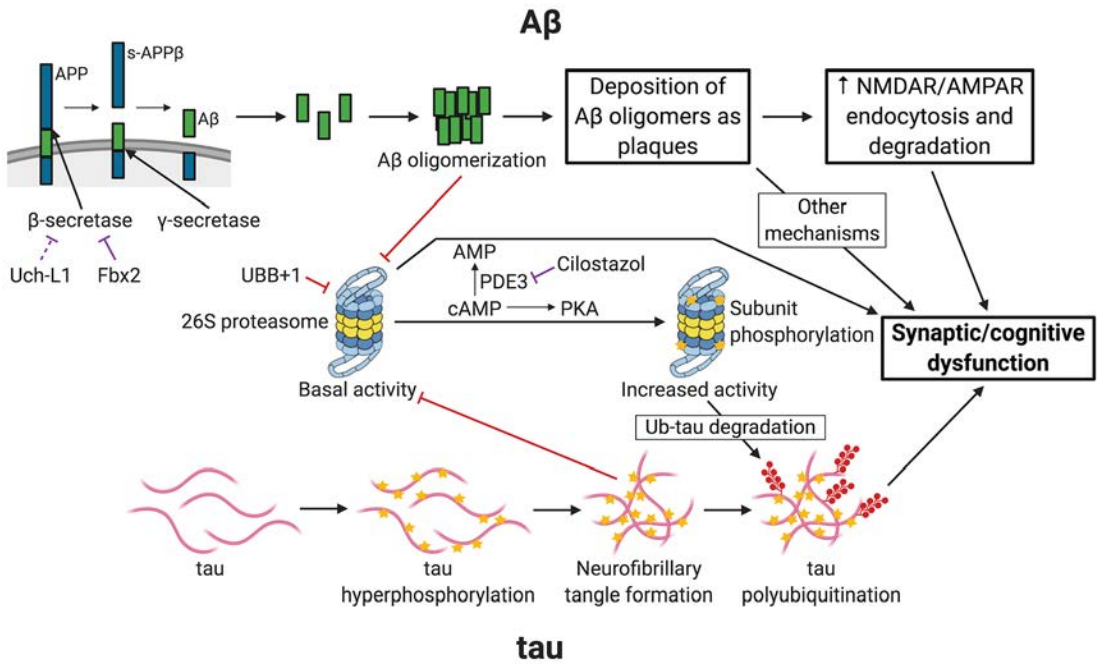

FIGURE 1 | Links between Alzheimer's disease (AD) and ubiquitin-proteasome-mediated degradation. The figure depicts the pathogenesis of AD and the resultant synaptic/cognitive dysfunction *via* Aβ and tau, the two main postulated mechanisms of AD. The connections between the ubiquitin-proteasome pathway (UPP) and the components of the two pathways are indicated. Only key relevant features of AD pathogenesis are shown. Processes with a purely inhibitory effect are shown by red connectors. Negative regulatory mechanisms with potential for therapeutic intervention are shown by purple connectors. APP, amyloid precursor protein; s-APPβ, soluble N-terminal APP fragment; PDE3, phosphodiesterase 3; PKA, Protein Kinase A (cAMP-dependent protein kinase); Ub-tau, tau with attachment of many ubiquitin (Ub) molecules (i.e., polyubiquitinated tau); stars indicate phosphorylation; shapes with filled red circles with tails represent polyubiquitin attachment. Created with biorender.com.

mice. The study also showed that proteasome activity in the neurons of mutant mice was reduced by about 50% (Almeida et al., 2006).

Other studies also support the idea of proteasome inhibition by $A\beta_{(1-42)}$. When cultured neurons were treated with $A\beta_{(1-42)}$, the peptide was found to enter the cells and inhibit proteasome activity as measured by the amount of a GFP-fused reporter construct. The same study also found decreased chymotrypsin-like activity of the proteasome in the hippocampus and cortex of AD model (Tg2576) mice (Oh et al., 2005). An investigation using triple transgenic AD model mice carrying PS1 (M146V), APP (Swe), and tau (P301L) transgenes showed that between 3 and 15 months of age, all three activities of the proteasome decreased in the brains of these mice. Additionally, when pre-pathological hippocampal slices from 3x-Tg mice were maintained in organotypic culture and were treated with the proteasome inhibitor epoxomycin, tau accumulation increased in neurons. Furthermore, the injection of epoxomycin into cerebral ventricles in 4-month-old 3x-Tg-AD mice increased Aβ and tau in the CA1 neurons of the hippocampus, as revealed by immunohistochemistry (Tseng et al., 2008). Earlier studies showed that in the 3x-Tg mice, LTP is reduced at 6 months of age before the development of plaques and tangles. Given that proteasome activity is impaired from 3 months in these mice (Oddo et al., 2003), it is possible that proteasome inhibition contributes to synaptic dysfunction in them.

Immunoproteasome and AD

The activity of the 20S core of the proteasome can be modulated by substitution of specific subunits. For example, in response to interferon-γ signaling, three subunits, β1, β2, and β5, can be substituted with β1i, β2i, and β5i to enable antigen presentation in the immune system (Hegde, 2004). This type of 20S is called the "immunoproteasome," which is also found in the brain. A study found an increase in the immunoproteasome in AD brains compared to the brains of the nondemented elderly. Additionally, polymorphisms in a substituted protein (Lmp2 aka β1i) in codon 60 (R/R or R/H) were found. Unaffected brain areas in AD patients with the R/R genotype showed increased proteasome activity compared to corresponding brain areas of AD patients with the R/H genotype (Mishto et al., 2006). Immunoproteasome activity is also increased in reactive glia surrounding plaques in AD model mice (APPswePS1dE9; Orre et al., 2013).

Enzymes of the UPP and AD

The enzymes of the UPP also interact with the pathological processes mediated by Aβ. For example, expression of a ubiquitin-conjugating enzyme, E2-25K/Hip-2, was upregulated when cultured neurons were exposed to Aβ. In addition, E2-

25K/Hip-2 activity was required for proteasome inhibition and for Aβ-mediated neurotoxicity (Song et al., 2003). Later studies found that in AD model mice, E2-25K/Hip-2 stabilizes Caspase-12 by inhibiting the proteasome and the active Caspase-12 mediates ER-stress-induced cell death. Cells that lack E2-25K/Hip-2 are resistant to Aβ-mediated neurotoxicity (Song et al., 2008).

Decrease in a deubiquitinating enzyme, Uch-L1, has been shown to increase the amounts of BACE1 (β-secretase 1), which in turn causes a rise in the levels of Aβ, as has been shown with experiments using AD model mice (5xFAD Tg mice; Guglielmotto et al., 2017).

LINK BETWEEN THE UPP AND TAUOPATHY

Accumulation of neurofibrillary tangles because of hyperphosphorylated tau is a key pathological process in AD as well as in some other diseases of the nervous system such as frontotemporal degeneration and is referred to as "tauopathy." Several studies have established that tau is a target of the UPP, and deficits in this pathway might play a role in the etiology of AD.

Previous studies found that proteasome activation by rolipram—a phosphodiesterase 4 (PDE4) inhibitor—decreased tau levels and improved cognition. Subsequently, an FDA-approved PDE3 inhibitor called cilostazol was tested on double transgenic rTg4510 mice overexpressing human mutant (P301L) tau. Experimental mice received intraperitoneal injections of cilostazol twice a day for 30 days, which significantly reduced disease-associated phosphorylated tau forms and the insoluble tau forms (Schaler and Myeku, 2018).

How does cilostazol exert its effect? Cilostazol administration increased the hydrolyzing activity of the proteasome, as shown by fluorogenic substrate kinetic analysis. Immunoblots revealed that the cilostazol-mediated increase of proteasome function was due to phosphorylation of serine and threonine residues by protein kinase A on multiple proteasome subunits. The levels of the proteasome subunits did not change (Schaler and Myeku, 2018).

Activation of the proteasome and the decrease in insoluble forms of tau seemed to ameliorate the cognitive impairment associated with tauopathy. When the rTg4510 mice were subjected to spatial learning testing using the Morris Water Maze (MWM), it was found that cilostazol administration significantly improved the cognitive performance as manifested by reduced escape latency in the MWM (Myeku et al., 2016). Another study showed that PKA-mediated phosphorylation of Rpn6 (part of 19S RP) can increase the activity of the proteasome and boost the degradation of misfolded proteins (Lokireddy et al., 2015).

ASSOCIATION BETWEEN Aβ, THE UPP, AND GLUTAMATE RECEPTORS CRITICAL FOR SYNAPTIC PLASTICITY

Studies investigating the possible connection between Aβ and the UPP have focused on the effect of Aβ on surface expression of NMDA and AMPA receptors. Because cognitive impairment precedes overt pathology in AD and Aβ oligomers cause synaptic dysfunction and memory impairment in AD model mice, it was logical to investigate the glutamate receptors critical for synaptic plasticity.

An investigation using cultured cortical neurons from APP_{Swe} mice and wildtype littermates showed decreased surface expression of NR1 NMDA receptors and reduced signaling to CREB. Also, Aβ was found to activate the tyrosine phosphatase STEP. Later studies found a connection between the UPP and internalization of NMDARs *via* tyrosine phosphatase striatal-enriched protein tyrosine phosphatase 61 (STEP61). Enhanced activity of STEP61 causes decreased surface expression of NR1 and NR2B subunits. STEP61 dephosphorylates a critical tyrosine residue (Tyr-1472) in NR2B subunits, leading to increased endocytosis of NR subunits. Normally, ubiquitin-proteasome-mediated degradation of STEP61 keeps the internalization of NR subunits in check. An increase in Aβ increases STEP61 activity and inhibits the degradation of STEP61. Thus, the overall effect is greatly enhanced STEP61 activity and augmented endocytosis of NMDAR, which leads to synaptic deficits (Kurup et al., 2010).

Previous reports have shown that AMPA receptor synaptic accumulation, trafficking, and turnover are altered in animal models of AD and human patients (Guntupalli et al., 2017). A mechanism responsible for the regulation of AMPA receptors is its proteolysis by the UPP. Specifically, AMPA receptor degradation is mediated by the E3 ligase Nedd4 and deubiquitinating enzyme USP46 (Lin et al., 2011; Huo et al., 2015). *Usp46* mutant mice exhibit alterations in cognitive behaviors (Tomida et al., 2009).

In a recent study, researchers exposed cultured mouse cortical and hippocampal neurons to Aβ and silenced Nedd4 with siRNAs. Aβ exposure for 24 h and 48 h elicited an overall reduction in the AMPA GluA1 subunit by 28% and 53%, respectively. Moreover, the expression of this subunit was significantly reduced in the dendrites of hippocampal neurons as a result of increased internalization (Zhang et al., 2018).

OTHER MODES OF CONNECTIONS BETWEEN THE UPP AND AD

Impairment in the UPP appears to contribute to AD pathology in ways that are not apparently connected to Aβ- or tau-mediated processes. This is evident in the case of frameshift mutation during the transcription of the ubiquitin-B gene, which results in UBB mRNA with the +1 frame (UBB+1). The UBB+1 protein has 20 extra amino acids at the C-terminus compared to normal ubiquitin. Cells can tolerate low levels of UBB+1 protein, but at high levels, UBB+1 inhibits the proteasome. Dysfunction of the proteasome in UBB+1 transgenic mice results in a proteomic profile that is similar to that of human AD brains and brains of several AD mouse models (Fischer et al., 2009). These mice show spatial memory deficits in a water maze as well as contextual fear conditioning (van Tijn et al., 2011).

MANIPULATING THE UPP TO AMELIORATE AD

Improving the function of UPP components should, in principle, ameliorate some of the symptoms of AD (Gong et al., 2016). Because synaptic dysfunction and cognitive impairment are seen early in AD and the UPP has a role in synaptic plasticity and memory, it might be possible to manipulate the UPP to rescue some deficits. One such attempt was made through the administration of Uch-L1 in a mouse model of AD. This was based on the earlier finding that Uch-L1 plays a role in long-term synaptic plasticity (Hegde et al., 1997). In the wildtype mice, Uch-L1 function was required for inducing hippocampal LTP. In AD model mice (APP/PS1), Uch-L1 function was reduced and could be restored by administering exogenous Uch-L1 protein. Exogenous Uch-L1 also restored LTP in APP/PS1 mice. Moreover, intraperitoneal injection of Uch-L1 fused with the transduction domain of HIV Tat protein (to render it membrane permeant and to allow it to cross the blood-brain barrier) improved contextual memory in APP/PS1 mice (Gong et al., 2006). Overexpression of Uch-L1 slows AD progression in APP/PS45 mice, providing additional evidence for the role of Uch-L1 (Zhang et al., 2014).

Another example is enhancing the degradation of a substrate by manipulating the ubiquitin ligase responsible for targeting it for degradation. BACE1 is a key enzyme in the production of Aβ. BACE1 is degraded by the UPP and is mediated by an SCF ligase. The substrate-binding protein in SCF ligases is the F-box protein. An F-box protein called Fbx2 was found to interact with BACE1. Overexpression of the Fbx2 protein through adenovirus-mediated delivery into neurons derived from AD model mice (Tg2576) reduced the expression of BACE1 and decreased Aβ production. When Fbx2 was overexpressed in the hippocampus of the Tg2576 mice at 12–14 months of age, LTP significantly improved relative to controls. Therefore, reducing BACE1 can potentially improve synaptic dysfunction caused by Aβ (Gong et al., 2010).

TARGETING COMPONENTS OF THE UPP FOR POTENTIAL THERAPEUTIC APPLICATION

Manipulating the components of the UPP might be beneficial in curtailing pathological development of AD at various stages. Generation of Aβ can potentially be reduced by activating the substrate-interacting component of E3 ligase (Fbx2) that degrades BACE1 (**Figure 1**). This can, in principle, be done by using small molecules. For example, chemicals that can selectively activate Uch-L1 could reduce the generation of Aβ. Administration of chaperones such as C-terminus of Hsc 70-interacting protein (CHIP) complexed with the heat-shock protein Hsc 70 can stimulate degradation of ubiquitinated tau (Petrucelli et al., 2004; Shimura et al., 2004; Sahara et al., 2005). Also, the design of molecules that increase the activity of the 26S proteasome directly or indirectly (for example, by stimulation of its phosphorylation) could help develop drugs that stimulate degradation of polyubiquitinated tau and enable the proteasome to overcome inhibition by Aβ.

FUTURE DIRECTIONS

Based on the research thus far, there is no clear-cut relationship between aging and impairment of the proteasome function. When individual molecules are studied (e.g., mahogunin), however, a clearer picture emerges.

In investigating the connections between the UPP and AD, many studies have focused on transgenic mouse models of AD based on the familial form of the human disease. These models were mainly based on the "Aβ hypothesis" of AD (Mullane and Williams, 2019). Many clinical trials aimed at reducing Aβ levels have not yielded any beneficial outcome for patients except for some encouraging results with drugs that target Aβ oligomers (Panza et al., 2019). There is an increasing realization in the field that alternative hypotheses need to be pursued (Morris et al., 2018). Because the link of the UPP to AD is not just through Aβ, it would be worth investigating how the UPP relates to other factors contributing to AD such as insulin resistance and inflammation in the brain. The development of animal models that recapitulate the sporadic form of the disease which affects most AD patients will be conducive to testing the role of the UPP by itself and in relation to other clearance mechanisms such as autophagy. While we await the development of models for sporadic AD, it might be worthwhile to study postmortem brain samples of patients with sporadic AD as well as neurons generated from patient-derived induced pluripotent cells to test alterations in key components of the UPP. Studies along these lines might be fruitful in years to come.

AUTHOR CONTRIBUTIONS

AH, SS, LD, AP and SV contributed to the search and assessment of the available literature. AH wrote the manuscript and other authors helped revise the text to the final form.

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Review

Recent developments in transcriptional and translational regulation underlying long-term synaptic plasticity and memory

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Formation of long-term synaptic plasticity that underlies long-term memory requires new protein synthesis. Years of research has elucidated some of the transcriptional and translational mechanisms that contribute to the production of new proteins. Early research on transcription focused on the transcription factor cAMP-responsive element binding protein. Since then, other transcription factors, such as the Nuclear Receptor 4 family of proteins that play a role in memory formation and maintenance have been identified. In addition, several studies have revealed details of epigenetic mechanisms consisting of new types of chemical alterations of DNA such as hydroxymethylation, and various histone modifications in long-term synaptic plasticity and memory. Our understanding of translational control critical for memory formation began with the identification of molecules that impinge on the 5′ and 3′ untranslated regions of mRNAs and continued with the appreciation for local translation near synaptic sites. Lately, a role for noncoding RNAs such as microRNAs in regulating translation factors and other molecules critical for memory has been found. This review describes the past research in brief and mainly focuses on the recent work on molecular mechanisms of transcriptional and translational regulation that form the underpinnings of long-term synaptic plasticity and memory.

Biochemical studies linking memory formation and consolidation began more than half a century ago (Flexner et al. 1965). The advent of molecular biology provided neuroscientists with many tools necessary to probe the molecular underpinnings of memory. As a result, substantial data have been accumulated on how protein synthesis plays a role in memory. This progress has paralleled advances in the knowledge of transcription and translation in non-neuronal systems. Beginning with the discovery of role of cAMP-responsive element binding protein (CREB), numerous studies have elucidated the role of transcription in memory formation (Yin and Tully 1996; Kandel 2012; Smolen et al. 2019). Similarly work on translation focused mainly on the molecules that regulate protein synthesis by interacting with the 5′ and 3′ untranslated regions (UTR) of mRNAs (Darnell and Richter 2012; Hinnebusch et al. 2016; Sossin and Costa-Mattioli 2018).

Reviewing a subject of this vast scope is a daunting task. Because many excellent reviews have been written on both transcriptional and translational mechanisms underlying memory, in this article we focus on relatively recent developments (research published mainly in the past two decades) in both of these fields and give a bird's eye view.

Early research on transcription underlying long-term synaptic plasticity and memory: role of CREB

Evidence for the role of CREB in long-term synaptic plasticity came from investigations on long-term facilitation (LTF) in *Aplysia*. In this invertebrate animal, serotonin (5-HT) is the neurotransmitter that functions to strengthen the synapses. The 5-HT receptors in *Aplysia* produce the second messenger cAMP via a G-protein-coupled pathway. Previous work had also established a requirement for macromolecular synthesis for development of LTF (Montarolo et al. 1986). Therefore, it was logical for researchers to look for a possible role of CREB in gene expression in sensory neurons of *Aplysia*, which is where the molecular changes important for the presynaptic LTF occur. When the oligonucleotides with cAMP-responsive element were injected into sensory neurons, LTF was significantly blocked (Dash et al. 1990). Subsequently, evidence for the role of CREB in long-term memory was obtained in the *Drosophila* model (Yin et al. 1994) using expression of a dominant-negative CREB. In the same year, evidence was also published showing that a mutation in the *CREB* gene causes deficiency in memory in mice (Bourtchuladze et al. 1994).

After CREB: other transcription factors that play a role in long-term synaptic plasticity and memory

Following the studies on CREB, several transcription factors have been shown to play a role in long-term synaptic plasticity and memory. Some of them are described below (See Table 1; Fig. 1).

Nr4 (nuclear receptor 4)

This belongs to a family of three transcription factors (Nr4a1, Nr4a2, and Nr4a3) encoded by immediate-response genes. Nr4a1 is also known by the names Nurr77/NGIB/TR3. Nr4a protein was originally described as an orphan nuclear receptor because of the lack of a known ligand. Expression of Nr4a increases in response to inhibitors of histone deacetylases (Hawk et al. 2012). Nr4a

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Table 1. Function of transcription factors in memory

Transcription factor	Model	Findings	Reference(s)
c-Rel	-	↑ nuclear accumulation in CA1 after CFC	Ahn et al. 2008
	c-Rel KO	↓ performance in CFC with mild training, but recovered with robust training ↓ performance in NOR ↓ facilitation of L-LTP, but not E-LTP	
CRTC1	-	↑ nuclear translocation during neural activity in HC excitatory neurons dependent on NMDAR, LVGCCs, and cAMP-mediated dephosphorylation ↑ levels at *Fgf1b* promoter after CFC causes ↑ expression independent of phospho-CREB	Ch'ng et al. 2012; Uchida et al. 2017
	Crtc1 KD	↓ *c-Fos*, *Arc*, *Egr4*, *Zif268*, and *Cyr61* expression in HC independent of phospho-CREB	Ch'ng et al. 2012
Foxp1	Foxp1 KO	↓ performance in MWM and T-maze ↓ facilitation of L-LTP, but not E-LTP	Araujo et al. 2017
Mef2	-	↑ phosphorylation in DG and CA1 after MWM and CFC causes ↓ Mef2 protein levels	Cole et al. 2012
	Mef2 OE	↓ spine density and performance in MWM via transcriptional regulation disruption (DNA binding domain is necessary) ↑ *Arc* expression leads to ↑ AMPAR endocytosis	
	Mef2 KD	↑ performance in MWM	
Npas4	-	↑ expression immediately after CFC in HC (especially CA3) ↑ levels at *Bdnf*-PI and *c-Fos* E2 after depolarization	Lin et al. 2008; Ramamoorthi et al. 2011
	Npas4 KO	↓ CA1 EPSP inter-event interval ↑ excitatory presynaptic release probability in HC ↓ performance in CFC ↓ expression of ERGs (*Bdnf*-PI and PIV, *Arc*, *c-Fos*, *Zif268*) ↓ RNA pol II colocalization at *Bdnf*-PI and *c-Fos* E2	
	Npas4 OE	↑ CA1 EPSP inter-event interval and magnitude ↓ excitatory presynaptic release probability in HC ↓ CA1 mIPSP inter-event interval via interaction with BDNF ↑ *Bdnf*-PI transcript expression ↑ performance in CFC caused by global KO	
Nr4a1/2/3	-	↑ *Nr4a2* expression in HC in young and cognitive intact aged, but ↓ expression in cognitive impaired aged rats after object recognition memory task	Kwapis et al. 2019
	Nr4a DN	↓ facilitation of L-LTP, but not E-LTP in HC due to ↓ interaction with HDACs	Bridi and Abel 2013; Bridi et al. 2017
	CBP KI	↓ *Nr4a2* and *Nr4a3* expression after CFC ↓ H3 acetylation at *Nr4a2* promoter	
	Nr4a C-DIM activation	↑ performance in CFC ↑ LTP magnitude, but not in Nr4a DN or CBP KI mutants	
Srf	Srf oligonucleotides	↓ performance in MWM	Dash et al. 2005
	Srf KO	↓ performance in NOR ↓ LTD magnitude ↓ *Bdnf*, *c-Fos*, and *Arc* expression	Etkin et al. 2006
XBP1	XBP1 KO	↓ performance in CFC and memory flexibility paradigm ↓ CA1 EPSP magnitude sustained into L-LTP ↓ *Bdnf* expression in HC	Martínez et al. 2016
	XBP1 OE	↑ performance in CFC and memory flexibility paradigm ↑ CA1 EPSP magnitude sustained into L-LTP	
Zif268	Zif268 OE	↑ performance in NOR ↑ DG LTP magnitude ↑ *synapsin II* and *PSMB9* expression in DG	Penke et al. 2014

CA1, cornu ammonis 1; CA3, cornu ammonis 3; CFC, contextual fear conditioning; DG, dentate gyrus; DN, dominant negative; E-LTP, early-phase LTP; EC, entorhinal cortex; ERG, early response genes; HC, hippocampus; KI, knockin; KO, knockout; L-LTP, late-phase LTP; LVGCC, L-type voltage-gated calcium channels; MWM, Morris water maze; NOR, novel object recognition task; OE, overexpression.

expression depends on signaling by the cAMP-dependent protein kinase and activity of CREB suggesting that Nr4a might be important for a second wave of transcription underlying long-term synaptic plasticity and memory. In hippocampal slices from mice expressing a dominant-negative *Nr4a* transgene, transcription-dependent late-phase long-term potentiation (L-LTP) is impaired (Bridi and Abel 2013). Synthetic ligands of Nr4a, para-phenyl substituted di-indolyl methane analogs or C-DIM compounds increase the duration of LTP and enhance contextual fear memory (Bridi et al. 2017). With respect to other Nr4a isoforms, knockdown experiments showed that Nr4a1 is necessary for memory for object location whereas Nr4a2 is necessary for long-term memory of both object location and object recognition (Table 1; Fig. 1; McNulty et al. 2012). It appears that the Nr4a isoform Nr4a2 is critical for preserving cognitive abilities in old age because it has been shown that histone deacetylase 3 (HDAC3)-mediated repression of Nr4a2 contributes to cognitive decline. Other Nr4a isoforms seem to contribute to the prevention of cognitive decline as well. Overexpression of *Nr4a1* and *Nr4a2* transcripts individually or together in the dorsal hippocampus of male mice can mitigate age-related impairment in object location memory (Kwapis et al. 2019).

Npas4 (neuronal PAS domain protein 4)

The PAS domain in Npas4 (and other proteins) is named after the structural motifs found in the proteins Period, Aryl hydrocarbon receptor and Single-minded which assist in protein–protein

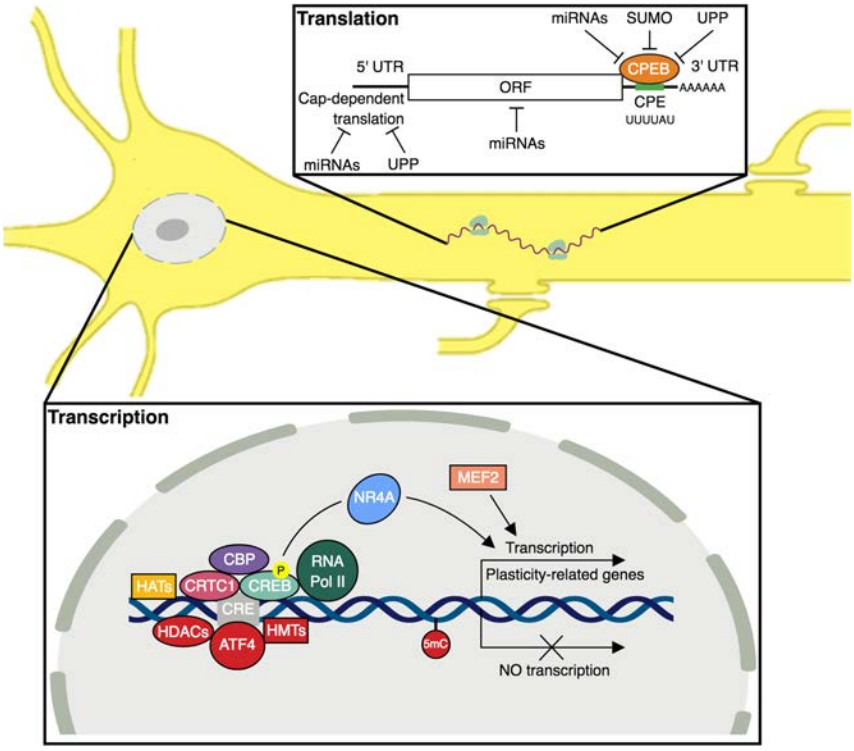

Figure 1. Molecules of transcription and translation underlying long-term synaptic plasticity and memory. Schematic figure showing a neuron with representative molecules that regulate transcription (below the neuron) and translation (above the neuron) during long-term synaptic plasticity and memory.

interactions. Npas4 is the protein product of an immediate-early gene whose transcriptional activity is required for inducing genes in the CA3 region of the hippocampus that play a role in contextual memory formation. Global knockout of *Npas4* impairs contextual fear conditioning (CFC). In addition, selective deletion of *Npas4* in CA3 but not CA1 of the hippocampus hinders CFC (Ramamoorthi et al. 2011). Npas4 was initially identified as a transcription factor required for inducing expression of activity-dependent genes during the development of inhibitory synapses (Lin et al. 2008). Therefore, the transcriptional program mediated by Npas4 might be important for tweaking the feedforward inhibition in the hippocampus in order to make the context of memory precise.

Foxp1 (forkhead box P1)

This is a transcription factor that belongs to the forkhead family of DNA-binding proteins previously known to be important for development of the brain and other organs. There has been a resurgence of interest in Foxp1 because heterozygous mutations and deletions in the human *FOXP1* gene are linked to autism spectrum disorder and intellectual disability. Experiments using mice with conditional knockout of *Foxp1* in pyramidal neurons of the neocortex and CA1 and CA2 subregions of the hippocampus show impairment in spatial memory but not generalized deficits in learning and memory. Moreover, in the hippocampal slices prepared from *Foxp1* knockout mice maintenance of CA1 LTP is impaired (Araujo et al. 2017).

Srf (serum response factor)

Initial work on Srf showed that it is critical for expression of immediate-early genes such as *c-Fos* and *Egr1* (a.k.a. *Zif268*) and for early-phase and late-phase LTP. This study suggested that the transcriptional program activated by Srf might run parallel to that of CREB (Ramanan et al. 2005). Later studies showed that Srf is also important for LTD and the formation of immediate memory in mice to a novel context (Etkin et al. 2006). A separate investigation showed that infusions of oligonucleotides containing binding sites for Srf into the rat hippocampus impaired spatial memory (Dash et al. 2005).

Mef2 (myocyte enhancer factor 2)

This is a transcription factor that restricts dendritic spine growth. Increasing Mef2 in the dentate gyrus and amygdala of mice impairs spatial and fear memory formation respectively. Conversely, decreasing the levels of Mef2 in the same brain regions enhances spatial and fear memory. Interfering with AMPA receptor (AMPAR) endocytosis rescues the adverse effect of Mef2 on memory formation. It is thought that Mef2 indirectly causes a decrease in surface expression of AMPARs through one of its target genes *Arc* (Cole et al. 2012). Based on the investigations conducted thus far, it appears that Mef2 does not have a connection to the CREB pathway of gene expression.

CRTC1 (CREB-regulated transcriptional coactivator 1)

This protein binds to the bZIP domain of CREB and works with it to regulate transcription. CRTC1 has been shown to reside in silent synapses and translocate to the nucleus in an activity-dependent manner. Its persistence in the nucleus requires cAMP signaling (Ch'ng et al. 2012). Subsequent studies showed that CRTC1 regulates the transcription of *Fgf1,* a gene that encodes fibroblast growth factor. With weak synaptic stimulation CRTC1 complexes with CREB-binding protein (CBP), however, it makes a complex with KAT5 upon strong stimulation. KAT5 is also a histone acetyltransferase like CBP. Its recruitment to the promoter of the *Fgf1* gene appears to be specifically associated with an increase in H4K12 acetylation (Uchida et al. 2017). It is tempting to speculate that flexibility of CRTC1 in associating with more than one factor might be indicative of combinatorial regulation of transcription factor assemblies which greatly increases their power in controlling the specificity of gene expression.

Other transcription factors such as c-Rel, XBP1and Zif268 also have a role in long-term synaptic plasticity and memory and are briefly described in Table 1 (Ahn et al. 2008; Penke et al. 2014; Martínez et al. 2016).

Transcriptional repressors: role in long-term synaptic plasticity and memory

Repressors of transcription can reduce the expression of synaptic plasticity-related genes and thus have a negative impact on memory formation. A corollary is that the removal of transcriptional repression should enhance memory. This is precisely what was found in mice expressing an *Atf4* transgene which showed an improvement in spatial memory (Chen et al. 2003). Atf4 (also called

Creb2) is a CREB repressor and down-regulates transcription when bound to CREs. In *Aplysia*, two CREB repressors have been shown to play a role in long-term synaptic plasticity, namely Creb1b and Creb2 (Bartsch et al. 1995, 1998). In order for CREB-mediated gene expression to go forward, repression has to be removed. This is likely to be accomplished by targeted degradation by the ubiquitin-proteasome pathway (UPP). Atf4 protein is degraded during long-lasting LTP in the mammalian hippocampus (Dong et al. 2008). CREB1b is subject to degradation by the UPP upon stimulation of sensory neurons by repeated application of the neurotransmitter 5-HT which induces long-term facilitation in *Aplysia* (Upadhya et al. 2004).

Another transcriptional repressor known to repress CREB-mediated transcription is called DREAM (downstream regulatory element antagonist modulator). DREAM binds to the leucine-rich domains located within the kinase-inducible domain of CREB and interferes with recruitment of CBP by phosphorylated CREB. The *Dream* (−/−) mutant mice show enhanced object recognition memory (Fontán-Lozano et al. 2009) and knocking out of *KChIP3*, which has 99% homology with *Dream,* improves contextual fear memory (Alexander et al. 2009).

In addition, transcriptional corepressors are known to exist. These tend to be large complexes of proteins often incorporating histone-modifying enzymes such as HDACs (Schoch and Abel 2014). Much of the evidence on these corepressors came from cancer research. It is interesting to note that a transcription factor with a role in memory Mef2 (described above) forms a corepressor complex with HDAC and SIN3A and blocks the expression of Nurr77 (Nr4a) another transcription factor that functions in memory formation. In Jurkat T cells, in response to Ca^{2+} signal, the repression is relieved and Mef2 associates with CBP to activate transcription of Nurr77 (Youn and Liu 2000). Therefore, it is likely that the activity-dependent regulation of transcriptional corepressors plays a role in controlling transcription underlying long-term synaptic plasticity and memory although the molecular details are likely to be different from those observed in non-neuronal cells.

Epigenetic control of transcription: DNA methylation and histone modification

DNA methylation

Methylation of cytosine residues at the fifth position (5-methyl cytosine) as an epigenetic modification was previously known to silence gene expression in the context of development of organisms and differentiation of tissues. DNA methylation came under the radar of scientists researching synaptic plasticity and memory only about a decade or so ago (Miller and Sweatt 2007). The 5-methyl cytosine modification of DNA occurs by the action of DNA methyltransferases of which several isoforms exist. The reaction can be reversed by DNA demethylases. A protein named Gadd45b (growth arrest DNA damage-inducible β) which promotes demethylation has been shown play a role memory formation. *Gadd45b* knockout mice showed impaired fear conditioning (Leach et al. 2012). These results make sense and conform to the previous notion that DNA methylation decreases gene expression and DNA demethylation promotes it. However, the situation may be more complex and depend on the pattern of DNA methylation (or demethylation) and may be modified by neuronal activity. Taken in that light, the memory-promoting activity of a DNA methyltransferase makes sense. For example, restoring age-related decline in a DNA methyltransferase Dnmt3a2 by adeno-associated virus-mediated expression improved fear memory as tested by contextual and trace fear conditioning paradigms (Oliveira et al. 2012).

Over the last few years, proteins that convert 5-methyl cytosine to 5-hydroxymethyl cytosine (5-hmc) have been discovered (Tahiliani et al. 2009). These proteins are called Ten-eleven translocation (Tet) methylcytosine dioxygenases. Based on high-throughput sequencing studies on embryonic stem cells as well as neurons from the fetal mouse hippocampus and cerebellum, 5-hmc as an epigenetic marker is thought to be associated with regulatory and intragenic regions of genes that are developmentally repressed but poised for activation (a.k.a. bivalent genes) (Szulwach et al. 2011; Gao et al. 2013). With regard to Tet enzymes, one study found that Tet1 was activity-regulated and was critical for expression of memory-associated genes and for contextual fear memory (Kaas et al. 2013). Another study, however, observed that *Tet1* knockout mice exhibited normal spatial memory but had impaired fear extinction (Rudenko et al. 2013). Other Tet isoforms appear to be critical for memory as well. For example, researchers found that Tet2 decreased in the neurons of the dentate gyrus (DG) with age and overexpressing Tet2 in DG prevented the decline in adult neurogenesis and improved CFC (Gontier et al. 2018). Another protein called Uhrf2, which is thought to be a 5-hmc reader, appears to have some role in memory formation. Mice lacking Uhrf2 exhibit partial impairment of spatial memory (Chen et al. 2017).

Other DNA modifications

Recent studies indicate that DNA modification at nucleotides other than cytosine also play a role in certain types of memory. For example, N6-methyl-2′-deoxyadenosine accumulates at promoters and coding regions of genes in prefrontal cortical neurons of mice trained in fear extinction. Furthermore, the enzyme responsible for this DNA modification, m6dA methyltransferase (N6amt1), binds to the genomic sequences and enhanced genome-wide occupancy of N6amt1 is associated with increased gene expression. Occupancy by N6amt1 also occurs at specific promoters of genes such as that of *Bdnf* exon 4, whose expression is correlated with extinction of conditioned fear (Li et al. 2019).

Epigenetic control by small noncoding RNAs

DNA methylation, especially of clusters of CpG sequences (CpG islands) in promoters of genes, can be brought about by small noncoding RNAs called piRNAs. Studies on *Aplysia* have shown that piRNAs have a role in regulating expression of genes important for long-term synaptic plasticity. The piRNAs are RNAs that were originally named because of their interaction with proteins called piwi (P-element Induced WImpy testis) in *Drosophila*. The piRNAs were mainly known for their role in posttranscriptional silencing of transposons in germline cells through DNA methylation. An investigation of RNA library generated from *Aplysia* neurons revealed the presence of piRNAs (Rajasethupathy et al. 2012). Additional studies carried out by silencing or overexpressing piwi proteins in *Aplysia* neurons showed that some piRNAs might silence expression of a CREB repressor called Creb2. A specific piRNA called aca-piR-F was shown to be a transcriptional regulator of Creb2. In addition, aca-piR-F was found to be up-regulated by 5-HT, the neurotransmitter critical for inducing long-term synaptic plasticity in *Aplysia*. It has been suggested (although not demonstrated) that aca-piR-F regulates *Creb2* promoter by methylation in response to 5-HT thus converting a transient stimulus into an enduring epigenetic change (Rajasethupathy et al. 2012). Later studies showed the presence of piRNAs in the mammalian brain (Nandi et al. 2016). Moreover, knockdown of piwi-like genes *Piwil1* and *Piwil2* in the dorsal hippocampus enhances contextual fear memory (Leighton et al. 2019).

Histone modification

Histone modification can be transcription-favoring or transcription-repressing type (Bach and Hegde 2016). Acetylation of histone on lysine residues opens the chromatin and facilitates transcription (Levenson et al. 2004). Conversely, the removal of acetyl groups by deacetylases inhibits transcription. Histone methylation on lysine residues can promote or block transcription depending on the number of methyl groups and the location of the lysine residue within the histone protein (Tables 2, 3; Gräff et al. 2012; Gupta-Agarwal et al. 2012; Bach et al. 2015).

With regard to the role of histone acetylation in memory formation, some of the early work indicated that histone acetyltransferase activity of CREB-binding protein (CBP), a transcriptional coactivator of CREB, plays a vital role in memory consolidation (Korzus et al. 2004). Subsequent evidence for a role of histone acetylation in memory came from the observations that the enzymes that remove the acetylation mark on chromatin, namely, histone deacetylases hinder long-term synaptic plasticity and impair memory (Guan et al. 2009).

Tri-methylation of histone 3 on lysine 4 (H3K4me3) is up-regulated in the hippocampus 1 h after CFC (Gupta et al. 2010). In addition, mice lacking *Mll*, a gene encoding a histone methyltransferase responsible for H3K4me3, exhibit impairment in CFC and performance in water maze (Kerimoglu et al. 2013). Recent studies indicate that specific histone methyltransferases control H3K4me3 in distinct genomic regions and are responsible for regulating distinct gene expression programs underlying memory consolidation (Kerimoglu et al. 2017). A transcription-repressing form of histone methylation (H3K27me3) has been found to have a role in memory as well. During reconsolidation of fear memory expression of Pten, a phosphatase that negatively regulates mTOR signaling, is reduced. This is achieved by an increase in H3K27me3 in the Pten promoter and coding regions (Jarome et al. 2018). The methyltransferase responsible for H3K27me3, Ezh2 has a role in adult neurogenesis and conditional knockout of the *Ezh2* gene impairs spatial learning and memory (Zhang et al. 2014). Pharmacological inhibition of SUV39H1, a methyltransferase that works to add a repressive mark on histone H3 (H3K9me3), improves dendritic spine formation, increases surface GluR1 levels on spines, and improves object location memory, CFC and performance in other complex spatial learning tasks (Snigdha et al. 2016). Another histone modification, phosphorylation of histone H3 on Ser-10 (H3S10ph), has been linked to an increase in transcription-dependent LTP mediated by stimulation of β-adrenergic receptors and consequent activation of Aurora kinase-B (Maity et al. 2016). Histone marks such as H3S10ph occur transiently in the hippocampus but persist in cortical areas in order to facilitate memory consolidation (Gräff et al. 2012). Some histone modifications such as demethylation of certain lysine residues (H3K9me2) function in memory consolidation by activating some genes and silencing other genes depending on whether the histone mark is at the promoter or the coding region of the gene (Table 2; Gupta-Agarwal et al. 2012). Given the role for histone modification in memory, logically the enzymes that are responsible for epigenetic marking of histones should have a role in memory-related synaptic plasticity. Indeed, numerous studies have demonstrated a role for histone-modifying and unmodifying enzymes in long-term synaptic plasticity and memory (Table 3; McQuown et al. 2011; Jing et al. 2017; Tang et al. 2017; Yamakawa et al. 2017; Schoberleitner et al. 2019; Zhu et al. 2019).

Other molecules that are part of the regulatory system for epigenetic modification have been shown to have a role in synaptic plasticity as well. Three classes of molecules called readers, erasers, and writers of chromatin modification are known to exist. A molecule belonging to the class of readers called L3mbt1 (lethal 3 malignant brain tumor-like 1) has a role in homeostatic synaptic downscaling (Mao et al. 2018). A key target of L3mbt1 is the *Ctnnb1* gene. In response to synaptic activity L3mbt1 positively regulates the expression of *Ctnnb1*. The protein product of the *Ctnnb1* gene is called β-catenin. One of the functions of β-catenin is in synaptic scaffolding at excitatory synapses. β-catenin interacts with cadherin, which bridges the pre- and postsynaptic parts of a synapse, and together they regulate synaptic structure and function.

Recent studies indicate that the accessibility of chromatin increases during learning and multiple noncoding regulatory regions are subject to modification (Koberstein et al. 2018).

Translation underlying long-term synaptic plasticity and memory: initial studies

Control of mRNA translation is one of the major ways by which the amount of protein product generated from a transcribed gene is

Table 2. Role of histone modifications in memory

Histone modification	Effect on transcription	Findings	Reference(s)
H3S10ph	Activate	↑ at *Zif268* promoter in HC after NOR	Gräff et al. 2012; Maity et al. 2016
		↑ in CA1 after HFS-induced LTP	
H3K9ac	Activate	↑ in CA1 immediately after cLTP and sustained until 30 min	Bach et al. 2015
H3K14ac	Activate	↑ in HC after CFC in an NMDAR- and ERK-dependent manner	Levenson et al. 2004
		↑ at *Zif268* promoter in HC after NOR	Gräff et al. 2012
		↑ in CA1 immediately after cLTP and sustained until 30 min	Bach et al. 2015
H3K4me3	Activate	↑ at *Zif268* and *Bdnf* promoters in HC after CFC	Gupta et al. 2010
		↑ in CA1 and EC after CFC training	Gupta-Agarwal et al. 2012
		↑ in CA1 immediately after cLTP	Bach et al. 2015
H3K9me3	Silence	↓ after CFC and other spatial learning	Snigdha et al. 2016
H3K27me3	Silence	↑ at select gene promoters (e.g. Pten) during memory reconsolidation	Jarome et al. 2018; Zhang et al. 2014
H3K36me3	Activate	↑ at *Zif268* promoter in HC after NOR	Gräff et al. 2012
H3K9me2	Silence	↑ at *Zif268*, *Dmnt3a*, *Bdnf*-PIV, and *c-Fos* promoters in CA1 and EC after CFC	Gupta-Agarwal et al. 2012
		↑ in HC after CFC	Gupta et al. 2010
H2BK120ub	Activate	↑ in CA1 immediately after cLTP and 30 min postinduction	Bach et al. 2015

CA1, cornu ammonis 1; CFC, contextual fear conditioning; cLTP, chemically induced LTP; EC, entorihinal cortex; HC, hippocampus; HFS, high frequency stimulation; NOR, novel object recognition task.

Table 3. Role of histone modifying proteins in memory

Molecule	Function	Model	Findings	Reference(s)
Aurora kinase B	Histone kinase	Chemical inhibition	↓ H3S10ph levels in CA1 associated with noradrenaline/HFS-induced LTP	Maity et al. 2016
Chd1	H3K4me regulation	*Chd1* deletion	↓ performance in NOR and Barnes maze ↓ *Egr1* and *Arc* expression in HC	Schoberleitner et al. 2019
Ezh2	Histone methyltransferase	Ezh2 KO	↓ performance in MWM, cued fear learning, and CFC	Zhang et al. 2014
G9a/GLP	Histone methyltransferase	CA1 chemical inhibition EC chemical inhibition	↓ LTP facilitation and performance in CFC ↑ LTP facilitation and performance in CFC ↓ H3K9me2 at *Zif268* and *Bdnf*-PII promoters in CA1	Gupta-Agarwal et al. 2012
HDAC1	Histone deacetylase	HDAC2 KO	↑ performance in CFC, cued fear learning, MWM, and T-maze ↑ number of functional excitatory synapses and spine density in CA1 ↑ LTP magnitude in HC ↑ histone acetylation at ERGs (*Bdnf*-PII, *c-Fos*, *Egr1*)	Guan et al. 2009; Morris et al. 2013
		Hdac2 KD	↑ EPSP in cultured neurons	Yamakawa et al. 2017
		HDAC2 KI	↓ performance in CFC, cued fear learning, MWM, and T-maze ↓ spine density in CA1 ↓ LTP magnitude in HC	Guan et al. 2009
HDAC3	Histone deacetylase	-	↓ *Nr4a2* expression in HC	Kwapis et al. 2019
		HDAC3 KO	↑ performance in object recognition memory task ↓ H4K8ac levels in HC ↑ *c-Fos* and *Nr4a2* expression in HC	McQuown et al. 2011
		HC chemical inhibition	↑ performance in object recognition memory task ↓ HDAC4 levels in HC ↑ H4K8ac levels in HC	
HDAC4/5	Histone deacetylase	- HDAC4/5-3SA mutation HDAC4/5 DKO	↑ HDAC4 nuclear translocation transiently after CFC in CA1 ↓ expression of ERGs (*Nra41*, *Nra43*, *Arc*, *Npas4*) due to impaired nuclear export ↓ performance in CFC and Barnes maze ↑ expression of ERGs after CFC in HC	Zhu et al. 2019
HDAC7	Histone deacetylase	- HDAC7 KO HDAC7 KI	↓ levels in HC after CFC via interaction with CBX4 E3 ligase ↓ performance in CFC ↑ performance in CFC	Jing et al. 2017
KMT2A	Histone methyltransferase	KMT2A KO	↓ performance in CFC and MWM ↓ H3K4me3 at ERG (functions in transcription, chromatin, and mRNA regulation and protein ubiquitination) promoters in HC	Kerimoglu et al. 2013, 2017
KMT2B	Histone methyltransferase	KMT2B KO	↓ performance in CFC, NOR, and MWM ↓ H3K4me3 at ERG (related to synaptic plasticity) promoters in HC	Kerimoglu et al. 2013
L3MBTL1	Regulator of methylated lysine histone residues	-	↓ levels during neural activity (PTX-induced) in HC by means of proteasome degradation	Mao et al. 2018
		L3MBTL1 KO	↓ *Ctnnb1* and *Gabra2* expression in HC causing impaired synaptic downscaling	
Sp3	HDAC2 regulation	*Sp3* KD HDAC2 C-terminal OE	↑ EPSP in cultured neurons ↑ ERG (role in ion transport and regulation of membrane proteins and receptors) expression ↓ HDAC2 binding to and facilitating H4K5ac and H2BK2ac levels at gene promoters	Yamakawa et al. 2017
SUV39H1	Histone methyltransferase	Chemical inhibition	↑ performance in object location memory task and CFC associated with ↓ H3K9me3 levels in HC	Snigdha et al. 2016
UTX	H3K27me3 demethylase	UTX KO	↓ performance in MWM ↓ PSD-95 levels and dendritic arborization ↓ ERG expression (functions in neurite elongation and dendritic synaptic) formation (*Egr3*, *Wnt4*) in CA1 ↑ H3K27me3 levels in HC leads to ↓ *Htr5b* expression, which is involved in neural structural formation	Tang et al. 2017

CA1, cornu ammonis 1; CFC, contextual fear conditioning; cLTP, chemically-induced LTP; EC, entorhinal cortex; ERG, early response genes; HC, hippocampus; HFS, high frequency stimulation; MWM, Morris water maze; NOR, novel object recognition task.

regulated. The bulk of early work on translational control of mRNAs relevant to synaptic plasticity and memory falls into two major lines of investigation. The first is modulation of mRNA translation through factors that interact with the 5′ UTR and the other is through pathways that regulate translation via interaction with the 3′ UTR.

Translational control at the 5′ UTR

Eukaryotic mRNAs possess a 7-methyl-Guanosine (m^7-G) cap. Translation of mRNAs can be controlled in a cap-dependent as well as cap-independent manner. Thus far, research on translational control underlying synaptic plasticity and memory has been mainly on the mechanisms that are cap-dependent.

A translation preinitiation complex consists of a tRNA that binds to the initiation codon for methionine (Met-tRNA) and a eukaryotic initiation factor eIF2 bound to guanosine triphosphate. The assembly of this complex is facilitated by other initiation factors. Attachment of this complex to the m^7-G cap is added by the eIF4F complex comprising eIF4E, eIF4G, and eIF4A (Hinnebusch et al. 2016).

eIF2 is phosphorylated by a protein kinase called Gcn2 which negatively regulates its function. As a result, general translation is inhibited but translation of specific mRNAs is facilitated. In the nervous system, this process facilitates the translation of Atf4 which is a CREB repressor. As expected in mice lacking *Gcn2*, L-LTP which is transcription-dependent, is induced with only one train of 100 Hz stimulation whereas in normal mice it takes four 100 Hz trains to induce L-LTP. The *Gcn2* (−/−) mice show memory improvement with weak training and memory impairment with strong training. Therefore, an interpretation of these results is that translational control indirectly regulates transcription and *Gcn2* (−/−) mice might have too much gene expression which has an adverse effect on memory (Costa-Mattioli et al. 2005).

A role for regulation by translational elongation factor has been described as well. In *Aplysia* sensory neurons, which make synapses with motor neurons, elongation factor eEF1A mRNA is transported along the axon to stimulated synapses. This kind of mRNA transport is thought to link transcription at the nucleus to local protein synthesis at the synapse to promote synaptic growth (Giustetto et al. 2003).

Translational control at the 3′ UTR

Translation of eukaryotic mRNAs depends on the extent of polyadenylation at the 3′ UTR. A key molecule that regulates lengthened Poly(A)-tail-dependent translation is Cytoplasmic Polyadenylation Element Binding Protein (CPEB). The mRNAs subject to regulation by CPEB contain Cytoplasmic Polyadenylation Element (CPE) which comprises a consensus sequence UUUUUAU. When the RNA transcribed in the nucleus is exported to the cytoplasm, CPEB binds to CPE and a ribonucleoprotein (RNP) assembly containing CPEB and other proteins forms. This RNP can keep the Poly(A) short because of the presence of a deadenylating enzyme. Alternatively, when CPEB is phosphorylated by Aurora kinase, the deadenylating enzyme is pushed out of the RNP complex which now elongates the Poly(A)-tail and thus promotes translation (Darnell and Richter 2012).

A role for CPEB in translation required for long-term synaptic plasticity was found in the invertebrate *Aplysia*. In the sensory-motor neuron synapses reconstituted in culture, the researchers found that injection of antisense oligonucleotides against an isoform of CPEB called CPEB2 inhibited long-term facilitation (Si et al. 2003a). It was proposed that CPEB might sustain its function because of its prion-like properties (Si et al. 2003b). A mammalian CPEB isoform, CPEB3, regulates protein synthesis required for hippocampus-based memory. Subsequent work showed that attachment of small-ubiquitin-like modifier (SUMO) to CPEB3 negatively regulates its prion-like aggregation (Drisaldi et al. 2015). It remains to be seen whether prion-like aggregation is a peculiarity of some CPEB isoforms because no other molecule with a role in memory with prion-like properties has been reported.

Regulation of translation by microRNAs during synaptic plasticity and memory

MicroRNAs are small noncoding RNAs typically 21-nt long. They bind to complementary sequences and negatively regulate translation by causing degradation of mRNAs or suppressing their translation. For example, miR-26a and miR-384-5p generally suppress the expression of ribosomal S6 kinase 3 which is a translational regulator. These microRNAs are down-regulated during NMDAR-dependent LTP thereby increasing the translation of S6 kinase which in turn boosts the translation required for making LTP last long. A microRNA regulated by activity, miR-188, decreases the expression of a glycoprotein Neuropilin-2 which functions as a receptor for semaphorin 3F, a negative regulator of spine development (Lee et al. 2012). Another example is miR-22 in presynaptic sensory neurons of *Aplysia* which negatively regulates CPEB. Upon stimulation that induces LTF, the miR-22-imposed constraint on CPEB translation is relieved and its expression increases. Augmented levels of CPEB lead to other molecular events, such as an increase in a specific atypical protein kinase C isoform, causing synapses to strengthen (Fiumara et al. 2015).

In terms of linking miRNAs to memory, global loss of miRNAs through deletion of *Dicer1,* which encodes a protein that is part of miRNA generation, causes enhancement of memory as measured by Morris water maze, trace fear conditioning and other tests of cognition (Konopka et al. 2010). Another microRNA is miR-132 which was originally found to control neuronal morphogenesis. Since then, a role of miR-132 in synaptic plasticity and memory has been shown. Transgenic mice expressing miR-132 at various levels showed that expression of this microRNA might be related to cognitive capacity (Hansen et al. 2013). Other studies showed a role for miR-132 in recognition memory that depends on synaptic plasticity in the perirhinal cortex (Scott et al. 2012).

Overexpression of miR-181c ameliorates cognitive impairment caused by chronic hypoperfusion in rats. This study found that miR-181c regulates the expression of TRIM2 which is a ubiquitin ligase that targets neurofilament light (NF-L) protein. Increased expression of miR-181c therefore ultimately led to enhanced expression of NF-L which in turn correlated with increased dendritic arborization and spine formation (Fang et al. 2017).

RNA-induced silencing complex (RISC) appears to play a role in synaptic protein synthesis in the circuitry underlying olfactory memory in *Drosophila*. Normally in the second order synapses mRNA translation is silenced by Armitage, an RNA helicase and a component of the RISC complex (Ashraf et al. 2006). During long-term memory formation, Armitage in degraded in a proteasome-dependent manner thus relieving the repression of translation by RISC. This mechanism seems to be evolutionarily conserved. When a key component of the RISC complex called Argonaute is down-regulated using a pool of siRNAs in the dorsal hippocampus of C57BL/6 mice, short- as well as long-term contextual memory is impaired (Batassa et al. 2010). From these studies, it is not clear how short-term memory is affected by reduction in translation. Additional investigation such as electrophysiological experiments to measure synaptic plasticity might help elucidate mechanistic links between RISC function and memory in mammals.

Regulation of miRNAs

miRNAs themselves are subject to transcriptional regulation. Previous studies showed that some miRNAs are subject to control by CREB. For example, miR-132 was identified as a target of CREB through a genome-wide screen (Vo et al. 2005). Expression of miR-132 has been shown to be regulated by neuronal activity in different brain regions and in the hippocampus by CFC (Nudelman et al. 2010). Although activation of microRNAs by specific transcription factors has not been studied widely, this type of activation might be important for expression of specific set of genes during memory formation.

miRNA expression might also be controlled more broadly as well. A gene implicated in schizophrenia *Satb2* encodes a transcriptional regulator which appears to control the expression of a large set of miRNAs. Satb2 protein is expressed in the cerebral cortex and

the CA1 region of the hippocampus. Forebrain-specific deletion of *Satb2* impairs maintenance of L-LTP and long-term contextual fear memory as well as object discrimination memory (Jaitner et al. 2016).

Role of long noncoding RNAs (lncRNAs) in translation

Recently, a nucleolus-specific long noncoding RNA (LoNA) has been identified by high-throughput sequencing. This LoNA is mainly expressed in neurons and plays a role in ribosomal RNA (rRNA) transcription and posttranscriptional modification. Normally, LoNA represses rRNA biogenesis and in response to neuronal activity its levels decrease leading to an increase in rRNA and translation of mRNAs. LoNA deficiency leads to improved learning and memory and conversely when its levels are increased it impairs memory (Li et al. 2018).

Local mRNA translation: latest findings

A key aspect of synapse-specificity of long-term plasticity that underlies memory is local translation of preexisting mRNAs. The importance of local translation during LTF in *Aplysia* and LTP in the hippocampus was shown several years ago (Frey and Morris 1997; Martin et al. 1997). Evidence for local translation continues to be accumulated. For example, a recent study showed the importance of Fragile X Mental Retardation Protein (FMRP) in regulating local dendritic translation of the α subunit of calcium-calmodulin-dependent protein kinase during an olfactory learning task that required the newborn neurons in the olfactory bulb (Daroles et al. 2016).

The mRNAs themselves are localized in dendrites which can provide spatial restriction of translation even within subregions of dendrites such as the shafts or spines (Van Driesche and Martin 2018). The recent data show that the mechanisms such as regulation through miRNAs can also be localized in dendrites as well. For example, Dicer and pre-miRNAs are localized at dendrites or in proximity to synapses. Investigation on miR-181a using a probe that increased its fluorescence upon processing by Dicer showed that miR-181a matured locally in dendrites in response to neuronal activity at individual synapses (Sambandan et al. 2017).

Translational control by protein degradation during formation of synaptic plasticity and memory

Apart from regulation by posttranslational modification the translation factors can be regulated by degradation by the UPP. During L-LTP, proteasome-mediated degradation controls the amounts of the initiation factor eIF4E and the elongation factor eEF1A. In addition, the proteasome also modulates the amount of translation repressors such as Paip2 and 4E-BP (Dong et al. 2014a).

Proteolysis also regulates translation by modulating the factors that interact with the 3′ UTR. Although CPEB is a substrate for the proteasome (Reverte et al. 2001), the effect of its degradation has not been studied so far. However, the proteasome has been shown to limit signaling in the cytoplasmic polyadenylation pathway during L-LTP (Dong et al. 2014b).

Other studies have shown that FMRP which acts as a translational repressor in the cytoplasm is a substrate for the ubiquitin ligase Cdh1-APC (anaphase promoting complex). In *Cdh1* knockout mice mGluR-dependent LTD is impaired (Huang et al. 2015).

Future prospects

Research on transcriptional and translational regulation is likely to advance on two fronts. One is the quest for additional proteins and regulatory mechanisms that are critical for transcription and translation during formation and maintenance of long-term synaptic plasticity and memory. The researchers might be aided by advances elsewhere. For example, analysis of public high-throughput data using machine learning led to the identification of a new gene called *Grunge/Atrophin* important for social learning in *Drosophila* (Kacsoh et al. 2017). The big data approach is being applied to the study of RNAs which should help researchers to ask new questions about translational regulation as well. For example, subcellular sequencing from single mouse neurons revealed the presence of 2225 dendritic RNAs (Middleton et al. 2019). The second front of advancement should be in making sense of the data on transcription and translation. Computational approach combined with pharmacological intervention is helping make testable predictions with regard to LTF in *Aplysia* sensory-motor neuron synapses. For example, researchers knocked down *Creb1* with siRNA and rescued the impaired LTF by using a training protocol predicted to be successful by computational approach (Zhou et al. 2015). Another example is a study using the *Caenorhabditis elegans* model in which investigators identified 757 CREB/memory-induced targets by combining memory-training and gene expression analysis (Lakhina et al. 2015). The challenge for the future is to devise such approaches to mammalian models and ultimately to humans to fully understand long-term synaptic plasticity and memory and possibly develop drugs to treat memory deficits.

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Research article

Proteasome limits plasticity-related signaling to the nucleus in the hippocampus

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This work was initiated at Wake Forest Univ. but the bulk of work was done at GCSU.

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ABSTRACT

Proteolysis by the ubiquitin-proteasome pathway has pleiotropic effects on both induction and maintenance of long-term synaptic plasticity. In this study, we examined the effect of proteasome inhibition on signaling to the nucleus during late-phase long-term potentiation. When a subthreshold L-LTP induction protocol was used, proteasome inhibition led to a significant increase in phosphorylated CREB (pCREB) in the nucleus. Inhibitors of cAMP-dependent protein kinase/protein kinase A, extracellular signal-regulated kinase and cGMP-dependent protein kinase/protein kinase G all blocked the proteasome-inhibition-mediated increase in nuclear pCREB after subthreshold stimulation. These results lay the groundwork for understanding a novel role for the proteasome in limiting signaling to the nucleus in the absence of adequate synaptic stimulation.

1. Introduction

The ability of the nervous system to change the strength of synapses, or synaptic plasticity, allows it to store information. Short-term synaptic plasticity depends on molecular mechanisms that alter existing proteins [1]. Long-term modification of the synapses requires new gene expression and protein synthesis [2]. Evidence gathered over the last two decades also supports a role for regulated proteolysis by the ubiquitin-proteasome pathway (UPP) in both short-term and long-term synaptic plasticity [3–5]. In this pathway, the proteins to be degraded are marked by enzymatically-mediated covalent attachment of a small protein ubiquitin. To the first ubiquitin a second ubiquitin is attached and thus a polyubiquitin chain forms. The polyubiquitinated protein is then recognized by a multi-subunit proteolytic complex called the proteasome [6]. Monoubiquitination can also serve as a degradation signal especially for short proteins and human proteins with less structural disorder than those that are targeted by polyubiquitination [7–10].

We previously showed that the UPP has differential roles in dendrites and the nucleus using late-phase long-term potentiation (L-LTP) in the murine hippocampus as a model system [11]. We observed that inhibition of the proteasome in dendrites enhances the early, induction phase of L-LTP whereas blockade of proteasome in the nucleus blocks the late, maintenance phase of L-LTP. Through a series of experiments we showed that the enhancement of the induction phase of L-LTP comes about by stabilization of locally translated proteins in dendrites. The inhibition of the late, maintenance phase is caused by inhibition of transcription. Furthermore, our previous data showed that proteasome inhibition stabilizes a CREB repressor called ATF4 (Dong, 2008).

There are additional ways in which proteasome inhibition could hinder transcription. One such way would be interference with signaling to the nucleus [12,13]. Therefore, we investigated this possibility. We found that proteasome inhibition did not block signaling to the nucleus as measured by its effect on phosphorylation of CREB. We then tested whether proteasome inhibition could have the opposite effect and enhance CREB phosphorylation. Indeed, we found that proteasome inhibition significantly increased CREB phosphorylation when we used a subthreshold LTP induction protocol to stimulate the Schaffer collateral pathway in the hippocampus. The increase in CREB phosphorylation was blocked with inhibition of cAMP-dependent protein kinase/protein kinase A (PKA), or extracellular signal-regulated kinase (ERK, also called mitogen-activated protein kinase), or cGMP-dependent protein kinase/protein kinase G (PKG).

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2. Materials and methods

2.1. Animals

Mice (C57/Bl6, male, age 6–12 weeks) were obtained from Charles River (Wilmington, MA) and used for experiments using a protocol approved by the Institutional Animal Care and Use Committee of Wake Forest University Health Sciences. Animals were housed (5 animals maximum per cage) and food and water were available *ad libitum*. Animal husbandry was done according to the Guide for the Care and Use of Laboratory Animals (8th edition; National Academies Press, Washington, D.C.).

2.2. Electrophysiology

Transverse hippocampus slices (400 μm) were prepared from 6–12-week old mice using a tissue chopper in oxygenated and chilled artificial cerebrospinal fluid (ACSF) containing 125 mM NaCl, 3 mM KCl, 2.3 mM CaCl2, 1.3 mM MgCl2, 25 mM NaHCO3, 1.25 mM NaH2PO4, and 10 mM glucose, pH 7.4. After recovery for 120 min in ACSF at 32 °C, field excitatory postsynaptic potentials (fEPSP) were recorded in the CA1 region of the hippocampus using a bipolar electrode to stimulate the Schaffer collateral pathway. The stimulation intensity was adjusted to give ~35% of the maximal fEPSP slope and the baseline responses were recorded at this intensity. To examine whether proteasome inhibition affects the phosphorylation of cAMP response element binding protein (CREB), slices were incubated with β-lactone (25 μM) for 30 min. The subthreshold LTP stimulation was given with 2 x 100 Hz trains spaced 5 min apart. Untreated slices were used as control. Slices were fixed in 4% paraformaldehyde immediately after stimulation and processed for immunohistochemistry and quantification of pCREB by confocal microscopy.

The stock solutions of pharmacological reagents PKA inhibitor (KT5720), specific PKA inhibitor (PKI), PKG (cGMP-dependent protein kinase) inhibitor KT5823 and MAP kinase inhibitor (U0126) were prepared in dimethylsulfoxide (DMSO) and diluted in ACSF and the final concentration was DMSO was no more than 0.02%. The same concentration of DMSO (without the drugs) was used in controls. The final concentrations of KT5720, U0126 and KT5823 were 1 μM, 20 μM and 2 μM respectively. All these reagents were applied to hippocampal slices before pre-incubation with β-lactone. Thereafter, the subthreshold LTP stimulation was given and slices were fixed for immunohistochemistry.

2.3. Immunohistochemistry (IHC)

IHC was performed as described previously (Dong, 2014). Briefly, free-floating 400 μm hippocampal slices were fixed in 4% paraformaldehyde for 1 h and washed six times with PBS at room temperature. A buffer containing 4% normal goat serum (Vector Laboratories, Burlingame, CA), 0.4% Triton-X-100, and 0.05% sodium azide in PBS was used to block hippocampal slices at 4 °C for 6 h. Polyclonal primary antibodies against pCREB (1:1,000, Cell Signaling Technology, Danvers, MA) or CREB (1:1,000, Abcam, Cambridge, MA) in blocking buffer were used to incubate slices at 4 °C overnight. After the overnight incubation, hippocampal slices were washed with 0.2% Triton-X-100 in PBS three times. Secondary Alexa 488-conjugated goat anti-rabbit antibody (1:300, Invitrogen, Grand Island, NY) and To-Pro-3 nuclear stain (1:500, Invitrogen) were then applied at 4 °C for 8 h. Following five washes with 0.2% Triton-X-100 in PBS, slices were mounted with Prolong Gold antifade reagent (Invitrogen) onto glass slides. Carl Zeiss LSM510 laser scanning confocal microscope was used to image the fluorescence and ImageJ (National Institutes of Health, Bethesda, MD) software was used to analyze fluorescence intensity. Hippocampal slices that received subthreshold LTP stimulation and/or chemical treatment were compared to their time-matched controls for quantification of fluorescence. The images were analyzed by an individual who was blind to the experimental conditions.

2.4. Statistical methods

The sample size was determined by power analysis (power = 0.8) in designing the experiments. The data are represented as mean ± standard error. The sample size (n) in each dataset corresponds to the number of animals (not slices) used to collect the data. We analyzed the data using one-way Analysis of Variance (ANOVA) followed by a post-hoc Tukey test.

3. Results

3.1. Proteasome inhibition increases the amount of phospho-CREB (pCREB) in the CA1 region in response to subthreshold stimulation to induce LTP

Phosphorylation of CREB on Ser-133 is thought to integrate signaling by multiple kinases to induce gene expression underlying long-term synaptic plasticity [12,13]. To test the role of proteasome-mediated proteolysis in regulating CREB phosphorylation, we prepared the hippocampal slices and after recovery incubated them in 25 μM *clasto* lactacystin β-lactone (henceforth β-lactone), an irreversible proteasome inhibitor. We subjected the β-lactone-treated and untreated slices to a subthreshold LTP stimulation protocol (2 x 100 Hz; henceforth referred to as the 2-train protocol). We then examined pCREB levels using immunohistochemistry with an antibody specific for phospho-Ser-133 (Fig. 1A). We found that neither the subthreshold LTP stimulation by itself did cause any significant changes in pCREB immunoreactivity nor did β-lactone alone led to an increase in pCREB immunoreactivity without any stimulation. When the 2-train stimulation and β-lactone were combined, however, we observed a significant increase in pCREB immunoreactivity (Control: 34.2 ± 5.0; 2 x 100 Hz: 35.6 ± 9.7; β-lactone + 2 x 100 Hz: 77.2 ± 6.8; $p < 0.01$; $F_{(3, 16)} = 8.028$; n = 5) (Fig. 1B, C).

To ascertain that the enhanced pCREB immunoreactivity was due to an increase in the amount of phosphorylated CREB rather than an increase in the amount of CREB protein, we carried out immunohistochemistry with anti-CREB antibodies after subjecting the hippocampal slices to the 2-train protocol with or without prior treatment with β-lactone. We did not observe any significant changes in the anti-CREB immunoreactivity (Fig. 1D–G) suggesting that there was indeed increase in pCREB amounts.

3.2. Proteasome-inhibition-mediated increase in pCREB depends on PKA

Previous research by many others has shown that PKA is one of main kinases that leads to the phosphorylation of CREB in the nucleus [14]. To test whether PKA plays a role in the enhancement of CREB phosphorylation mediated by proteasome inhibition, we stimulated hippocampal slices with the 2-train LTP protocol after treatment with β-lactone alone or in combination with a PKA inhibitor, KT5720 (1 μM) (Fig. 2A). As before, β-lactone-treated slices showed a marked increase in CREB phosphorylation whereas in slices that were treated with KT5720 prior to β-lactone exposure, the pCREB immunoreactivity was significantly lower (Fig. 2B, C) (Control: 37.4 ± 3.4; β-lactone + 2 x 100 Hz: 62.4 ± 6.0; β-lactone + KT5720 + 2 x 100 Hz: 27.0 ± 3.9; $p < 0.0001$; $F_{(5, 30)} = 9.12$; n = 6).

Next, we tested the effect of Myristoylated PKI (200 nM), which is a membrane-permeant peptide that specially inhibits PKA [15]. We found that treatment with PKI before β-lactone treatment greatly reduced pCREB immunoreactivity compared to treatment with β-lactone alone (Fig. 2D, E) (Control: 23.8 ± 2.3; β-lactone + 2 x 100 Hz: 71.7 ± 11.5; β-lactone + PKI + 2 x 100 Hz: 38.5 ± 8.0; $p < 0.0001$; $F_{(5, 42)} = 9.79$; n = 6).

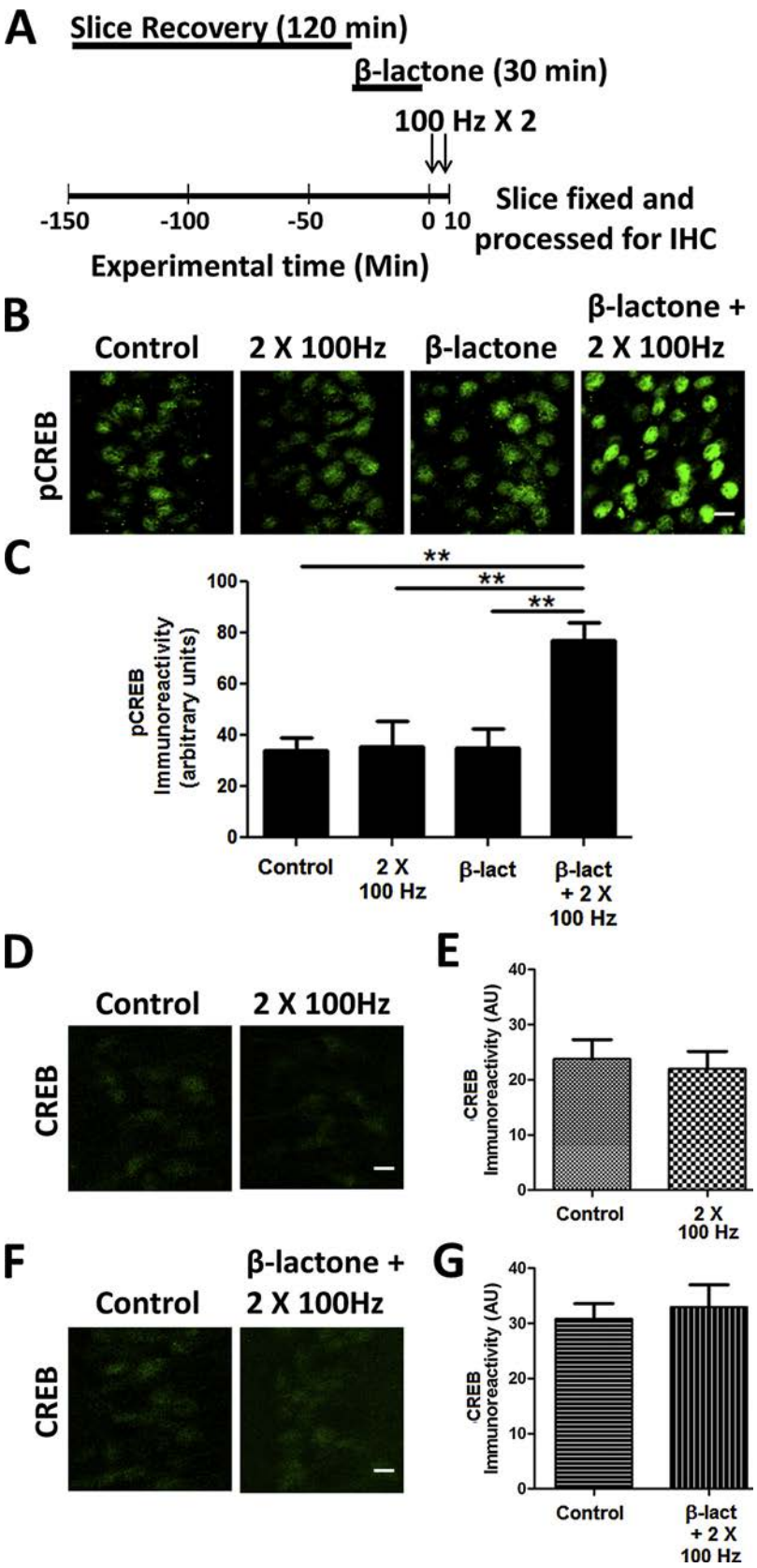

Fig. 1. β-lactone treatment increases CREB phosphorylation (pCREB).
(A) Schematic outline of the experiment: after the recovery period of 120 min, hippocampal slices were treated with β-lactone for 30 min. The beginning of electrophysiological stimulation is designated as 0 min at which point the slices were subjected to subthreshold LTP induction (2 X 100 Hz). (B) Confocal images of pCREB immunoreactivities in the CA1 region of hippocampal slices without any treatment (control) or after subthreshold LTP induction (2 X 100 Hz) alone, after β-lactone treatment alone, after β-lactone treatment followed by subthreshold LTP induction (β-lactone + 2 X 100 Hz). Scale bars: 20 μm. (D) Quantification of pCREB immunoreactivities shows that β-lactone treatment significantly increases CREB phosphorylation. (D) CREB immunoreactivities in the CA1 region with no treatment (control) or after subthreshold LTP induction (2 X 100 Hz). (F) CREB immunoreactivities in the CA1 region with no treatment (control) or after β-lactone treatment followed by subthreshold LTP induction (β-lactone + 2 X 100 Hz). (E, G) Quantification of CREB immunoreactivities of results shown in panels D & F. $^{**}p < 0.01$ comparison between two groups as indicated by horizontal lines.

3.3. MAP kinase and PKG play a role in proteasome-inhibition-mediated increase in pCREB

Signaling from synapses to the nucleus that increases phospho-CREB amounts during development of L-LTP does not depend just on PKA. Rather, previous research suggests that there is cross-talk between kinases. MAP kinase (ERK) is one of the main kinases implicated in signal transduction to the nucleus in hippocampal neurons [16]. To test the

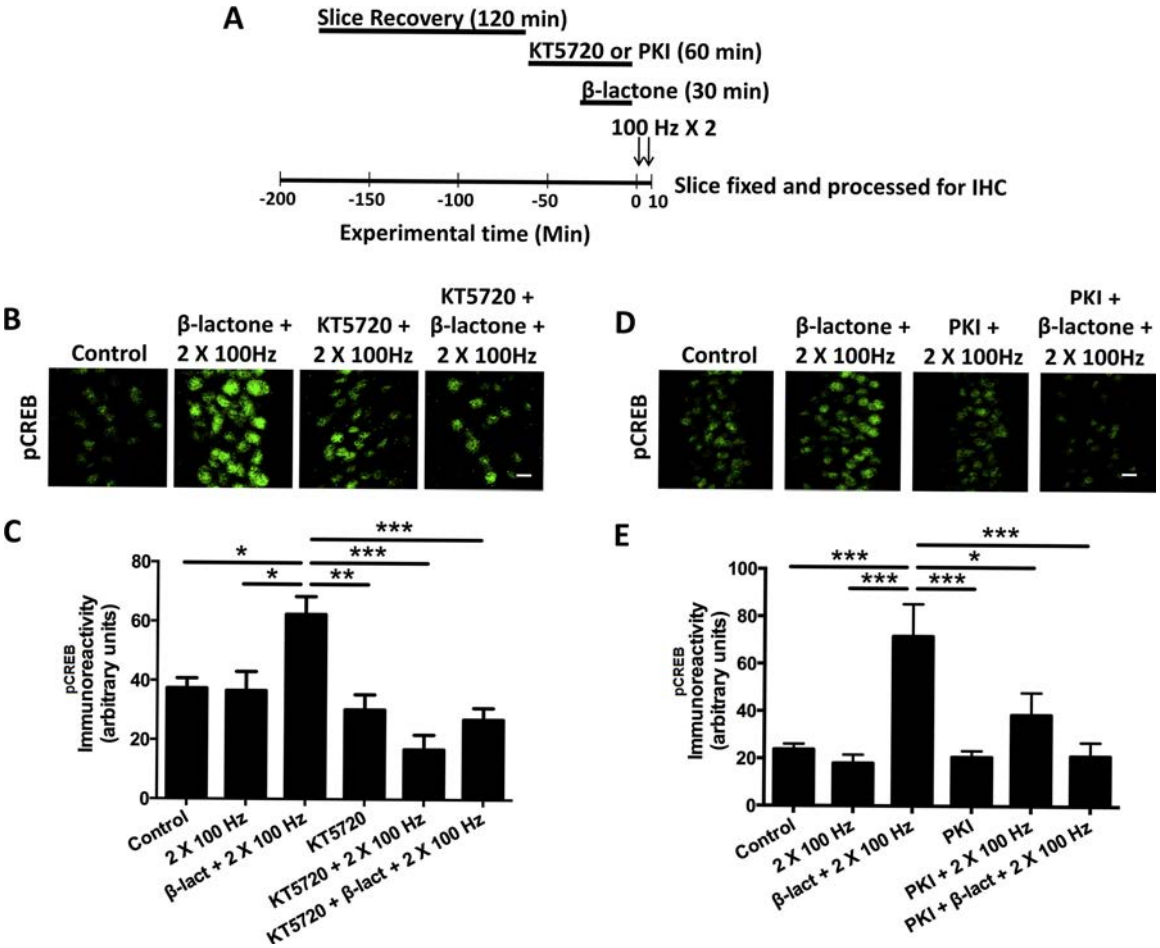

Fig. 2. Pretreatment with PKA inhibitors, KT5720 and PKI, blocks β-lactone-mediated enhancement in amounts of pCREB.
(A) Schematic outline of the experiment: after the recovery period of 120 min, hippocampal slices were treated with a PKA inhibitor (KT5720 or PKI) for 60 min. For the last 30 min of the PKA inhibitor treatment, β-lactone was added to the incubation solution. After chemical treatment, slices were transferred to the recording chamber and the slices were subjected to the 2 X 100 Hz protocol. Slices were then fixed and processed for immunohistochemistry.
(B, D) Confocal images of pCREB immunoreactivities in the CA1 region of hippocampal slices without any treatment (control) or after β-lactone treatment followed by subthreshold LTP induction (β-lactone + 2 X 100 Hz), after PKA inhibitor treatment followed by subthreshold LTP induction (KT5720 or PKI + 2 X 100 Hz), or after PKA inhibitor and β-lactone pretreatment followed by subthreshold LTP induction (KT570 or PKI + β-lactone + 2 X 100 Hz). Scale bars: 20 μm. (C, E) Quantification of pCREB immunoreactivities shows that PKA inhibitor pretreatment blocks the β-lactone-mediated enhancement of CREB phosphorylation after subthreshold LTP. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ comparison between two groups as indicated by horizontal lines.

role for MAP kinase, we carried out the subthreshold L-LTP protocol after pre-treatment of the hippocampal slices with β-lactone or with the MAP kinase inhibitor U0126 (20 μM) followed by application of β-lactone. Enhancement in pCREB immunoreactivity was much lower with 'U0126 + β-lactone' compared to treatment with β-lactone alone (Fig. 3A, B) (Control: 28.3 ± 3.4; β-lactone + 2 x 100 Hz: 62.8 ± 9.1; U0126 + β-lactone + 2 x 100 Hz: 23.6 ± 9.6; $p < 0.001$; $F_{(5, 39)} = 5.49$; n = 6).

Another protein kinase that is known to have a role in signaling to the nucleus in hippocampal neurons is PKG [17]. Previous work showed that PKG phosphorylates CREB and increases CREB-mediated transcription in neurons [18]. We tested the possible role of PKG by prior treatment of the slices with a PKG inhibitor, KT5823 (2 μM), before treating with β-lactone. We found that phospho-CREB immunoreactivity was significantly lower when β-lactone-treatment was preceded by application of KT5823 (Fig. 3C, D) (Control: 19.7 ± 2.0; β-lactone + 2 x 100 Hz: 54.3 ± 7.3; KT5823 + β-lactone + 2 x 100 Hz: 19.1 ± 6.2; $p < 0.0001$; $F_{(4, 37)} = 11.29$; n = 5).

4. Discussion

As a result of many studies, the role of protein degradation by the UPP in synaptic plasticity is now firmly established [5,19]. It has also been clear that the role of proteolysis in synaptic plasticity is complex and exact mechanistic functions of the UPP remain to be understood. Our previous work showed differential roles for proteasome-mediated degradation in different parts of the neuron [11]. In dendrites, proteasome limits accumulation of locally translated proteins and therefore proteasome inhibition boosts the early induction part of L-LTP [11,20]. In contrast, the proteasome facilitates transcription in the nucleus by degrading the negative regulators of transcription such as ATF4 and possibly through modulation of histone modification. Proteasome inhibition leads to blockade of transcription and inhibition of the late, maintenance phase of L-LTP [11].

The present study began as an attempt to test whether proteasome inhibition blocks signaling to the nucleus and contributes to blockade of transcription, which we had observed earlier [11]. To assess signaling to the nucleus, we measured accumulation of phospho-Ser-133 CREB (pCREB) in the nuclei in the CA1 region of the hippocampus. Previous studies by others have established that the 4-train protocol (4 x 100 Hz, 5 min apart) causes an increase in nuclear pCREB [17]. Our pilot studies also showed an increase in pCREB when the 4-train protocol (4 x 100 Hz, 5 min apart) was used to induce L-LTP, but prior proteasome inhibition with β-lactone revealed no adverse effect on quantities

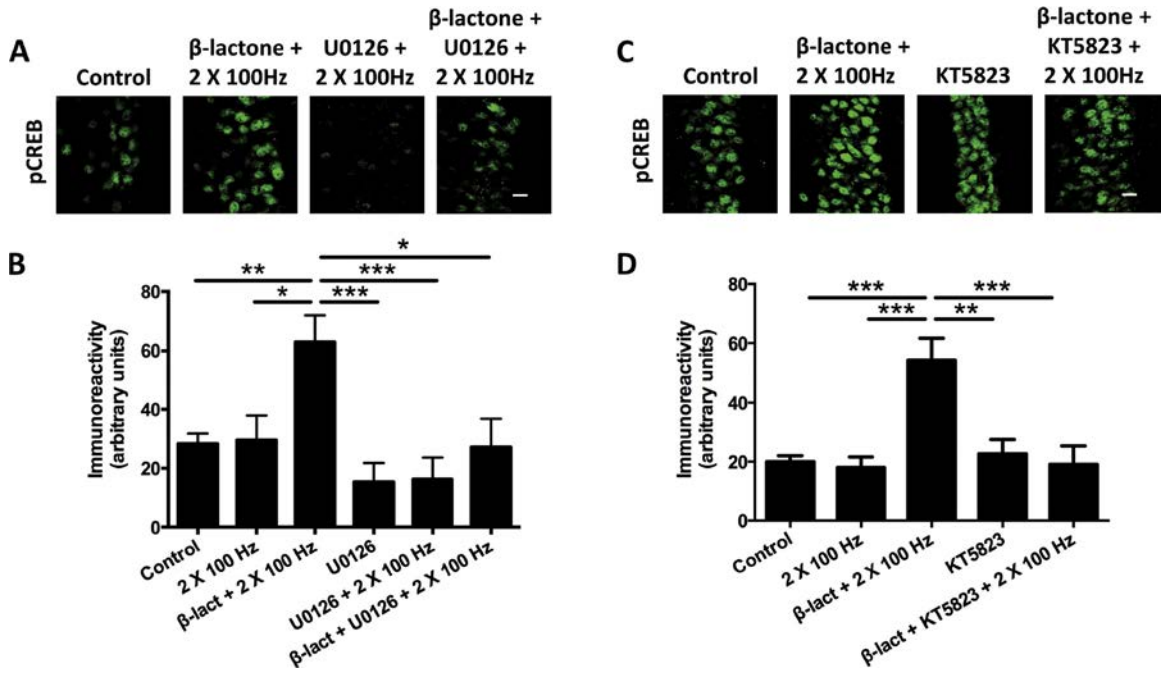

Fig. 3. Pretreatment with inhibitors of ERK (U0126) and PKG (KT5823) blocks β-lactone-mediated pCREB enhancement.
(A) Confocal images of pCREB immunoreactivities in the CA1 region of hippocampal slices without any treatment (control) or after β-lactone treatment followed by subthreshold LTP induction (β-lactone + 2 X 100 Hz), after ERK inhibitor treatment followed by subthreshold LTP induction (U0126 + 2 X 100 Hz), and after ERK inhibitor and β-lactone treatment followed subthreshold LTP induction (β-lactone + U0126 + 2 X 100 Hz). Scale bars: 20 μm. (B) Quantification of pCREB immunoreactivities shows that ERK inhibitor pretreatment blocks the β-lactone-mediated enhancement of CREB phosphorylation after subthreshold LTP. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ comparison between two groups as indicated by horizontal lines.
(C) Confocal images of pCREB immunoreactivities in the CA1 region of hippocampal slices in without any treatment (control) or after β-lactone treatment followed by subthreshold LTP induction (β-lactone + 2 X 100 Hz), after PKG inhibitor treatment followed by subthreshold LTP induction (KT5823 + 2 X 100 Hz), and after PKG inhibitor and β-lactone treatment followed by subthreshold LTP (β-lactone + KT5823 + 2 X 100 Hz). Scale bars: 20 μm. (D) Quantification of pCREB immunoreactivities shows that PKG inhibitor pretreatment blocks the β-lactone-mediated enhancement of CREB phosphorylation after subthreshold LTP. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ comparison between two groups as indicated by horizontal lines.

of pCREB (data not shown). Therefore, we concluded that hindrance of signaling to the nucleus is not likely to be the reason for the decrease in transcription that we had previously observed with proteasome inhibition [11]. During the course of investigation, we tested whether proteasome inhibition can enhance pCREB levels. With subthreshold 2-train stimulation protocol (2 x 100 Hz, 5 min apart), we consistently found an increase in nuclear pCREB levels in the presence of a highly specific proteasome inhibitor, β-lactone. Our data also show that under these conditions, the amount to total CREB is not changed. Although CREB is known to be a substrate for the proteasome based on the studies in non-neuronal cells [21,22], in the induction stage of subthreshold L-LTP perhaps it is not phosphorylated within the DSVDTS motif (which is separate from the sequence within which Ser-133 resides) that targets it to the proteasome. It is likely that initially, when conditions are adequate to induce gene expression through CREB in hippocampal neurons, CREB is phosphorylated on Ser-133, which leads to transcriptional activation. Once transcription is completed, CREB is phosphorylated on a serine residue within the DSVTDS motif and is degraded by the proteasome. The time window for transcription required for L-LTP maintenance is 2 h [23]. Therefore CREB degradation, if it were to occur, would happen at much later time point than we investigated. In addition, it is possible that CREB degradation does not occur under subthreshold L-LTP conditions. Therefore, it is unlikely that proteasome inhibition is stabilizing CREB leading to an increase in pCREB.

Another possibility for increase in pCREB is that blockade of the proteasome prevents degradation of pCREB. In other words, if basal pCREB were to turn over rapidly, in principle, β-lactone could prevent this from happening and thus cause accumulation of pCREB. This is also highly unlikely because inhibiting protein kinases such as PKA, ERK and PKG prior to proteasome inhibition blocks the increase in pCREB caused by proteasome inhibition. Therefore, it is highly likely that proteasome inhibition is enhancing signaling to the nucleus by PKA, ERK and PKG. Whether these kinases act in series or parallel is not known and will have to await detailed future investigations. It must be noted that we chose not to test another kinase implicated in L-LTP, namely calcium-calmodulin-dependent kinase IV [24] because at present drugs that only inhibit CaMKIV (and not other CaM kinases such as CaMKII) are not available.

How does proteasome inhibition cause an increase in nuclear signaling? The most likely possibility is that proteasome normally limits one or more components of the pathway(s) that links NMDA receptor activation to phosphorylation of CREB. These components could be neurotransmitter receptors themselves or any of the downstream components of the kinase signaling cascade.

Proteasome could in principle limit signaling to the nucleus by limiting accumulation of a critical signaling protein that is locally translated in dendrites. Although this scenario is possible, we think the simplest mechanistic explanation for our results is that proteasome inhibition stabilizes AMPA and NMDA receptors. Stabilizing the AMPA receptor should facilitate opening of NMDA receptor channels and stabilization of the NMDA receptors should further enhance signaling to the nucleus *via* a cascade initiated by the elevation of Ca^{2+} entering through the NMDA receptor channels.

Both AMPARs and NMDARs are known to be degraded by the UPP [25,26]. The number of AMPARs in the plasma membrane is determined by the rate of its turnover. AMPARs are endocytosed in after attachment of ubiquitin. It is thought that linkage to one, two, or three ubiquitin molecules traffics the endocytosed AMPARs to the lysosome whereas attachment of four or more ubiquitin molecules

(polyubiquitination) causes AMPARs to be degraded by the proteasome [27]. Polyubiquitination of AMPARs can be reversed by the action of two deubiquitinating enzymes USP8 and USP46 [28,29]. Removal of polyubiquitin tag caused the AMPARs to be recycled back to the plasma membrane which increases the overall number of the AMPARs in the postsynaptic membrane. We favor the idea that in the presence of a proteasome inhibitor, the 2-train protocol leads to insertion of additional AMPARs in the postsynaptic membrane thus setting the stage for an increase in NMDAR-mediated signaling.

NMDARs are known to be targets of ubiquitin-proteasome-mediated proteolysis. NMDARs are retrotranslocated and degraded by the UPP in an activity-dependent fashion [30]. Ubiquitination of the NR1 subunit of NMDARs by an F-box protein called Fbx2 is critical for this process [30] suggesting that an SCF-type ligase targets the NR1 subunits for ubiquitination. Subsequent studies showed that another NMDAR subunit NR2B is targeted for ubiquitination by an E3 ligase called Mind bomb-2 in a phosphorylation-dependent manner [31].

Based on our results, we envisage the following scenario. In the absence of adequate stimulation, the turnover of both AMPARs and NMDARs is high and thus the signaling to the nucleus is weak. Therefore, CREB phosphorylation occurs at basal levels. This situation is mimicked by our 2-train protocol. With sufficient stimulation, however, the AMPARs and NMDARs are stabilized and signaling to the nucleus is strengthened and phosphorylation of CREB is increased. Experimentally this situation occurs with the 4 X 100 Hz protocol, which induces L-LTP, which depends on transcription mediated by CREB. With prior proteasome inhibition (as with application of β-lactone in this study) in hippocampal slices AMPARs and NMDARs should be stabilized and even a weak subthreshold (2 X 100 Hz) stimulation should increase CREB phosphorylation which indeed is what we observed. Although we have focused on phosphorylation of CREB in this study, proteasome inhibition may also affect phosphorylation of other transcription factors.

It is possible that proteasome inhibition also boosts signaling to the nucleus by having an effect directly on the kinases. All the three kinases we tested PKA, ERK and PKG have been implicated in L-LTP [32–35]. In addition, cross-talk between PKA and ERK [16] as well as PKG and ERK [17] have been reported. Therefore, at present no simple explanation exists as to how proteasome blockade might enhance kinase function and therefore must await future research.

5. Conclusion

Our investigations suggest a hitherto unknown role for proteasome-mediated proteolysis, namely, regulation of signaling for CREB-phosphorylation in the nucleus. These initial results form the basis for further exploration of proteasome's role which might include controlling nuclear signaling to modulate other transcription factors.

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frontiers in Molecular Neuroscience

EDITORIAL
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Editorial: Ubiquitin and the Brain: Roles of Proteolysis in the Normal and Abnormal Nervous System

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Keywords: proteasome, synaptic plasticity (LTP/LTD), neurodegenerative diseases, Alzheimer disease, Parkinson's disease, Huntington's disease, glutamate receptors, local protein degradation

Editorial on the Research Topic

Ubiquitin and the Brain: Roles of Proteolysis in the Normal and Abnormal Nervous System

Proteolysis by the ubiquitin-proteasome pathway (UPP) is now widely recognized as a major molecular mechanism playing a role in numerous normal functions of the nervous system as well as in malfunctions of the brain in several neurodegenerative diseases. In the UPP, attachment of a small protein, ubiquitin, tags the substrates for degradation by a multi-subunit complex called the proteasome. Linkage of ubiquitin to protein substrates is highly specific and occurs through a series of well-orchestrated enzymatic steps. Protein degradation has key functions in the nervous system including fine-tuning of synaptic connections during development and synaptic plasticity in the adult organism (Hegde, 2017). In neurons, several physiological processes are regulated by proteolysis. From gene transcription to posttranslational modification of proteins, several quality checks are essential before the protein is ready for biological action, locally in dendrites or distantly via axonal transport. Beyond regulation at the RNA level, proteolysis by the UPP provides a quick and efficient way to regulate the amount of protein in neurons. In addition, accurate folding and control over levels of many proteins must be tightly regulated both spatially and temporally. To achieve this function, the cell possesses a network of different protein quality control systems (PQC) for protein folding via molecular chaperones as the first line of defense against protein misfolding and aggregation (Ciechanover and Kwon, 2017). Subsequently accurate protein degradation by the UPP and the autophagosomal-lysosomal system is the second line of defense. In addition, recent data suggest an additional PQC pathway in which misfolded proteins are excreted actively after encapsulation at the endoplasmic reticulum, a process dubbed MAPS (Misfolded Associated Protein Secretion) (Lee et al., 2016). It is even speculated that a dysfunctional PQC contributes to the process of proteopathic seeding in Alzheimer's disease (AD) (Gentier and van Leeuwen, 2015). In addition, dysfunction of the UPP is linked to Parkinson's, Huntington's, and other neurodegenerative diseases (Hegde, 2017). Perturbation in the UPP is also believed to play a causative role in mental disorders such as Angelman syndrome (Jiang and Beaudet, 2004).

Many questions pertaining to the UPP in the nervous system remain unanswered. How is the UPP-mediated degradation regulated spatially and temporally in neurons? What is the role of local protein degradation? How does the interplay between proteolysis and protein synthesis affect synaptic plasticity and memory? Do perturbations in the UPP have a role in the pathology of neurodegenerative diseases?

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The papers in this *Frontiers in Molecular Neuroscience* Research Topic-Special Issue on *Ubiquitin and the Brain: Roles of Proteolysis in the Normal and Abnormal Nervous System* cover a wide range of topics from development of methods and primary research studies to reviews. These articles from researchers working on yeast, neuronal and glial cell culture and mouse models, often validated in postmortem human brain tissue, cover a wide array of topics such as receptor endocytosis and synaptic plasticity in the normal nervous system to abnormalities of the nervous system such as AD and Huntington's disease.

The paper by Pinto et al. reports the results of studies on visualizing a specific type of ubiquitin linkage to substrate proteins in which ubiquitin molecules are linked to each other through a covalent linkage to Lysine-48 (K48) in the ubiquitin sequence. The K-48 linkage targets substrate proteins for degradation. Pinto et al. study adapted a technique to monitor K-48-liked ubiquitin molecules in cultured rat hippocampal neurons. In this technique, a yellow fluorescent protein called Venus is split into non-fluorescent N- and C- termini and fused to sequences containing ubiquitin-interacting-motifs (UIMs). These parts come together when they bind to closely spaced ubiquitin molecules in a polyubiquitin chain assembled through K-48 linkage. Using this technique, the authors show that proteins tagged with K-48-linked ubiquitin chains accumulate in presynaptic terminals when synapses newly form.

The studies by Scott Wilson and colleagues (Vaden et al.) builds on their previous work on the role of a deubiquitinating (DUB) enzyme called USP14 in the mammalian neuromuscular junction (NMJ). Their studies suggest that the role of USP14 in maintaining the free ubiquitin levels is critical for NMJ structure but the function of NMJ (such as muscle coordination) is likely to be regulated by USP14 through a separate pathway. Apart of USP14, many other DUBs play a role in the nervous system. Ristic et al. argue in their review that looking at the UPP from the point of view of DUBs can provide novel insights into the exquisite regulation of proteolysis in the nervous system and might be helpful in devising therapeutic approaches.

Goo et al. take a look at the role of ubiquitin in regulating trafficking of AMPA-type glutamate receptors. After internalization AMPA receptors can either be recycled back to the plasma membrane or degraded by the lysosome or the proteasome. For example, Nedd4-1, a ubiquitin ligase, is responsible for targeting AMPA receptors to proteasome-mediated degradation. Two deubiquitinating enzymes called Usp8 and Usp46 remove the ubiquitin attachment on AMPA receptors and thus appears to be important for rescuing the receptors from degradation and recycling them back to plasma membrane.

Rodriguez et al. examine the question of increased longevity in female mice with chronic rapamycin treatment and the effect of the drug on proteasome activity and expression of chaperone proteins. They show that rapamycin treatment has different effects on various tissues of the body and increases the amount of proteasome-interacting chaperone proteins in female but not in male mice.

Stojkovic et al. review the key role of ubiquitination in modifying the clock proteins which determine the circadian rhythm. They propose that ubiquitination is a key part of the posttranslational modification "code" that determines the fate and function of a particular clock protein.

Hegde et al. review the accumulated evidence on local roles of protein degradation in induction and maintenance of long-term synaptic plasticity. Because neurons are highly polarized cells, understanding local proteolysis is likely to be important both from a basic science point of view and translational research. Local neuronal function is also emphasized in the mini-review by Zhao et al. with respect to clearance of mutant huntingtin (mHtt) protein. Tagging of mHtt by ubiquitin plays a role in clearance by the proteasome as well as through autophagy. The authors present strategies to study local clearance and accumulation in neuronal sub-compartments.

Jarome and Helmstetter review the evidence for the roles of protein degradation in long-term memory (LTM) storage. They discuss the interplay between protein degradation and protein synthesis in the hippocampus and amygdala and other cortical areas during formation and consolidation of LTM.

The coverage of the role of proteolysis by the UPP in abnormalities of the nervous system ranges from drug abuse to neuroinflammation and neurodegenerative diseases. Massaly et al. review the evidence for role of the UPP in mediating the effects of drug abuse on the nervous system. They details the role of the UPP in regulating molecules that mediate drug-abuse-related neuroplasticity. In addition, they present evidence from the literature regarding how drugs of abuse regulate amounts and function of the various components of the UPP. The review by Figueiredo et al. discusses the role in neuroinflammation of a specific class of prostaglandins called J2 which are the toxic products of cyclooxygenases. Among the pleiotropic effects of J2 prostaglandins is perturbation of UPP. The authors suggest that the targeting neuroinflammatory pathways stimulated by J2 prostaglandins might be beneficial in treating several neurodegenerative diseases such as AD, Parkinson's and amyotrophic lateral sclerosis. Gong et al. discuss the role of components of the UPP such as the deubiquitinating enzyme Uch-L1, F-Box protein Fbx2, and the proteasome in development of AD and spinal cord injury. They suggest pharmacological manipulations of these components could be developed as a therapeutic strategy. In addition, the role of UPP is highlighted in neurodegenerative diseases such as AD, Parkinson's disease and Huntington's disease (papers by Ortega and Lucas; Atkin and Paulson; Jueneman et al.).

Until recently, the investigation with regard to the role of proteolysis in neurodegenerative diseases largely focused on neurons and role of the UPP in glia was largely ignored. This has been true also of other diseases with protein conformational abnormalities such as Amyotrophic Lateral Sclerosis and Parkinson's disease. The review by Jansen et al. tackles this issue and discusses among other things the differences between neurons and glia in how the two cell types react to impaired proteolysis by the UPP.

The studies on the role of the UPP in neurodegenerative diseases can be greatly benefited by work on simple model systems such as yeast. This model system provides an easy readout of perturbations in the UPP and allows quick analysis of

action of numerous chemicals on the UPP (see review by Brau), which can be subsequently validated using mammalian model systems. The group of Dantuma has developed many tools to detect proteasomal activity (e.g., constructs with GFP and YFP) *in vitro* as well as *in vivo*. The authors (Dantuma and Bott) discuss "the paradigm shift that has repositioned the UPS from being a prime suspect in the pathophysiology of neurodegeneration to an attractive therapeutic target that can be harnessed to accelerate the clearance of disease-linked proteins" (review by Dantuma and Bott). For example, downregulation misframed ubiquitin (UBB^{+1}) that interacts in AD with γ-secretase (Gentier and van Leeuwen, 2015) could be a potential therapeutic target to restore neuronal function perturbed by abnormalities in the UPP. Based on accumulating evidence, it is clear that the neuronal-glial network of the brain functions effectively when protein degradation is normal and impairments in the UPP contribute to the early cellular abnormalities seen in AD.

The research articles and reviews in this research topic now collected as an e-book highlight the complex role of ubiquitin-proteasome-mediated proteolysis in the neuronal and glial physiology and pathology. In spite of the considerable progress made over the last two decades, many challenges remain. For example, with respect to normal roles of the UPP in the nervous system, the roles of the UPP in memory formation especially in relation to the role of protein synthesis need to be better understood. The exact contributions and the interplay of the UPP with other protein-clearance systems such as autophagy in various neurodegenerative diseases also need to be delineated. As many new technological developments such as CRISPR/Cas9, allow precise testing of hypotheses, we may expect to see many additional interesting discoveries on both these fronts.

AUTHOR CONTRIBUTIONS

Both authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Research report

Age-dependent changes in brain hydration and synaptic plasticity

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ABSTRACT

For this collaborative work, Dr. Hegde contributed his expertise in Electrophysiology which was an essential part of this research project. His GCSU affiliation is shown at bottom left of this page.

Aging in humans and animals is associated with gradual and variable changes in some cognitive functions, but what causes them and explains individual variations remains unclear. Hydration decreases with aging but whether dehydration contributes to cognitive dysfunction is not known. The brain hydration of aging mice was determined by colloidosmotic-pressure titration. Dehydration increased with age from ~76 mmHg at 6 weeks to ~105 mmHg at 40 weeks, or a progressive ~10 percent loss of brain water but seemed to level off afterward. When we adjusted dehydration in hippocampal slices of <8-week-old mice to the levels seen in mice 40 weeks and older, their basal synaptic responses were amplified at all stimulus voltages tested, but induction of late-phase long-term potentiation was impaired. Our results document progressive brain dehydration with age in inbred mice to levels at which *in vitro* synaptic plasticity appears dysregulated. They also suggest that dehydration contributes to some of the changes in synaptic plasticity observed with aging, possibly due to adjustments in neuronal excitation mechanisms.

1. Introduction

With age, some biological mechanisms thought to underlie cognition change at the cellular level; for example, dysregulation of excitation and of signaling pathways in hippocampal neurons have been documented (Hansen and Zhang, 2013; Oh et al., 2016; Whalley, 2001). At the same time, the proportion of body water (Hooper et al., 2014) and brain parenchyma volume decrease, but no mechanistic relationship has been established between these cognitive and hydration changes.

The human brain progressively shrinks. The ventricular volume increases, while certain areas, including the caudate, cerebellum, hippocampus, and the association cortices, show substantial, albeit localized, diminution, which could be due, in part, to a decrease in gray matter, perhaps from neuronal loss or shrinkage, reduced synaptic spines, or fewer synapses (Deary et al., 2009; Fjell and Walhovd, 2010; Kempton et al., 2011; Raz and Rodrigue, 2006; Walhovd et al., 2005). However, neuronal loss, in particular in the hippocampus and neocortex, is not found in healthy aging and cannot account for the reduced volume measured in these areas (Burke and Barnes, 2006; Rapp and Gallagher, 1996). Water loss from cells and the interstitial spaces might be a factor, but we do not know its precise contribution to brain volume.

Because many forces balance interstitial hydration, the volume and/or total weight of brain could change, at least in theory, without concomitant changes in water content and vice versa. Our earlier work developed the concept of *hydration potential* as a useful parameter to explore hydration changes in interstitial microenvironments (McGee et al., 2009, 2011, 2012, 2014). The present study measured it in the aging brain to document any age-related changes in hydration that might explain the previously documented volumetric reduction and the physiologically relevant ranges. We must first know the ranges typical for healthy aging brain to test whether physiologic ranges of brain dehydration influence neural plasticity.

Aging has been associated with dysregulated excitability in hippocampal pyramidal neurons mainly due to enhanced hyperpolarization (Foster and Kumar, 2002). In addition to changes in the intrinsic excitability of hippocampal neurons, a variety of differences between old and young brain function have been described (Burke and Barne, 2006). They include both low-level cognitive functions, such as attention, working memory, long-term memory, and perception, and high-level cognitive functions, such as speech and language, decision making, and executive control (Glisky, 2007; Whalley, 2001). Animal studies have associated aging with certain apparent deficits in consolidating short-term into long-term memory. Synaptic function in L-LTP and LTD (late-phase long-term potentiation and long term depression, respectively; *ex-vivo* models of neuronal plasticity) appear impaired in old compared to young rodents and contribute to cognitive changes (Disterhoft and Oh, 2006; Bach et al., 1999; Huang and Kandel,

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2006). LTP impairments and accelerated decay (Rosenzweig and Barnes, 2003, Rogers et al., 2017) as well as reduced synaptic strength, paired pulse facilitation, and spatial memory have also been reported (Weber et al., 2015).

This study tested the hypothesis that changes in hydration influence neuronal pathways. To determine the physiologic range of hydration we measured hydration potential in brain tissue from mice aged 6 to 60 weeks. To determine whether hydration influences neuronal function we modified the hydration potential of hippocampus slices from young inbred mice and measured the resultant changes in basal synaptic transmission and L-LTP induction.

2. Results

2.1. Water transfers from ACSF to brain interstitium

The *hydration potential* of mouse brain tissue was derived *ex vivo* from changes in the explants' weight. In all age groups, water flowed into the brain tissue when it was immersed in ACSF (artificial cerebrospinal fluid) without colloids. Influx rates were faster initially, approaching a maximum in approximately 30 min, and remaining stable for at least 1 h. Routinely, initial rates, *Ri*, were calculated from the first derivative of second-degree polynomials fitted to the progress curves. When the colloidosmotic pressure in ACSF was varied, R_i changed in proportion; $\Delta R_i = K \cdot \Delta P_i$ where ΔP_i is the change in fluid-driving pressure induced at each colloid concentration (Fig. 1A–D). In regression analyses, plots of the initial rate versus pressure were consistent with a linear model giving $r^2 > 0.8$.

The slope K of fitted lines, $\Delta R_i = K\ \Delta P_i$, with units of μl/min/mmHg/g, reflects the *hydraulic conductance* of the brain tissue. The ΔP_i in mmHg when $Ri = 0$ is the fluid driving pressure and numerically equals the *hydration potential* (Fig. 2A–F). This value depends on all the hydrating interactions when fluid-driving forces are balanced and reflects the competition for water in brain tissue. The higher the potential, the more dehydrated the tissue and the stronger the interstitial suction forces generated by the matrix.

In the 6-week-old animals used as a reference, the hydration potential was 75 ± 5 mmHg relative to ACSF, and the hydraulic conductance was 0.060 ± 0.007 μl/mmHg/min/g. The hydration potential increased with age up to 40 weeks (Fig. 2E summarizes mean values and statistical analyses). Compared to the 6-week-old mice, the change in hydration potential observed in 40- and 60-week-old mice corresponds to an approximately 10 percent decrease in relative water content (dehydration). Since the literature reports that the total brain weight of the C57BL/6 strain changes little with age, and the hippocampal weight decreases slightly only after 112 weeks (Lessard-Beaudoin et al., 2015), the dehydration we detected may be associated to relative increases in the weight of some other tissue component.

In contrast to these hydration potential changes, no significant changes in the hydraulic conductance were detected in aging mice. The hydraulic conductance, *K*, reflects the resistance to, and power for, fluid transfer across the brain extracellular matrix. Since it depends on matrix structure and cell mechanical activity, physiologic variations in its value are expected to be small under the conditions of these experiments. Statistical analyses comparing hydration parameters among all age groups tested are summarized in Fig. 2E and F.

The observed increase in hydration potential implies decreased water activity relative to ACSF and therefore increased activity of all hydrated molecules and surfaces in the brain tissue. It also indicates an increase in the interstitial suction that must be balanced in the intact brain by blood and lymphatic hydrostatic and colloidosmotic potentials.

2.2. Basal synaptic transmission increases in dehydrated brain tissue

Hydration levels in the brains of young mice were manipulated *ex vivo* by equilibration in baths at potentials set from Δ0 to Δ200 mmHg, which includes the physiologic 75–102 mmHg range found in mice 6–60 weeks old. After equilibration, the basal synaptic transmission strength increased with the osmotic pressure of the equilibrating bath, indicating that the level of hydration in the micro-environment influenced neuronal responses to a single stimulus. The fEPSP (field excitatory postsynaptic potentials) recorded in response to stimulating amplitudes from 10 to 35 V were higher in dehydrated slices than those at the physiologic hydration level; dehydration induced no apparent saturation. Fig. 3B shows the changes in fEPSP/voltage with changes in hydration, summarizing the results and statistics from linear regression analyses.

The changes in synaptic strength were further analyzed using the amplitudes and parameters derived from Eq. (2) (Section 4.4, Fig. 3C and D), including apparent stimulation threshold (i.e., the x-axis intercept), the A_{max}, and the B factor. While the apparent stimulation threshold did not change with hydration level, both A_{max} and B increased with dehydration. Fig. 3C illustrates the convergence of thresholds, and Fig. 3D, the nonlinear relationships between the increases in basal A_{max} and B with dehydration. Large changes in synaptic transmission strength are detected when hydration is below the physiologic levels of 6 weeks-old mice, but little change is noted when it is above.

Overall, the relationships between the hydration potential and either the A_{max} or B parameter are better described by exponential than linear equations, which suggests that the measured variations in neuronal responses depend on the a_w reached at equilibrium rather than the potentials driving water flow during equilibration (Eq. (1)). If that is the case, in the slices equilibrated at increasing levels above physiologic, the functional changes proportional to Δa_w are expected to become progressively smaller. Alternatively, sensing may be linear with the potential, but the observed nonlinear response reflects other intervening processes; for example, changes in the velocity of reactions with hydration/dehydration rate-limiting steps (McGee et al., 2002).

The increase in synaptic transmission was unrelated to the physicochemical characteristics of the polymer used to decrease water activity in the bath. The observed change was similar with dextran 10 or PEG, both adjusted to a colloidosmotic pressure of ~100 mmHg (Fig. 4A). These inert polymers are similar in size and molecular weight, but their very different structure and chemistry impart different properties in solution, including their viscosity. Furthermore, increases in basal synaptic transmission were fully reversible, excluding the possibility of permanently altered neuronal function, as shown in experiments where slices equilibrated at Δ100 mmHg colloidosmotic pressure returned to the expected basal levels after re-equilibration in the ACSF solution at Δ0 mmHg (Fig. 4B).

2.3. Dehydration inhibits induction of late-phase, long-term potentiation

Late-phase, long-term potentiation was induced in slices equilibrated and perfused with physiologic ACSF solution at 0 mmHg colloidosmotic pressure (Dong et al., 2008). After 30 min of stable baseline recording, when stimulated by 4 × 100 Hz trains spaced 5 min apart, L-LTP was induced and maintained for three hours. When the procedure was repeated in slices equilibrated within a 54–196 mmHg pressure range, L-LTP induction was significantly impaired at 100 and 196 mmHg compared to that in slices at 0 and 54 mmHg (0 mmHg: 159.80 ± 5.08%, 54 mmHg: 155 ± 8.96%, 101 mmHg: 112.50 ± 1.77%; $p < .001$, 196 mmHg: 97.55 ± 2.29%;

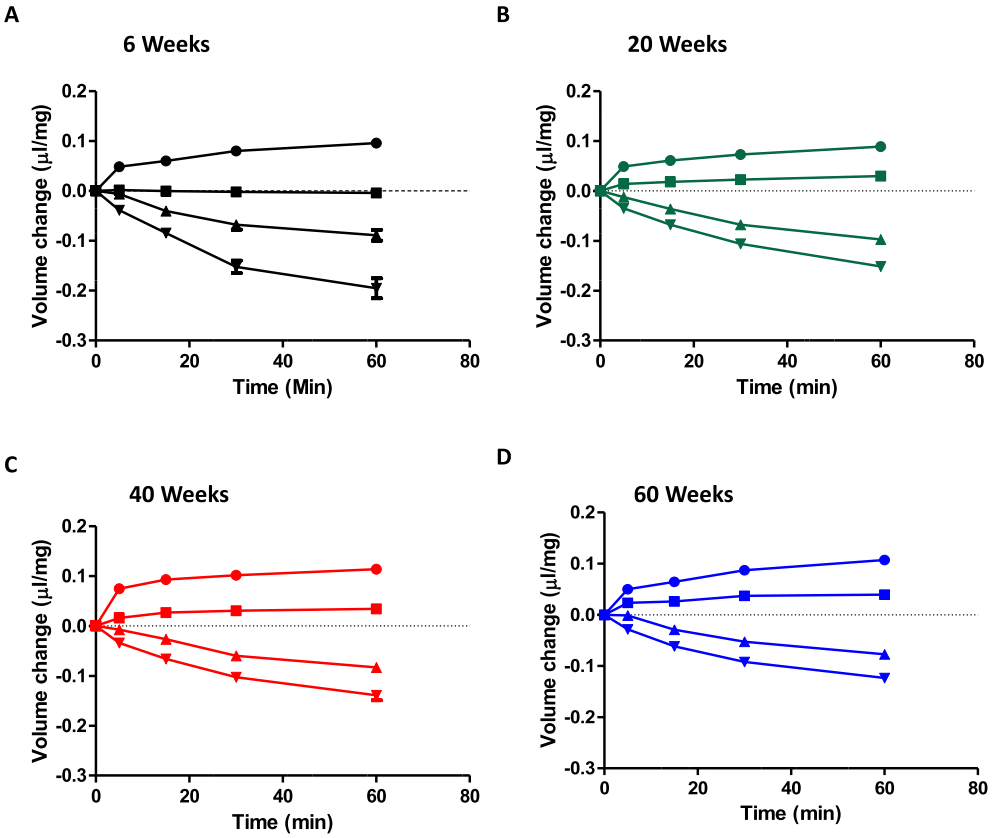

Fig. 1. Water flux across brain explants changes with age. Freshly isolated brain sections from mice aged 6 (black), 20 (green), 40 (red), and 60 (blue) weeks were immersed in baths with colloidosmotic pressure set at ● – 0, ■ – 54, ▲ – 101, and ▼ – 196 mmHg. Brain-volume changes were measured at 0, 5, 15, 20, 30, and 60 min. The fluid influx in µl, normalized per mg of brain-tissue weight, is represented by the positive half of the y-axis (●, ■), and efflux by the negative half (▲, ▼).

$p < .001$; Fig. 5). As noted above, hippocampal slices equilibrated at 0 and 54 mmHg gain water and are hyperhydrated by ~75 and 25 mmHg, respectively, while slices equilibrated at 100 and 196 mmHg are dehydrated by ~25 and ~120 mmHg, respectively, relative to the physiologic hydration of young mouse brain. Thus, the defect in L-LTP is related directly to dehydration level and inversely to the increases in synaptic strength seen in the basal synaptic transmission protocol.

3. Discussion

In the present studies, we modified neural activity by controlling cortical hydration *ex vivo*. We used objective parameters that revealed and quantified hydration changes in neuronal microenvironments during aging and measured a progressive decrease in hydration from 6 to 40 weeks that appears to level off by 60 weeks. *Ex-vivo* dehydration of hippocampal slices from juvenile mice to the same levels observed in the older mice induced changes in neuronal activity. A single depolarizing stimulus applied to Shaffer collateral axons resulted in field potentials at the CA1 pyramidal neurons that were inversely related to hydration in the hippocampus; that is, as hydration decreased, basal responses increased up to ~200 percent over those in brain tissue at physiologic and supraphysiologic hydration levels. In contrast to this enhanced excitability, slices dehydrated >5 percent were refractory to induction of L-LTP in standard protocols; that is, trains of tetanic stimulation did not further modify synaptic strength beyond the effects achieved by dehydration alone. Dehydration probably *saturates* synaptic strength, so no additional modifications are possible. However this saturation may be related to the tetanic stimulation since the same neurons responded to increased voltages in single stimulating depolarization protocols. Note that this pattern of dysregulation may not translate to other regions of the brain or even of the hippocampus. Reduced and increased neuronal excitability, respectively, have been described in the CA1 and CA3 regions in aged animals. Dysregulation of CA1 responses in preparations from old rodents showed an increase in voltage- and calcium channel-dependent forms of LTP and a concomitant decrease in N-methyl-D-aspartate receptor-dependent forms (Shankar et al., 1998). At the cellular level, dysregulation of calcium homeostasis in aged CA1 neurons, with post-burst hyperpolarization after repeated action potentials, has been proposed to explain age-associated synaptic dysfunctions (Oh et al., 2016). An increased response to synaptic activation was also suggested as a compensatory mechanism for the age-associated loss of synapses in hippocampal granule cells (Barnes and McNaughton, 1980; Shankar et al., 1998).

To the extent that L-LTP is a valid model to study synaptic plasticity mechanisms, our findings point to a possible association between dehydration and some cognitive changes observed with aging. These *ex-vivo* observations make highly improbable any alternative hypotheses stating that brain dehydration does not influence mechanisms underlying cognition. However, they do not exclude mechanistic age-dependent differences not caused by dehydration. A number of previous studies have described impairment of synaptic plasticity in association with some types of hyperexcitability, suggesting that similar phenomena can occur under a variety of experimental conditions, including overexpression of brain-derived neurotrophic factor (BDNF) (Croll et al., 1999); methamphetamine treatment (Swant et al., 2010); and deficiency of extracellular matrix tenascin-R (Saghatelyan et al., 2001).

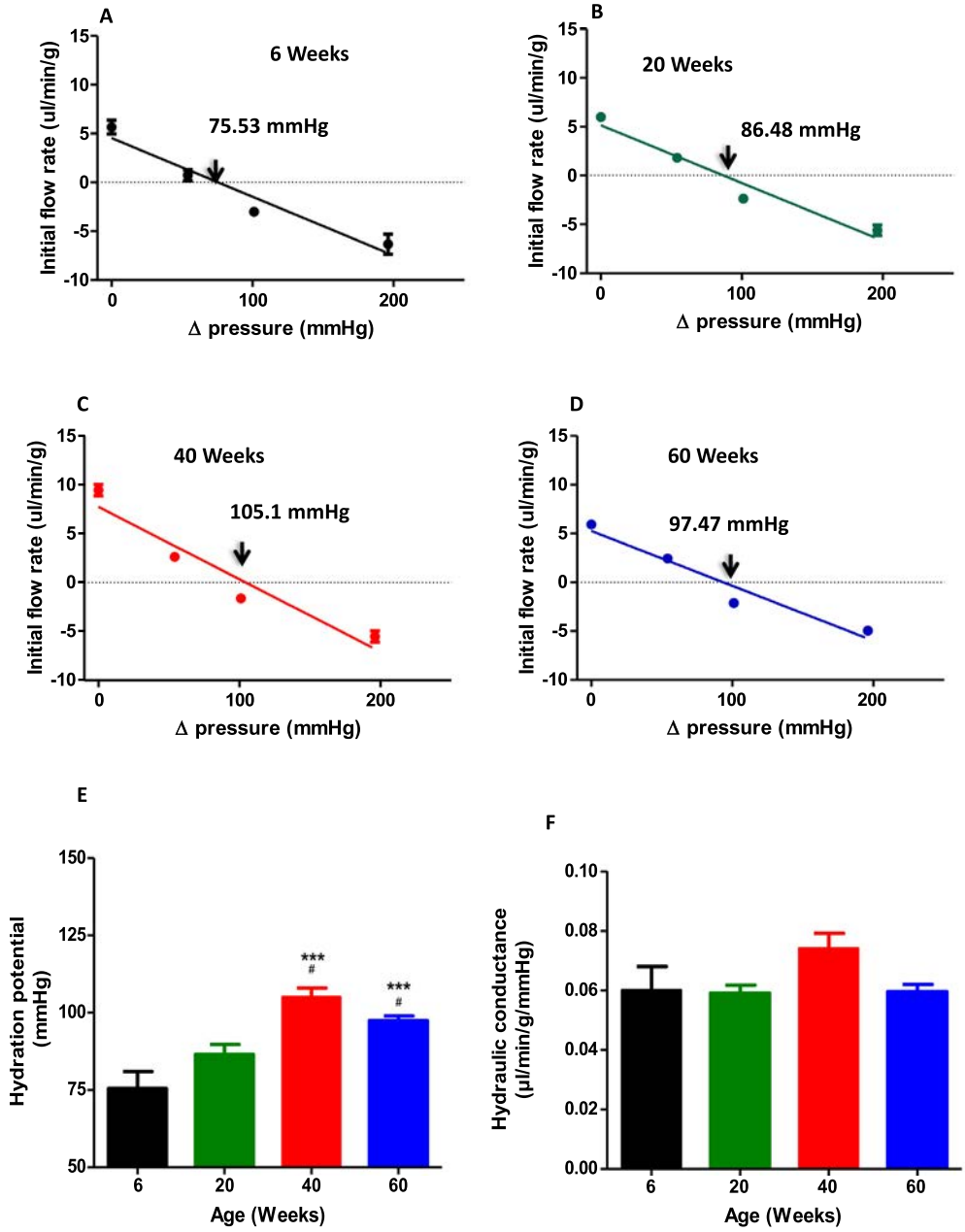

Fig. 2. Brain hydration decreases with age. Initial rates of fluid influx or efflux are plotted as a function of bath colloidosmotic pressure: panels A–D. Hydration parameters were determined by linear regression analyses from straight lines fitted to rate/pressure data points, with r^2 >0.8. (Initial rates were derived from second-degree polynomials fitted to the trajectories shown in Fig. 1.) The *hydration potential* is derived as the pressure at which there is no influx or efflux of water, so the initial rate = 0 (indicated by arrows in panels A–D), while conductance values are derived from the slope. Panels E and F show mean and standard errors for hydration potential and *conductance* values, respectively (n = 6–10 mice per age group; Differences between 6 and 20 weeks and between 20 and 40 weeks were statistically significant, P < .05. In the figure statistically significant differences P < .0001 relative to 6-week-old mice, are indicated by ***).

The use of a very well-characterized model for studying synaptic plasticity and an inbred strain reduced individual and experimental variability, allowing us to demonstrate a significant correlation between hydration parameters and cortical responses *ex vivo*. It indicates the possibility that *in vivo*, superimposed water losses cause gradated effects and at least partially explain the observed individual variability during cognition testing. Systemic dehydration has been associated with cognitive and behavioral changes in both young and middle-aged individuals (Lieberman, 2007; Lindseth et al., 2013).

The molecular mechanisms by which dehydration modulates synaptic strength may be related to local responses of the brain interstitial matrix to a_w changes. Reducing the fraction of water available for hydration implies an increase in the effective concentration of all the solutes and a spatial approximation of all reactive molecular surfaces. While any imbalance in ion concentration in the neuropil is likely to be rebalanced in milliseconds (Hoffman et al., 2009), changes in the effective concentration and diffusion distances of larger molecules can, in theory, persist long enough to modify the kinetics of neuronal processes; for example, by increasing the local density of neurotransmitters in narrowed synaptic clefts (Araque et al., 2014). The hydration potential of brain tissue indicates lower water activity and therefore more competition for water in the extracellular matrix than in cerebrospinal fluid. As water activity decreases, all molecular structures shift toward more dehydrated conformations (McGee and

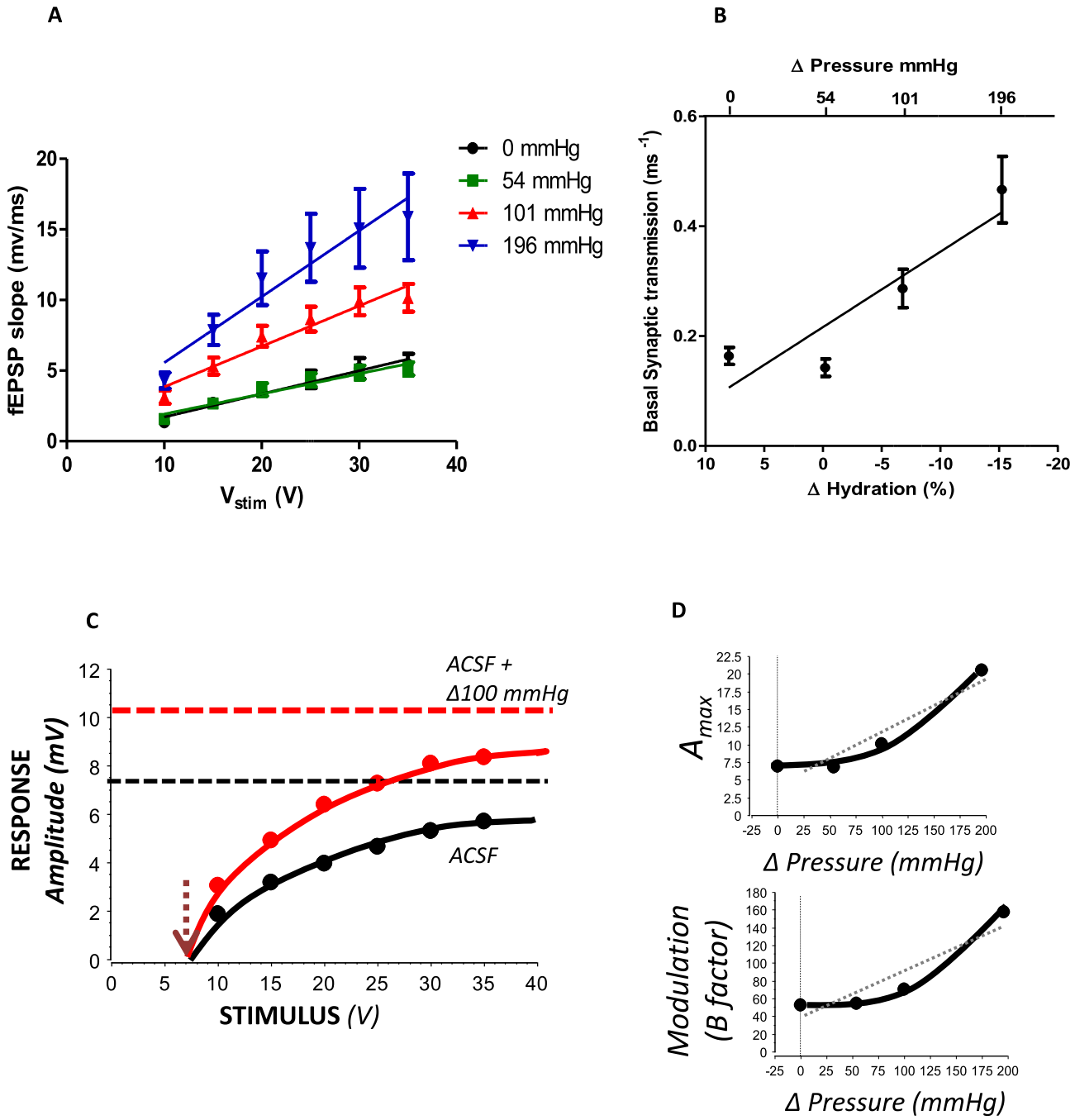

Fig. 3. Basal synaptic transmission strength changes as a function of brain tissue hydration. The hydration of hippocampal slices was adjusted within a range that includes hydration levels measured in aging mice. A. Responses (expressed as fEPSP slopes mv/ms) to a single depolarizing stimulus increasing from 10 to 35 V were measured at each hydration level, and the paired response/stimulus data fitted with straight lines. B. The change in response with the stimulus, a measure of basal synaptic transmission strength, is plotted as a linear function of tissue hydration, expressed as % of the physiologic level. The corresponding equilibrating colloidosmotic pressure in the incubation baths is indicated in the top scale. Data points are mean and standard errors for 6 mice per hydration level. C. Differences in amplitudes measured in slices equilibrated either in ACSF or ACSF in which colloidosmotic pressure was increased 100 mmHg. Data points are fitted with an inverse function (*Amplitude* = A_{max} − *B 1/Stimulus*) to compare theoretical maximal amplitudes, A_{max}, and the *B* factor. Variations in *B* factor represent response/stimulus deviations from a linear relationship and reflect the regulatory adjustments. D. Amplitude (upper panel) and *B* factor (lower panel) values are plotted as a function of the colloidosmotic pressure. Data points are fitted with either exponential (solid line) or linear (dotted line) functions. The regression coefficients (r^2) were 0.94 and 0.90 for the exponential and linear fits, respectively.

Teuschler, 1995; Parsegian et al., 2000, McGee et al., 2002). Consequently, in the aging brain the rate of reactions linked to water transfer will change and these reactions may include receptor-ligand binding and/or ion channel opening. In this regard, certain ionic channels can be activated by suction pressures in the range of the hydration potential changes (Zaelzer et al., 2015) shown here to occur with aging.

3.1. Conclusion and perspectives

The results presented here document and quantify brain hydration changes as mice age. They also demonstrate that ex vivo, neuronal functions in young brains are exquisitely sensitive to dehydration within ranges that reflect the dehydration levels that mark aging. The dehydration-induced hyperexcitability and defi-

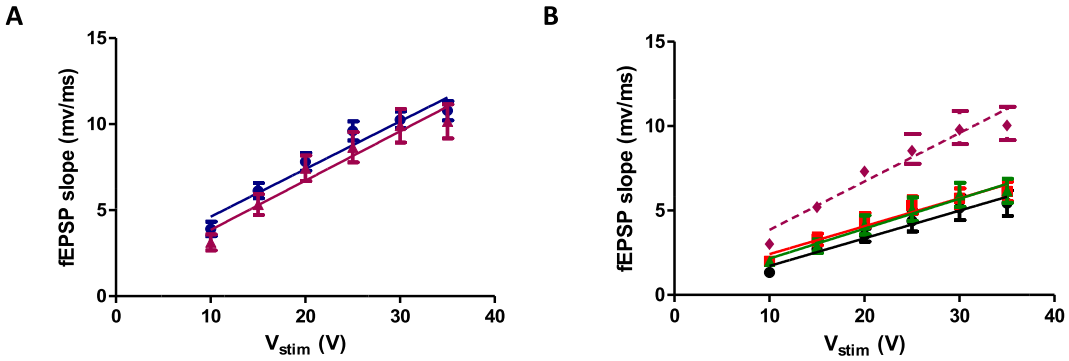

Fig. 4. Changes in basal synaptic transmission are independent of the physicochemical properties of the colloid used to control hydration and fully reversible. A. Field excitatory postsynaptic potentials (fEPSP) were measured in hippocampal slices dehydrated ~7 percent by equilibration with artificial cerebrospinal fluid solutions. The colloidosmotic pressure (Δ100 mmHg) of the solutions was adjusted with either PEG 8000 (▲) or dextran 10,000 (▲). B. fEPSP were measured in hippocampal slices after dehydration or overhydration relative to the physiologic level; (♦) dehydrated by ~7 percent (ACSF, Δ100 mmHg with PEG 8000, 30 min); (♦) overhydrated by ~7 percent (ACSF, Δ0 mmHg 30 min); (▼) dehydrated 30 min, then overhydrated 30 min; (●) overhydrated 60 min.

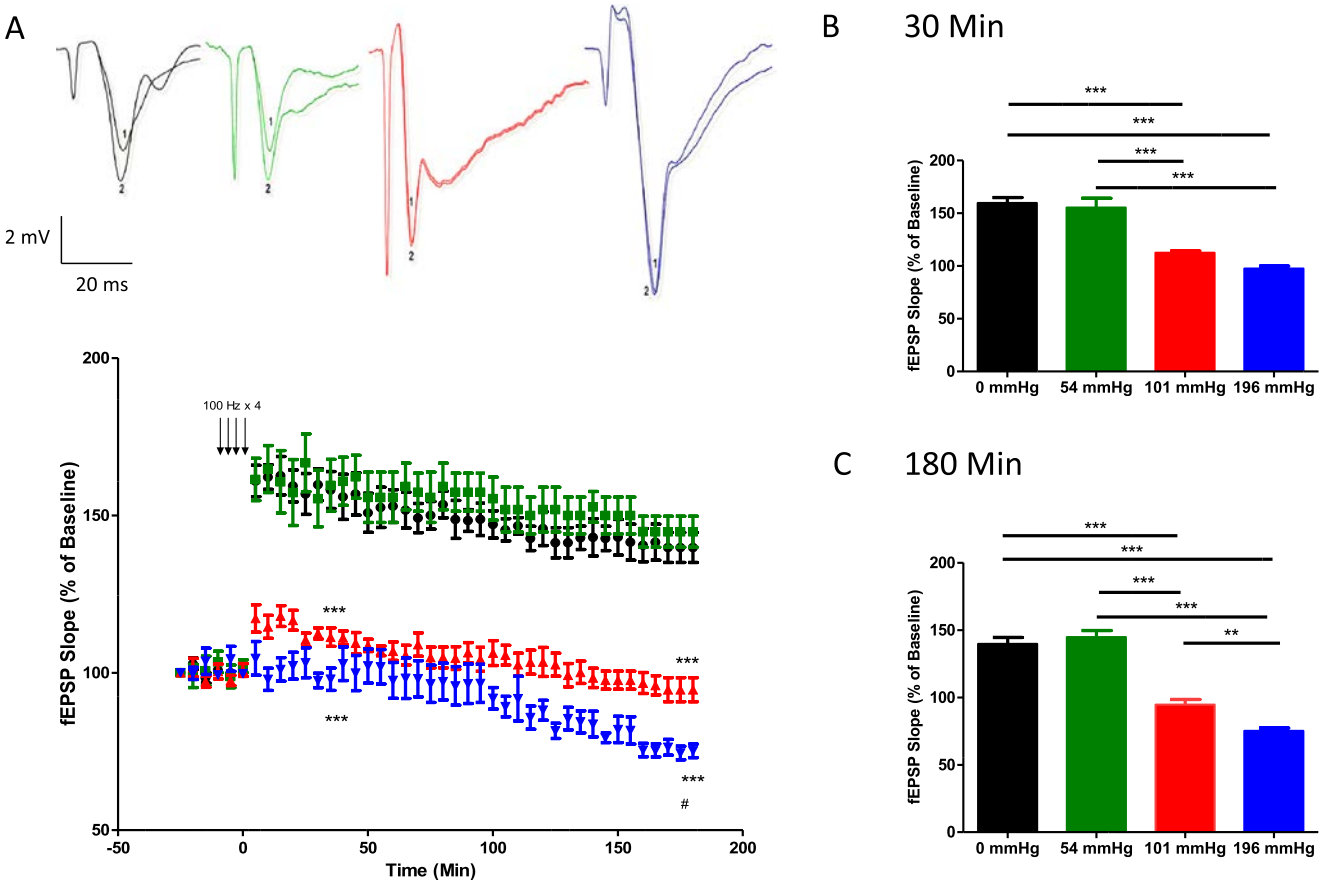

Fig. 5. Tissue dehydration impairs late-phase long-term potentiation. Hippocampal slices were equilibrated to achieve hydration levels over ●: 75.53 mmHg; and ■: 21.53 mmHg; and under ▲: −25.47 mmHg; and ▼: −120.47 mmHg the mean equilibrium hydration potential of brain tissue. Long-term potentiation was induced by 4 × 100 Hz train stimulation at 5 min intervals. Amplitudes and fEPSP were followed for three hours (Panel A). The insets show typical tracings observed before (1) and 30 min after (2) the tetanic stimulation for slices equilibrated at 4, 54, 100, and 196 mmHg. The bar graphs in Panels B and C represent the means and standard errors of slopes measured 30 and 180 min after tetanization, respectively. (***Indicates statistically significant differences, P < .001.)

cient L-LTP also observed suggest an explanation for at least some cognitive changes in older individuals. The brain hydraulic conductance was not significantly different in young and old animals indicating interstitial fluid transport rates and the factors that determine it do not change with aging. However, more subtle changes may occur and further studies should explore the possibility of age-dependent differences in neuronal responses under controlled hydration/ dehydration *ex vivo*. Perhaps moderate dehydration in the mature brain is an adaptive response to other age-dependent changes; for example, decreased receptor turnover and other alterations for which decreased water activity and consequent increases in the activity of all solutes could compensate. This explanation is consistent with the idea that, physiologically, optimization of brain function over the individual lifespan may require changes in the efficiency of some cognitive processes (Whalley, 2001; Gutches, 2014).

4. Experimental procedures

4.1. Animals

C57BL/6 mice of different ages (6 weeks-old, n = 6; 20 weeks-old, n = 10; 40 weeks-old, n = 10; 60 weeks-old; n = 10) obtained from Harlan Laboratories were used in experimental protocols approved by the Institutional Animal Care and Use Committee.

4.2. Brain tissue preparation and fluid-transfer measurement

Brains were removed immediately after euthanasia. Medulla and spinal cord connections were excised, and the remaining cerebrum was divided in four approximately equal sections with approximately equal volumes of grey and white matter.

To measure the hydration potential, brain sections were immersed in ACSF containing 125 mM NaCl, 3 mM KCl, 2.3 mM $CaCl_2$, 1.3 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, and 10 mM glucose (pH 7.4). The solution's water activity, a_w, was progressively reduced with inert polymers (0–12% w/w), either polyethylene glycol 8000 (PEG-8000; ~8000 Da and ~26 Å radius; henceforth termed PEG) or dextran (Sigma, ~10,000 Da and ~23 Å radius). Colloidosmotic pressure was determined by reference to standard curves constructed using a membrane osmometer (molecular weight cutoff 5000, Wescor 4420) calibrated with a 5 percent solution of human albumin set at 20 mmHg. The relative changes in water chemical potential, $\Delta\mu_w$, and activity, a_w, resulting from each incremental addition of macromolecular solute can be approximated from the corresponding changes in colloidosmotic pressure, π:

$$\Delta\mu_w = RTlna_w = V_w\pi \quad (1)$$

where μ_w and V_w are the chemical potential and molar volume of water, respectively; R is the gas constant (8.314 J/mol K); and T, the absolute temperature (Bergethon and Simons, 1990).

The rate of fluid transfer between the brain interstitium and bulk fluids was measured at 25 °C by precision weighing of explants at regular intervals. Volume change (V) was derived from the weight differences between the explants at time = 0 and subsequent intervals. In the brain interstitium considered as an open system, the work of fluid transfer includes forces arising from its structural and functional complexity. Hence, we evaluate hydration potential, the net force measured in the interstitial matrix of excised tissues, as a thermodynamic grand potential that includes not only pressure-volume work and water chemical potential but also entropic, interfacial, and mechanical components (Evans and Bettolo, 1987, McGee et al., 2012). The hydration potential measures the difference in thermodynamic grand potential between the excised tissue and the bulk fluid in the bath. It is defined empirically as the bath pressure at which explant weight is stable; that is, when the initial potential in explant and bath are equal.

4.3. Extracellular recording in hippocampus slices

Transverse hippocampus slices (400 μm) were prepared from 6 to 12-week-old mice (n = 6) using a tissue chopper in oxygenated and chilled ACSF. Slices were allowed to recover for about one hour in oxygenated ACSF and maintained at 32 °C. The fEPSP were measured in the CA1 region of the hippocampus recording in the stratum radiatum using a bipolar electrode to stimulate the Schaffer collateral pathway. Prior to fEPSP recording, hydration potential in the tissue was fixed by equilibration for 30 min in ACSF solution with colloidosmotic pressures of 0, 50, 100 and 196 mmHg.

4.4. Basal synaptic transmission and LTP measurements under controlled hydration

The slices were stimulated with increasing voltage, starting at 10 V, and the fEPSP recorded until the slope (amplitude/time) approached a plateau at each one of the four hydration levels.

Using separate groups of animals two sets of control experiments were designed to determine whether hydration effects were reversible and independent of the physicochemical properties of the polymer used to control hydration. In the first set, the slices equilibrated at Δ100 mmHg were re-equilibrated at Δ0 mmHg for an additional 30 min and recordings compared to those from slices maintained at 0 mmHg. In the second set, slices were equilibrated at Δ100 mmHg for 30 min but dextran instead of PEG was used as the inert solute.

To measure L-LTP the slices were maintained at fixed pressure levels during the entire induction and the recording protocol. The stimulation voltage was adjusted to give ~35 percent of the maximal recorded fEPSP slope at each hydration level, and the voltage of test pulses maintained at this intensity. L-LTP was induced by administering four 100-Hz rains 5 min apart, and the responses were recorded every 5 min for 3 h (Dong et al., 2008). The extent of LTP was expressed as percent increase in the fEPSP slope relative to baseline.

4.5. Modeling and statistical analyses

To analyze fluid-transfer kinetics in and out of the brain tissue, we fitted second-degree polynomials to the volume/time data points using the commercially available software packages GraphPad Prism and StatView (SAS Institute, Cary, NC). Initial fluid-transfer rates were determined from the slope of progression curves at time 0. The rate changes with changes in bath colloidosmotic pressure were modeled as a linear relationship, and the hydration parameters, including hydration potential, and conductance were determined by regression analyses. The hydration parameters for different age groups were compared with one-way ANOVA followed by a post-hoc Fisher's PLSD test.

Data from the extracellular recording conducted in the L-LTP protocols, including the fEPSP slopes, were analyzed by, first, one-way ANOVA and, second, post-hoc Student–Newman–Keuls test at two time points, 30 min and 180 min after the four-train protocol (100 Hz × 4). The values are expressed as mean ± SE. The sample size (n) in each dataset corresponds to the number of animals (not slices) used. In all of the experiments presented here, $p < .05$ was considered significant.

Changes in basal synaptic strength under various experimental conditions were examined by determining both fEPSP slopes and amplitude. Amplitude/stimulus data points were fitted with a simple equation:

$$A_{obs} = A_{max} - B(1/Stimulus) \quad (2)$$

where A_{obs} and A_{max} are the observed and theoretical maximal responses, respectively. The theoretical A_{max} is the response expected for high stimulus intensities. At the range used in these experiments, the difference between A_{max} and A_{obs} is approximated by B $(1/stimulus)$, where variations in B reflect all the processes that modulate response magnitude when experimental conditions change. This relationship fits the data with r^2 values between 0.95 and 0.97 and was used to evaluate and compare results under the present range of experimental conditions. It serves here as a convenient interpolation formula and does not imply any general mechanism. At $A_{obs} = 0$, $A_{max} = B$ $(1/Stimulus)$, and $B/A_{max} = threshold$.

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Proteolysis, synaptic plasticity and memory

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ABSTRACT

Protein degradation has many critical functions in the nervous system such as refinement of synaptic connections during development and synaptic plasticity and memory in the adult organisms. A major cellular machinery of proteolysis is the ubiquitin-proteasome pathway (UPP). The UPP precisely regulates proteolysis by covalently attaching ubiquitin, a small protein, to substrates through sequential enzymatic reactions and the proteins marked with the ubiquitin tag are degraded by complex containing many subunits called the proteasome. Research over the years has shown a role for the UPP in regulating presynaptic and postsynaptic proteins critical for neurotransmission and synaptic plasticity. Studies have also revealed a role for the UPP in various forms of memory. Mechanistic investigations suggest that the function of the UPP in neurons is not homogenous and is subject to local regulation in different neuronal subcompartments. In both invertebrate and vertebrate model systems, local roles have been found for enzymes that attach ubiquitin to substrate proteins as well as for enzymes that remove ubiquitin from substrates. The proteasome also has disparate functions in different parts of the neuron. In addition to the UPP, proteolysis by the lysosome and autophagy play a role in synaptic plasticity and memory. This review details the functions of proteolysis in synaptic plasticity and summarizes the findings on the connection between proteolysis and memory mainly focusing on the UPP including its local roles.

1. Introduction

The quest for understanding how the nervous system stores information has led to the exploration of synaptic plasticity and memory in several model systems: from worms to human beings. Many decades of research in the 20th century focused on the role of protein synthesis in long-term synaptic plasticity and memory. Research that began in the 1990s revealed a role for regulated proteolysis in long-term synaptic plasticity. Protein degradation that functions to sculpt synapses and thus in aiding memory formation occurs mainly through the ubiquitin-proteasome pathway. Evidence over the last few years has also indicated a role for other types of proteolysis that occur through the lysosome and autophagy. This review mainly focuses on ubiquitin-proteasome-mediated degradation and provides brief descriptions of the functions of the lysosome and autophagy.

2. The ubiquitin-proteasome pathway

In the ubiquitin-proteasome pathway (UPP), covalent attachment of ubiquitin, a highly conserved 76-amino acid protein, to substrate proteins marks them for degradation by a proteolytic complex called the proteasome. The attachment of ubiquitin (ubiquitination) to proteins requires sequential activity of three enzymes (E1, E2, and E3) (Fig. 1). There are two E1s in many organisms but multiple genes encoding E2s exist.

In the UPP, an E1 activates ubiquitin and passes it onto an E2 which can transfer ubiquitin to the substrates directly or through generation of E3∼ubiquitin thioester intermediates. The substrate-specificity of ubiquitin ligation is largely determined by E3s. The first ubiquitin is covalently attached to the ε amino group of lysine residues in the substrate. After these enzymes attach the first ubiquitin to the substrate protein, to an internal lysine residue a second ubiquitin is attached and thus several ubiquitin molecules are attached to the growing chain which is termed "polyubiquitin". Substrates that are destined for degradation by the proteasome carry a specific polyubiquitin linkage. Every successive ubiquitin is attached to the 48th lysine residue in the previous ubiquitin (Glickman & Ciechanover, 2002; Hegde, 2010a). It must be noted, however, that ubiquitin attachment to other ubiquitin molecules could occur through any of the seven lysine residues in ubiquitin. For marking the substrate for ubiquitin-proteasome-mediated degradation, additional ubiquitin are attached to the first ubiquitin at its 11th or 48th Lys residue. Lys-63 linked polyubiquitin chains modulate protein function such as NFκ-B activation (Deng et al., 2000). There are instances when polyubiquitin chains are formed through second ubiquitin linkage to Lys-6, Lys-27, Lys-29 and

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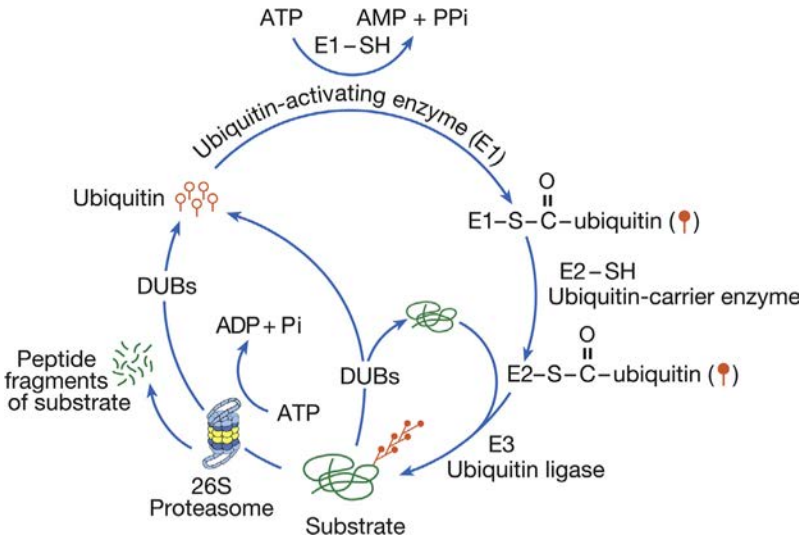

Fig. 1. The ubiquitin-proteasome pathway. In this proteolytic pathway, ubiquitin (single ubiquitin molecule is represented by open circles with straight tails) is selectively and covalently attached to the substrate. The enzymatic process of attaching ubiquitin to substrates depends on the action of three different classes of enzymes E1, E2 and E3. First, ubiquitin is activated by E1 to form a ubiquitin-AMP intermediate. Activated ubiquitin (closed circles with straight tails) is passed on to E2 (ubiquitin carrier enzymes). E2s transfers ubiquitin to an E3 (ubiquitin ligase) which ligates the activated ubiquitin to the substrate. To the ubiquitin attached to substrate another ubiquitin is attached and thus through successive linkages of ubiquitin a polyubiquitin chain forms. Polyubiquitinated substrates are degraded by a multi-subunit proteolytic complex called the 26S proteasome in an ATP-dependent reaction. Ubiquitin is not degraded but the polyubiquitin chain is disassembled and ubiquitin is recycled by deubiquitinating enzymes (DUBs). Before being committed to be degraded by the proteasome, ubiquitination is reversible. DUBs can disassemble the polyubiquitin chain if a substrate is ubiquitinated erroneously and prevent the degradation of the substrate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Lys-33 of the first ubiquitin attached to the substrate are known to occur (Komander, 2009; Ye & Rape, 2009). Polyubiquitin chains contain mixed type of linkage between ubiquitin molecules such as through Lys-11 and Lys-48 in the same chain. Furthermore, ubiquitin itself can be posttranslationally modified through acetylation and phosphorylation (Ohtake et al., 2015; Swaney, Rodriguez-Mias, & Villen, 2015).

The E3 enzymes that ligate ubiquitin to substrate proteins are the most diverse in the UPP. There are two major classes of E3s: (1) HECT (homologous to E6-AP carboxyl-terminus) domain E3s, (2) RING (really interesting new gene) finger E3s. The RING finger E3s in turn can be divided into two classes SCF (SKP1-cullin-F-Box protein) and APC (Anaphase promoting complex). The specificity of the ubiquitin conjugation reaction, although largely occurs at the E3 ligation step, specific interactions between E2s and E3s and the type of ubiquitin linkage (Lys-48, Lys-63 and so on as described above) all add to the "combinatorial coding" of specificity in the ubiquitin conjugation reaction (Hegde, 2010b).

The protein substrate marked by polyubiquitin attachment is then degraded by the proteasome to small peptides and amino acids (Fig. 1). The polyubiquitin chains are not degraded but disassembled by deubiquitinating enzymes (DUBs) and the free ubiquitin molecules are recycled (Fig. 1). There are two types of DUBs. The category called ubiquitin C-terminal hydrolases (UCHs) is characterized by low molecular weight. The second class is that of high molecular weight DUBs which are called ubiquitin-specific proteases (UBPs or USPs). Apart of structural differences, UCHs and UBPs functionally differ with respect to substrates on which they act (Wilkinson, 2000).

The proteasome that functions to degrade the substrate proteins marked by polyubiquitin chain attachment is called the 26S proteasome based on its sedimentation coefficient during ultracentrifugation. It comprises a cylindrical catalytic 20S core and two regulatory complexes (RC) that are attached to either end of the 20S. The 20S consists of two outer rings with seven α subunits (α1 to α7) in each ring and two inner rings consisting of seven β subunits (β1 to β7). The catalytic activity of the proteasome is conferred by three of the seven β subunits (β1, β2 & β5). The catalytic sites in these β subunits are located at their N-termini which are inside the catalytic cavity which has a narrow opening of 13 Å in diameter (Cheng, 2009). Because of this, only an unfolded substrate can enter the catalytic core. It is thought that the unfolding activity is provided by the ATPases that are present in the base of the 19S RC which contains six ATPase subunits Rpt1-Rpt6 (Regulatory particle ATPase 1–6) and four non-ATPase subunits Rpn1, Rpn2, Rpn10 & Rpn13 (Regulatory particle non-ATPases 1, 2, 10 & 13). The 19S RC also consists of the 'lid' which includes only non-ATPase subunits (Rpn3, Rpn5, Rpn6-9, Rpn11, Rpn12, & Rpn15) (Hegde, 2010a; Marques, Palanimurugan, Matias, Ramos, & Dohmen, 2009).

Among the Rpn subunits, Rpn11 (also called Poh1) and Rpn13 (also called Uch37) are DUBs that are integral part of the 19S RC that assist in deubiquitination of the substrate as it is unfolded and threaded into the catalytic chamber of the 20S core. Another DUB called Usp14 (also known as Ubp6) reversibly associates with the Rpn1 and stimulates substrate degradation through deubiquitination (Leggett et al., 2002; Peth, Besche, & Goldberg, 2009). Two Rpn subunits, Rpn10 (S5) and Rpn13, have a role in recognizing the polyubiquitin chain (Baboshina & Haas, 1996; Husnjak et al., 2008; van Nocker, Deveraux, Rechsteiner, & Vierstra, 1996).

In neurons, the proteasome has widespread roles as will be explained later. Although there have not been extensive studies of individual subunits of the proteasome, at least one ATPase subunit, Rpt6, is known to have a role in activity-dependent growth of dendritic spines and the function of Rpt6 is regulated by NMDA receptor (NMDAR)- and CaMKII- mediated phosphorylation (Hamilton et al., 2012).

3. The UPP and long-term synaptic plasticity

Ubiquitin was familiar to researchers as a marker for brain pathology such as neurofibrillary tangles in Alzheimer's disease

and Lewy bodies in Parkinson's disease (Mori et al., 1987; Lowe et al., 1988) but no physiological or pathological role for ubiquitin in the nervous system was found until the 1990s. The first discovery of degradation by the UPP of a substrate critical for synaptic plasticity in the nervous system was that of regulatory (R) subunits of cAMP-dependent protein kinase (PKA) (Hegde, Goldberg, & Schwartz, 1993). Since then, several substrates of the ubiquitin-proteasome pathway in the nervous system have been identified (Hegde, 2010a).

3.1. Degradation of R subunits of PKA and proteolytic removal of a CREB repressor

Initial discovery on the role of the UPP in synaptic plasticity came during studies on persistent activation of PKA. Investigations on the biochemical mechanism of long-term facilitation (LTF) (Greenberg, Castellucci, Bayley, & Schwartz, 1987) in *Aplysia* indicated that PKA was persistently activated in the absence of elevated cAMP. LTF underlies behavioral sensitization of defensive reflexes in *Aplysia* which is a simple form of memory (Abrams, 1985). How is PKA activated in the absence of sustained increase in cAMP? It was found that the R subunits of PKA were decreased without any change in the catalytic (C) subunit during induction of LTF. Because there was no change in mRNA for either the R subunit or the C subunit, the inference was that quantity of R subunits was reduced probably through proteolysis. What is the mechanism of R subunit degradation? Hegde et al. (1993) found through a series of biochemical experiments that R subunits were substrates for ubiquitin conjugation and degradation by the proteasome. In addition, it was found that in response to LTF-causing stimuli (such as five pulses of the neurotransmitter 5-HT on to sensory neurons), a UCH (*Aplysia* ubiquitin C-terminal hydrolase, Ap-uch) that interacts with the proteasome was induced (Fig. 2). Electrophysiological experiments showed that Ap-uch was critical for induction of LTF (Hegde et al., 1997). Subsequently Chain et al. demonstrated that at sensory-motor neuron synapses, injection of lactacystin, a specific proteasome inhibitor blocked induction of LTF (Chain et al., 1999). Since R subunits inhibit the activity of C subunits of PKA, the results suggested that the ubiquitin-proteasome pathway operates to remove inhibitory constraints on formation of long-term memory.

Work on the *Aplysia* model provided further evidence that the UPP might have a role in degrading proteins that normally inhibit long-term synaptic plasticity. Experimental protocols that induce LTF in *Aplysia*, cause ubiquitination and degradation of a CREB repressor called CREB1b (Upadhya, Smith, & Hegde, 2004) (Fig. 2). These observations appear to hold true in vertebrates as well. The mammalian orthologue of CREB1b, ATF4 is degraded by the UPP during induction of long-term synaptic plasticity in the murine hippocampus (Dong, Upadhya, Ding, Smith, & Hegde, 2008).

3.2. Modulation and essential function of a DUB in long-term synaptic plasticity

Subsequent to the finding on ubiquitin-proteasome-mediated degradation of R subunits of PKA, crucial role in LTF for a neuronal specific Ap-uch was discovered. Ap-uch is the homolog of human UCH-L1 and is induced by stimuli that produce LTF but not stimuli that lead to short-term facilitation. Injection of antibodies or antisense oligonucleotides specific to Ap-uch into sensory neurons synapsing onto motor neurons in culture blocked induction of LTF (Hegde et al., 1997). Investigation on biochemical functions of Ap-uch indicated that Ap-uch is capable of cleaving small attachments to linearly attached ubiquitin molecules such as ubi quitin–ubiquitin–cysteine but not large attachments like GST in substrates like ubiquitin–GST. Interestingly, additional biochemical analyses showed that Ap-uch associates with the proteasome. The association of Ap-uch increases the rate of degradation by the proteasome. For example, addition of recombinant Ap-uch to

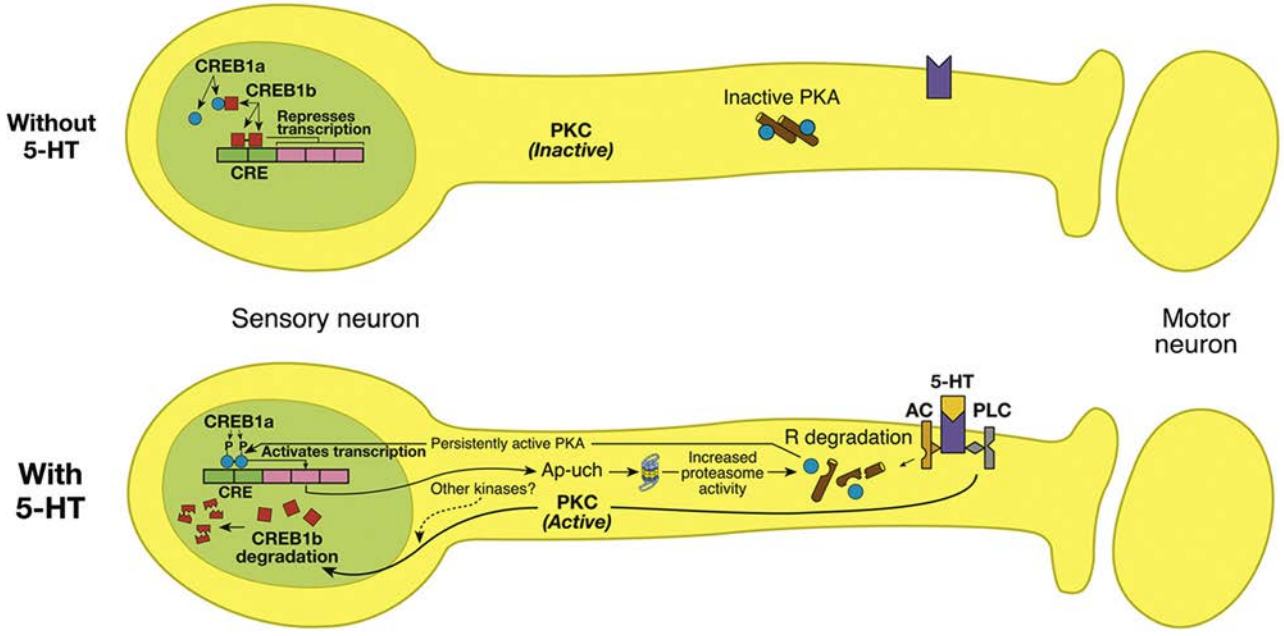

Fig. 2. Role of the ubiquitin–proteasome pathway in long-term facilitation in *Aplysia*. When sensory neurons are stimulated with the neurotransmitter serotonin (5-HT), which induces long-term facilitation, R subunits of protein kinase A (PKA) are ubiquitinated and degraded by the proteasome, making the kinase persistently active. The catalytic subunit of PKA (blue circle) translocates to the nucleus and phosphorylates cAMP response element binding protein 1a (CREB1a), the activator form of CREB. Concomitantly, the repressor form of CREB, CREB1b, is degraded by the ubiquitin-proteasome pathway. Protein kinase C (PKC), which is also activated by 5-HT-mediated signaling, stimulates ubiquitin conjugation to CREB1b and subsequent degradation. AC, adenylyl cyclase; PLC, phospholipase C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in vitro degradation systems showed that there was approximately a twofold increase in degradation of R subunit of PKA. Since persistent activation of PKA has been shown to be critical for induction of LTF and R subunits of PKA were found to be substrates for the ubiquitin-proteasome pathway, the experiments on Ap-uch provided some molecular explanation for the role of regulated proteolysis in LTF (Hegde et al., 1997). Computational modeling has provided support for the idea that persistently active PKA induces Ap-uch which in turn provides a positive feedback loop for increasing PKA activity through enhancement of R subunit degradation (Song, Smolen, Av-Ron, Baxter, & Byrne, 2006).

How does Ap-uch enhance the rate of degradation by the proteasome? Using recombinant ubiquitin with its Lysine-48 mutated to Arg that cannot support Lys-48 type of polyubiquitin linkage to protein substrate, it was shown that Ap-uch stimulates the release of ubiquitin from substrates in the presence of the proteasome (Hegde et al., 1997). Ubiquitin with Arg-48 can form single or multiple monoubiquitin linkages on the substrate. Therefore, it can be inferred that Ap-uch perhaps cleaves the first ubiquitin in the polyubiquitin chain attached to the peptide remnant of the substrate. Such a function of Ap-uch has to occur after the DUBs that are tightly associated with the proteasome finish bulk of the polyubiquitin chain disassembly as the unfolding of the substrate and its degradation progresses. Function of UCHs in synaptic plasticity appears to be evolutionarily conserved. It was found that the mammalian counterpart of Ap-uch, Uch-L1 is required for normal synaptic plasticity and memory. The same study also showed a link between UCH-L1 and R subunit degradation in the mouse hippocampus (Gong et al., 2006).

Other studies have expanded the role of Ap-uch and the proteasome in *Aplysia* to Ltd. In *Aplysia,* sensory-motor neuron synapses undergo transcription-dependent LTD in response to treatment with the neuropeptide Phe-Met-Arg-Phe-NH2 (FMRFa). Application of the proteasome inhibitor lactacystin blocked FMRFa-induced Ltd. Also, FMRFa was found to upregulate *Ap-uch* mRNA (Fioravante, Liu, & Byrne, 2008). Thus Ap-uch could have a role in LTD in *Aplysia* perhaps through its action on a different set of downstream targets compared to those affected by Ap-uch during LTF. Subsequent studies indicate that proteasome has a role in mammalian LTD as well. In rat hippocampal neurons, NMDAR-dependent LTD was shown to be independent of proteasome-mediated degradation whereas mGluR-dependent LTD was limited by ubiquitination and proteasome-mediated degradation (Citri, Soler-Llavina, Bhattacharyya, & Malenka, 2009). These results, however, contradict earlier studies showing that proteasome inhibition reduced the magnitude of LTD dependent on NMDARs (Colledge et al., 2003) or mGluRs (Hou et al., 2006). The role of the UPP in LTD may be more complex than previously thought. Recently, Sajikumar and colleagues showed that in rat hippocampus, proteasome activity is necessary for protein synthesis-independent early-LTD and inhibition of the proteasome converts early-LTD into protein synthesis-dependent late LTD (Li, Korte, & Sajikumar, 2015). In addition, work from another group showed that precise coordination between protein synthesis and proteasome-mediated degradation is essential in regulating induction of mGluR-dependent LTD (Klein, Castillo, & Jordan, 2015).

3.3. The roles of the UPP in histone modification underlying synaptic plasticity

Recent studies indicate that, in addition to modulating transcription factors, the UPP has other roles in regulating transcription. For example, a novel role of the proteasome in modulation of epigenetic histone modifications was described. This study demonstrated that trimethylation of histone 3 at lysine 4 (H3K4me3), acetylation of histone H3 at lysines 9 and 14 (H3K9/14ac), and monoubiquitination of histone H2B at lysine 120 (H2BK120ub) are enhanced immediately after cLTP induction and their enhancement is blocked by β-lactone pretreatment (Bach, Tacon, Morgan, & Hegde, 2015).

H3K4me3 and H3K9/14ac are transcription-favoring epigenetic modifications previously shown to be important for learning and memory in rodents (Day & Sweatt, 2011; Jarome & Lubin, 2013; Zovkic, Guzman-Karlsson, & Sweatt, 2013). The Bach et al. study reported that these modifications were dynamic. They showed that both H3K4me3 and H3K9/14ac were upregulated soon after cLTP induction and returned to baseline after 30 min (Bach et al., 2015). The experiments reported earlier described histone modifications that lasted hours or days after synaptic stimulation or behavioral training (Gupta-Agarwal et al., 2012; Levenson et al., 2004). It has also been shown, however, that histone modifications can occur rapidly, in minutes (Buro, Chipumuro, & Henriksen, 2010; Lopez-Atalaya, Ito, Valor, Benito, & Barco, 2013; Riffo-Campos et al., 2015). Some researchers have postulated that lasting cellular changes in synaptic plasticity can be triggered by a transient histone modification signal (Levenson & Sweatt, 2005). Evidence from the *Aplysia* model indicates that transient acetylation of histone H3 is critical during long-term synaptic plasticity (Guan et al., 2002). Therefore, short-lived proteasome-dependent histone acetylation and methylation may be sufficient to trigger long-lasting upregulation of plasticity-related genes.

The Bach et al. study also investigated the role of the transcription-favoring H2BK120ub in synaptic plasticity. This investigation showed that H2BK120ub levels oscillate after the induction of cLTP: an increase in histone H2B monoubiquitination was observed immediately after cLTP induction and at 30 min after cLTP induction, but not at 15 min (Bach et al., 2015). This finding is consistent with previous studies of histone H2B monoubiquitination in yeast transcriptional regulation, where multiple rounds of histone ubiquitination and deubiquitination are required for transcription initiation and elongation, respectively (Minsky et al., 2008; Weake & Workman, 2008; Wyce et al., 2007). Histone H2B mono-ubiquitination has also been described as a precursor to other histone modifications (Gonzalez, Delahodde, Kodadek, & Johnston, 2002; Lee et al., 2007). The dynamic nature of global histone modifications in cLTP suggests that the role of histone modifications in synaptic plasticity may be more complex than previously believed.

4. The UPP, short-term plasticity and synaptic transmission: presynaptic and postsynaptic roles

Proteolysis by the UPP regulates key proteins at the synaptic terminals (presynaptic) as well as in the postsynaptic compartment. The UPP has been shown to control synaptic transmission as well as short-term synaptic plasticity.

4.1. Presynaptic roles of the UPP

The UPP, in addition to its role in regulating molecules such as PKA which are critical for long-term synaptic plasticity, also has a function in acute modulation of proteins which affects synaptic transmission and short-term synaptic plasticity. For example, a protein Dunc-13, which is critical in priming the synaptic vesicles, is ubiquitinated and degraded by the proteasome in *Drosophila* neuromuscular synapse. Administration of proteasome inhibitors and the dominant-negative mutation in a core subunit (β6) of the *Drosophila* proteasome both lead to an increase in the quantity of Dunc-13 protein in presynaptic terminals. In addition, application of the proteasome inhibitors lactacystin and epoxomycin cause an increase in the excitatory junctional current suggesting

that stabilization of Dunc-13 and the resultant increase in the net Dunc-13 quantity leads to increased synaptic transmission (Speese, Trotta, Rodesch, Aravamudan, & Broadie, 2003).

The UPP seems to have a wider role in controlling short-term synaptic plasticity and has been found to regulate the amounts of other presynaptic proteins. For example, synaptic vesicle proteins such as syntaxin 1 and RIM1α are degraded by the UPP. Syntaxin 1 is a presynaptic protein that has a role in synaptic vesicle exocytosis. Evidence for ubiquitin-proteasome-mediated degradation of syntaxin 1 was obtained through identification of a ubiquitin ligase called staring (syntaxin 1-interacting RING finger protein) using the yeast two-hybrid system (Chin, Vavalle, & Li, 2002). Co-expression of staring with syntaxin 1 in HeLa cells increases the degradation of syntaxin 1 which can be inhibited by the proteasome inhibitor MG132. The physiological effect of ubiquitin-proteasome-mediated degradation of syntaxin 1 remains to be determined. RIM1α functions to form a presynaptic scaffold that links synaptic vesicle with fusion machinery. A ubiquitin ligase named SCRAPPER (an acronym whose derivation is not clearly defined) has been shown to regulate the amount of RIM1α (Rab3-interacting molecule 1α). Studies using miniature postsynaptic current (mEPSC) measurements established that SCRAPPER regulates synaptic transmission. It was also found that in mice lacking SCRAPPER short-term synaptic plasticity was impaired (Yao et al., 2007).

The proteasome has been shown to function in recycling of synaptic vesicles in hippocampal neurons in primary culture. Proteasome inhibition causes an increase in the size of the recycling pool of vesicles. Blockade of neuronal activity significantly reduces the effect of proteasome inhibition, decreasing vesicle numbers. Inhibition of the proteasome, however, does not increase transmitter release probability. Therefore, it seems that in vertebrate neurons, the proteasome functions to maintain vesicle homeostasis (Willeumier, Pulst, & Schweizer, 2006). Results from later experiments add another layer to the complexity of UPP function in neurons. In cultured mammalian hippocampal neurons, proteasome inhibitors increase mEPSC frequency without any effect on the amplitude indicating a presynaptic role for the UPP. Although expected, stabilization of the presynaptic proteins (RIM1 or Munc13) was not observed (Rinetti & Schweizer, 2010). A different study, however, found a decrease in Rim 1 and Munc 13 during persistent presynaptic silencing induced by depolarization (Jiang et al., 2010). The results from these two sets of investigations seem to be at odds with each other even though both used postnatal rat hippocampal neurons in culture and antibodies against Rim 1 and Munc 13 from the same commercial sources. Perhaps the discrepancy was due to the fact that Jiang et al. study measured Rim 1 and Munc 13 after K^+-induced depolarization whereas Rinetti and Schweizer study tested Rim 1 and Munc 13 levels in relation to changes in mEPSCs and spontaneous EPSCs. Therefore, it is likely that degradation of Rim 1 and Munc 13 is triggered by neuronal depolarization rather than baseline activity.

4.2. Postsynaptic roles of the UPP

Several studies have indicated that the UPP modulates neurotransmitter receptors, structural proteins and regulatory molecules in the postsynaptic compartment. Regulation of the neurotransmitter receptors mainly occurs through ubiquitination that marks proteins for endocytosis which is mainly mediated by attachment of a single ubiquitin (monoubiquitination) or a Lys-63-linked polyubiquitin chain. The ubiquitinated protein that is endocytosed may be recycled back to the plasma membrane if the ubiquitin is removed by DUBs or targeted to the lysosome via the multivesicular body. Some membrane proteins, upon endocytosis are degraded by the proteasome instead of being routed to the lysosome for degradation.

Earlier investigations on *Caenorhabditis elegans* showed a role for ubiquitin in endocytosis of GLR-1 type of glutamate receptor (Burbea, Dreier, Dittman, Grunwald, & Kaplan, 2002). In mammalian hippocampal neurons, treatment with the proteasome inhibitor MG132 blocks agonist-induced endocytosis of AMPA-type glutamate receptors (Patrick, Bingol, Weld, & Schuman, 2003). In addition, NMDA-induced AMPA receptor internalization is prevented by application of the proteasome inhibitor. Later studies showed that AMPA receptor endocytosis and In support of this idea, a postsynaptic density protein PSD-95 was shown to be regulated by ubiquitin-proteasome-mediated degradation (Colledge et al., 2003). PSD-95 is a major component of the postsynaptic scaffold which through interaction with another protein called stargazin provides a docking site for AMPA receptors (Schnell et al., 2002). Proteolytic removal of PSD-95 leads to AMPA receptor internalization and mutations that block PSD-95 ubiquitination block NMDA-induced AMPA receptor endocytosis (Colledge et al., 2003). Furthermore, application of the proteasome inhibitor MG132 to hippocampal slices reduces the magnitude of hippocampal long-term depression (LTD) (Colledge et al., 2003). Because the transient, protein-synthesis independent LTD (Sajikumar & Frey, 2003) requires a net reduction in synaptic AMPA receptors (Malenka & Bear, 2004), these data further support a role for the proteasome in decreasing AMPA receptor amount at synaptic sites. The signal for regulating AMPA receptor internalization and degradation has been investigated in the last few years. Studies showed activity-dependent ubiquitination of the GluA1 subunit in hippocampal neurons. This ubiquitination was mediated by ubiquitin ligase Nedd4-1(neural-precursor cell-expressed developmentally downregulated gene 4-1) (Schwarz, Hall, & Patrick, 2010). A recent investigation demonstrated that ubiquitination of GluA1 and GluA2 was critical in directing the internalized AMPA receptors to late endosomes and then into lysosomes for degradation (Widagdo et al., 2015). Given that monoubiquitination on one or multiple sites in a protein generally directs the substrates to late endosomes and lysosome, and polyubiquitination directs substrates to the proteasome, how AMPA receptors are degraded may have consequences on synaptic plasticity.

A role for the proteasome in short-term synaptic plasticity has also been obtained through experiments on LTP. A type of LTP called early phase LTP (E-LTP), which is independent of protein synthesis, is enhanced by pre-incubation of hippocampal slices with the proteasome inhibitor β-lactone (Dong et al., 2008). Although the mechanisms by which E-LTP is enhanced by proteasome inhibition have not been elucidated, it is likely that AMPA receptor stabilization and consequent increase in AMPA receptor number at postsynaptic sites might contribute to the increase in E-LTP.

It is likely that the UPP has a broad role in regulating neurotransmitter receptors. NMDARs are retrotranslocated and degraded by the UPP in an activity-dependent fashion. Ubiquitination of the NR1 subunit of NMDARs by an F-box protein called Fbx2 is critical for this process (Kato, Rouach, Nicoll, & Bredt, 2005) suggesting that an SCF-type ligase targets the NR1 subunits for ubiquitination. Subsequent studies showed that another NMDAR subunit NR2B is targeted for ubiquitination by an E3 ligase called Mind bomb-2 in a phosphorylation-dependent manner (Jurd et al., 2008). Endocytosis of other neurotransmitter receptors might be regulated by ubiquitination. Glycine receptor has been shown to be internalized upon ubiquitination (Buttner et al., 2001). A protein Plic-1, which is associated with $GABA_A$ receptors, indirectly controls removal of $GABA_A$ through endocytosis (Bedford et al., 2001). It was shown that proteasome inhibitors prevent degradation of internalized $GABA_A$ receptors. Later studies showed that $GABA_A$ receptor

ubiquitination is controlled by neuronal activity. Chronic blockade of neuronal activity by tetrodotoxin increases the level of $GABA_A$ receptor ubiquitination and increase in neuronal activity decreases $GABA_A$ receptor ubiquitination and improves insertion of these receptors into the plasma membrane (Saliba, Michels, Jacob, Pangalos, & Moss, 2007). $GABA_A$ receptors are heteropentameric proteins typically consisting of two α subunits, two β subunits and one γ subunit. In the brain, the β subunits of the $GABA_A$ receptors are either β2 or β3 (Rudolph & Mohler, 2006). The site of ubiquitination is the β3 subunit of the receptor. Activity blockade reduces the insertion of β3-containing $GABA_A$ wild type receptor but not of the receptor containing mutant β3 that cannot be ubiquitinated (Saliba et al., 2007).

The UPP also degrades several other proteins in addition to PSD-95 in the postsynaptic density including several structural proteins. For example, Shank, GKAP and AKAP79/150 are degraded through the ubiquitin-proteasome pathway. Unlike for the degradation of PSD-95, physiological relevance of proteolytic removal of Shank, GKAP and AKAP79/150 is not clear because the studies were correlative and a direct link between ubiquitin-proteasome-mediated degradation of the PSD proteins and structural remodeling was not established (Ehlers, 2003).

There is also evidence that the UPP controls a protein that regulates spine shape. SPAR controls dendritic spine shape by reorganizing the actin cytoskeleton. During activity-dependent remodeling of synapses, SPAR was shown to be degraded by the ubiquitin-proteasome pathway. Degradation of SPAR is stimulated by serum inducible kinase (SNK). Activity induces SNK mRNA in the cell body and the induced SNK is targeted to the dendritic spines. Because of the time required for SNK mRNA to travel to the spines, the conjecture is that SPAR may function to oppose synaptic remodeling after elevated activity (Pak & Sheng, 2003).

5. Local proteolysis and synaptic plasticity: Roles for spatial control of proteasome-mediated degradation

I previously proposed a role for local ubiquitin-proteasome-mediated degradation in synaptic plasticity (Hegde, 2004). Many studies carried out since then provide support to this idea. Others working in this field are embracing the idea of local degradation as well (Segref & Hoppe, 2009). It appears that local, regulated degradation of substrate proteins plays an important role in synaptic plasticity as well as many other aspects of the nervous system such as development and fine-tuning of synaptic connections. Spatially restricted degradation can achieve synapse-specific effects. Cell-wide degradation would have consequences on all synapses made by a given neuron (Hegde, 2004).

How might local protein degradation be achieved in neurons? A simple way would be to restrict the substrate to a subcellular location. For example, proteins whose expression is largely restricted to the synapses could be locally degraded because all the requisite components of the UPP are present at the synapse. Also substrates can be made vulnerable (or resistant) to ubiquitination by phosphorylation or glycosylation which can be locally controlled in neurons. For example, NR2B subunit of NMDAR is phosphorylated by Fyn tyrosine kinase and made susceptible for ubiquitination by the E3 ligase Mind Bomb which is localized to the apical dendrites (Jurd et al., 2008). A transcription factor critical for LTF in *Aplysia* called C/EBP is made resistant to ubiquitin-proteasome-mediated degradation upon phosphorylation by MAP kinase (Yamamoto, Hegde, Chain, & Schwartz, 1999). Other posttranslational modifications of substrates add another level to the regulation of substrate ubiquitination. For example, attachment of O-linked N-acetylglucosamine (O-GlcNAc) is known regulate phosphorylation of substrates and their consequent ubiquitination (Guinez et al., 2008; Ruan, Nie, & Yang, 2013).

Activation (or inactivation) of ubiquitin ligases by phosphorylation or other posttranslational modifications can be locally controlled as well. Phosphorylation can either positively or negatively regulate E3 ligase activity (Im & Chung, 2015; Stacey, Breen, & Jefferies, 2012). Linkage to O-GlcNAc of an E3 has been reported to occur which can potentially modify ligase activity (Zaro, Yang, Hang, & Pratt, 2011). Moreover, specific E3 ligases can also be sequestered to specific cellular compartments. The removal of attached ubiquitin by DUBs has been found to be locally regulated as well. Experimental evidence has been obtained for local regulation of E3 s as well as that of DUBs, mainly from work on neuronal development in Drosophila (Hegde, 2010b). Accumulating evidence indicates that proteasome activity is also differentially regulated in different neuronal compartments, which is the main focus of discussion here.

5.1. Local roles of the proteasome in synaptic plasticity

Modulation of the proteasome adds another level to regulation of proteolysis by the UPP. Even though it was not previously appreciated, the data from the last several years indicate that proteasome is not homogenous throughout the neuron. Hints for local functions of the proteasome initially came from work on LTF in Aplysia. Later studies on hippocampal late phase of long-term potentiation (L-LTP) provided strong evidence for local roles of the proteasome in long-term synaptic plasticity.

5.1.1. Local roles of the proteasome in LTF in Aplysia

The impetus for closely looking at the regulation of the neuronal proteasome came from conflicting results obtained with proteasome inhibitors on LTF in *Aplysia*. Originally, it was found that proteasome inhibitors block induction of LTF (Chain et al., 1999). Later studies on LTF, however, showed that bath application of the active form of lactacystin, *clasto*-lactacystin β-lactone, to sensory-motor neuron synapses resulted in enhanced LTF and an increase in neurite outgrowth in isolated sensory neuron (Zhao, Hegde, & Martin, 2003). The increase in neurite elongation is consistent with results obtained in PC12 and Neuro2A cells in which lactacystin induces neurite outgrowth (Fenteany, Standaert, Reichard, Corey, & Schreiber, 1994). Both sets of results can be reconciled if one postulates that proteasome has different roles in different cellular compartments (Hegde, 2004). In the same neuron, the proteasome is likely to carry out different tasks in different subcellular compartments resulting in different physiological consequences at different loci. Therefore, blocking different roles of the proteasome during induction of memory would lead to distinct and even opposite effects on synaptic strength. For example, the proteasome is known to degrade transcription repressors. Degradation of transcription repressors should allow transcription activators to induce gene expression which in turn leads to development of LTF. If the proteasome is inhibited only in the nucleus before the repressors are degraded, gene expression and hence induction of LTF should be blocked. Degradation of the CREB repressor CREB1b by the UPP in response to LTF-inducing protocols (Fig. 2) (Upadhya et al., 2004) supports this idea. On the other hand, if the degradation of proteins needed at the synapse for developing LTF is inhibited by the proteasome, LTF should be enhanced. As previously proposed, the main purpose of transcription during induction of LTF or other forms of long-term memory is to provide mRNAs for synthesis of 'rapidly turning over proteins' needed for memory formation (Hegde, 2004). If the degradation of these proteins is prevented, then long-term memory formation becomes independent of transcription. In support of this idea, Zhao et al. found that

proteasome inhibitor-induced synaptic strengthening depends on translation but not transcription (Zhao et al., 2003).

The biochemical experiments on the proteasome also support differential function of the proteasome in different neuronal compartments. The results of these experiments showed that both in *Aplysia* nervous system and mouse brain, proteasome activity in the synaptic terminals is significantly higher than that of the nuclear proteasome. Moreover, the proteasome activity in the two compartments is differentially regulated by protein kinases relevant to synaptic plasticity such as PKA, PKC, and MAP kinase (Upadhya, Ding, Smith, & Hegde, 2006). Later, others found that CaMKII can stimulate proteasome activity in cultured hippocampal neurons (Djakovic, Schwarz, Barylko, DeMartino, & Patrick, 2009).

5.1.2. Local regulation of the proteasome in L-LTP in murine hippocampus

As discussed above, differential activity of the proteasome in *Aplysia* might explain conflicting results obtained in different studies. Does differential proteasomal activity affect synaptic plasticity differentially in vertebrates? It has been found that the proteasome has differential roles during induction and maintenance phases of hippocampal late phase of long-term potentiation (L-LTP) (Dong et al., 2008).

Evidence for functional significance of local roles of the proteasome came from studies on hippocampal late phase LTP (L-LTP). Investigations by Dong et al. showed that the proteasome inhibitor application to hippocampal slices prior to induction of L-LTP caused an increase in the magnitude of the early, induction phase but an inhibition of the late, maintenance phase (Dong et al., 2008). What is the basis of these differential effects of the proteasome on phases of L-LTP? The enhancement of the early, induction phase (This early part of L-LTP is referred to as Ep-L-LTP for convenience) by the proteasome inhibitor β-lactone is blocked by prior application of the translation inhibitor anisomycin but not by a transcription inhibitor actinomycin D. The increase in Ep-L-LTP caused by β-lactone is also prevented by prior application of rapamycin which blocks signaling that controls translation of a subset of mRNAs (Gingras, Raught, & Sonenberg, 2001). Moreover, Ep-L-LTP is augmented in dendrites isolated from the cell body by means of a surgical cut. These lines of evidence suggest that proteasome inhibition enhances Ep-L-LTP by stabilizing proteins locally translated from pre-existing mRNAs (Dong et al., 2008) (Fig. 3 top).

How does proteasome inhibition block maintenance of L-LTP? The proteasome inhibitor β-lactone blocks maintenance of L-LTP only if applied prior to induction of L-LTP but not if applied 2 h after induction of L-LTP. Previous studies by others have established that the critical time window for transcription required for maintenance of L-LTP is 2 h (Nguyen, Abel, & Kandel, 1994). These results suggest that proteasome inhibition blocks maintenance of L-LTP by inhibiting transcription. Additional molecular evidence supports this notion. Application of β-lactone to hippocampal slices significantly reduced induction of *BDNF* (*Brain-derived neurotrophic factor*) mRNA by chemically induced LTP (cLTP) or L-LTP induced by a theta-burst protocol (Dong et al., 2008). *BDNF* is a CREB-inducible gene linked to maintenance of L-LTP (Barco et al., 2005).

What is the mechanism of transcription blockade caused by inhibition of the proteasome? One possibility is that normally the UPP aids the degradation of transcription repressors. Hence proteasome inhibition would result in accumulation of these repressors thus blocking transcription. In support of this idea, it was found that a CREB repressor ATF4 is degraded by the UPP during cLTP and β-lactone application to hippocampal slices prevents degradation of ATF4. Furthermore, ATF4-ubiquitin conjugates accumulate during cLTP when proteasome is inhibited (Dong et al., 2008) (Fig. 3 bottom).

These studies have also revealed the changing role of the proteasome even in dendrites through progression of L-LTP. Application of β-lactone to isolated dendrites also blocks maintenance of the dendritic L-LTP (Dong et al., 2008). Under these conditions, there is no supply of newly transcribed mRNA from the cell body. Thus blockade of transcription by proteasome inhibition does not explain this phenomenon. The most likely possibility is that proteasome inhibition leads to a slow accumulation of translation repressors in dendrites. Buildup of translation repressors would also occur in the cell body which would hinder translation of newly transcribed mRNAs. Thus late stages of translation in both dendrites and the cell body would be blocked by stabilization of translation repressors by proteasome inhibition. In support of this idea, confocal microscopy experiments at various time points after L-LTP induction showed that proteasome inhibition causes accumulation of translational activators eukaryotic initiation factors 4E (eIF4E) and eukaryotic elongation factor 1A (eEF1A) early during L-LTP (Dong, Bach, Haynes, & Hegde, 2014). Translational repressors such as polyadenylate-binding protein interacting protein 2 (Paip2) and eukaryotic initiation factor 4E-binding protein 2 (4E-BP2) buildup at later stages of L-LTP in response to proteasome inhibition (Dong et al., 2014). Other negative regulators of translational repressors such as Mov10 might be stabilized by proteasome inhibition as well. For example, in cultured hippocampal neurons Mov10, which inhibits translation of key plasticity-related mRNAs such as that of *CaMKIIα*, is degraded by the proteasome in an NMDA- and activity- dependent manner (Banerjee et al., 2009). The requirement for coordination between protein synthesis and proteasome-mediated degradation has also been reported for induction of mGluR-dependent LTD (Klein et al., 2015).

Other studies have investigated the effect of proteasome inhibition on LTP. Although these studies reported reduction in magnitude of LTP by proteasome inhibitors, they failed to discover differential dendritic and nuclear functions of the proteasome in LTP perhaps because one study used MG-132 (Karpova, Mikhaylova, Thomas, Knopfel, & Behnisch, 2006) which is not a highly specific proteasome inhibitor (Chain et al., 1999; Tang & Leppla, 1999) and the other used proteasome inhibitors lactacystin and epoxomycin at nanomolar concentration (Fonseca, Vabulas, Hartl, Bonhoeffer, & Nagerl, 2006) which is significantly lower than the effective concentration (micromolar) required to block proteasome activity.

5.1.3. Evidence for local roles of the proteasome in other model systems

Evidence from other studies using cultured rat hippocampal neurons showed dynamic local regulation of the proteasome at the dendrites. It was found that proteasome is redistributed from dendritic shafts to synaptic spines in an NMDAR-dependent manner. How does the redistribution of the proteasome occur? The experiments showed that activity only modestly increased the entry of the proteasome into dendritic shafts but significantly reduced their exit. Furthermore, the results suggested that the proteasome was sequestered persistently in the spines through association with cytoskeleton (Bingol & Schuman, 2006). Subsequent studies showed that a protein called NAC1, which is induced by psychostimulants, modulates the recruitment of the proteasome into the dendritic spines (Shen et al., 2007). Since the bulk of the evidence in this study is for the catalytic 20S core of the proteasome, it remains to be seen whether the recruitment of the full proteasome complex (26S) that degrades polyubiquitinated proteins is also regulated by NAC1. Another study has suggested that CaMKIIα subunit acts as a scaffold for the proteasome (Bingol et al.,

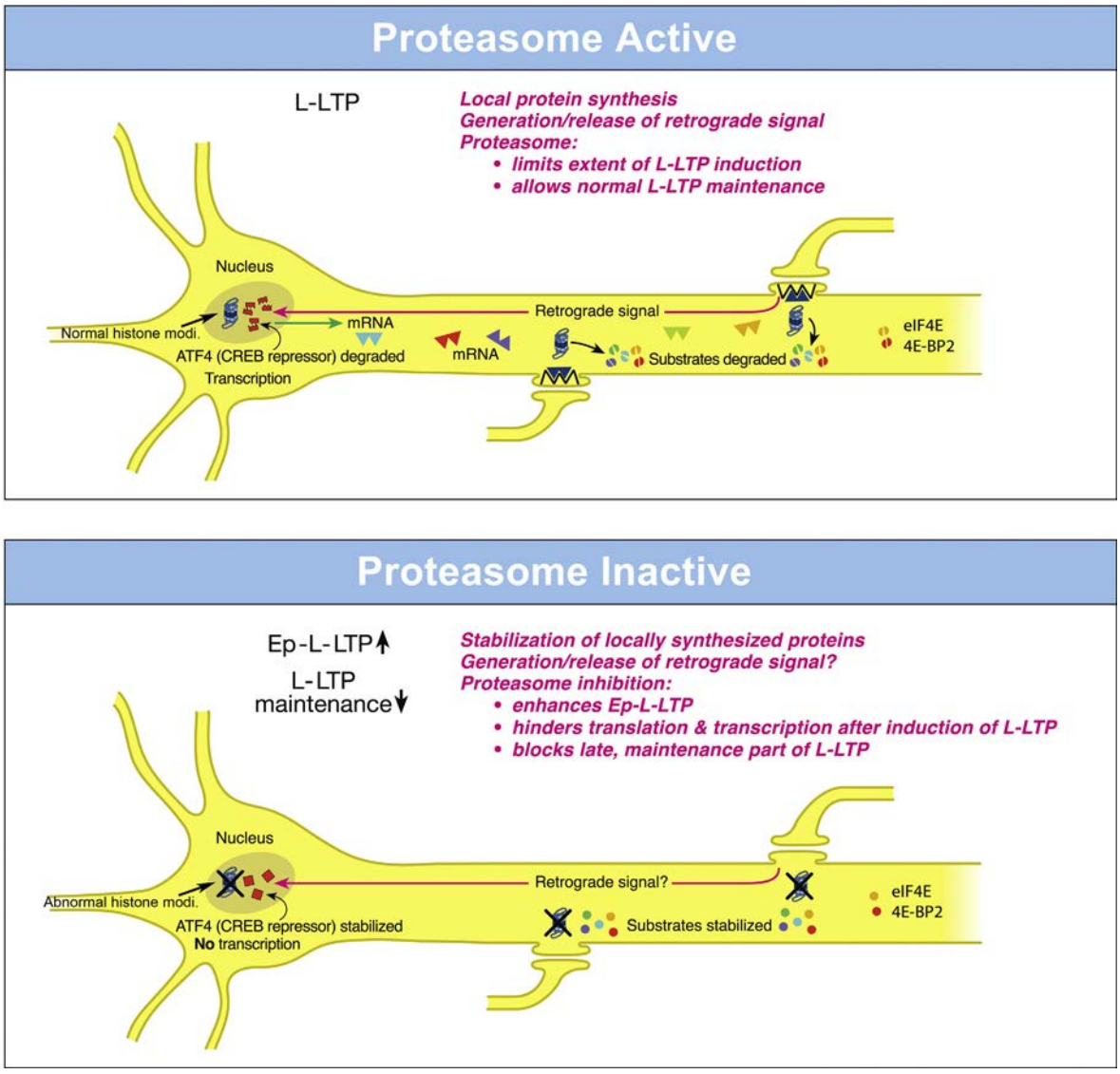

Fig. 3. Dissimilar local roles of the proteasome in dendrites and in the nucleus during L-LTP. (Top)-Proteasome Active: The proteasome in dendrites is highly active, translational activators such as eIF4E are degraded (broken green spheres) and protein substrates that positively regulate L-LTP are degraded (broken spheres). Therefore extent of L-LTP is limited and only normal L-LTP ensues. A retrograde signal is likely transmitted to the nucleus. Proteasome aids transcription of genes by degrading the CREB repressor ATF4 (broken squares in the nucleus) thus allowing for normal L-LTP maintenance. The proteasome also enables histone modifications (Normal histone modi.). Transcribed mRNAs (triangles) travel to activated synapses. (Bottom)-Proteasome Inactive: When the proteasome is inhibited (indicated by X marks on the proteasome), translational activators are stabilized (intact green spheres) leading to increased protein synthesis in dendrites. Also the newly synthesized proteins in dendrites are stabilized (intact spheres) and L-LTP-inducing stimulation protocols dramatically increase (upward arrow) the early part of L-LTP (Ep-L-LTP). Proteasome inhibition obstructs CREB-mediated transcription by preventing the degradation of transcription repressor ATF4 (intact squares in the nucleus). Proteasome inhibition could inhibit the generation of the retrograde signal as well. Therefore, L-LTP is not maintained but decays (downward arrow). Proteasome inhibition also perturbs histone modifications (Abnormal histone modi.). In addition, proteasome inhibition causes failure of sustained translation because of stabilization of translation repressors such as 4E-BP (intact red spheres) which accumulate after induction of L-LTP thus contributing to blockade of L-LTP maintenance [Modified from (Hegde, Haynes, Bach, & Beckelman, 2014)].

2010). It is not clear how or if the functions of NAC1 and CaMKIIα relate to each other in sequestering the proteasome.

Proteasome might function to locally regulate other processes required for synaptic plasticity such as translation of mRNA. For example, fragile X mental retardation protein (FMRP), which is thought to regulate translation of a subset of mRNAs in dendrites, is regulated by the proteasome. Furthermore, regulation of FMRP by the proteasome appears to be critical for metabotropic glutamate receptor-dependent LTD (Hou et al., 2006).

6. The UPP and memory

Considering the role of the UPP in synaptic plasticity, it can be expected that the UPP would have a role in memory. Accordingly, experimental evidence has been accumulating in support for roles of the UPP in various stages memory including induction, consolidation and reconsolidation. Initial results were obtained from experiments on the rat hippocampus. Lopez-Salon and co-workers demonstrated that bilateral infusion of lactacystin to the CA1 region of the rat hippocampus caused total retrograde amnesia for a one-trial avoidance learning. They also showed that total ubiquitination increases in the hippocampus 4 h after the training (Lopez-Salon et al., 2001). These results are consistent with the idea that a decrease in some critical inhibitory proteins during long-term memory formation (Abel, Martin, Bartsch, & Kandel, 1998) is mediated by the ubiquitin-proteasome pathway.

Later studies on vertebrates suggest that the UPP may have much broader and more complex roles than just degrading the inhibitory constraints on long-term synaptic plasticity and

memory such as R subunits and the CREB repressor. For example, infusion of the proteasome inhibitor β-lactone into the CA1 region of the hippocampus prevents extinction of contextual fear memory (Lee et al., 2008). Also, using infusion of lactacystin into the CA3 region of the hippocampus it was shown that protein degradation is important for consolidation as well as reconsolidation of spatial memory (Artinian et al., 2008). The mechanistic details as to how the UPP contributes to the extinction of fear memory or reconsolidation of spatial memory are not clearly understood.

In the past few years, there has been a resurgence of interest in the connection between the UPP and memory. Several studies have examined the role of the UPP in memory. Helmstetter and colleagues showed that infusion of a specific proteasome inhibitor β-lactone into amygdala of rats blocks fear memory. These investigators also showed a global increase K-48-linked polyubiquitination of proteins in response to NMDAR stimulation suggesting that protein degradation is required (Jarome, Werner, Kwapis, & Helmstetter, 2011). These researchers, in a different study, demonstrated the importance of protein degradation by the UPP in the prefrontal cortex in development of trace fear memory (Reis, Jarome, & Helmstetter, 2013). Subsequently they also found that during development of long-term fear memory, Serine-120 phosphorylation of an ATPase subunit of the proteasome namely Rpt-1 occurs. They also found increase in 20S proteasome activity (Jarome, Kwapis, Ruenzel, & Helmstetter, 2013). Given that Rpt1 is a subunit in the 19S RC, it is not clear how Rpt-1 phosphorylation leads to enhancement of 20S proteasome activity. One possibility is allosteric regulation of the 20S by the 19S RC although this is yet to be tested. Earlier, others had established that Rpt-1 phosphorylation decreases the amplitude of miniature postsynaptic currents in cultured rat hippocampal neurons and suggested that Rpt-1 phosphorylation may be important for homeostatic synaptic plasticity (Djakovic et al., 2012).

There have been other studies on the role of the UPP both in vertebrates and invertebrates. For example, in the conditioned taste aversion model, long-term memory is impaired by infusion of the proteasome inhibitor lactacystin into amygdala and the insular cortex (Rodriguez-Ortiz, Balderas, Saucedo-Alquicira, Cruz-Castaneda, & Bermudez-Rattoni, 2011). Others infused protein synthesis inhibitors anisomycin and proteasome inhibitor β-lactone into the CA1 region of the hippocampus of rats and tested for memory using novel object recognition task. This study showed that the proteasome inhibitor did not have an effect on memory consolidation and reconsolidation but was able to reverse the impairment of these processes caused by inhibition of protein synthesis (Furini et al., 2015). Other investigations have implicated a role for a deubiquitinating enzyme USP14 (Jarome, Kwapis, Hallengren, Wilson, & Helmstetter, 2014) and the ubiquitin ligase APC (Kuczera et al., 2011) in memory in vertebrates. A study carried out on honey bees showed that drugs that inhibit the proteasome enhance memory formation (Felsenberg et al., 2014) which is an opposite result compared to that obtained in vertebrate model systems. This may be a case of the drugs affecting proteasome in one part of the circuitry more than the others (i.e. local effects). Given the complex nature of the role of the UPP in synaptic plasticity and memory, only deep mechanistic investigations can resolve such discrepancies.

How might the UPP regulate memory consolidation? One possibility is that the UPP regulates gene expression critical for memory. For example, ubiquitin-proteasome-mediated degradation of transcription repressors might facilitate initiation of transcription. For example, gene expression pathway mediated by CREB requires the removal of repressors. In *Aplysia* a CREB repressor called CREB1b is degraded by the UPP during LTF (Upadhya et al., 2004) as described elsewhere in this review. A second CREB repressor called CREB2 is an important negative regulator of LTF (Bartsch et al., 1995). The mammalian counterpart of CREB2 called ATF4 is polyubiquitinated and is degraded by the proteasome during chemically induced long-lasting LTP in the mouse hippocampus (Dong et al., 2008). A repressor of another transcriptional pathway mediated by NF-κB called IκB is degraded by the proteasome (Yaron et al., 1998). Although transcription by the NF-κB pathway has been implicated in some forms of memory (Meffert, Chang, Wiltgen, Fanselow, & Baltimore, 2003), its contribution to memory-forming gene expression relative to the CREB pathway remains unclear.

The UPP might have other roles in memory such as reconsolidation. It has been argued that protein degradation by the UPP is necessary for making memories labile before the reconsolidation process occurs (Sol Fustinana, de la Fuente, Federman, Freudenthal, & Romano, 2014). Although this argument makes intuitive sense, much remains to be learned about how proteolysis relates to the lability of memory. Progress in this regard is beginning to be made. A recent study showed that phosphorylation of Rpt6 by the proteasome by CaMKII is critical for memory destabilization after retrieval (Jarome, Ferrara, Kwapis, & Helmstetter, 2016). To advance towards a thorough mechanistic understanding of the role of the UPP in memory, additional studies pursuing specific substrates and integrating molecular, electrophysiological and behavioral approaches in the same experimental model system would be necessary.

7. The UPP and diseases of the synapse

Apart from the numerous roles of in normal synaptic function, the UPP has also been linked to synaptic malfunction observed in many diseases and disorders of the brain. Deficiencies in the UPP are believed to play some role in development of Alzheimer's disease (AD) (Gentier & van Leeuwen, 2015; Upadhya & Hegde, 2007), Parkinson's disease (PD) (Atkin & Paulson, 2014) and Huntington's disease (HD) (Ortega & Lucas, 2014). Because of its role in synaptic plasticity the UPP may also play a role in synaptic defects underlying cognitive impairment observed in these diseases.

Deficits in synaptic plasticity and its association with the UPP is better understood in AD compared to PD and HD. Cognitive defects observed early in AD likely occur because of synaptic failure (Selkoe 2002). In mouse models of AD, deficits in LTP and memory are known to occur and have been shown to correlate well with accumulation of Aβ (Hsiao et al. 1996). Ubiquitin immunoreactivity is found in plaques and tangles of AD brains. Blockade of the UPP in the neurons of AD brains might be responsible for accumulation of ubiquitinated proteins (Upadhya & Hegde, 2005, 2007). Although how the UPP connects to AD pathology and cognitive impairment is not understood, some hints regarding the role of the UPP in AD have been discovered. For instance, application of oligomeric Aβ inhibits LTP which can be rescued by treatment with exogenous Uch-L1 (mammalian homolog of Ap-uch). In AD model mice carrying amyloid precursor protein and presenilin1 transgenes, deficits in LTP and memory can also be rescued by treatment with exogenous Uch-L1 (Gong et al., 2006).

Evidence for directly connecting the UPP to pathogenesis of AD came from the observation that the brains of some AD patients contained an aberrant form of ubiquitin that has 20 additional amino acids at its C-terminus (UBB^{+1}) (van Leeuwen et al., 1998). Postnatal expression of UBB^{+1} in neurons of transgenic mice showed proteasome dysfunction and deficits in contextual memory (Fischer et al., 2009). Given that proteasome inhibition throughout the neuron blocks maintenance of L-LTP because it hinders transcription and sustained translation (Dong et al., 2008), it is interesting to speculate that the memory deficits in the UBB^{+1} mice result from impaired synaptic plasticity owing to neuron-wide proteasome dysfunction.

Another type link between the UPP and neurodegeneration has also been reported. It was shown that another cellular degradative process, autophagy, utilizes an enzymatic pathway similar to ubiquitin conjugation which attaches ubiquitin-like proteins such as Atg12 to some proteins that regulate the autophagic process (Nakatogawa, Suzuki, Kamada, & Ohsumi, 2009). In autophagy, a double membrane vesicle (called the autophagosome) engulfs parts of the cytoplasm or organelles and delivers it to the lysosome. Ubiquitination has also been found to be linked to autophagy. In SH-SY5Y cells, Lys-63-linked polyubiquitination promotes inclusion bodies which are cleared by autophagy (Tan et al., 2008). In addition, it was observed that parkin, which is an E3 ligase, promotes Lys-63-linked polyubiquitin chain attachment to misfolded proteins. Lys-63-polyubiquitin chain seems to serve as a signal to couple the misfolded proteins to dynein motor complex through histone deacetylase 6 (which serves as an adaptor) and thus aiding in sequestration of misfolded proteins into specialized inclusion bodies called aggresomes which are cleared by autophagy (Olzmann & Chin, 2008). A subsequent study found that autophagy promotes synapse development in *Drosophila* (Shen & Ganetzky, 2009). The UPP and autophagy might work in concert to remove aggregated proteins observed in many neurodegenerative disease (Ciechanover & Kwon, 2015). It remains to be seen whether autophagy is connected to synaptic malfunction observed in neurodegenerative diseases.

8. Other proteolytic pathways that play a role in synaptic plasticity and memory

In addition to the UPP, two other cellular proteolytic mechanisms are known to play a role in synaptic plasticity and memory: the lysosomal pathway and autophagy. It must be noted that both of these degradation mechanisms do have connection to the UPP.

8.1. Roles of the lysosome in synaptic plasticity and memory

A major way in which the lysosome participates in synaptic plasticity and memory is through control of the number of neurotransmitter receptors on the plasma membrane. As described elsewhere in this article, the receptors that undergo endocytosis through ubiquitin signaling can either be recycled back to the plasma membrane or routed to the lysosome for degradation. Therefore, any effect on neurotransmitter receptors such as glutamate receptors or GABA receptors would affect synaptic strength and plasticity. A study carried out several years ago showed that in cultured rat hippocampal neurons, kainate receptors (KRs) undergo endocytosis in response to stimulation by kainate (Martin & Henley, 2004). The internalization of KRs in response to kainate stimulation is Ca^{2+}- and PKC- dependent and the internalized KRs are targeted to lysosomes for degradation. Activation of NMDARs, however, results in Ca^{2+}- and PKA- dependent endocytosis of KRs and recycling of the KRs back to the plasma membrane (Martin & Henley, 2004).

There have been some mechanistic studies using stimulation of cultured rat neurons NMDA and other reagents. It was found that stimulation of NMDARs caused dephosphorylation of a DUB called Usp8. This leads to recycling of the AMPARs that have been endocytosed as a result of ubiquitination by the ligase Nedd4-1. The same study also showed that increased synaptic activity through prolonged bicuculline treatment reduced Usp8 levels and caused an increase in the recruitment of Nedd4-1 at synapses and consequent enhancement in ubiquitination of AMPARs and trafficking of the endocytosed receptors to the lysosome for degradation (Scudder et al., 2014).

The number of inhibitory neurotransmitter receptors such as $GABA_A$ receptors at synaptic sites is controlled by lysosomal degradation as well. This was demonstrated using behavioral adaptation in the worm *C. elegans* to acute exposure of $GABA_A$ agonist muscimol (Davis et al., 2010). Initially the worms are paralyzed because of hyperpolarization of postsynaptic cells. After several hours of exposure to muscimol the worms adapt. Using electrophysiological recordings and visualization through immunofluorescence it was shown that during adaptation $GABA_A$ receptors are selectively removed from synaptic sites and are routed to the lysosome for degradation. The role of lysosome in $GABA_A$ receptor degradation was established based on the fact that the mutant worms with a defect in lysosomal function have elevated levels of synaptic $GABA_A$ receptors (Davis et al., 2010).

The role of the lysosome in memory has not been investigated much. There is a study in *Drosophila*, however, showing a link between lysosomal degradation and long-term olfactory memory. A genetic screen to identify new genes with a role in memory revealed that a gene called *debra* is linked to long-term memory (Kottler, Lampin-Saint-Amaux, Comas, Preat, & Goguel, 2011). Previous results had established that debra protein functions to induce polyubiquitination of a protein called Ci and directs it to the lysosome for degradation (Dai, Akimaru, & Ishii, 2003). The exact role of debra in lysosomal trafficking of Ci is not clear because debra in neither a ligase not does it have any homology to any other enzymes of the UPP. It has been suggested that Ci ubiquitination is mediated by Slimb, a protein containing F-box/WD40 repeats. The vertebrate homolog of Slimb is βTrCP which is part of an SCF ligase. Dai et al. suggested that debra possibly binds to the ligase complex that ubiquitinates Ci (Dai et al., 2003). Even though these results provide a hint for possible function of debra in the Drosophila mushroom bodies in mediating long-term memory, much remains to be learned regarding the molecular mechanisms by which debra contributes to long-term memory.

8.2. Roles of autophagy in synaptic plasticity and memory

There are three main types of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy. Macroautophagy is an elaborate process comprising the formation of a double-membraned structure called the autophagosome which can fuse with the lysosome. In microautophagy, the lysosome directly takes up cytosolic components through invagination of its membrane. Chaperone-mediated autophagy denotes translocation of unfolded proteins across the lysosomal membrane with the aid of chaperones such as heat shock proteins. The focus in this article is on macroautophagy (henceforth referred to as "autophagy") which requires the action of many proteins some of which are functionally akin to the enzymes of the UPP. The molecules that regulate autophagy (Atg proteins) were originally discovered in yeast (Mizushima, Yoshimori, & Ohsumi, 2011). Since then, orthologues of Atg proteins have been discovered in other organisms.

Although there have not been many systematic investigations on the roles of autophagy in synaptic plasticity, some studies indicate a role for autophagy in phenomena such as LTP and Ltd. Investigations on streptozotocin-induced diabetes in mice showed that LTP impairment in diabetic mice was exacerbated in the presence of an inhibitor of autophagy called 3-methyl adenine (Li, Hao, Yin, Gao, & Yang, 2016). In the context of chemically induced LTD, it has been shown that neuronal stimulation induces autophagy (Shehata, Matsumura, Okubo-Suzuki, Ohkawa, & Inokuchi, 2012). When chemical LTD is induced by low doses of NMDA, a marker of autophagosome formation called light chain protein 3-II (LC 3-II) increases along with the number of auophagosomes. The increase in LC 3-II is coincides with dephosphorylation of Akt

and mammalian target of rapamycin and degradation of GluR1, an AMPA receptor subunit. RNA interference-mediated knockdown of ATG7, an enzyme which is like E1 of the UPP and is critical for formation of autophagosomes, blocks autophagy induced by chemical LTD (Shehata et al., 2012).

There have been some studies on relating autophagy to memory. A CNS-specific knockout of the *Wdr45* gene (one of the orthologues of yeast *Atg18*), whose protein product has an essential function in autophagosome formation, leads to memory impairment as judged by performance in Morris water maze and contextual fear conditioning. Mutations in the human *WDR45* cause a type of neurodegeneration called β-propeller associated neurodegeneration which is characterized by cognitive impairment (Haack et al., 2012; Hayflick et al., 2013; Saitsu et al., 2013). Therefore, it is likely that autophagy has a role in memory formation in humans as well. In the study on diabetic mice, it was found that spatial memory was not impaired but spatial reversal memory was and the memory impairment was exacerbated by inhibition of autophagy (Li et al., 2016). Even though these results are suggestive of the link between autophagy and memory, the caveat is that the studies were conducted in the context of diabetes. Definitive conclusion on the role of autophagy in memory will have to await CNS-specific and conditional suppression of molecules critical for autophagy.

The connection between autophagy and memory was observed in another model organism, *Drosophila*. It was found that spermidine ameliorates age-related memory impairment and this beneficial effect on memory requires autophagy (Gupta et al., 2013). Spermidine is a naturally occurring polyamine with pleiotropic effects on cells. Previous research showed that spermidine promotes longevity in yeast cells and one of the mediators of spermidine's effect is autophagy (Eisenberg et al., 2009). Although these results suggest a role for autophagy in memory and the mechanisms are likely to be evolutionarily conserved, it would be necessary to test the effects of compounds such as spermidine in vertebrate model systems of memory.

9. Looking ahead

Despite numerous investigation into the roles of ubiquitin-proteasome-mediated proteolysis in synaptic plasticity and memory, there are many open questions. An important area of for future investigation is likely to be elucidation of mechanisms that determine the spatial and temporal regulation of proteolysis in the nervous system. Although many substrates in the nervous system have been identified, roles for many more are likely to be revealed. Function of the UPP in wiring and fine-tuning the nervous system has not been investigated much and needs to be explored. Studies on the roles of the UPP in memory would benefit from genetic manipulations such as gene editing. Connections between the UPP and neurodegenerative diseases will also likely to be a fruitful area of research because of the potential for therapeutic development.

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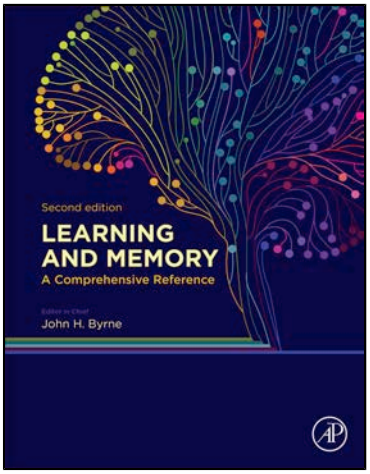

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4.12 Proteolysis and Synaptic Plasticity☆

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4.12.1 Introduction

Understanding mechanisms underlying synaptic plasticity has been an intense area of research in neuroscience. As a result of many years of research, we now know that short-term synaptic plasticity requires posttranslational modification of existing proteins,

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whereas long-term synaptic plasticity entails new gene expression and protein synthesis. Since the early 1990s, proteolysis by the ubiquitin–proteasome pathway has attained prominence as a new molecular mechanism underlying synaptic plasticity.

4.12.2 The Ubiquitin–Proteasome Pathway

Although the ubiquitin–proteasome pathway was originally thought to function in degradation of abnormal proteins, a large number of studies indicate that this pathway plays a major role in regulated proteolysis of short-lived regulatory protein substrates under physiological conditions. In this pathway, the process of degradation of a substrate protein can be divided into two steps: (1) covalent attachment of ubiquitin to the substrate protein, forming the polyubiquitin chain attached to the substrate (ubiquitin conjugation) and (2) degradation of the polyubiquitinated substrate and disassembly of the polyubiquitin chain and recycling of free ubiquitin (Fig. 1).

When a protein is to be specifically degraded in the cell, it is marked by covalent attachment of ubiquitin to an ε-amino group of lysine residues. The ubiquitin conjugation step is a highly regulated step catalyzed by the action of three classes of enzymes called E1, E2, and E3. E1, the ubiquitin-activating enzyme, activates the free ubiquitin, a small protein of 76 amino acids, in an adenosine triphosphate (ATP)–dependent reaction. Activated ubiquitin is then transferred to an E2, which is generally referred to as a ubiquitin-carrier enzyme. An enzyme belonging to a class of enzymes called E3s (ubiquitin ligases) then ligates the activated ubiquitin to the substrate. A second ubiquitin is attached to an internal lysine residue in the first ubiquitin (Lys-48), and thus by sequential linkages of monoubiquitins, a polyubiquitin chain grows (Glickman and Ciechanover, 2002; Hegde, 2004, 2010b).

The polyubiquitinated substrate is then recognized by a large multisubunit proteolytic complex called the 26S proteasome and is degraded to small peptides and amino acids (Hegde and DiAntonio, 2002; Marques et al., 2009). Ubiquitination also plays a role in endocytosis. Attachment of a single ubiquitin to a lysine residue (monoubiquitination) or single ubiquitin molecules to multiple lysine residues in a substrate (multiple monoubiquitination) usually marks the protein substrates in the plasma membrane for endocytosis. The disassembly of polyubiquitin chains or removal of monoubiquitins is carried out by deubiquitinating enzymes (DUBs) (Hegde, 2004, 2010b).

4.12.3 Ubiquitin-Conjugating Enzymes: E1, E2, and E3

Among the three classes of ubiquitin-conjugating enzymes, E1 is the least physiologically regulated. E1 activates ubiquitin in an ATP-dependent step. Activated ubiquitin is then attached to all the substrates degraded by the ubiquitin–proteasome pathway; there

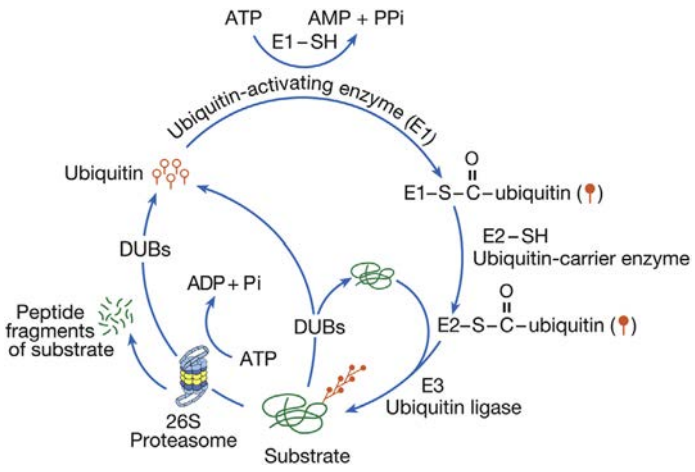

Figure 1 The ubiquitin–proteasome pathway. In this proteolytic pathway, ubiquitin (a single ubiquitin molecule is represented by *open circles with straight tails*) is selectively and covalently linked to the substrate. The enzymatic process of attaching ubiquitin to substrates is called ubiquitination, or ubiquitin conjugation, and depends on the action of three different classes of enzymes: E1, E2, and E3. First, ubiquitin is activated by E1 to form a ubiquitin-adenosine monophosphate (AMP) intermediate. Activated ubiquitin (*closed circles with straight tails*) is passed onto E2 (ubiquitin carrier enzymes). E2s transfer ubiquitin to an E3 (ubiquitin ligase), which ligates the activated ubiquitin to the substrate. To the ubiquitin attached to substrate, another ubiquitin is attached and thus through successive linkages of ubiquitin a polyubiquitin chain forms. Polyubiquitinated substrates are degraded by a proteolytic complex called the 26S proteasome in an adenosine triphosphate (ATP)–dependent reaction. Ubiquitin is not degraded but the polyubiquitin chain is disassembled and ubiquitin is recycled by deubiquitinating enzymes (DUBs). Before being committed to being degraded by the proteasome, ubiquitination is reversible. DUBs can disassemble the polyubiquitin chain if a substrate is ubiquitinated erroneously and prevent the degradation of the substrate.

is no substrate specificity in this process. E2s are more selective and are believed to interact with specific E3s. Based on our knowledge of ubiquitination reaction, it can be asserted that E3s are the enzymes that possess substrate specificity.

Originally, E2s were believed only to carry the activated ubiquitin and transfer it onto the E3s. Recent studies, however, suggest that at least some E2s can directly conjugate ubiquitin to substrates. E2s are structurally and functionally diverse. Simple eukaryotes such as yeast (*Saccharomyces cerevisiae*) have 13 genes potentially encoding E2s. The number of E2s in mammals is estimated to be in the range of 25–30. Most E2s have a core domain of around 14 K that is ~35% conserved between different E2s. The other part of the enzymes appears to be variable. Although most E2s are small, some notable exceptions exist. For example, an E2 called BIR-repeat-containing ubiquitin-conjugating enzyme (BRUCE) is a gigantic 528-K protein. The diversity of E2s generates some degree of specificity in the ubiquitin-conjugating reaction. E2s bind to E3s selectively. Since the diversity of E3s is even greater than that of E2s, the combination of E2s and E3s potentially can generate a high degree of specificity. The heterogeneity of E2s is reflected in their subcellular localization as well. Although several E2s are present in the cytosol, some E2s are localized to other subcellular compartments. For example, BRUCE is localized to the Golgi apparatus, and a yeast E2 called Ubc6 is anchored to the membrane of endoplasmic reticulum (Glickman and Ciechanover, 2002; Hegde, 2010a).

E3s are the enzymes that specifically recognize the substrates. E3s can be single proteins or a complex of proteins. Single-subunit E3s can accept ubiquitin in a thioester linkage from E2s and ligate ubiquitin to the substrate. When E3 contains multiple subunits, it is generally believed that the enzyme brings the E2 and the substrate together and facilitates the transfer of ubiquitin to substrate. However, E3s are the most diverse among the ubiquitin-conjugating enzymes and the least characterized. Therefore, it is possible that E3 catalytic mechanisms other than those described below might exist in nature. There are two major classes of E3s: (1) HECT (homologous to E6-AP carboxyl-terminus) domain E3s and (2) RING (really interesting new gene) finger E3s (Fig. 2).

4.12.3.1 Homologous to E6-AP Carboxyl-Terminus Domain E3s

The typical example in this class of E3 is the ubiquitin ligase called E6-AP, which ligates ubiquitin to the tumor suppressor protein p53. Viral protein E6 associates with a cellular protein called E6-AP (E6-associated protein). The C-terminal region of E6-AP contains the catalytic domain of the ubiquitin ligase. E6-AP ligase can function with either of the E2s called UbcH5 and UbcH7 (Fig. 2). Later studies found that a family of proteins, ubiquitin ligases, with homology to the catalytic domain of E6-AP exists. These ubiquitin ligases are called HECT domain E3s. In addition to the HECT domain, there is another domain in many E3s called the WW domain. The WW domain–containing E3s also tend to have a C2 domain. The presence of the C2 domain is highly relevant to nervous system function because the C2 domain responds to elevation of intracellular Ca^{2+} and helps in translocation to the

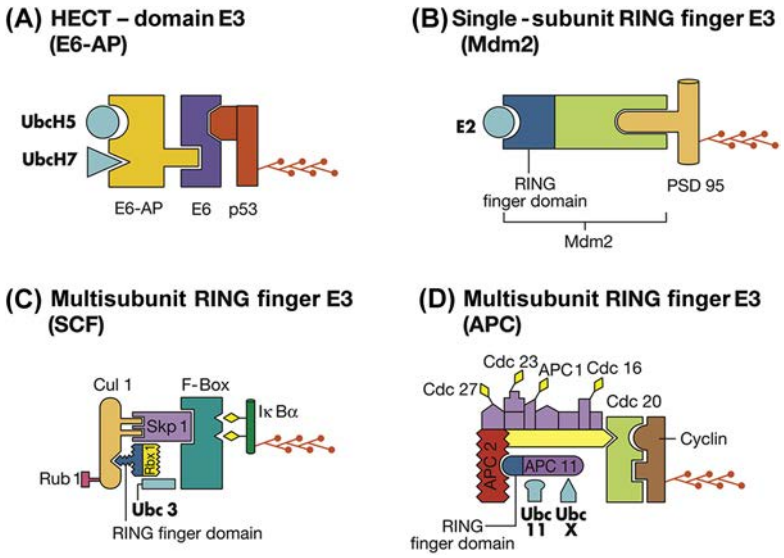

Figure 2 Classes of ubiquitin ligases (E3s). (A) Homologous to E6-AP carboxyl-terminus (HECT) domain E3. E6-AP ubiquitin ligase in combination with E6 protein and one of the two E2s (UbcH5 or UbcH7) ligates ubiquitin to the p53 tumor suppressor protein. (B) Single-subunit RING finger E3. Mdm2 ligates ubiquitin to postsynaptic density 95 (PSD 95) with the help of an E2 enzyme. (C) Multisubunit RING finger E3. SKP1-cullin-F-Box protein (SCF) ligases contain the substrate recognition site on an F-box protein. Skp1 is an adaptor that joins the F-box protein to Cul1. Ring finger domain is on Rbx 1. The E2 is Ubc3. Cul 1 is modified by Rub1, another ubiquitin-like protein, leading to an increase in the activity of the ligase complex. The substrate is phosphorylated (*diamonds*) IκBα. (D) Multisubunit RING finger E3. Anaphase-promoting complex (APC) is a more complex example of multisubunit RING finger E3s and has a subunit composition distinct from that of SCF. Cdc20 protein in APC has the substrate (*Cyclin*) recognition site. The RING finger domain is on APC11. The E2s Ubc11 or UbcX can function with the APC ligase. In addition, several adaptor proteins, some labeled (Cdc27, Cdc23, APC 1, Cdc16) and some unlabeled, interact with Cdc20 and APC11. *Diamonds* on the adaptor subunits indicate phosphorylation. The polyubiquitin chain is shown on the substrates in each panel.

plasma membrane. Therefore, presence of this domain in neuronal HECT E3s might be critical in ligating ubiquitin to neurotransmitter receptors or proteins associated with them (Hegde, 2004, 2010b).

4.12.3.2 RING Finger E3s

These E3s are called RING finger E3s because they contain a RING finger domain, which consists of seven cysteine residues and one histidine residue, forming a single folded domain binding two zinc ions. The arrangement of metal-binding residues in the RING finger domain contrasts with the tandem arrangement in the zinc finger domain found in many proteins. Although numerous other proteins were found to have the RING finger motif, the biological function of these proteins remained elusive. During the past few years, several ubiquitin ligases were found to contain the RING finger. The RING finger motif in ubiquitin ligases is critical for transfer of ubiquitin to substrates or to RING finger proteins themselves. The RING finger category of E3s can be subdivided into (1) single-subunit RING finger E3s and (2) multisubunit RING finger E3s.

4.12.3.2.1 Single-Subunit RING Finger E3s

Single-subunit RING finger E3s contain the RING finger domain and the substrate recognition site in the same protein. One of the well-characterized single-subunit RING finger E3s is Mdm2, which ubiquitinates p53 in normal cells. As discussed previously, E6-AP, a HECT ubiquitin ligase, ubiquitinates p53 in human papilloma virus (HPV)–infected cells. A recent study showed that in HPV-infected cells, E6-AP ubiquitinates p53. Although Mdm2 is present in HPV-infected cells, it does not mediate ubiquitination of p53. Other studies using antisense oligonucleotides directed against E6-AP showed that E6-AP is essential for degradation of HPV-positive cells but not HPV-negative cells. Conversely, decreasing Mdm2 expression or expression of Mdm2-inactivating peptides decreased p53 degradation in HPV-negative cells but not in HPV-positive cells. Interestingly, the structural determinants on p53 that are recognized by E6-AP and Mdm2 are different from each other. For example, p53 that is a substrate for E6-AP has an asparagine at position 268, whereas p53 that is a substrate for Mdm2 has an aspartate at position 268 (Hengstermann et al., 2001).

4.12.3.2.2 Multisubunit RING Finger E3s

4.12.3.2.2.1 The SKP1-Cullin-F-Box Protein Complex

The SKP1-cullin-F-Box protein (SCF) complex contains at least four proteins: Skp1, Cul1, Roc1/Rbx1/Hrt1, and an F-box protein (**Fig. 2**). In the center of the SCF complex is the RING finger domain–containing protein Rbx1. The SCF type ligases have another invariant protein called cullin. The theme appears to be that the cullins interact with linker proteins such as Skp1 to recruit substrate-interacting proteins such as the F-box proteins. There are at least five different cullins in mammals. There are several F-box proteins as well. Although it is not clear how many F-box proteins exist in mammals, the budding yeast genome comprises 17 F-box proteins. Therefore, with just with the cullin and F-box combination alone, it would be theoretically possible to generate close to 100 E3s with differing specificities. Although the regulation of SCF ligases is not completely understood, two mechanisms of posttranslational regulation have been discovered thus far. One is the covalent linkage of ubiquitin-related protein Rub1 to a cullin (Cul1 in **Fig. 2**). The second mode of regulation appears to be regulation of levels of F-box proteins through ubiquitin-mediated degradation via an autocatalytic mechanism (Petroski and Deshaies, 2005). A well-characterized substrate of the SCF complex is IκBα.

4.12.3.2.2.2 Anaphase-Promoting Complex

Although anaphase-promoting complex (APC) has a subunit with a RING finger domain (APC11), this ubiquitin ligase is distinct from the SCF ligase in overall subunit combination (**Fig. 2**). For example, instead of one adaptor found in SCF ligases (such as Skp1), APC has multiple subunits that serve as adaptors. Also, unlike SCF ligases, substrate phosphorylation is not an important determinant for specific substrate recognition by the APC ligase. Rather, substrate specificity of APC ligases appears to be modulated by incorporation of specificity factors into the ligase complex. For example, Cdc20 (**Fig. 2**) enables APC to degrade substrates at the onset of anaphase such as the anaphase inhibitor Pds1p, whereas substitution of Cdc20 with another specificity factor called Hct1 enables APC to degrade a different set of substrates such as mitotic cyclins late in the anaphase. APC acts together with an E2 Ubc11 or Ubc X. One of the most studied substrates of APC is mitotic cyclin. This substrate has a short stretch of nine amino acids called the destruction box, which is critical for recognition by the APC ubiquitin ligase (Page and Hieter, 1999).

4.12.3.2.2.3 Von Hippel-Lindau-Elongin B-Elongin C Complex

The VBC (VHL-elongin B-elongin C) complex was identified in association with the VHL (von Hippel-Lindau) tumor-suppressor protein. The structure of this complex is similar to that of the SCF complex. The substrate (transcription factor hypoxia-inducible factor 1α, HIF1-α) binds to the VHL protein or SOCS box–containing proteins. The VHL protein binds to the Skp1 homologue elongin C and a ubiquitin homologue elongin B. Cul2 and the Roc1/Rbx1 RING finger protein are associated with the complex (Jackson et al., 2000).

Another class of proteins called E4s that elongate the polyubiquitin chain has been discovered (Koegl et al., 1999). The protein product of a gene previously known as ubiquitin fusion degradation protein 2 (UFD2) in yeast was found to catalyze ubiquitin chain assembly along with E1, E2, and E3 and was named E4. E4s contain a modified version of the RING finger, designated as U-box. Since there have not been many studies on E4s and the first E4 discovered functions as cofactor for an E3, it might be premature to conclude that E4s belong to a special class of E3s.

4.12.4 The Proteasome

The term proteasome is used to describe two kinds of multisubunit proteolytic complexes, the 26S and 20S, based on their sedimentation coefficient. The 26S proteasome degrades ubiquitinated protein substrates. The 26S complex contains the 20S as a core and regulatory caps on either end like a dumbbell. Each cap of the 26S proteasome is called the 19S regulatory complex (19S RC). The 20S core is a cylindrical structure consisting of the catalytic part of the proteasome (Hegde, 2004, 2010b; Pickart and Cohen, 2004).

4.12.4.1 The Catalytic 20S Core

Our knowledge of the proteasome organization comes from the studies on the crystal structure of the proteasome from the archaebacterium *Thermoplasma acidophilum* (Lowe et al., 1995) and the yeast *S. cerevisiae* (Groll et al., 1997). It appears that the proteasome is more ancient than ubiquitin because archaebacteria have the proteasome but not ubiquitin. *T. acidophilum* has two genes encoding α and β subunits. The subunits are arranged in four stacked rings to form the catalytic cylinder with the two middle rings consisting of β subunits, which are sandwiched between two rings of α subunits. In the *T. acidophilum* proteasome, both the α and β subunits are present in seven copies each and assembled in a symmetrical fashion $\alpha_7\beta_7\beta_7\alpha_7$. This general structure is preserved in eukaryotes, but the α and β subunits have diverged into seven different subunits each. In yeast, the 20S core is made up of two outer rings with seven α subunits (α1–α7) in each ring and two inner rings consisting of seven β subunits each (β1–β7) (Marques et al., 2009).

The catalytic core of the proteasome is a threonine protease. Based on the crystal structure of the proteasome, it was concluded that proteasome functions through a new kind of proteolytic mechanism. In this mechanism, the active site nucleophile is the hydroxyl group on the threonine residue at the N-terminus of the β subunit. This catalytic mechanism seems to be conserved across evolution. The antibiotic lactacystin and the active form β-lactone, which are specific and irreversible inhibitors of the proteasome, bind to the N-terminal threonine residue in the β subunit of the mammalian proteasome. The 20S proteasome can exist not only as a core of 26S, but also as a separate population that cannot degrade ubiquitinated proteins. However, the 20S proteasome by itself has chymotrypsin-like, trypsin-like, and postglutamyl peptidase activities, which cleave after hydrophobic, basic, and acidic residues, respectively. The peptide hydrolyzing activity of the 20S proteasome can itself be modulated by an 11S regulatory cap (Glickman and Ciechanover, 2002; Hegde, 2004).

4.12.4.2 19S Regulatory Complex

The 19S RC recognizes the polyubiquitinated substrate and channels the substrate into the catalytic 20S core of the proteasome. It also has the capacity to regulate the activity of the catalytic core and determine the nature of the degradation process. Usually one 19S RC is attached to either end of the catalytic core. The subunits of the 19S RC are highly conserved across evolution. Two subcomplexes can be recognized within the 19S RC called the base and the lid.

4.12.4.2.1 The Base of the Proteasome

The base consists of six ATPase subunits (Rpt1–Rpt6) and two non-ATPase subunits (Rpn1 and Rpn2). The Rpt subunits are highly conserved through evolution (up to 75% identical between yeast and humans). The ATPase subunits have a domain called the AAA (ATPases associated with different cellular activities) domain in the center. These subunits are homologous to each other, with the highest degree of homology in the AAA domain. Some of the Rpt subunits contact the α ring in the catalytic core of the proteasome and are believed to channel the substrate into the catalytic chamber for degradation (Hegde, 2004, 2010b).

4.12.4.2.2 The Lid of the Proteasome

The lid, which comprises eight subunits, can attach itself to the base of the 19S RC or detach as a complex from the base. All the subunits in the lid are non-ATPase subunits. The exact function of the lid subunits is not known. Degradation of polyubiquitinated proteins requires lid attachment, indicating that the lid performs an essential function in ubiquitin–proteasome-mediated degradation. Also, in archaebacteria that lack ubiquitin, the proteasome is devoid of the lid. The lid subunits of the proteasome share a characteristic sequence of 200 amino acids with COP9 signaling complex and translation initiation factor 3. This sequence is called the PCI (proteasome, COP9, initiation factor 3) domain. In addition, the subunits of the lid have a 120-amino-acid-long MPN (Mpr1p/Pad1p N-terminus) domain that is important for the structure of Rpn8 and Rpn11 subunits. In addition, a subset of MPN domain–containing proteins have a motif of five polar residues called the MPN+ motif. The MPN+ motif has been shown to be critical for the function of Rpn11 (Hegde, 2004, 2010b).

4.12.5 Deubiquitinating Enzymes

Ubiquitination reaction is reversible before the ubiquitinated protein is committed to degradation by the proteasome. The reversibility is less clear with respect to endocytotic degradation, i.e., internalization of plasma membrane proteins through endocytosis and their degradation through the lysosome. Based on knowledge of the endocytotic pathway, it is reasonable to assume that

ubiquitination is reversible until the endocytosed membrane proteins such as neurotransmitter receptors are routed to the multivesicular body (MVB) for lysomal degradation or prior to its entry into invaginating vesicles of MVB. However, if the machinery responsible for recognizing ubiquitinated cargo and initiating its entry into the MVB vesicles is defective, recycling of activated receptors might occur because of the action of DOA4, a DUB required for the removal of ubiquitin from the MVB cargo proteins.

Ubiquitin is removed from substrates by enzymes called DUBs. Based on protein sequence and the molecular size, DUBs can be classified into two general classes: (1) low-molecular-weight (20–30 K) ubiquitin C-terminal hydrolases (UCHs) and (2) high-molecular-weight (~100 K) ubiquitin-specific proteases (UBPs; also called USPs) (Hegde, 2004). There are numerous DUBs in almost every eukaryotic organism studied. Among the DUBs, UBPs belong to a large family containing diverse genes, whereas the UCH family has fewer genes. For example, in yeast *S. cerevisiae* there are 17 UBPs and one UCH (Amerik et al., 2000). In the human genome there are 63 genes encoding UBPs and four genes that code for UCHs. UCHs and UBPs subserve different functions in the eukaryotic cell. Although the current name for these enzymes—DUBs—emphasizes the removal of ubiquitin from substrates, some DUBs, particularly UCHs, function to process linearly linked ubiquitin precursors and generate monoubiquitin. UCHs are cysteine proteases in that the critical residue in the catalytic site is a cysteine. In addition, histidine and aspartate residues are critical for catalytic activity. All UCHs contain these residues even if they do not share a high degree of homology elsewhere in the sequence. UCHs cleave small peptide chains linked to the carboxyl-terminus of ubiquitin. UBPs can cleave the isopeptide bond between ubiquitins in a polyubiquitin chain as well as the isopeptide bond between ubiquitin and the substrate. DUBs are important for generating free ubiquitin at various steps of the ubiquitin–proteasome pathway. Ubiquitin is encoded by the tandemly linked polyubiquitin gene. In the cell, linear polyubiquitin protein molecules are not detected. When polyubiquitin and UCH-L1 are coexpressed in bacterial cells, polyubiquitin is cotranslationally processed to generate monoubiquitin. Therefore it is possible that polyubiquitin is processed by UCH or other DUBs to generate monoubiquitin in eukaryotic cells as well. In addition to the polyubiquitin gene, ubiquitin is also encoded by fusion to two ribosomal subunits called L40 and S27. These gene products are also believed to be processed by DUBs. Cleavage of isopeptide bond in the ubiquitin chains linked through Lys-48 of ubiquitin serves two purposes. One is to recycle ubiquitin after it has been used for marking a substrate for ubiquitination. Another function is to edit the errors made by the ubiquitin-conjugating enzymes and reverse the ubiquitination reaction so that the substrate is no longer degraded (Wilkinson, 2000). Also, the editing function of DUBs probably serves to reverse the monoubiquitin attachment that marks membrane proteins for endocytosis.

4.12.6 Regulation of the Ubiquitin–Proteasome Pathway

Proteolysis by the ubiquitin–proteasome pathway can be regulated at the ubiquitin-conjugation step or at the proteasome step. Since the specificity of ubiquitination lies at the conjugation step, clearly regulation of the conjugation process is important in determining whether or not a substrate is targeted for degradation. Regulation of proteasome has a global effect on degradation of cellular substrates.

4.12.6.1 Regulation of Ubiquitin Conjugation

Ubiquitin needs to be conjugated to the right substrate, at the right place in the cell, and at the right time to control physiological processes properly. Commitment of a substrate protein to ubiquitin–proteasome-mediated degradation is regulated by (1) modification of the substrate; (2) modulation of ubiquitin ligase activity; and (3) removal of ubiquitins.

4.12.6.1.1 Modification of the Substrate

Protein substrates are degraded in the cell at specific times in response to physiological stimuli. In addition, degradation of substrates is likely to be spatially restricted within a cell. Based on the accumulated evidence, it appears that the vulnerability or resistance to ubiquitin–proteasome-mediated degradation is regulated usually by a posttranslational modification. The protein substrates are modified in two main ways: (1) by posttranslational modification such as phosphorylation or (2) by allosteric modifications.

4.12.6.1.1.1 Phosphorylation of the Substrate

Phosphorylation of a substrate can make it vulnerable to ubiquitination or resistant to ubiquitination. For example, yeast cyclins Cln2 and Cln3, the cyclin-dependent kinase inhibitor p27Kip1, and transcriptional regulators IκBα and β-catenin are ubiquitinated after phosphorylation. In neurons, ubiquitination of p35, a neuronal-specific activator of cyclin-dependent kinase 5 (Cdk5), is stimulated when the protein is phosphorylated by Cdk5 within the active kinase complex (Patrick et al., 1998).

It is instructive to consider examples of some substrates as to how the regulation at the level of substrate works. The transcription factor nuclear factor kappa B (NF-κB) is inhibited by IκBα, which binds to NF-κB and keeps it in an inactive form in the cytosol. NF-κB is activated by numerous external stimuli such as cytokines, ionizing radiation, or neuronal injury. NF-κB is activated by proteolysis of IκBα, which releases NF-κB to be translocated to the nucleus, where it initiates transcription. Ubiquitination of IκBα requires phosphorylation on Ser-32 and Ser-36. Upon phosphorylation, IκBα is recognized by a specific multisubunit RING finger ligase complex called $SCF^{\beta-TrCP}$. Activity of $SCF^{\beta-TrCP}$ ligase seems to be constitutive, while the kinases that phosphorylate IκBα are activated by the stimuli known to induce NF-κB-mediated transcription. It must be noted that phosphorylation of a substrate can

make a stable protein vulnerable to degradation, as we saw earlier, or phosphorylation can have the opposite effect of stabilizing a short-lived protein. In the *Aplysia* nervous system, for example, CCAAT/enhancer binding protein, a transcription factor critical for long-term synaptic plasticity, is made resistant to ubiquitin–proteasome-mediated degradation when it is phosphorylated by mitogen-activated protein kinase (MAPK) (Yamamoto et al., 1999).

4.12.6.1.1.2 Allosteric Modification of the Substrate

Although less well studied, a mechanism for making a substrate susceptible to ubiquitination is allosteric modification by ligands. A physiological example is that of degradation of regulatory (R) subunits of cyclic adenosine monophosphate (cAMP)–dependent protein kinase (PKA) (Hegde et al., 1993). R subunits are substrates for ubiquitination and degradation by the proteasome. Degradation of R subunits leads to persistent activation of PKA without persistence in cAMP elevation and bridges the short-term action of the neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) to gene expression (Hegde et al., 1997). The R subunit has two cAMP-binding sites. Without cAMP binding, R subunits are resistant to ubiquitination. Mutation studies have revealed that for ubiquitination of R subunits, binding of cAMP to both sites is essential. For example, R subunit mutants that bind cAMP to only one site are not efficiently degraded (Chain et al., 1999).

4.12.6.1.2 Modulating the Activity of Ubiquitin Ligases

Ubiquitin ligases largely control the substrate specificity of the ubiquitin conjugation reaction. The temporal specificity of ubiquitin conjugation to substrates by these enzymes is provided by regulation of the ligase activity. The activity of ubiquitin ligases can be modulated by posttranslational modification such as phosphorylation and by allosteric modification of the enzyme, or by attachment to ubiquitin-like (UbL) proteins.

4.12.6.1.2.1 Modulation of Ubiquitin Ligases by Phosphorylation

Regulation of ubiquitin ligase activity by phosphorylation has been shown in the studies on a multisubunit ligase, APC. As the name implies, this complex is critical for cell cycle progression into anaphase. A recent study shows that a form of APC is also expressed in postmitotic neurons.

APC can be activated by Cdc2 kinase, which appears to exert its effect by activating another protein kinase called polo-like kinase. In *Xenopus* and humans, phosphorylation of four different APC subunits, APC1, CDC16, CDC23, and CDC27, has been shown to be increased during mitosis. In neurons, APC might have a role in ubiquitinating different substrates from the ones ubiquitinated during cell cycle progression. An observation that lends credence to this notion is that levels of polo-like kinases Fnk and Snk dramatically increase with stimuli that produce long-term potentiation (LTP) and other forms of synaptic plasticity (Kauselmann et al., 1999). Activity of at least one single-subunit RING finger ubiquitin ligase, c-Cbl, is known to be regulated by phosphorylation. c-Cbl ubiquitinates the epidermal growth factor receptor (EGFR). Tyrosine phosphorylation of c-Cbl stimulates the ligase to ubiquitinate the EGFR at a site next to the RING finger domain (Glickman and Ciechanover, 2002).

Phosphorylation of ubiquitin ligases could have an inhibitory effect as well. In the fission yeast *Schizosaccharomyces pombe*, PKA blocks APC activity. Moreover, the inhibitory effect of PKA seems to be dominant over the stimulatory effect of the polo-like kinase. Even if APC has been activated by polo-like kinase, addition of mammalian PKA to APC-containing fractions inhibits ubiquitination of the substrate cyclin B. Phosphorylation of ubiquitin ligases is also regulated by phosphatases. For example, type I protein phosphatases (PP1) are necessary for progression into anaphase. Also, in *S. pombe*, mutations in *dis2+*, a gene that encodes a catalytic subunit of PP1, have a deleterious effect. Although the exact mechanism of phosphatase action in promoting activity of APC is not clear, perhaps the phosphatases act by counteracting the negatively regulating protein kinases such as PKA (Hegde, 2004).

4.12.6.1.2.2 Allosteric Modification of Ubiquitin Ligases

In addition to phosphorylation, ubiquitin ligase activity can be stimulated by allosteric activation. A ubiquitin ligase called Ubr1 targets transcription factor Cup9, which is a negative regulator of di/tri-peptide transporter *Ptr1* gene. Ubr1 has three sites at which it can bind other molecules. It is believed that site III on Ubr1 binds to the substrate Cup9. Peptides that bind to site I or II can allosterically stimulate ligase activity of Ubr1 toward Cup9 (Turner et al., 2000).

4.12.6.1.2.3 Modulation of Ubiquitin Ligase Activity by Attachment of Ubiquitin-Like Proteins

Activity of the ligases is also modified by posttranslational modification by covalent linkage of UbL proteins. Linkage of a UbL protein to an E3 ubiquitin ligase appears to modulate the activity of the ligase. For example, a UbL protein called Rub1 (related to ubiquitin 1) is conjugated to proteins of the cullin family, which are part of the SCF ligase complex. Conjugation to Rub1 is required for maximal activity of the SCF ligase 14 (Hegde, 2004).

4.12.6.1.3 Removal of Ubiquitins

Ubiquitin–proteasome-mediated degradation can be regulated by removal of ubiquitin. When a protein is polyubiquitinated, it is targeted to the proteasome for degradation unless the ubiquitin chains are removed by the action of DUBs. Deubiquitination by DUBs serves two purposes: (1) reversing ubiquitination of a protein or (2) disassembling the polyubiquitin chains before the ubiquitinated proteins are channeled to the 26S proteasome. Disassembly of polyubiquitin chains at the proteasome step is likely to be a rate-limiting step for degradation. Since the pore of the proteasome catalytic chamber is small (13 Å), the polyubiquitin tag needs

to be removed before the substrate is fed into the catalytic core. Otherwise the catalytic chamber is likely to be clogged, thereby reducing the rate of degradation. The DUBs, called UBP6 (called USP14 in yeast) and UCH37, are associated with the proteasome. Also, a subunit in the lid of the 19S RC, Rpn11, has been shown to possess deubiquitinating activity (Yao and Cohen, 2002).

4.12.7 Achieving Specificity of Ubiquitin Linkage: Combinatorial Coding by E2s and E3s

Marking substrates by ubiquitin attachment is a highly specific reaction. How is this specificity achieved? Conjugating ubiquitin to a substrate requires the action of three enzymes: E1, E2, and E3. E1 is common to all ubiquitination reactions because this enzyme activates ubiquitin. There is some degree of specificity at the E2 step, which mainly derives from specific E2–E3 interactions. E2s interact with E3s through two loops (named L1 and L2) and an N-terminal α-helix 1 in the three-dimensional structure of E2s. Variations in amino acid sequence in these structural elements contribute to the specificity of E2s binding to E3s (Ye and Rape, 2009). In addition to the structural elements described previously, unique parts of some E2s contribute to the specificity of E3 binding as well. For example, the C-terminal tail of a yeast E2 called Cdc34, which is dissimilar to that of a closely related E2 Ubc4, confers specificity of Cdc34 binding to an SCF ligase (Kolman et al., 1992; Silver et al., 1992). The E3s are the most specific to a given substrate, however. Previously it was thought that there is a specific E3 for each substrate. Having a dedicated E3 for each substrate would be untenable because of the coding burden it places on the genome. Instead, the specificity is derived from a combination of recognition modules. Interaction between an E3 and its substrate is believed to be specific. In some cases, an E3 ligates ubiquitin to only one substrate. In other instances, an E3 ligates ubiquitin to more than one substrate. In the latter case, specificity of E3-substrate interaction might be determined by other factors such as posttranslational modification of the substrate (Hegde, 2010a). A genome-wide study estimated that the human genome contains 617 genes encoding putative E3s (Li et al., 2008). The diversity of E3s is further increased by the fact that some E3s such as SCFs and APCs are modular and by mixing and matching subunits additional unique E3s could be generated. The estimate for number of genes coding for E2s is around 25–30. Given that there are about 21,000 genes in the human genome (Harrow et al., 2012), E2s and E3s together potentially could generate a unique combination for nearly every protein substrate. Besides the unique E2–E3 combinations, specificity can be generated by the state of the substrate (susceptible or resistant to degradation) as well as regulation of E3s through posttranslational modification such as phosphorylation (Hegde, 2010a; Hegde and DiAntonio, 2002). Thus, with the combination of all the factors, the ubiquitin conjugation machinery can be highly specific to a given substrate.

Additional combinatorial coding capacity of the ubiquitin conjugation reaction derives from the type of ubiquitin linkage because the way ubiquitin is attached to a given substrate determines its fate. Ubiquitin is covalently linked to the side chain (called the ε-amino group) of Lys residues in the substrate. The first ubiquitin is attached to the substrate through the C-terminus of ubiquitin. Attachment of a single ubiquitin at one site in the substrate (monoubiquitination) or at multiple sites (multimonoubiquitination) generally determines binding of the ubiquitinated substrate to other proteins and is utilized for functions such as endocytosis and modulation of protein activity. Linkage of many ubiquitin molecules to the substrate (polyubiquitination) serves diverse purposes, the main one of which is marking the protein for degradation by the proteasome. Polyubiquitin chains are built through successive addition of single ubiquitin molecules to an internal lysine residue in the previously attached ubiquitin. Ubiquitin linkage to other ubiquitin molecules could occur through any of the seven lysine residues in ubiquitin. For targeting the substrate for ubiquitin–proteasome-mediated degradation, additional ubiquitins are attached to the first ubiquitin at its 11th or 48th Lys residue. Lys-63–linked polyubiquitin chains modulate protein function such as NFκ-B activation (Deng et al., 2000). Polyubiquitin chains can also be formed through second ubiquitin linkage to Lys-6, Lys-27, Lys-29, and Lys-33 of the first ubiquitin attached to the substrate (Komander, 2009; Ye and Rape, 2009). Polyubiquitin chains contain mixed type of linkage between ubiquitin molecules such as through Lys-11 and Lys-48 in the same chain. Furthermore, ubiquitin itself can be posttranslationally modified through acetylation and phosphorylation (Ohtake et al., 2015; Swaney et al., 2015). Thus, a combination of all these factors provides exquisite specificity of ubiquitin attachment to substrate proteins.

4.12.8 Regulation of the Proteasome

Proteasome activity can be regulated in two main ways. One is regulation by cofactors or proteins that are loosely associated with it and by induction or phosphorylation of the subunits, particularly those of the 19S RC. The proteasome can also be regulated by a change in the composition of the intrinsic subunits.

4.12.8.1 Regulation by Cofactors and Loosely Associated Factors

In addition to the intrinsic subunits, proteins that interact with the proteasome complex regulate its activity. Often the cofactors are components of the ubiquitin pathway. Proteins such as chaperones and heat shock proteins also assist in proteasome-mediated degradation of proteins. Both E2s and E3s have been found to interact with the proteasome. For example, ubiquitin conjugating enzymes (E2s) Ubc1, Ubc2, and Ubc4 coimmunoprecipitate with the proteasome. E3s such as Ubr1 and Ufd4 have been shown to physically interact with subunits of the 19S RC. APC and SCF ubiquitin ligases copurify with the 19S RC. A ubiquitin ligase called Hul5 is associated with the proteasome as well. The cofactors interact with the proteasome directly or via other proteins such as Cic1

and hPLICs that recruit the cofactors to the proteasome. For example, hPLIC-1 and hPLIC-2, the human counterparts of yeast Dsk2, interact with the proteasome as well as specific E3s E6-AP and β-TrCP. Two other proteins, Rad23 and BAG1, are also known to interact with the proteasome (Hegde, 2004).

In addition, the DUBs interact with the proteasome. A DUB called Ubp6 is known to interact with the regulatory complex of the proteasome. Since binding to the proteasome increases the activity of Ubp6 300-fold, Ubp6 is thought to assist in the removal of polyubiquitin chain just before the substrate is degraded and aid in recycling ubiquitin for further use. Other DUBs have also been reported to associate with the proteasome. A yeast DUB that is related to the human tre-2 oncogene associates with the proteasome and assists in the disassembly of the polyubiquitin chain. The *Aplysia* homologue of UCH-L1 (Ap-uch) associates with the proteasome and improves proteolytic activity. In in vitro experiments, it was shown that Ap-uch cleaves the first ubiquitin attached to the substrate (Hegde et al., 1997). Among the factors that interact with the proteasome are heat shock proteins and chaperones. It has been known for a long time that the ubiquitin–proteasome pathway degrades misfolded or unfolded proteins. The general belief is that the heat shock proteins refold the misfolded proteins into the right conformation. Misfolded proteins not rescued by the heat shock proteins are thought to be substrates for the ubiquitin–proteasome pathway. It was not clear, however, which ubiquitin ligase (E3) specifically recognizes misfolded proteins. Recently, a chaperone called BAG1 has been shown to interact both with the proteasome and the heat shock protein Hsp70. CHIP (carboxyl terminus of the Hsc70-interacting protein) is known to ubiquitinate unfolded proteins. CHIP contains a protein sequence motif called the U-Box and a RING finger domain. CHIP can interact with either Hsp70 or Hsp90, and together with either of the heat shock proteins it can ligate ubiquitin to misfolded proteins. The cooperation between heat shock proteins and the proteasome is also required to degrade aberrant membrane proteins. For example, the cystic fibrosis transmembrane conductance regulator (CFTR) is a membrane protein which is often misfolded. CHIP and Hsc70 together recognize the CFTR protein and target it to proteasome-mediated degradation (Hegde, 2004).

Proteasome activity can also be regulated by substitution of subunits of the 20S core with subunits such as the ones induced by interferon γ (IFNγ). Furthermore, alternative regulatory complexes with different composition than that of the 19S RC can be attached to the 20S core, which alters the substrate specificity and activity of the proteasome (Glickman and Ciechanover, 2002).

4.12.8.2 Regulation of the Proteasome by Induction and Phosphorylation of Subunits and Subcellular Distribution

The subunits of the 26S proteasome are not fixed but change in response to the physiological condition of the cell. The capacity of the 26S proteasome to degrade ubiquitinated proteins can be regulated by changes in (1) the total amount of the proteasome, (2) its subunit composition, and (3) its subcellular distribution. The changes in the total amount of 26S can be brought about by the extent of 19S cap binding to the 20S core. This could occur by an increase in the amount of 19S as well as by increased association of the existing 19S with the 20S core. During metamorphosis of *Manduca*, flight muscles develop and intersegmental muscles are destroyed. The destruction of intersegmental muscles is brought about by an increase in ubiquitin-dependent proteolysis as a result of extensive hormone-dependent reprogramming of the 19S RC. It has been shown that the multiubiquitin binding subunit (MBP/S5a) and the ATPases MSS1 (S7) and S4 are induced significantly during this period. An increase in the amount of 26S by enhanced binding of 19S to 20S without an increase in the total amount of 20S occurs during the metaphase–anaphase transition in the meiotic cell cycle. In addition to the regulation of total proteasome content, the activity of the proteasome also appears to be regulated by alterations in its subcellular distribution. For example, during ascidian (marine animals commonly called sea squirts) embryonic development, the distribution of the proteasome changes in a cell-cycle-dependent manner. The proteasome localized in the nucleus during interphase disappears from the nucleus during prophase, and in telophase the proteasome is again localized in the newly formed nucleus (Hegde, 2004).

Proteasome activity can also be regulated by phosphorylation. For example, it has been shown that phosphorylation events are necessary for the assembly of the 26S proteasome. Several subunits, including MSS1, S4, S6, and S12 of the 19S RC, have been shown to be phosphorylated. Recently it was demonstrated that assembly of the proteasome requires phosphorylation of Rpt6, an ATPase subunit. Our recent studies showed modulation of the proteasome activity by protein kinases in the nucleus and synaptic terminals. Interestingly, we observed that protein kinases differentially modulate the proteasome activity in the two subcellular compartments of the neuron (Upadhya et al., 2006).

4.12.9 Ubiquitin–Proteasome Pathway and Synaptic Plasticity

Although ubiquitin was used a marker for brain pathology, no physiological or pathological role for ubiquitin in the nervous system was found until the 1990s. The first discovery of ubiquitin–proteasome-mediated degradation of a physiologically relevant substrate in the nervous system was that of R subunits of PKA (Hegde et al., 1993). Since then many substrates of the ubiquitin–proteasome pathway in the nervous system have been identified.

4.12.9.1 Degradation R Subunits of PKA and Proteolytic Removal of a cAMP Response Element Binding Protein Repressor

A role for the ubiquitin–proteasome pathway in synaptic plasticity was discovered during the investigation of persistent activation of PKA. Studies on the biochemical mechanism of long-term facilitation (Greenberg et al., 1987) in *Aplysia* indicated that PKA was persistently activated in the absence of elevated cAMP. How is PKA activated in the absence of sustained increase in cAMP? It was

found that the R subunits of PKA were decreased without any change in the catalytic (C) subunit during induction of long-term facilitation. Since there was no change in mRNA for either the R subunit or the C subunit, it was concluded that R subunits were diminished perhaps through proteolysis. What is the mechanism of R subunit degradation? In a series of biochemical experiments, Hegde et al. (1993) found that R subunits were substrates for ubiquitination and proteasome-mediated degradation. Moreover, a UCH (Ap-uch) that interacts with the proteasome was found to be induced by 5-HT, the neurotransmitter that induced long-term facilitation. Ap-uch was found to be critical for induction of long-term facilitation (Hegde et al., 1997; **Fig. 3**). Subsequently, Chain et al. (1999) showed that at sensorimotor neuron synapses, injection of lactacystin, a specific proteasome inhibitor, blocked induction of long-term facilitation. Since the R subunit inhibits the activity of C subunits of PKA, the results were interpreted to suggest that the ubiquitin–proteasome pathway operates to remove inhibitory constraints on formation of long-term memory. This has been corroborated by work carried out on the rat hippocampus. Lopez-Salon et al. (2001) demonstrated that bilateral infusion of lactacystin to the CA1 region of the rat hippocampus caused total retrograde amnesia for a one-trial avoidance learning. They also showed that total ubiquitination increases in the hippocampus 4 h after the training. These results are consistent with the idea that a decrease in some critical inhibitory proteins during long-term memory formation is mediated by the ubiquitin–proteasome pathway.

Additional evidence that the ubiquitin–proteasome pathway might function to degrade proteins that normally inhibit long-term synaptic plasticity has also been obtained using the *Aplysia* model. Stimulation protocols that induce long-term facilitation in *Aplysia* cause ubiquitination and degradation of a cAMP response element binding protein (CREB) repressor called CREB1b. Both ubiquitination and degradation of CREB1b are increased by protein kinase C (**Fig. 3**; Upadhya et al., 2004). Ubiquitin–proteasome-mediated degradation of CREB repressors occurs in vertebrates as well. For example, a mammalian CREB repressor called ATF4 (which despite being called activating transcription factor 4, often acts a repressor; also called CREB-2) is ubiquitinated and degraded by the proteasome in long-term synaptic plasticity in the murine hippocampus. Beyond degrading proteins that inhibit synaptic plasticity, the ubiquitin–proteasome pathway seems to have a broader role in regulating other forms of synaptic plasticity such as long-term depression (LTD). In the *Aplysia* model, transcription-dependent LTD requires the activity of the proteasome and the transcription associated with LTD requires CREB2 as a transcriptional activator (Fioravante et al., 2008).

4.12.9.2 Modulation and Essential Function of a Deubiquitinating Enzyme in Synaptic Plasticity

Subsequent to the finding on ubiquitin–proteasome-mediated degradation of R subunits of PKA, a crucial role in long-term facilitation for a neuronal-specific UCH was discovered. *Aplysia* UCH (Ap-uch) is the homologue of human UCHL1 and is induced by stimuli that induce long-term facilitation but not stimuli that induce short-term facilitation. Injection of antibodies or antisense oligonucleotides specific to Ap-uch into sensory neurons that synapse onto motor neurons in culture blocked induction of long-term facilitation (Hegde et al., 1997). Investigation of biochemical functions of Ap-uch indicated that Ap-uch is capable of cleaving small attachments to linearly attached ubiquitin molecules such as ubiquitin–ubiquitin–cysteine but not large attachments such as glutathione-S-transferase (GST) in substrates such as ubiquitin–GST. More interestingly, additional biochemical analyses showed that Ap-uch associates with the proteasome. The association of Ap-uch increases the rate of degradation by the proteasome. For example, addition of recombinant Ap-uch to in vitro degradation systems showed that there was approximately double the degradation of the R subunit of PKA. Since persistent activation of PKA has been shown to be critical for induction of long-term facilitation and R subunits of PKA were found to be substrates for the ubiquitin–proteasome pathway, the experiments on Ap-uch provided some molecular explanation for the role of regulated proteolysis in long-term facilitation (Hegde et al., 1997). How does Ap-uch increase the rate of degradation by the proteasome? Using recombinant ubiquitin with its Lysine-48 mutated to

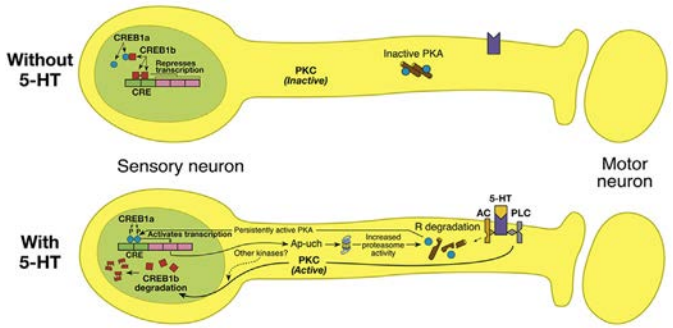

Figure 3 Role of the ubiquitin–proteasome pathway in long-term facilitation. When sensory neurons are stimulated with the neurotransmitter serotonin (5-HT), which induces long-term facilitation, R subunits of protein kinase A (PKA) are ubiquitinated and degraded by the proteasome, making the kinase persistently active. The catalytic subunit of PKA (*blue circle*) translocates to the nucleus and phosphorylates cAMP response element binding protein 1a (CREB1a), the activator form of CREB. Concomitantly, the repressor form of CREB, CREB1b, is degraded by the ubiquitin–proteasome pathway. Protein kinase C (PKC), which is also activated by 5-HT-mediated signaling, stimulates ubiquitin conjugation to CREB1b and subsequent degradation. *AC*, adenylyl cyclase; *PLC*, phospholipase C.

Arg, which can support multiple monoubiquitin attachments to the protein substrate, it was shown that Ap-uch stimulates the release of ubiquitin from substrates (Hegde et al., 1997). Therefore, it can be inferred that Ap-uch perhaps cleaves the first ubiquitin in the polyubiquitin chain attached to the substrate. Ap-uch might have a role in other forms of long-term synaptic plasticity in Aplysia. For example, stimuli that induce transcription-dependent LTD upregulate *Ap-uch* mRNA and protein (Fioravante et al., 2008). The function of UCHs in synaptic plasticity seems to be evolutionarily conserved. The mammalian homologue of Ap-uch, Uch-L1, has been shown to play a role in long-term synaptic plasticity memory in mice.

4.12.9.3 Differential Role of the Proteasome in Different Compartments of Neurons

Although previous investigations found that proteasome inhibitors block induction of long-term facilitation, recent analysis of long-term facilitation at *Aplysia* synapses obtained different results. Bath application of the active form of lactacystin, *clasto*-lactacystin β-lactone, to sensorimotor neuron synapses resulted in strengthening the synapses and increasing neurite outgrowth in isolated sensory neurons. Furthermore, application of β-lactone increased 5-HT–induced long-term facilitation. The increase in neurite elongation is consistent with results obtained in PC12 and Neuro2A cells in which lactacystin induces neurite outgrowth. Interestingly, the increase in synaptic strength using the *Aplysia* sensorimotor neuron synapses observed by application of β-lactone was blocked in the presence of the translation inhibitor anisomycin but not by the transcription inhibitor actinomycin D (Zhao et al., 2003). This result is highly surprising, since innumerable studies have documented the requirement for transcription in the induction of long-term facilitation in *Aplysia* as well as several vertebrate forms of long-term memory (Chen and Tonegawa, 1997; Kandel, 2001).

Both sets of results can be reconciled if one postulates that proteasome has different roles in different cellular compartments. In the same neuron, the proteasome is likely to carry out different tasks in different subcellular compartments, resulting in different physiological consequences at different loci. Therefore, blocking different roles of the proteasome during induction of memory would lead to distinct and even opposite effects on synaptic strength. For example, the proteasome is known to degrade transcription repressors. Degradation of transcription repressors should allow transcription activators to induce gene expression, which in turn leads to development of long-term facilitation. If the proteasome is inhibited only in the nucleus before the repressors are degraded, gene expression and hence induction of long-term facilitation should be blocked. On the other hand, if the degradation of proteins needed at the synapse for developing long-term facilitation is inhibited by the proteasome, long-term facilitation should be enhanced. Although it might sound radical, I think the main purpose of transcription during induction of long-term facilitation or other forms of long-term memory is to provide mRNAs for the synthesis of the rapidly turning over proteins needed for memory formation. These proteins are likely to be degraded by the ubiquitin–proteasome pathway. If the degradation of these proteins is prevented, then long-term memory formation becomes independent of transcription. In support of this idea, Zhao et al. (2003) found that proteasome inhibitor–induced synaptic strengthening depends on translation but not transcription. Differential temporal regulation of the proteasome could occur within the same subcellular compartments. In the nucleus, the proteasome has to degrade transcription repressors to allow transcription activators to induce gene expression, which in turn leads to development of long-term facilitation. If the proteasome is inhibited during the time the repressors are degraded, gene expression and hence induction of long-term facilitation should be blocked. In support of this idea, we have found that a transcription repressor called CREB1b is degraded by the ubiquitin–proteasome pathway in response to long-term facilitation-inducing protocols (Upadhya et al., 2004).

Our biochemical experiments on the proteasome also support differential function of the proteasome in different neuronal compartments. Our results showed that in both *Aplysia* nervous system and mouse brain, proteasome activity in the synaptic terminals is significantly higher than that of the nuclear proteasome. Moreover, the proteasome activity in the two compartments is differentially regulated by protein kinases relevant to synaptic plasticity such as PKA, PKC, and MAPK (Upadhya et al., 2006).

As discussed, differential activity of the proteasome in *Aplysia* might explain conflicting results obtained in different studies. Does differential proteasomal activity affect synaptic plasticity differentially in vertebrates? Investigations carried out on late phase of long-term potentiation (L-LTP), a widely used mammalian model of long-term synaptic plasticity, found that proteasome inhibition enhances the early phase of L-LTP but inhibits the late, maintenance phase of L-LTP, which is described in detail later (see section Local Proteolysis and Synaptic Plasticity).

4.12.10 Roles of the Ubiquitin–Proteasome Pathway at the Synapse

Ubiquitin–proteasome-mediated proteolysis has been found to have both presynaptic and postsynaptic roles at the synapse.

4.12.10.1 Presynaptic Roles of Proteolysis

The earlier observations on the role of the ubiquitin–proteasome pathway in synaptic plasticity mainly focused on long-term effects. Recent studies, however, indicate that the ubiquitin–proteasome pathway also functions in regulating short-term synaptic plasticity. For example, a protein, Dunc-13, which is critical in priming the synaptic vesicles, is ubiquitinated and degraded by the proteasome in *Drosophila* neuromuscular synapse. Application of proteasome inhibitors and the dominant-negative mutation in a core subunit (β6) of the *Drosophila* proteasome both lead to an increase in presynaptic accumulation of Dunc-13 protein. Also, application of the proteasome inhibitors lactacystin and epoxomycin causes an increase in the excitatory junctional current, suggesting that

accumulation of Dunc-13 and the resultant increase in the net Dunc-13 quantity leads to enhanced synaptic transmission (Speese et al., 2003).

Is it likely that the quantity of other synaptic proteins with a key role in neurotransmission is regulated by the ubiquitin–proteasome pathway? If so, regulated proteolysis may have a wider role in controlling short-term synaptic plasticity. As of now, two other synaptic vesicle proteins, synaptophysin and syntaxin, have been shown to be substrates for ubiquitin–proteasome-mediated degradation. Vulnerability of synaptophysin for ubiquitin–proteasome-mediated degradation was discovered indirectly by looking for proteins that bind to synaptophysin in the yeast two-hybrid system. Two genes encoding for proteins Siah-1A and Siah-2 were isolated from the rat brain two-hybrid cDNA library. These two genes are closely related to each other and are homologues of *Drosophila seven* in absentia gene. Siah proteins are single-subunit RING finger ubiquitin ligases with the RING finger domain in the N-terminal region. It is not clear which ligase (Siah1-A or Siah-2) is responsible for degradation of synaptophysins in vivo in neurons. Because the experiments were carried out in nonneuronal cells (Chinese hamster ovary cells) and PC12 cells, the physiological consequences of synaptophysin degradation remain to be determined.

Another presynaptic substrate for the ubiquitin–proteasome pathway is syntaxin 1. The group that isolated the Siah ligases also investigated ubiquitin–proteasome-mediated degradation of syntaxin 1 using the yeast two-hybrid strategy. The ligase that ubiquitinates syntaxin 1 is called staring (syntaxin 1-interacting RING finger protein). Coexpression of staring with syntaxin 1 in HeLa cells increases the degradation of syntaxin 1, which can be inhibited by the proteasome inhibitor MG132. Again, the physiological effect of ubiquitin–proteasome-mediated degradation of syntaxin 1 remains to be determined.

Proteolysis also regulates other presynaptic proteins. For example, amount of RIM1α (Rab3-interacting molecule 1α), which is critical for regulating neurotransmitter release, is a target for a ubiquitin ligase named SCRAPPER (an acronym whose derivation is not clear). Studies using miniature postsynaptic current (mEPSC) measurements established that SCRAPPER regulates synaptic transmission. It was also found that in mice lacking SCRAPPER short-term synaptic plasticity was impaired (Yao et al., 2007).

Other presynaptic processes such as recycling of synaptic vesicles are also regulated by proteasome-mediated proteolysis. In hippocampal neurons in primary culture, proteasome inhibition causes an increase in the size of the recycling pool of vesicles. Blockade of neuronal activity significantly reduces the effect of proteasome inhibition, decreasing vesicle numbers. Inhibition of the proteasome, however, does not increase transmitter release probability. Therefore, it seems that in vertebrate neurons, the proteasome functions to maintain vesicle homeostasis (Willeumier et al., 2006).

4.12.10.2 Modulation of Postsynaptic Structure and Function by Proteolysis

Ubiquitin and proteasome regulate proteins such as neurotransmitter receptors and proteins that are part of the synaptic structure such as postsynaptic density proteins. Neurotransmitter receptor levels on the postsynaptic membrane are regulated through ubiquitination that targets proteins for endocytosis, which is mediated by monoubiquitination (attachment of a single ubiquitin) or a Lys-63-linked polyubiquitin chain. The ubiquitinated protein that is endocytosed may be recycled back to the plasma membrane if the ubiquitin is removed by DUBs or targeted to the lysosome via the MVB. Some membrane proteins, on endocytosis, are degraded by the proteasome instead of being routed to the lysosome for degradation.

Studies on *Caenorhabditis elegans* showed a role for ubiquitin in endocytosis of GLR-1 type of glutamate receptor (Burbea et al., 2002). In vertebrates, treatment with the proteasome inhibitor MG132 blocks agonist-induced endocytosis of AMPA-type glutamate receptors as determined from experiments on hippocampal neurons (Patrick et al., 2003). Furthermore, NMDA-induced AMPA receptor internalization is prevented by application of the proteasome inhibitor. Subsequent studies showed that AMPA receptor endocytosis, and in support of this idea, a postsynaptic density protein PSD-95 was shown to be regulated by ubiquitin-proteasome–mediated degradation (Colledge et al., 2003). PSD-95 is a major component of the postsynaptic scaffold, which through interaction with another protein called stargazin provides a docking site for AMPA receptors (Schnell et al., 2002). Degradation of PSD-95 leads to AMPA receptor internalization, and mutations that block PSD-95 ubiquitination block NMDA-induced AMPA receptor endocytosis (Colledge et al., 2003). In addition, application of the proteasome inhibitor MG132 to hippocampal slices reduces the magnitude of hippocampal LTD (Colledge et al., 2003). Because the transient, protein synthesis–independent LTD (Sajikumar and Frey, 2003) requires a net reduction in synaptic AMPA receptors (Malenka and Bear, 2004), these data further support a role for the proteasome in decreasing AMPA receptor amount at synaptic sites. Investigations carried out over the last few years have identified the signal for regulating AMPA receptor internalization and degradation. GluA1 subunit was found to be ubiquitinated in hippocampal neurons in an activity-dependent manner. This ubiquitination was mediated by ubiquitin ligase Nedd4-1(neural-precursor cell-expressed developmentally downregulated gene 4-1) (Schwarz et al., 2010). A subsequent study demonstrated that ubiquitination of GluA1 and GluA2 was critical in directing the internalized AMPA receptors to late endosomes and then into lysosomes for degradation (Widagdo et al., 2015). Because monoubiquitination on one or multiple sites in a protein generally directs the substrates to late endosomes and lysosome, and polyubiquitination directs substrates to the proteasome, how AMPA receptors are degraded might have different effects on synaptic plasticity.

Studies have shown has postsynaptic role for the proteasome in short-term synaptic plasticity using experiments carried out on LTP in the CA1 region of the hippocampus (where LTP is induced through mainly postsynaptic mechanisms). A type of LTP called early phase LTP (E-LTP), which is independent of protein synthesis, is enhanced by preincubation of hippocampal slices with the proteasome inhibitor β-lactone (Dong et al., 2008). Even though the mechanisms by which E-LTP is enhanced by proteasome inhibition have not been elucidated, it is likely that AMPA receptor stabilization and consequent increase in AMPA receptor number at postsynaptic sites might contribute to the increase in E-LTP.

It appears that the UPP has a broad role in regulating neurotransmitter receptors. NMDA receptors are retrotranslocated and degraded by the UPP in an activity-dependent fashion. An F-box protein called Fbx2 is critical for this process (Kato et al., 2005) suggesting that an SCF-type ligase targets the NMDA receptors for ubiquitination. Endocytosis of other neurotransmitter receptors might be regulated by ubiquitination. For example, glycine receptor is internalized on ubiquitination (Buttner et al., 2001). A protein Plic-1, which is associated with $GABA_A$ receptors, indirectly controls removal of $GABA_A$ through endocytosis (Bedford et al., 2001). It was shown that proteasome inhibitors prevent degradation of internalized $GABA_A$ receptors. Subsequently, it was shown that $GABA_A$ receptor ubiquitination is controlled by neuronal activity. Chronic blockade of neuronal activity by tetrodotoxin increases the level of $GABA_A$ receptor ubiquitination and increase in neuronal activity decreases $GABA_A$ receptor ubiquitination and improves insertion of these receptors into the plasma membrane (Saliba et al., 2007). $GABA_A$ receptors are heteropentameric proteins typically consisting of two α subunits, two β subunits, and one γ subunit. In the brain, the β subunits of the $GABA_A$ receptors are either $\beta2$ or $\beta3$ (Rudolph and Mohler, 2006). Ubiquitin is attached to the $\beta3$ subunit of the receptor. Activity blockade reduces the insertion of $\beta3$-containing $GABA_A$ wild-type receptor but not of the receptor containing mutant $\beta3$ that cannot be ubiquitinated (Saliba et al., 2007).

Ubiquitin–proteasome-mediated proteolysis also degrades several other proteins in addition to PSD-95 in the postsynaptic density including several structural proteins. For example, Shank, GKAP, and AKAP79/150 are degraded through the ubiquitin–proteasome pathway. Unlike for the degradation of PSD-95, physiological relevance of proteolytic removal of Shank, GKAP, and AKAP79/150 is not clear because the studies were correlative and a direct link between ubiquitin–proteasome-mediated degradation of the PSD proteins and structural remodeling was not established (Ehlers, 2003).

Proteolysis also controls a protein that regulates spine shape. SPAR controls dendritic spine shape by reorganizing the actin cytoskeleton. During activity-dependent remodeling of synapses, SPAR was shown to be degraded by the ubiquitin–proteasome pathway. Degradation of SPAR is stimulated by serum inducible kinase (SNK). Activity induces SNK mRNA in the cell body and the induced SNK is targeted to the dendritic spines. Because of the time required for SNK mRNA to travel to the spines, the conjecture is that SPAR may function to oppose synaptic remodeling after elevated activity (Pak and Sheng, 2003).

4.12.11 Local Proteolysis and Synaptic Plasticity

Previously I proposed a role for local ubiquitin–proteasome-mediated degradation in synaptic plasticity (Hegde, 2004). Many studies carried out since then provide support to this idea. Other researchers working in this field are embracing the idea of local degradation as well (Segref and Hoppe, 2009). Based on numerous lines of evidence, it seems that regulated local degradation of substrate proteins plays an important role in synaptic plasticity as well as many other aspects of the nervous system such as development and refinement of synaptic connections. Spatial restriction of degradation allows synapse-specific effects. Cell-wide degradation would have an impact on all synapses of a neuron (Hegde, 2004).

What is the mechanistic basis of local proteolysis in neurons? One possibility is to restrict the substrate to a subcellular location. For instance, proteins whose expression is largely restricted to the synapses could be locally degraded because all the requisite UPP components are present at the synapse. In addition, substrates can be made vulnerable (or resistant) to ubiquitination by phosphorylation, which can be locally controlled in neurons. Likewise, activation (or inactivation) of ubiquitin ligases by phosphorylation or other posttranslational modifications (such as attachment of UbL protein Nedd8 to Cul1 that activates SCF ligases; see **Fig. 2C**) can be locally controlled as well. Furthermore, specific E3 ligases can also be sequestered to specific subcellular compartments. Evidence from various experimental model systems has been obtained for some of these possibilities. Also, evidence that suggests that proteasome activity is differentially regulated in different subcellular compartments of neurons has been accumulating. A few examples of local roles of ubiquitination and local roles of the proteasome in neuronal compartments are discussed in the following section.

4.12.11.1 Local Roles of Ubiquitination and Deubiquitination

As described earlier, the specificity of ubiquitination is mostly controlled at the level of E3 ubiquitin ligases. The specificity of ubiquitination could also be regulated at the level of E2s because of diversity in E2s and unique E2–E3 combinations. Results from several studies provide evidence for local roles of E2s and E3s as well as for DUBs during development of synaptic connections.

4.12.11.1.1 E2s

An E2 called ubcD1 controls dendritic pruning in *Drosophila* neurons, where local degradation appears to be crucial. In this insect, most of the larval neurons die during metamorphosis but a subpopulation of neurons including a group of peripheral sensory neurons called the class IV dendritic arborization (C4da) neurons survive to adulthood (Kuo et al., 2005). These neurons extensively remodel their dendrites by completely degrading the old arborizations and by developing a new intricate set of dendrites. During remodeling of dendrites, axons are kept in their original form. Therefore, the molecular processes have to be spatially restricted. Experimental disruptions of ubiquitin–proteasome-mediated proteolysis by overexpression of an exogenous DUB called UBP2 from yeast or mutations in E1 or a 19S proteasome subunit all hindered dendritic pruning. Later studies identified the essential role of ubcD1 in this process (Kuo et al., 2005). Mutations in ubcD1 led to inhibition of dendritic pruning and retention of larval dendrites in C4da neurons. Based on other experiments it was concluded that ubcD1 targets DIAP1 (*Drosophila* inhibitor of apoptosis 1) an E3 ubiquitin ligase. DIAP1 is required for degradation of a caspase called Dronc. Therefore, degradation of

DIAP1 enables local activation of the Dronc caspase in dendrites. Because the Dronc caspase is critical for severing dendrites of C4da neurons, restricted dendritic activation of this caspase allows preservation C4da neurons while removing their dendrites (Kaneko-Oshikawa et al., 2005; Kuo et al., 2005).

4.12.11.1.2 E3s

In the *Drosophila* neuromuscular junction, number of synapses significantly increases with loss-of-function mutation in a gene called *highwire* (*hiw*). The *hiw* gene encodes an enormous protein with 5233 amino acids. Highwire protein contains a RING finger domain, which is a distinguishing feature of some ubiquitin ligases (Wan et al., 2000).

Later work carried out on the *C. elegans* homologue of *hiw* gene called RPM-1 found that the ligase functions to regulate presynaptic differentiation. RPM-1 protein is localized to the periactive zone, a presynaptic region excluded from the active zone and synaptic vesicles. RPM-1 combines with an F-box protein called FSN-1 and the *C. elegans* homologues of SKP1 and Cullin to form an SCF-like ubiquitin ligase complex. The spatially restricted function of this ubiquitin ligase in the periactive zone is critical for presynaptic differentiation in *C. elegans* (Liao et al., 2004). The downstream target of RPM-1 in *C. elegans* is an MAP kinase kinase kinase (MAPKKK) called DLK-1 (Delta-like homolog 1), which is also localized to the periactive zone like RPM-1. Inactivation of the DLK-1 pathway suppresses RPM-1 loss-of-function phenotypes, whereas overexpression of DLK-1 causes synaptic abnormalities similar to the ones caused by RPM-1 mutations (Nakata et al., 2005). In *Drosophila*, the downstream target of highwire is an MAPKKK encoded by a gene called *wallenda* (Collins et al., 2006). Although the downstream effectors of DLK-1 and wallenda proteins are different, reduction in the signaling mediated by these proteins inhibits synaptic growth in similar ways (Fulga and Van, 2008).

The function of the highwire protein in development of neuronal connections appears to be conserved through evolution. During discovery of new genes critical for axon navigation in mice, mutation in *Phr1*, a gene encoding a ubiquitin ligase, which is a vertebrate homologue of *highwire*, was identified. Studies using mice with a mutation in the *Phr1* gene (a mutation called *Magellan*) that lacks the C-terminal ligase domain showed that the Phr1 protein is localized to the axon shaft and no appreciable amounts of it are present in growth cones and distal processes. The substrate of Phr1 is most likely DLK in mice as well. Distribution of DLK does not overlap with that of Phr1; DLK is present in growth cones and not much is found in the axon shaft (Lewcock et al., 2007).

Results from subsequent studies indicate that RPM-1 may have additional roles in the postsynaptic compartment. It was found that RPM-1 regulates AMPA receptor trafficking in interneurons in *C. elegans*. RPM-1 works by negatively regulating the levels of DLK-1 (MAPKKK) (Park et al., 2009).

Local regulation of other E3 ligases has also been observed. For example, in hermaphrodite-specific motor neurons of *C. elegans*, an SCF ligase containing the protein SKR-1 and an F-box protein called SEL-10 mediates elimination of synapses during development. A synaptic adhesion molecule called SYG-1 binds to SKR-1 and inhibits the assembly of the SCF complex, which protects the neighboring synapses (Ding et al., 2007).

4.12.11.1.3 Deubiquitinating Enzymes

A substrate targeted for degradation by ubiquitination can be rescued by removal of the attached ubiquitin molecules by DUBs. Thus DUBs provide vital negative regulation of protein degradation. Similar to ubiquitin ligases, DUBs can act locally to reverse ubiquitination. In *Drosophila*, a screen for molecules that control the size and strength of synapses found that a DUB encoded by the *fat facets* (*faf*) gene is critical for synapse formation. Overexpression of *faf* in the developing nervous system causes synaptic overgrowth and disrupts synaptic transmission. Comparable phenotype is observed when a yeast DUB is expressed in *Drosophila* CNS (DiAntonio et al., 2001).

Mammalian DUBs also have a spatially limited synaptic role. A DUB called Usp14 is necessary for synaptic development and function in the mouse neuromuscular junction. The discovery on the role for Usp14 was originally made during studies on mice with the *ataxia* (ax^j) mutation, a recessive mutation characterized by severe tremors, hind limb paralysis, and postnatal lethality (Wilson et al., 2002). The ax^j gene encodes the Usp14 protein, which associates with the proteasome and is thought to help disassemble polyubiquitin chains and recycle ubiquitin (see **Fig. 1**), thus maintaining ubiquitin amounts in the cell. Therefore, loss of Usp14 results in reduced ubiquitin levels in many tissues of the ax^j mice including the brain (Anderson et al., 2005). Transgenic Usp14 rescues the motor defects of the ax^j mice and restores viability indicating that Usp14 shortage is the cause of neurological defects in these mice (Crimmins et al., 2006). Later studies showed that in Usp14-deficient ax^j mice ubiquitin loss occurred in spinal cord and sciatic nerve. Additional experiments showed that greatest loss of ubiquitin occurred in synaptosomal fractions, suggesting Usp14 at synaptic sites was crucial. Loss of Usp14 caused presynaptic defects such as nerve terminal sprouting and poor arborization of motor nerve terminals, and transgenic expression of Usp14 rescued these defects. Thus it appears that local Usp14 function is essential for maintaining ubiquitin levels and hence protein degradation at the synapse (Chen et al., 2009).

4.12.11.2 Local Role of the Proteasome in Synaptic Plasticity

Regulation of the proteasome allows additional control of proteolysis by the UPP. Although it was not previously appreciated, experimental results from the last several years indicate that proteasome is not homogenous throughout the neuron. Locally controlled proteasomal activity is likely to have an important role in synaptic plasticity in both invertebrates and vertebrates. For example, studies carried out on the mouse hippocampus showed that the proteasome has differential roles during induction and maintenance phases of the L-LTP (Dong et al., 2008), which is discussed in detail in the next section.

Results from other studies using cultured rat hippocampal neurons showed dynamic local regulation of the proteasome at the dendrites. It was found that proteasome is redistributed from dendritic shafts to synaptic spines in an NMDA receptor–dependent manner. How does the redistribution of the proteasome occur? The studies showed that activity only modestly increased the entry of the proteasome into dendritic shafts but significantly reduced their exit. Moreover, the data suggested that the proteasome was sequestered persistently in the spines through association with cytoskeleton (Bingol and Schuman, 2006). Later studies showed that a protein called NAC1, which is induced by psychostimulants, modulates the recruitment of the proteasome into the dendritic spines (Shen et al., 2007). Since the bulk of the evidence in this study is for the catalytic 20S core of the proteasome, it is not clear whether the recruitment of the full proteasome complex (26S) that degrades polyubiquitinated proteins is also regulated by NAC1. Another study has suggested that CaMKIIα subunit acts as a scaffold for the proteasome (Bingol et al., 2010). It is not clear how or if the functions of NAC1 and CaMKIIα relate to each other in sequestering the proteasome.

The proteasome might also function to locally regulate other processes required for synaptic plasticity such as translation of mRNA. For example, fragile X mental retardation protein (FMRP), which is believed to regulate translation of a subset of mRNAs in dendrites, is regulated by the proteasome. In addition, regulation of FMRP by the proteasome was shown to be critical for metabotropic glutamate receptor–dependent LTD (Hou et al., 2006).

4.12.11.2.1 Dissimilar Function of the Proteasome in Induction and Maintenance of L-LTP: Evidence for Opposing Local Roles in Dendrites and the Nucleus

Support for functional significance of local roles of the proteasome came from studies on hippocampal late phase LTP (L-LTP). Studies by Dong et al. (2008) showed that the application of a proteasome inhibitor to hippocampal slices prior to induction of L-LTP caused an increase in the magnitude of the early, induction phase but an inhibition of the late, maintenance phase. What mechanisms underlie these differential effects of the proteasome on phases of L-LTP? The enhancement of the early, induction phase (henceforth, the early part of L-LTP is referred to as Ep-L-LTP) by the proteasome inhibitor β-lactone is blocked by prior application of the translation inhibitor anisomycin but not by a transcription inhibitor actinomycin D. The increase in Ep-L-LTP caused by β-lactone is also prevented by prior application of rapamycin, which blocks signaling that controls translation of a subset of mRNAs (Gingras et al., 2001). Furthermore, Ep-L-LTP is augmented in dendrites isolated from the cell body by means of a surgical cut. These data indicate that proteasome inhibition enhances Ep-L-LTP by stabilizing proteins locally translated from preexisting mRNAs (Dong et al., 2008; **Fig. 4** top).

How does proteasome inhibition block maintenance of L-LTP? The proteasome inhibitor β-lactone hinders maintenance of L-LTP only if applied prior to induction of L-LTP but not if applied 2 h after induction of L-LTP. Previous studies by others have established that the critical time window for transcription required for maintenance of L-LTP is 2 h (Nguyen et al., 1994). These data suggest that proteasome inhibition blocks maintenance of L-LTP by inhibiting transcription. Evidence from molecular experiments supports this idea. Treatment of hippocampal slices with β-lactone significantly reduced induction of *Bdnf* (*brain-derived neurotrophic factor*) mRNA by chemically induced LTP (cLTP) or L-LTP induced by a theta-burst protocol (Dong et al., 2008). *Bdnf* is a CREB-inducible gene linked to maintenance of L-LTP (Barco et al., 2005).

What is the basis of transcription blockade caused by inhibition of the proteasome? One possibility is that normally the UPP aids the degradation of transcription repressors. Therefore, proteasome inhibition would result in accumulation of these repressors thus blocking transcription. In support of this notion, it was found that a CREB repressor ATF4 is degraded by the UPP during cLTP and that β-lactone application to hippocampal slices prevents degradation of ATF4. In addition, ATF4-ubiquitin conjugates accumulate during cLTP when proteasome is inhibited (Dong et al., 2008; **Fig. 4** bottom).

These investigations have also discovered the changing role of the proteasome even in dendrites through progression of L-LTP. Application of β-lactone to isolated dendrites also blocks maintenance of the dendritic L-LTP (Dong et al., 2008). Under these conditions, there is no supply of newly transcribed mRNA from the cell body. Therefore, blockade of transcription by proteasome inhibition does not explain this phenomenon. The most likely possibility is that proteasome inhibition leads to a slow accumulation of translation repressors in dendrites. Buildup of translation repressors would also occur in the cell body, which would hinder translation of newly transcribed mRNAs. Thus late stages of translation in both dendrites and the cell body would be blocked by stabilization of translation repressors by proteasome inhibition. In support of this idea, confocal microscopy experiments at various time points after L-LTP induction showed that early during L-LTP proteasome inhibition causes accumulation of translational activators eukaryotic initiation factor 4E (eIF4E) and eukaryotic elongation factor 1A (eEF1A) (Dong et al., 2014). At later stages of L-LTP, translational repressors such as polyadenylate-binding protein interacting protein 2 (Paip2) and eukaryotic initiation factor 4E-binding protein 2 (4E-BP2) build up in response to proteasome inhibition (Dong et al., 2014). Other negative regulators of translational repressors such as Mov10 might be stabilized by proteasome inhibition as well. For example, in cultured hippocampal neurons Mov10, which inhibits translation of key plasticity-related mRNAs such as that of *CaMKIIα*, is degraded by the proteasome in an NMDA- and activity-dependent manner (Banerjee et al., 2009).

Although two other studies have tested proteasome inhibition on LTP, neither study reported differential function of proteasome on LTP (Fonseca et al., 2006; Karpova et al., 2006). Both of these studies only showed inhibition of LTP with proteasome inhibitor. The study by Karpova et al. (2006) used MG132, which is not a highly specific proteasome inhibitor. MG132 has been known to strongly inhibit other proteases such as calpain and cathepsin B (Chain et al., 1999; Tang and Leppla, 1999). Because calpain inhibition is known to block LTP (del et al., 1990; Denny et al., 1990), the results of experiments that use only MG132 are not strictly attributable to proteasome inhibition. In the study Fonseca et al. (2006), the most likely reason the authors failed to discover the enhancement of the early phase of LTP with proteasome inhibition is because they used nanomolar concentrations

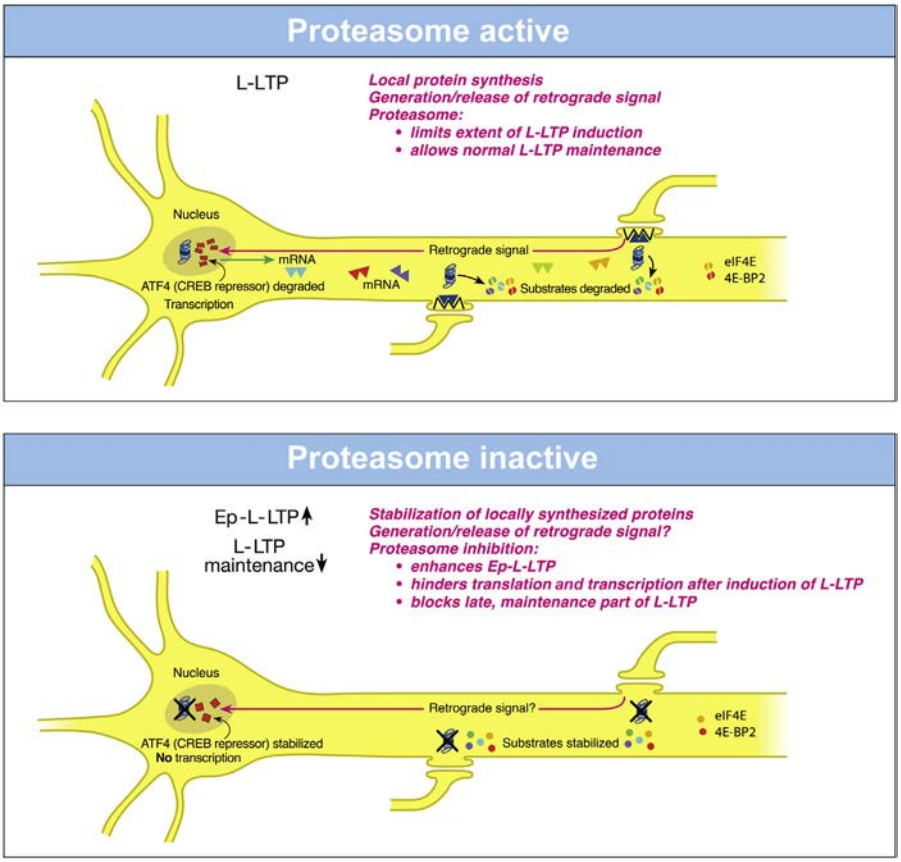

Figure 4 Dissimilar local roles of the proteasome in dendrites and in the nucleus during L-LTP. (Top) *Proteasome active*: The proteasome in dendrites is highly active, translational activators such as eIF4E are degraded (*broken green spheres*), and protein substrates that positively regulate L-LTP are degraded (*broken spheres*). Therefore extent of L-LTP is limited and only normal L-LTP ensues. A retrograde signal is likely transmitted to the nucleus. Proteasome aids transcription of genes by degrading the CREB repressor ATF4 (*broken squares* in the nucleus), thus allowing for normal L-LTP maintenance. Transcribed mRNAs (*triangles*) travel to activated synapses. (Bottom) *Proteasome inactive*: When the proteasome is inhibited (indicated by X marks on the proteasome), translational activators are stabilized (*intact green spheres*), leading to increased protein synthesis in dendrites. Also the newly synthesized proteins in dendrites are stabilized (*intact spheres*) and L-LTP-inducing stimulation protocols dramatically increase (*upward arrow*) the early part of L-LTP (Ep-L-LTP). Proteasome inhibition obstructs CREB-mediated transcription by preventing the degradation of transcription repressor ATF4 (*intact squares* in the nucleus). Proteasome inhibition could also inhibit the generation of the retrograde signal. Therefore, L-LTP is not maintained but it decays (*downward arrow*). Proteasome inhibition also causes failure of sustained translation because of stabilization of translation repressors such as 4E-BP (*intact red spheres*), which accumulate after induction of L-LTP, thus contributing to blockade of L-LTP maintenance. Adapted from Hegde, A.N., Haynes, K.A., Bach, S.V., Beckelman, B.C., 2014. Local ubiquitin-proteasome-mediated proteolysis and long-term synaptic plasticity. Front. Mol. Neurosci. 7, 96.

of lactacystin and epoxomycin. Numerous studies, including the one cited in the aforementioned study (Dick et al., 1996), have established that micromolar concentration of lactacystin is required for efficient proteasome inhibition. All three catalytic activities of the proteasome are inhibited only with 100 μmol/L lactacystin or 50 μmol/L β-lactone (Fenteany et al., 1995).

4.12.12 Ubiquitination and Endocytosis

As a result of earlier research on nonneuronal cells and recent studies on neurons, it has become clear that ubiquitination plays a vital role in targeting membrane proteins for endocytosis. Some general principles regarding the role of ubiquitin in endocytosis have emerged. A membrane protein, say, a neurotransmitter receptor, becomes a target for endocytosis when it is ubiquitinated. Unlike the polyubiquitin chain that marks substrate for proteasome-mediated degradation, endocytosis appears to be mainly mediated by monoubiquitination. The ubiquitinated receptor binds to adaptor proteins called epsins that couple the receptor to the clathrin-coated pits (Carbone et al., 1997). The ubiquitinated receptor then undergoes endocytosis and is incorporated into endosomes, which in turn are sequestered into the MVB. The membrane of the MVBs becomes continuous, with lysosomes leading to degradation of the receptor. It has been shown that targeting the internalized receptor to the MVB also requires ubiquitination. Moreover, a 350-kDa complex called endosomal sorting complex that is required for transport (ESCRT-I) and that recognizes

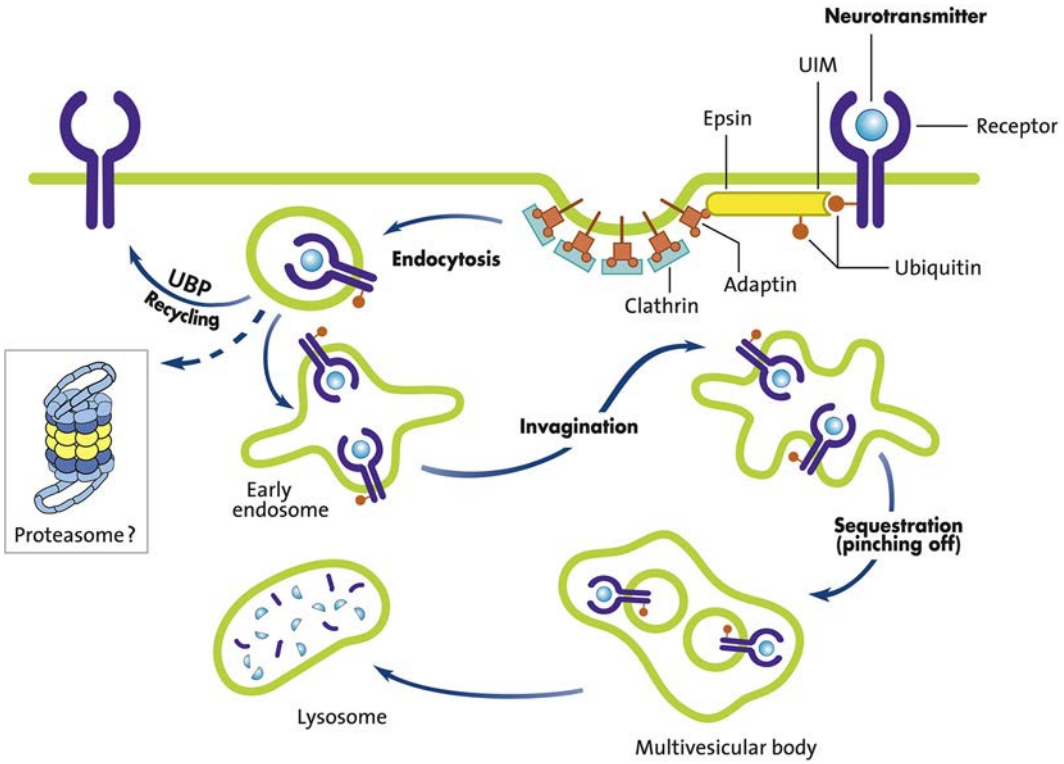

Figure 5 Ubiquitin and endocytosis. Receptors on the plasma membrane undergo monoubiquitination as a result of ligand (e.g., neurotransmitter). Ubiquitinated receptors bind to proteins called epsins, which in turn interact with adaptor proteins (adaptin) bound to clathrin-coated pits. Ubiquitination also functions to sort the internalized membrane protein into early endosomes, which directs them to degradation by lysosome via the multivesicular body. If ubiquitin from the endocytosed receptors is removed by a ubiquitin-specific protease (UBP), the receptor recycles back to the membrane. Proteasome inhibitors block endocytotic degradation of some proteins such as glutamate receptor subunits, indicating a possible role for the proteasome. *UIM*, ubiquitin-interacting motif.

the ubiquitinated receptors has been identified. Function of ESCRT-I is essential for sorting the endocytosed receptor into the MVB (Fig. 5; Katzmann et al., 2001). Two other complexes, ESCRT-II and ESCRT-III, are thought to be necessary for the continued sorting into the MVB (Babst et al., 2002a,b). If the ubiquitin from the endocytosed receptor is removed, the receptor recycles back to the plasma membrane. Although deubiquitination of endocytosed receptors is less well studied, UBPs are likely to carry out the ubiquitin removal function (Fig. 5).

4.12.12.1 Endocytosis and Synaptic Function

Internalization through ubiquitin-mediated signaling and sorting into the MVB is likely to play a critical role in controlling the neurotransmitter receptor number on the plasma membrane (hence synaptic function) in the nervous system. In support of this idea, Burbea et al. (2002) found in *C. elegans* that GLR-1, a homologue of the mammalian GluR1, which is part of the AMPA-type glutamate receptor, is internalized through a ubiquitin-mediated mechanism. GLR-1 was found to be ubiquitinated in vivo. When ubiquitin was overexpressed, the quantity of GLR-1 on the neuronal surface was reduced. The effect of ubiquitin overexpression was blocked by mutations in the unc-11 gene, which encodes a clathrin adaptin protein (AP180). Mutation of a specific lysine residue to arginine in the cytoplasmic tail of GLR-1 reduced internalization of GLR-1. All these results together suggest that GLR-1 is endocytosed through a ubiquitin- and clathrin-dependent mechanism. Furthermore, mutation of the lysine residues that prevents ubiquitination of GLR-1 affected locomotion in *C. elegans*. These observations indicate that prevention of ubiquitin-mediated GLR-1 endocytosis leads to an increase in synaptic strength resulting from a higher number of GLR-1 on the neuronal surface. Since the cytoplasmic tails of four mammalian AMPA receptor subunits (GluR1–GluR4) and *C. elegans* GLR-1 have a stretch of 16 conserved amino acids homologous to the region in the yeast Ste2p and Ste6p proteins that have been shown to be signals for ubiquitination and endocytosis, it is highly likely that GluR subunits in other species are endocytosed through a ubiquitin-dependent mechanism as well. Moreover, it has been found that internalization of GluR1 and GluR2 subunits of the AMPA receptor is inhibited by introduction of ubiquitin chain elongation mutant (Lys-48 mutated to Arg-48) (Patrick et al., 2003). Intriguingly, it was observed that a proteasome inhibitor, MG132, reduced internalization of AMPA receptors. One would expect the ubiquitinated GluRs to be either recycled back to the plasma membrane or routed to the lysosome for degradation. Involvement of the proteasome, if proven, would

add a new twist to the process of endocytosis. A similar effect of MG132 on degradation of internalized vasopressin receptors (Martin et al., 2003) as well as the quantity of GABA receptors (Bedford et al., 2001) has been reported (see the following text for description). Other neurotransmitter receptors have been shown to be internalized through a ubiquitin-dependent process as well. Using the *Xenopus* oocyte expression system, Buttner et al. (2001) demonstrated that inhibitory glycine receptors are ubiquitinated at the plasma membrane and internalized. The internalization process generates fragments of 35 and 13 kDa. In their experiments, application of concanamycin, which blocks acidification of lysosomal and endosomal compartments by inhibiting vesicular H^+-ATPases, prevents cleavage, whereas the proteasome inhibitor lactacystin has no effect on generation of the smaller fragments of glycine receptor. The GABAA receptor number on the neuronal membrane appears to be determined by a ubiquitin-mediated process as well. Evidence for a possible role of ubiquitin in GABAA receptor internalization is indirect. The GABAA receptor interacts with the UbL protein Plic-1, which stabilizes the GABAA receptor. Application of the proteasome inhibitor lactacystin leads to a significant increase in the steady-state levels of a1 and b3 subunits of the GABAA receptor (Bedford et al., 2001). The inference drawn with respect to GABAA internalization is that association with Plic-1 prevents the receptor from being ubiquitinated and routed to either the lysosome or the proteasome for degradation. It is not clear what roles proteasome and lysosome play in GABAA receptor degradation (Luscher and Keller, 2001). The Plic-1 UbL domain contains a proteasome-interacting motif (Upadhya and Hegde, 2003). Since Plic-1 also binds the GABAA receptor a and b subunits through a different domain (ubiquitin-associated domain, which is different from UbL domain), it is possible that the UbL domain in Plic-1 is utilized for routing the internalized GABAA receptor subunits for degradation through the proteasome.

Ubiquitination of plasma membrane receptors is likely to have a widespread role in the brain. Recently, mammalian G protein–coupled receptors (GPCRs) have been shown to be endocytosed through a ubiquitin-mediated mechanism. Two receptors, the β2-adrenergic receptor (β2-AR) and the V2-type vasopressin receptor (V2-VR), have been studied in detail (Martin et al., 2003; Shenoy et al., 2001). The endocytosis of GPCRs mediated by the ubiquitin signal differs from the endocytosis of other receptors in that an adaptor protein called β-arrestin plays a role. β-arrestin is ubiquitinated as well. The endocytosed β2-AR is routed to the lysosome for degradation if the β-arrestin is deubiquitinated or dissociated from β2-AR. If β-arrestin remains ubiquitinated, β2-AR recycles back to the plasma membrane. In contrast to the β2-AR example, endocytosis of V2-VR typifies the regulated ubiquitin-mediated endocytotic removal and destruction of the receptor, which effectively reduces the receptor number on the plasma membrane. In response to simulation by the agonist, arginine-vasopressin V2-VR is internalized. Agonist stimulation induces ubiquitination of V2-VR as well as ubiquitination of β-arrestin2. Ubiquitination of β-arrestin2 as well as ubiquitination of V2-VR is persistent. Unlike other receptors, V2-VR appears to be polyubiquitinated. How is endocytosed V2-VR degraded? Is it routed to the lysosome? Although earlier studies showed that V2-VRs were delivered to the lysosome after internalization, it was found that ubiquitinated V2-VR could only be detected in the presence of a proteasome inhibitor, MG132 (Martin et al., 2003), thus suggesting that the ubiquitinated V2-VR was degraded by the proteasome. Is it possible, however, that both the proteasome and lysosome have a role in degradation of V2-VR? Requirement for both proteasome- and lysosome-mediated degradation has been reported for other plasma membrane receptors such as insulin-like growth factor receptor (Vecchione et al., 2003), estrogen receptor (Nawaz et al., 1999), and growth hormone receptor (van et al., 2000).

4.12.12.2 Fate of Proteins After Endocytosis: Lysosomal Versus Proteasomal Degradation

The traditional view of endocytotic degradation is that the degradation occurs in the lysosome. Also, as discussed, ubiquitin appears to target the endocytosed proteins to the MVB for eventual degradation in the lysosome. A role for the proteasome is also indicated based on several studies. For example, endocytotic degradation of interleukin-2 receptor complex (Yu and Malek, 2001), growth hormone receptor (van et al., 2000), δ opioid receptor (Chaturvedi et al., 2001), vasopressin receptor (Martin et al., 2003), and GABAA receptor subunits (Bedford et al., 2001) is blocked by proteasome inhibitors. In addition, proteasome inhibitors have been shown to block endosomal sorting of membrane proteins to the lysosome (van et al., 2001). There is a caveat to these studies, however. Prolonged application of proteasome inhibitors leads to depletion of the ubiquitin pool (Patrick et al., 2003). Therefore, proteasome inhibitors might have an adverse effect on ubiquitination of receptors and hence indirectly block lysosomal degradation of receptors. The proteasome might also degrade some proteins that otherwise block routing to the lysosome through the MVB. Since proteasome is known to act on retrotranslocated endoplasmic reticulum proteins, it might act on the proteins in the early endosome in a similar fashion. In support of this idea, some investigations suggest that proteasome inhibitors might have a global effect on intracellular trafficking (Rocca et al., 2001). Later studies have shown that proteasome through an adaptor protein Ecm9 associates with a subpopulation of endosomes possibly to degrade endocytosed signaling proteins facing the cytoplasmic side (Gorbea et al., 2010). Other investigations have added another layer to the already complicated story of endocytic sorting. It has been found that ubiquitin also has a role in "selective autophagy," which specifically degrades protein aggregates and cellular structures (as opposed to nonspecific sequestration of cytosolic material). Autophagy of protein aggregates requires ubiquitin-binding receptors p62 and NBR1, which attach to ubiquitin as well as autophagosome-associated UbL proteins such as LC3 and help dock aggregated proteins to autophagosomes and enable their degradation (Kirkin et al., 2009; Wooten et al., 2008).

What determines whether the endocytosed substrate is targeted to the lysosome through the MVB or to the lysosome via autophagasome or degraded by the proteasome? Particular ubiquitin linkages (such as Lys-63 linked ubiquitin) in combination with the specific proteins that act as ubiquitin receptors are likely to sort the endocytosed ubiquitinated protein to its destination (Clague and Urbe, 2010).

4.12.13 Unanswered Questions and Future Directions

The research on the role of the ubiquitin–proteasome pathway in synaptic plasticity and other physiological functions in the nervous system has made much progress in the last two decades. Nonetheless, many details remain to be elucidated. For example, we have only rudimentary understanding of the spatial and temporal regulation of ubiquitin–proteasome-mediated degradation of substrates in the nervous system. It is likely that the local protein degradation is responsible for strengthening or weakening specific synapses (Hegde et al., 2014). To make further progress, precise manipulations in the nervous system of molecules critical for ubiquitin conjugation and proteasome-mediated degradation will be necessary. We can envisage many exciting future discoveries on how proteolysis helps sculpt and modify synapses.

See also: 4.02 A Comparative Analysis of the Molecular Mechanisms Contributing to Implicit and Explicit Memory Storage in *Aplysia* and in the Hippocampus. 4.03 Long-Term Potentiation: A Candidate Cellular Mechanism for Information Storage in the CNS. 4.22 Presynaptic Mechanisms of Plasticity and Memory in *Aplysia* and Other Learning-Related Experimental Systems. 4.23 Cellular and Molecular Mechanisms of Memory in Mollusks.

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Review

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The proteasome and epigenetics: zooming in on histone modifications

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Abstract: The proteasome is a structural complex of many proteins that degrades substrates marked by covalent linkage to ubiquitin. Many years of research has shown a role for ubiquitin-proteasome-mediated proteolysis in synaptic plasticity and memory mainly in degrading synaptic, cytoplasmic and nuclear proteins. Recent work indicates that the proteasome has wider proteolytic and non-proteolytic roles in processes such as histone modifications that affect synaptic plasticity and memory. In this review, we assess the evidence gathered from neuronal as well as non-neuronal cell types regarding the function of the proteasome in positive or negative regulation of post-translational modifications of histones, such as acetylation, methylation and ubiquitination. We discuss the critical roles of the proteasome in clearing excess histone proteins in various cellular contexts and the possible non-proteolytic functions in regulating transcription of target genes. In addition, we summarize the current literature on diverse chromatin-remodeling machineries, such as histone acetyltransferases, deacetylates, methyltransferases and demethylases, as targets for proteasomal degradation across experimental models. Lastly, we provide a perspective on how proteasomal regulation of histone modifications may modulate synaptic plasticity in the nervous system.

Keywords: epigenetics; histone modifications; proteasome; synaptic plasticity; ubiquitin.

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Introduction

The proteasome is a cellular complex that degrades proteins marked by covalent attachment to several molecules of ubiquitin. The linkage of ubiquitin to substrates is precisely regulated by an enzyme that mediates activation of ubiquitin (E1), enzymes that carry the activated ubiquitin (E2s) and enzymes that ligate it to the protein substrate (E3s). The proteolytic portion of the proteasome resides in its 20S (named thus because of its sedimentation coefficient) core to which two 19S regulatory caps (RCs) are attached. The 19S RCs remove the polyubiquitin tag, unfold the substrate protein and thread it through the narrow aperture of the 20S core for degradation into polypeptide fragments (Figure 1) (1).

Protein degradation by the ubiquitin-proteasome pathway (UPP) plays numerous roles in the nervous system. Originally, the role of the UPP was discovered in synaptic plasticity in the invertebrate *Aplysia californica* (2), a marine slug utilized for pioneering discoveries of molecular mechanisms underlying long-term facilitation (3). Subsequently, the role of the UPP was shown in late-phase long-term potentiation in the murine hippocampus, a well-studied model of synaptic plasticity in vertebrates (4, 5). Since then, numerous other investigations have shown that the proteasome is critical for memory formation. For example, proteasome inhibition in the hippocampus hinders consolidation of inhibitory avoidance memory, while blocking proteasome activity in the amygdala interferes with long-term fear memory (6, 7).

What are the mechanisms by which UPP contributes to synaptic plasticity and memory? Although the role of the UPP and the proteasome have been elucidated in regulating protein kinases, transcription factors, neurotransmitter receptors and other molecules critical for changing synaptic strength, much remains to be understood. Recent studies show that the proteasome modulates transcription by regulating epigenetic modifications of the N-terminal tails of histone proteins, such as histone acetylation, methylation and ubiquitination, which are critical for synaptic plasticity and memory.

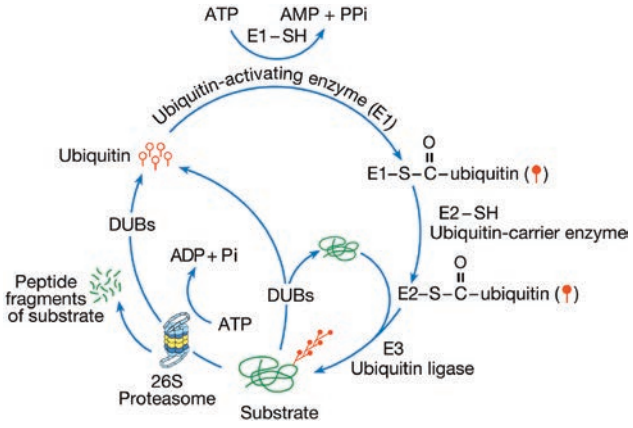

Figure 1: The ubiquitin-proteasome pathway.
In this pathway, ubiquitin (represented by open circles with straight tails) is selectively and covalently liked to the substrate. The enzymatic process of attaching ubiquitin to substrates (called ubiquitination or ubiquitin conjugation) depends on the action of three different classes of enzymes E1, E2 and E3. Initially, ubiquitin is activated by E1 to form a ubiquitin-AMP intermediate. Activated ubiquitin (closed circles with straight tails) is passed on to E2 (ubiquitin carrier enzymes). E2 transfers ubiquitin to an E3 (ubiquitin ligase) which ligates the activated ubiquitin to the substrate. A series of other ubiquitin molecules are attached to the substrate-linked ubiquitin and, thus, a polyubiquitin chain forms. The substrates marked with polyubiquitin tags are degraded by a proteolytic complex called the 26S proteasome in an ATP-dependent reaction. Ubiquitin is not degraded, but the polyubiquitin chain is disassembled and ubiquitin is recycled by deubiquitinating enzymes (DUBs). Prior to being committed for degradation by the proteasome, ubiquitination is reversible. DUBs can disassemble the polyubiquitin chain and prevent the degradation of the substrate.

Roles of the proteasome and ubiquitin

The proteasome is traditionally known to degrade proteins that are marked by attachment of ubiquitin. A single ubiquitin is covalently linked to the side chain of lysine (K) residues in the substrate. Once the first ubiquitin molecule is attached, a second ubiquitin molecule is linked to the K residue in the 48th amino acid sequence position of the first ubiquitin. This process is repeated several times and thus a polyubiquitin chain grows. The polyubiquitin chain is recognized by the proteasome for degradation.

Attachment of ubiquitin molecules to substrate proteins (ubiquitination) is a highly regulated step performed by three specialized enzymes (Figure 1), as explained above. The E1 is the least physiologically regulated enzyme, while the E2 is highly selective for its E3 binding partner and the E3 displays a high degree of substrate specificity. Although the kinetics of ubiquitination have not been extensively investigated, one study showed that transfer of the first ubiquitin by a ligase is the rate-limiting step (8). Genes encoding just one E1, dozens of E2s and hundreds of E3s have been identified in the mammalian genome (9, 10).

Substrates can also be posttranslationally modified with a single ubiquitin (monoubiquitination), or a single ubiquitin attached to several different lysine residues (multi-monoubiquitination). In addition, the type of polyubiquitin linkage determines how a substrate protein is degraded. Ubiquitin molecules covalently liked to each other through their 48th K residues mark a substrate protein for degradation by the proteasome, whereas ubiquitin linkage via K11 leads to endoplasmic reticulum-mediated substrate degradation (11) and linkage via K29 leads to endosomal substrate degradation (12). Monoubiquitination usually causes a conformational change within the protein and is not a signal for substrate degradation, but is associated with regulation of protein activity and protein-protein interactions (13). For example, a monoubiquitin tag attached to histones is an epigenetic modification that changes the landscape of chromatin and alters gene transcription (14, 15). In addition, attachment of a single ubiquitin to different K residues of a substrate protein marks a protein for endocytosis (16). Polyubiquitination via unusual ubiquitin linkage has also been described to regulate signaling pathways and kinase activity; however, its role is not very well understood (17–19). The ubiquitination process can be reversed by deubiquitinating enzymes (DUBs) (Figure 1) (9, 20).

Structure of the proteasome

To appreciate the proteolytic and non-proteolytic roles of the proteasome in histone modifications, it is instructive to describe the structure of the proteasome in detail. The proteasome is a large complex, the parts of which perform different functions not only in proteolysis but also in other functions such as histone modification. Originally, components of the proteasome were characterized by their sedimentation coefficient (21). The full complex is called the 26S proteasome and the catalytic core is termed the 20S proteasome. The catalytic core is a narrow cylinder to the either end of which two 19S RCs are attached. In eukaryotic cells, the 20S core is made up of two outer rings with seven α subunits (α1–α7) in each ring and two inner rings comprising seven β subunits (β1–β7). Three of the seven β subunits (β1, β2 and β5) are responsible for the catalytic activity of the proteasome. The catalytic sites

in these β subunits are located at their N-termini and are positioned inside the catalytic chamber, which has a narrow opening of 13Å in diameter (22). Therefore, only an unfolded substrate can pass through this opening. It is thought that the ATPases that are present in the base of the 19S RC provide the unfolding activity (23).

There are six ATPase subunits in the 19S RC, named regulatory particle ATPase 1–6 (Rpt1–6), which are collectively called the ATPases independent of 20S (APIS) complex. The 19S RC also contains four non-ATPase subunits regulatory particle non-ATPases 1, 2, 10 and 13 (Rpn1, Rpn2, Rpn10 and Rpn13). In addition, the 19S RC has a 'lid' which consists of only non-ATPase subunits (Rpn3, Rpn5, Rpn6–9, Rpn11, Rpn12, and Rpn15) (24, 25). Among the Rpn subunits, Rpn11 (also called Poh1) and Rpn13 (also called Uch37) are DUBs that are an integral part of the 19S RC. Rpn11 and Rpn13 assist in deubiquitination of the substrate as it is unfolded and threaded into the catalytic chamber of the 20S core. One DUB called Usp14 is known to stimulate substrate degradation through deubiquitination by reversibly associating with the Rpn1 subunit in the base (23, 26) and is known to be a critical regulator of long-term memory formation (27).

The 20S proteasome can exist without the 19RC attachment, in which case it cannot degrade ubiquitinated proteins (28). The 20S proteasome by itself possesses chymotrypsin-like, trypsin-like and postglutamyl peptidase activities that cleave after hydrophobic, basic, and acidic residues, respectively (25). The 19S RC recognizes the polyubiquitinated substrate, and its ATPases channel the substrate into the catalytic 20S core of the proteasome. The catalytic core then cleaves the ubiquitinated protein into small peptides. The peptides thus generated are likely to be later hydrolyzed to generate free amino acids by other proteases and amino peptidases (24, 25). The studies carried out so far indicate that the proteolytic activity of the 26S proteasome and the non-proteolytic activity of the 19S RC both have a role in histone modifications, as will be discussed later.

Histone posttranslational modifications (PTMs) and transcription

How do cells containing identical genetic makeup express different sets of genes and differentiate into various cell types with distinct structures and functions? In the past few decades, it has become clear that heritable genetic information is not limited to the DNA sequence and that other processes, which alter the structure of DNA-protein complexes (or chromatin), are crucial in guiding gene expression (29–31). Such dynamic chromatin alterations that determine the spatial and temporal sequence of gene expression in response to environmental factors are referred to as epigenetic modifications. Epigenetic mechanisms include PTMs of N-terminal tails of histone proteins, DNA methylation at cytosine residues, and noncoding RNAs, which collectively remodel chromatin and regulate gene expression. This review will focus on histone PTMs to examine their relationship with the proteasome and the UPP.

Histone proteins can be divided into two groups: replication-dependent (or canonical) and replication-independent (or variant histones) (32). Canonical histone proteins are encoded by a family of replication-dependent genes expressed rapidly during the S phase of the cell cycle. The genes that encode canonical histones are arranged in long clusters containing multiple copies of core histones (H2A, H2B, H3 and H4) and the linker histone H1. Replication-dependent histone mRNAs are the only known mRNAs in eukaryotes that lack a 3′ polyadenylated tail (33). Instead, they end in a 3′ stem-loop sequence that plays an important part in their regulation. On the other hand, replication-independent histone genes are expressed throughout the cell cycle and are polyadenylated. Some of the most commonly studied histone variants include the H3.3 and the H2A.Z, which are known to mark actively remodeled chromatin regions (34, 35).

In eukaryotic cells, DNA wraps around octamers of histones to form DNA-protein structural units called nucleosomes. Each nucleosome is composed of 147 bp of DNA wrapped around two molecules of each core histone (H2A, H2B, H3 and H4) with linker H1 histones occurring in between the nucleosomes (29). The tight packaging allows for space conservation needed to accommodate millions of base pairs of DNA into a small space of the nucleus. This tight packaging, however, restricts the accessibility of DNA by transcriptional machinery and serves as an additional regulatory step in the transcription process (29, 36–38).

The double-stranded DNA can assume two folding states based on how closely it is associated with histone-packaging proteins. In a heterochromatic state, strong DNA-protein interactions lead to tightly coiled chromatin that is transcriptionally inactive (Figure 2). When the DNA assumes its euchromatic state, the chemical interactions between DNA and proteins are weakened, producing loosely coiled chromatin that is transcriptionally active (Figure 2). These different packaging states depend largely

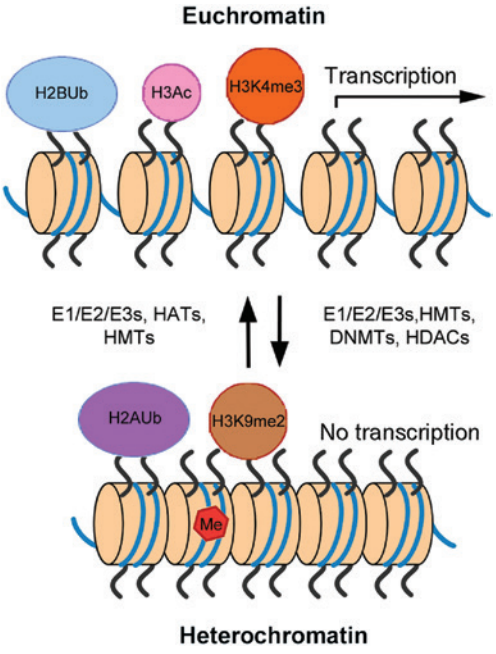

Figure 2: Histone modifications that regulate transcription. Top: In the open 'euchromatin' state, histone H2B is ubiquitinated, histone H3 is acetylated and tri-methylated on lysine 4. These modifications allow the transcription machinery to bind to the chromatin and genes are transcribed. Bottom: In the closed 'heterchromatin' state, histone H2A is ubiquitinated and histone H3 is dimethylated on lysine 9. DNA is methylated at cytosine residues. These modifications prevent the transcription machinery from binding the chromatin and transcription of genes does not occur. E1/E2/E3s: Enzymes that ubiquitinate H2B or H2A; HATs, histone acetyltransferases; HMTs, histone methyltransferases; DNMTs, DNA methyltransferases; HDACs, histone deacetylases.

on PTMs of histone N-terminal tails that protrude from the nucleosomes. Covalent PTMs of histone tails induce conformational changes within the chromatin, allowing it to adopt condensed or relaxed states that inhibit or stimulate transcription, respectively (29, 36, 39, 40) (Figure 2).

Most commonly studied histone PTMs are acetylation and methylation, both of which have been implicated in memory (41–43). During histone acetylation, an acetyl group from an acetyl-CoA molecule is transferred to a K residue within the histone tails. This transfer neutralizes a positively charged histone protein and weakens its affinity for the negatively charged DNA, promoting euchromatin formation (44). Histone acetyltransferase (HAT) enzymes catalyze histone acetylation and stimulate transcription, while histone deacetylase (HDAC) enzymes remove the acetyl groups from histone tails and repress transcription (44). Histone methylation occurs on both K and arginine (R) side-chain groups of H3 and H4 histone tails. Histone methylation is catalyzed by histone methyltransferase (HMT) enzymes and their removal is facilitated by histone demethylase (HDM) enzymes (37, 45, 46). K residues can be mono, di, and trimethylated whereas R residues can be mono and dimethylated. Depending on the methylation site and the number of methyl groups transferred, histone methylation can repress or stimulate transcription. For example, transcriptionally silent genes contain di and trimethylated histone H3 at lysine 9 (H3K9) whereas actively transcribed genes contain di and trimethylated histone H3 at lysine 4 (H3K4) (45, 47–49). On the other hand, R methylation serves for transcriptional activation only (36). A wide variety of HDMs, specific to certain methylation sites and numbers of methyl groups, have been identified (45).

During histone methylation, a methyl group is transferred from a high-energy donor, S-adenosyl methionine. Unlike histone acetylation, this transfer does not neutralize a positive charge on the histone protein and, therefore, does not necessarily cause direct conformational changes within nucleosomes. It is hypothesized that histone methylation can cause chromatin modifications through binding of effector proteins (36). A particular histone methylation code could serve as a binding surface for effector proteins that recruit other transcription cofactors to regulate transcription (36). For example, studies in yeast have demonstrated that methylation of H3K4 recruits the SAGA protein complex that possesses acetyltransferase enzymes. Therefore, methylation of H3K4 promotes histone acetylation and specific gene transcription (36, 50).

In addition to acetylation and methylation, less well-characterized PTMs of histones include phosphorylation and monoubiquitination. Histone phosphorylation occurs at serine and threonine residues and has been observed in numerous cellular processes such as transcription, mitosis, DNA repair and apoptosis (36, 37, 51). Histone phosphorylation facilitates both condensation and relaxation of chromatin; therefore, it can repress or induce transcription depending on the cellular context. One well-characterized histone phosphorylation event occurs on histone H3 at serine residues 10 and 28 (37, 52). This histone phosphorylation has been correlated to the activation of protein S6 kinase 2 (RSK2), extracellular signaling-regulated kinase (ERK) and mitogen-activated protein kinase 1 (MAPK1) signaling pathways in hippocampal-dependent memory (36, 37, 51, 53).

During histone monoubiquitination, one ubiquitin molecule is attached to the ε-amino group of a K residue. Histone monoubiquitination occurs at histones H2A, H2B and H3 (13, 36, 37). The addition of a large and bulky moiety, such as ubiquitin, to histone proteins leads to radical structural changes within the chromatin and, depending on the monoubiquitination site, recruits

different co-factors to facilitate up or downregulation of transcription. For example, monoubiquitination of histone H2A is associated with silenced gene expression (54), while monoubiquitination of histone H2B may recruit transcriptional co-activators and promote histone acetylation, methylation and gene transcription (55, 56).

Interestingly, transcription of replication-dependent histones themselves is also regulated by epigenetic histone modification. For instance, in yeast, H2B phosphorylation at tyrosine 37 occurs in a region of chromatin upstream of the histone gene cluster called *Hist1* (or *HIST1* in mammalian cells) and serves to inhibit transcription of multiple histone genes during the late S phase (57). In *Drosophila*, a molecular switch, called histone gene-specific epigenetic repressor in late S phase (HERS), binds to histone regulatory elements and blocks transcription of histone genes by inducing H3K9 methylation (58). In human mammary epithelial cells, a histone modifying enzyme, Pygopus 2, binds to promoters of histone genes and upregulates the acetylation of H3K56, previously associated with transcriptional activation (59). Moreover, the enzyme Set8, known to monomethylate H4K20 and repress histone gene expression during the late S phase of the cell cycle, is ubiquitinated and degraded by the proteasome, indicating the importance of the UPP in the regulation of histone gene expression and cell cycle regulation (60).

Many transcription factors and co-factors such as HATs, HDACs and HMTs are ubiquitinated and degraded by the proteasome (61). Moreover, histone proteins as well as their variants have been identified as targets of the UPP in different cellular contexts, ranging from DNA damage repair (62) to synaptic plasticity (34). Detailed investigations of the mechanisms behind how key regulatory proteins are targeted for proteasomal degradation in the nucleus to regulate transcription are still lacking. In addition to protein degradation, some studies suggest an alternative, non-proteolytic role of the UPP in the regulation of chromatin folding and transcription (56). In this review, we discuss key studies across different fields and model systems that describe a strong relationship between the UPP and histone modifications, specifically focusing on the nervous system.

Histone degradation by the proteasome

Several studies demonstrate that histone proteins themselves can be degraded by the proteasome, although the underlying mechanisms of this process are still unclear (63). *In vitro* analysis of histone H3 degradation by the UPP indicates that it may be independent of polyubiquitin chain formation or even E3 activity (64). Other *in vitro* studies report ATP- and ubiquitin-independent proteasomal degradation of histones, damaged by oxidative stress that occurs during antitumor chemotherapy (65). In K562 human hematopoietic cells, a nuclear proteasome-activating pathway that specifically targets oxidatively damaged histones has been identified (65, 66).

Later studies confirmed the ATP- and polyubiquitination-independent degradation of histones by the proteasome and identified that histone degradation is dependent upon histone acetylation (67). This mechanism was found to be important for DNA repair mechanisms in developing sperm (67). Qian and colleagues showed that the special type of proteasomes, containing the PA200 activating complex bound to the 20S particle that are predominant in sperm and, therefore, termed ‘spermatoproteasomes’, are inefficient at degrading polyubiquitinated proteins. PA200 binds acetylated core histone proteins and targets them for degradation by the proteasome. This process is promoted by DNA double-stranded breaks induced by γ-irradiation in yeast (67).

In addition to the PA200-dependent proteasomal degradation of histone proteins, some studies also describe polyubiquitination-dependent histone degradation by the UPP. Some argue that in order for new histone incorporation to be possible, old evicted histones must be degraded (63); however, the mechanistic details of that process are still poorly understood. Studies in the budding yeast, *Saccharomyces cerevisiae*, show the importance of tight regulation of excess histones by the UPP. In their yeast model, Singh and colleagues demonstrated that non-chromatin bound histones undergo phosphorylation, polyubiquitination and proteasome-dependent degradation (68). In addition, they identified specific E2s (Ubc4 and Ubc5) as well as E3s (Tom1, Pep5, Snt2, Hel1 and Hel2) associated with the ubiquitination of excess histones (68, 69). These studies highlight the importance of clearing excess histone proteins, as non-chromatin-bound histones are known to interfere with cell viability and promote cytotoxicity (70). The corresponding mechanism of histone clearing in the mammalian cells is not yet apparent.

One recent study found that histone variant H3.3 is polyubiquitinated and degraded by the proteasome in mouse embryonic neurons (34). In neurons and glial cells, the H3.3 variant has been found to rapidly accumulate with age, replacing most of the canonical H3 histone (34). H3.3 incorporation produced highly dynamic and

transcriptionally active chromatin in both humans and rodents (34). This histone turnover was identified to be essential for controlling cell type-specific gene expression as well as synaptic connectivity (71). In addition, efficient H3.3 incorporation and eviction from chromatin were dependent upon clearing of histones by the proteasome, as H3.3 turnover was significantly reduced by inhibition of the proteasome. Therefore, this suggests that active histone degradation regulates activity-dependent gene expression and may provide life-long synaptic and behavioral plasticity (34).

Another histone variant, H2A.Z, has been identified as a target for UPP-dependent degradation and has been examined in clinically relevant work. Studies in rat cardiac myocytes and prostate cancer cell lines identified an upregulated level of H2A.Z variant in both diseases (62, 72). Both studies suggest that H2A.Z is a target of the UPP and that a class III HDAC, sirtuin 1 (Sirt1), promotes deacetylation and subsequent degradation of H2A.Z in both disease models (62, 72). Such studies provide evidence for the therapeutic potential of compounds, which enhance HDAC activity (such as resveratrol), in combination with other chromatin-remodeling compounds that increase H2A.Z degradation by the proteasome. Alternatively, compounds aimed to upregulate proteasomal activity directly could be useful for the same purposes.

Recently, Zovkic and colleagues described an important role of H2A.Z in relation to cognitive function (35). H2A.Z was identified as a negative regulator of memory consolidation in the hippocampus and cortex, by showing that it is evicted from transcriptionally active chromatin in response to fear conditioning, a widely used behavioral paradigm of learning and memory. Even though the proteolytic degradation of the H2A.Z variant was not investigated in this learning context, the authors did suggest that the reduction of the H2A.Z protein after fear conditioning might be mediated by the UPP (73), therefore implicating the role of the UPP in chromatin remodeling necessary for memory consolidation.

Proteasome-dependent degradation of epigenetic remodeling machinery

Histone acetylation is intricately linked to proteasomal degradation of proteins in regulating gene expression. The proteasome has been found to degrade key regulatory proteins required for histone acetylation, such as HATs (74, 75) and HDACs (76). For example, the transcriptional co-activator with HAT activity, cyclic adenosine monophosphate (cAMP) response element-binding (CREB) binding protein (CBP), that is essential for cell proliferation and embryonic development is a known target for UPP-mediated degradation in specialized nuclear compartments, called the promyelocytic leukemia (PML) bodies (74, 75, 77). Moreover, CBP's homolog p300 displays nucleo-cytoplasmic shuttling and has been identified as a target of the UPP both in the cytoplasm and the nucleus, allowing for even more stringent regulation of its activity by the proteasome (78).

Emerging studies, describing many transcriptional co-factors that are targets of the UPP, are rapidly accumulating. Among such chromatin remodeling proteins that associate with HATs are mortality factor on human chromosome 4 (MORF4), a cellular senescence factor that regulates cell division (79, 80) and p300/CBP-associated factor (PCAF), a protein important for the transcriptional regulation of p53 and many other genes (77). Further investigation of the UPP-targeted transcriptional activating complexes will help decipher the mechanisms by which the proteasome regulates transcriptional activation in many cellular processes.

The UPP plays an equally important role in regulating transcriptional-silencing enzymes, such as HDACs and HMTs. One of the most fascinating examples of how the proteasome regulates histone deacetylation and gene expression is found in the immune system, as some viruses manipulate gene expression of the host through proteasomal degradation of HDACs. A recent study found that an HIV-1 accessory protein, Vpr, physically interacts with the class I HDACs, HDAC1, 2, 3 and 8, to direct them for proteasome-dependent degradation (76). This removal of HDACs facilitates histone hyperacetylation at the HIV-1 promoter and drives infection in primary macrophages (76).

Additional indirect evidence for the function of UPP-mediated proteolysis of transcriptional-silencing complexes has been obtained by the use of an HDAC inhibitor, valproic acid (VPA). Over the past three decades, VPA has been used for treatments of seizures (74), some cancers (81) and, more recently, has been described as a potent memory enhancer (82). VPA inhibits HDAC activity by either direct binding or by the stimulation of HDAC degradation by the UPP. Interestingly, the facilitation of UPP to degrade HDACs has been described as a selective process, specifically affecting HDAC2, but not other class I HDACs, suggesting a possible utility for targeted application of this drug (83). Moreover, HDAC2 has been shown to be subject to NEDD8 conjugation, or NEDDylation, which is a prerequisite for its degradation by the UPP (84). NEDDylation-activating enzymes have

been proposed as novel therapeutic targets for upregulating proteasomal activity (84).

Transcriptional-silencing histone mono and dimethyl transferases, such as G9a and GLPs, are targeted for proteasome-mediated degradation in response to DNA damage in human fibroblasts (85). A role for the proteasome has also been found in degrading HDMs associated with heterochromatin, such as JARD1C (86). Degradation of polyubiquitinated JARD1C/SMCX by the proteasome promotes H3K4 trimethylation and gene expression. The earlier studies were carried out in yeast and the observations on the UPP-mediated degradation of Jhd2 (the yeast counterpart of JARD1C) such as the requirement for the ligase Not4 hold true for human cells as well (86). Mutations in the human *SMCX* gene are linked to mental retardation (87–89) and therefore it is likely that the regulation of HDMs by the UPP plays a significant physiological role in the nervous system.

It seems counterintuitive that the UPP is responsible for the degradation of transcription-silencing histone modifiers, such as HDACs, HMTs and HDMs, as well as proteolysis of transcription-promoting histone modifiers such as HATs. It is highly likely, therefore, that the cellular context and signaling affects what molecules are to be degraded by the proteasome and, thus, determines the transcriptional outcome.

The 19S RC and histone modifications

The best described role of the 19S ATPases, outside of their function as a part of the proteasome, is in the transcriptional regulation of genes. Studies in yeast and cancer cells showed that ATPase subunits bind to promoters of active genes and physically interact with chromatin-remodeling transcriptional machinery (87, 90–95). The 19S ATPases, Rpt4 and Rpt6, are known to regulate epigenetic histone PTMs and control gene expression (96). These studies led to the hypothesis that ATPase subunits facilitate transcription independently of the 20S catalytic core. Other studies, however, found that both the 19S cap and the 20S core are recruited to active chromatin (97). The APIS complex and the 20S proteasomal subunits bind at promoters and gene bodies, independently of one another (98). Also, the entire 26S proteasome has been shown to co-immunoprecipitate with RNA polymerase II, supporting both the proteolytic and the non-proteolytic roles of the proteasome in transcriptional regulation (99).

The proteasome, histone modifications and synaptic plasticity

Numerous studies across various model systems have yielded a wealth of information supporting the role of the UPP in the regulation of synaptic plasticity, learning and memory (25, 100–103). Our previous studies showed that the maintenance of murine hippocampal late phase of long-term potentiation (L-LTP), that underlies long-term memory, is blocked by a specific proteasome inhibitor β-lactone (4, 104). We showed that proteasome inhibition with β-lactone stabilizes translational activators early, followed by translational repressors later in L-LTP, illustrating the proteasome's dual role in mediating signaling pathways in synaptic plasticity at the level of dendrites (104) (Figure 3). A recent study in cultured hippocampal neurons showed that only 20% of the proteasomes are engaged in substrate processing at baseline, leaving ample room for activity-dependent increase in protein degradation, upon sufficient environmental stimulation (105). Taken together, these studies suggest an activity-triggered system in which the proteasome regulates plasticity-related proteins to produce an appropriate synaptic response within the hippocampal neurons after synaptic stimulation.

The proteasome also plays a critical role in mediating gene expression in synaptic plasticity. In the nucleus, proteasome inhibition blocks gene expression induced by CREB, a transcription factor that is crucial for long-term synaptic plasticity and memory (106). Treatment of hippocampal slices with β-lactone prior to chemically induced LTP (cLTP) or electrically induced L-LTP with theta-burst protocol (TBP) blocks the upregulation of a CREB-inducible gene, *brain-derived neurotrophic factor* (*Bdnf*), necessary for the maintenance of L-LTP (4, 107). This observation was supported by the finding that a CREB repressor, activating transcription factor 4 (ATF4), is degraded by the proteasome during cLTP (4). Thus, proteasome inhibition causes a buildup of transcriptional repressors, such as ATF4, which blocks the upregulation of *Bdnf* and other plasticity-related genes, and blocks the maintenance of L-LTP (4). The connection between proteasome-dependent protein regulation in the dendrites and the nucleus during synaptic plasticity remains uncharacterized. Future studies addressing proteasome-mediated retrograde signaling in synaptic plasticity will perhaps provide some mechanistic details.

Furthermore, we identified a novel role of the proteasome in modulating transcription-favoring epigenetic

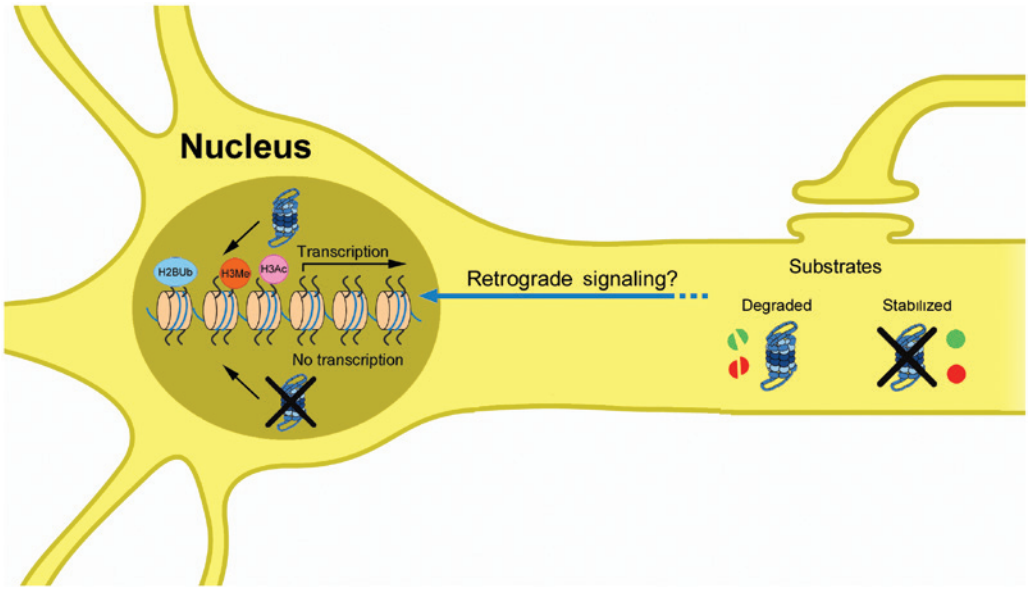

Figure 3: Histone-modifying roles of the proteasome in synaptic plasticity.
In the nucleus, when the proteasome is active (depicted at the top), proteasome facilitates histone acetylation, methylation and ubiquitination. When the proteasome is inactive (depicted with an X mark at the bottom), histone modifications are blocked. The proteasome might also have an indirect effect on histone modification through the regulation of protein degradation in or near dendrites (broken circles), which in turn is expected to affect retrograde signaling to the nucleus. When the proteasome is inactive and the substrates are stabilized (solid circles), retrograde signaling to the nucleus may not occur.

histone modifications, which are known to control gene transcription in synaptic plasticity, learning and memory. Our study demonstrated that the trimethylation of histone 3 at lysine 4 (H3K4me3), acetylation of histone H3 at lysines 9 and 14 (H3K9/14ac), and monoubiquitination of histone H2B at lysine 120 (H2BK120ub) are enhanced immediately after cLTP induction and their enhancement is blocked by β-lactone pretreatment (108) (Figure 3).

H3K4me3 and H3K9/14ac are transcription-favoring epigenetic tags that have been identified as critical regulators of learning and memory in behavioral rodent models (46, 109–112); however, the mechanisms behind their addition and removal are still poorly understood. Our study illustrated the dynamic nature of these modifications, since both H3K4me3 and H3K9/14ac were upregulated immediately after cLTP induction and returned back to baseline after 30 min of recovery. This histone remodeling time-course was surprisingly fast. Transient histone modifications have been studied in the timescale of hours or days after synaptic stimulation or behavioral training (113, 114). It is also known, however, that histone modifications can occur much more rapidly, in the timescale of minutes (115–117). It has been previously hypothesized that lasting cellular changes in synaptic plasticity can be triggered by a transient histone modification signal (37). Previous results in *Aplysia* show that transient acetylation of histone H3 is critical during long-term synaptic plasticity (118). Therefore, transient spikes of proteasome-dependent histone acetylation and methylation may be sufficient to trigger long-lasting upregulation of plasticity-related genes.

Furthermore, we investigated the role of the transcription-favoring H2BK120ub in synaptic plasticity. We demonstrated that H2BK120ub levels oscillate after the induction of cLTP, as an increase of monoubiquitination was observed immediately after cLTP induction and at 30 min after cLTP induction, but not at 15 min. This finding is consistent with previous studies of histone H2B monoubiquitination in yeast transcriptional regulation, where multiple rounds of histone ubiquitination and deubiquitination are required for transcription initiation and elongation, respectively (119–121). Histone H2B monoubiquitination has also been described as a precursor for other histone modifications (55, 95). If H2BK120ub is the first link in a chain of events that precede the initiation of transcription in synaptic plasticity, manipulating histone monoubiquitination at promoters of active genes may serve as a therapeutic target for memory impairments.

Collectively, these studies are in agreement with the idea that a combination of all epigenetic tags at promoters of genes, brought upon by environmental stimulation, control gene expression and modify behavior (109, 122). In the hippocampus, the regulation of chromatin structure through PTMs of histones may represent a ‘molecular

code' for long-term memory (37). This flexible 'molecular code' may mediate long-term physiological and behavioral changes by controlling the transcription of genes (37). In our work, we observed the dynamic nature of global histone modifications in cLTP, which suggests that the role of histone modifications in synaptic plasticity may be more complex than previously thought. It appears that the proteasome (and the UPP) has a role in regulating the process of histone modifications in synaptic plasticity underlying memory. Many key questions regarding the exact nature of the proteasome's role remain to be answered.

Numerous lines of evidence support the importance of both protein degradation by the proteasome and histone modifications in behavioral learning and memory models. Proteasome inhibition in the hippocampus and the amygdala is associated with an impaired consolidation of an inhibitory avoidance memory and long-term fear memory, respectively (6, 7). In addition, retrieval of either an auditory or a contextual fear memory results in an increase of the degradation-specific protein polyubiquitination in the amygdala (7). Epigenetic histone modifications, such as acetylation and methylation, are likely to be part of the molecular mechanisms necessary for persistent gene expression to support long-term memory formation and promote memory storage (109, 123, 124). Studies delineating how the two critical systems – the UPP and histone modification – may interact to drive memory formation *in vivo* are still lacking.

Future directions

In the past several years, parallel lines of research have found evidence for the roles of the UPP and histone modifications in synaptic plasticity and memory. It is only recently that the investigations on the UPP and epigenetics have begun to intersect. The main challenge for the future is to elucidate the mechanistic details on how different components of the UPP, the ubiquitin conjugating enzymes and the proteasome regulate histone modification. Given that the UPP can control both transcription-favoring and transcription-repressing types of histone modifications, it would be important to investigate the signaling pathways in neurons that control these opposing effects. There is also a necessity to understand the dynamic nature of some of the histone modifications (such as H2BK120ub) and how they relate to gene expression underlying synaptic plasticity and memory. Temporal regulation of histone PTMs is beginning to be understood in non-neuronal systems (125) and the same kind of in-depth analysis would be beneficial for understanding the physiological functions of the nervous system, including synaptic plasticity and memory.

Conclusion

In this review, we provided a brief summary on the current knowledge connecting two seemingly unrelated cellular processes, the UPP and epigenetic histone modifications, in multiple cell types and model systems. We discussed studies describing proteolytic degradation of canonical histones as well as their variants. We also described studies on proteolytic degradation of histone-remodeling machinery, such as HATs, HDACs, HMTs and HDMs, as well as transcriptional co-factors that form complexes with epigenetic remodeling enzymes. Although not very well described in the literature, we attempted to summarize the current knowledge of non-proteolytic roles of the proteasomal APIS complex in modulating transcription from yeast studies. Finally, we provided a perspective on proteasomal regulation of histone modifications in the nervous system by discussing studies on synaptic plasticity, learning and memory. As the relatively new and still developing field of neuroepigenetics continues to grow, we look forward to exciting future studies that incorporate the roles of the proteasome in modulating synaptic plasticity by regulating epigenetic modifications.

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List of abbreviations

ATF4	Activating transcription factor 4
ATP	adenosine triphosphate
APIS	ATPases independent of 20S
Bndf	Brain-derived neurotrophic factor
cAMP	cyclic adenosine monophosphate
CREB	cAMP response element-binding
cLTP	chemically induced long-term potentiation
CBP	CREB binding protein
DUB	deubiquitinating enzyme
E1	ubiquitine activating enzyme

E2	ubiqutine carrier enzyme
E3	ubiquitine ligase enzyme
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDM	histone demethylase
H2BK120ub	histone H2B monoubiquitinated at lysine 120
H3K9/14ac	histone H3 acetylated at lysine 9 and 14
HMT	histone methyltransferase
K	lysine
L-LTP	late phase of long-term potentiation
PTM	posttranslational modification
R	arginine
RC	regulatory cap
Rpt	regulatory particle ATPase
Rpn	regulatory particle non-ATPase
TBP	theta-burst protocol
H3K4me3	trimethylated histone H3 at lysine 4
UPP	ubiquitin-proteasome pathway
VPA	valproic acid

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Expression of the Longest *RGS4* Splice Variant in the Prefrontal Cortex Is Associated with Single Nucleotide Polymorphisms in Schizophrenia Patients

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The *Regulator of G protein signaling 4* (*RGS4*) gene is a candidate susceptibility gene for schizophrenia (SCZ). Previous studies showed that the mRNA level of the longest splice variant *RGS4-1* was decreased in the dorsolateral prefrontal cortex (DLPFC) of SCZ patients compared with healthy controls. In this pilot study, we examined the possible mechanisms of *RGS4-1* mRNA reduction in SCZ. We genotyped SNP1 (rs10917670), rs2661347, SNP4 (rs951436), SNP7 (rs951439), SNP18 (rs2661319), and rs10799897 (SNP9897) and tested the methylation status of CpG islands of the *RGS4* gene in the postmortem DLPFC samples obtained from subjects with SCZ and bipolar disorder as well as healthy controls. *RGS4-1* mRNA level was associated with five SNPs (SNP1, rs2661347, SNP4, SNP7, and SNP18) and their haplotypes but not with SNP9897. In addition, this study revealed that *RGS4-1* mRNA was low in subjects with specific genotypes of SNP1, rs2661347, SNP4, SNP7, and SNP18. Lower *RGS4-1* mRNA expression in the DLPFC of SCZ is associated with SNPs in the 5′ regulatory region of the *RGS4* gene but not with the methylation status of its CpG islands.

Keywords: genotype, bipolar disorder, psychiatric, allele, postmortem, regulatory region

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INTRODUCTION

The *Regulator of G protein signaling 4* (*RGS4*) gene is associated with susceptibility to schizophrenia (SCZ). A previous microarray study showed that *RGS4* mRNA levels were decreased in prefrontal cortex (PFC) of SCZ patients as compared to healthy subjects (1). The four common single-nucleotide polymorphisms (SNP1, 4, 7, and 18) of the *RGS4* gene, localized between the 5′ upstream sequence and the second intron, as well as two haplotypes derived from these SNPs may confer SCZ risk (2). Some other SNPs in the 20 kb genomic region of *RGS4* are associated with SCZ (2), and the G-allele of SNP1 is associated with non-deficit SCZ (3). Other linkage and association studies also indicated a connection between *RGS4* polymorphisms and SCZ risk (4–6). *RGS4* polymorphisms (SNP1, 4, 7, and 18) were associated with differences in the dorsolateral prefrontal cortex (DLPFC) volume in first episode SCZ patients (7). Several *RGS4* SNPs are associated with clinical symptoms of SCZ. For example, Positive and Negative Symptoms Scale (PANSS) total and global psychopathology scores were associated with SNP4 (8). SNP18 (rs2661319) and rs2842030 were associated

with more severe baseline PANSS total score (9). *A*t the baseline status, the A/A genotype at SNP7 of *RGS4* was associated with a poorer social function compared with the G/G genotype (10). SNP1 (rs10917670) was associated with the depression factor in SCZ (11).

We previously cloned five splice variants of *RGS4* mRNA expressed in the DLPFC of the human brain and three splice variants of *Rgs4* from the mouse brain and found that the transcriptional regulation of human *RGS4* differs from that of the mouse *Rgs4*. Our data showed that mouse *Rgs4* encodes one protein while human *RGS4* encodes four different proteins, three of which are unique to humans (12). We also found that the mRNA level of the longest isoform *RGS4-3* was specifically deceased in DLPFC of SCZ patients but not in healthy controls (NC) or subjects with bipolar disorder (BPD) (13). Since then, *RGS4-3* has been renamed *RGS4-1* (NM_001102445.2), and we use the new nomenclature here.

How is *RGS4-1* decreased in DLPFC of SCZ? One possibility is through epigenetic regulatory mechanisms such as DNA hypermethylation. Bioinfomatics analysis found CpG islands (GC% >50%, >200 bp and the ratio of observed/expected CpG >0.6) (14) spanning 246 bp located in the first intron and second exon of the *RGS4* gene. Hypermethylation of CpG islands leads to decrease in gene expression (15, 16), which might explain the reduction in *RGS4-1* mRNA level. To test whether the decrease in *RGS4-1* mRNA expression in the DLPFC of postmortem SCZ brains is due to epigenetic mechanisms, such as DNA hypermethylation, we investigated the methylation status of CpG islands of *RGS4.*

RGS4-1 might also be decreased through mechanisms in which the regulatory region of the *RGS4* gene plays a role. Such a possibility might be revealed by testing whether the SNPs in the regulatory region of the *RGS4* gene are associated with *RGS4* mRNA expression. Therefore, we genotyped a few SNPs in the regulatory region of the *RGS4* gene including SNP1 (rs10917670), rs2661347, SNP4 (rs951436), SNP7 (rs951439), SNP18 (rs2661319), and rs10799897 (SNP9897) to test for possible associations between the SNPs and *RGS4* mRNA expression. We present results showing that expression of *RGS4-1* is associated with five SNPs and that *RGS4-1* expression is not correlated with the methylation status of CpG islands in the *RGS4* gene.

MATERIALS AND METHODS

RNA and DNA Samples

RNA samples (RNA Array Collection) from the DLPFC of postmortem brains of the patients with SCZ, BPD, and normal controls (NC) ($N = 35$ in each group) were obtained from Stanly Medical Research Institute (SMRI) (see Tables S1 and S2 in Supplementary Material for demographic and other details), and mRNA expression levels in these samples were measured by quantitative real-time PCR (qPCR) (13). The corresponding DNA samples from the DLPFC and occipital cortex from the postmortem brains of these patients and NC were also obtained from SMRI.

The samples used in this study were all from post-mortem brain tissue, and therefore a protocol approved by the Institutional Review Board was not necessary as per institutional guidelines.

SNP Genotyping Assays and Haplotype Determination

We used pre-designed Taqman SNP genotyping assays for SNP18 (rs2661319) and SNP9897 from Applied Biosystems (AB). Custom Taqman SNP genotyping assays for SNP4 (rs951436) and SNP7 (rs951439) (Table S3 in Supplementary Material) were designed using AB web site.[1] We chose these SNPs because of their known association with SCZ. All the four SNPs were identified at the Genotyping Core of University of North Carolina (UNC) Chapel Hill. To verify the genotyping accuracy, 12 randomly selected samples were also genotyped again by Roche Light Cycler 480 using the same TaqMan assays. The two genotyping results completely match. Primers and probe sequences used for genotyping are shown in Table S4 in Supplementary Material. SNP1 (rs10917670) was identified by PCR and sequencing since it did not comply with the ABI custom Taqman assay design requirement. All analyses were carried out blind with respect to diagnosis. Haploview 4.2 (Broad Institute of MIT and Harvard)[2] was used to perform Linkage Disequilibrium (LD) analysis. Based on population genotype, the program PHASE[3] was used to reconstruct haplotype and assign diplotype for each individual and calculate haplotype frequency (17).

Bioinformatics Analysis, Primer Design, and DNA Methylation Analysis

To identify possible CpG islands in the regulatory region and introns of the *RGS4* gene, a 3.2-kb putative promoter region including the 5′ regulatory region upstream of the transcription start site (TSS), exon1, 2 and intron 1 of *RGS4* were searched, and a 246 bp region containing nine CpG islands was found. The 105 DNA samples from SMRI were treated with bisulfite, and then used for PCR amplification and PCR products sequencing (18). Briefly, 125 ng of DNA was treated by bisulfite using EZ DNA methylation kit (Zymo Research Corporation, Irvine, CA, USA). Human Methylated and Non-Methylated DNA Set (Human HCT116 DKO Methylated and non-methylated DNAs) were used as positive and negative controls (Zymo Research Corporation). The bisulfite PCR primers for DNA methylation analysis of RGS4 were designed by using MethPrimer.[4]

The primers were: *RGS4*-BSP_fd 5′ TAGAGGGAGATAGA GGAGTTGGTATT 3′ and RGS4-BSP_rev 5′ ACAAACCTACAA ACCCTTTACACAT3′. ZymoTaq™ DNA Polymerase was used to amplify bisulfite-treated DNA.

Statistical Analysis

SAS software 9.2 (SAS Institute Inc., Cary, NC, USA) was used to perform statistical analysis. Pearson's Correlation analysis was performed to identify possible correlations between the

[1] https://www5.appliedbiosystems.com/tools/cadt/

[2] http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview

[3] http://stephenslab.uchicago.edu/software.html

[4] http://www.urogene.org/

mRNA expression levels of two different splice variants. The association analysis of SNPs with SCZ or BPD was performed by using Haploview and PROC LOGISTIC regression analysis in the SAS software. The general linear model was used to analyze the relationship of the mRNA expression level with SNPs by using SAS software. When analyzing the association of genotypes with mRNA expression level of *RGS4* splice variants, an additive model was used, in which the phenotype of heterozygotes is intermediate to those of the two homozygotes. Previously, we found that the mRNA expression level of *RGS4-1* was significantly decreased in the DLPFC of SCZ compared to normal controls and was correlated with brain weight, and mRNA expression level of *RGS4-2* was correlated with brain pH (13). We did not, however, find a correlation between mRNA expression of *RGS4* isoforms and RNA integrity number (RIN), age, gender, postmortem interval, race, refrigerator interval, age of onset and duration of illness, lifetime use of alcohol, or antipsychotics. Therefore, to compare the effect of genotype or diplotype on mRNA level of *RGS4-1* in among all groups (including SCZ, NC, and BP groups), univariate analysis of covariance (ANCOVA) was performed, in which mRNA expression of *RGS4-1* or *RGS4-2* was a dependent variable, while genotype or diplotype was used as an independent factor and brain weight or brain pH as a covariate. To detect a possible interaction between SNP genotype and groups, SNP genotype and group were taken as two factors, and brain weight as a covariate.

When analyzing the relationship of SNPs with mRNA expression level of *RGS4-1* between groups, group (SCZ, BPD, or NC) was an independent variable, brain weight was a covariate, and *post hoc* test was used to identify the differences in *RGS4-1* mRNA levels between different groups. To determine the possible differences in the ratio between two splice variants among groups, general linear model was used, with the ratio as a dependent factor and the group as independent variable. To test whether the SNPs were related to ratios, the ratio was taken as a dependent variable and the genotype of SNPs and group were two factorial independent variables. Then *post hoc* Bonferroni test was applied.

RESULTS

Association of mRNA Expression of RGS4 Splice Variants with RGS4 SNPs

Although we successfully genotyped six SNPs in the 5′ regulatory region and introns of *RGS4* in all samples from SMRI (see Table S1 in Supplementary Material for demographics), some RNA samples had to be excluded because of insufficient quantity and other limitations (reasons for which are detailed under Table S2 in Supplementary Material). Therefore, statistical analysis of the association between SNPs and the mRNA expression levels of *RGS4* splice variants was performed on samples from 27 individuals in each group (13). All DNA samples were from Caucasian subjects except for one from a Hispanic subject in the SCZ group and another from a Native American subject in the BPD group. Regardless of whether these two cases were included or not in the analysis, the statistical outcome remained the same. The mRNA expression levels of the isoforms *RGS4-2* to *RGS4-5* were not associated ($p > 0.05$) with the six SNPs of the *RGS4* gene that we examined. However, the mRNA expression of the longest isoform *RGS4*-1 was associated with the five SNPs (SNP1, rs2661347, SNP4, SNP7 and SNP18, $p < 0.05$) but not with SNP9897 ($p > 0.05$).

When the three groups were considered together including all samples from SCZ, normal controls (NC), and BPD, *RGS4-1* mRNA expression level was associated with the genotype of SNP1 [$F(3,77) = 4.20$, overall $p = 0.0083$, SNP1 genotype $p = 0.0263$] and rs2661347 [$F(3,77) = 4.69$, overall $p = 0.0046$, rs2661347 genotype $p = 0.0141$]. The mRNA expression levels of *RGS4-1* in the carriers with homozygous genotype TT of SNP1 or with allele T of SNP1 were lower than that from subjects with genotype CC (adjusted $p = 0.0220$) or allele C of SNP1 (adjusted $p = 0.0338$). The genotype TT of rs2661347 or allele T of rs2661347 was associated with lower amounts of *RGS4-1* transcript compared with genotype AA of rs2661347 ($p = 0.0117$) (**Figure 2**) or allele A ($p = 0.0106$). *RGS4-1* mRNA expression level was also associated with the genotype of SNP4 [$F(3,77) = 4.93$, overall $p = 0.0035$, SNP4 genotype $p = 0.0104$], SNP7 [$F(3,77) = 4.20$,

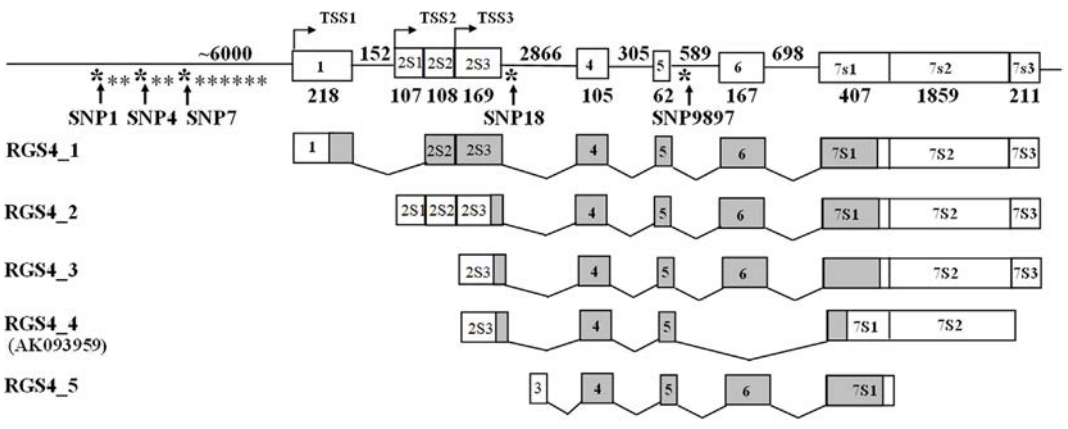

FIGURE 1 | Location of SNPs in the *RGS4* gene. SNP1, SNP4, and SNP7 are located in the 5′ regulatory region of *RGS4*. SNP18 is in the second intron and SNP9897 is in the fourth intron of the *RGS4* gene.

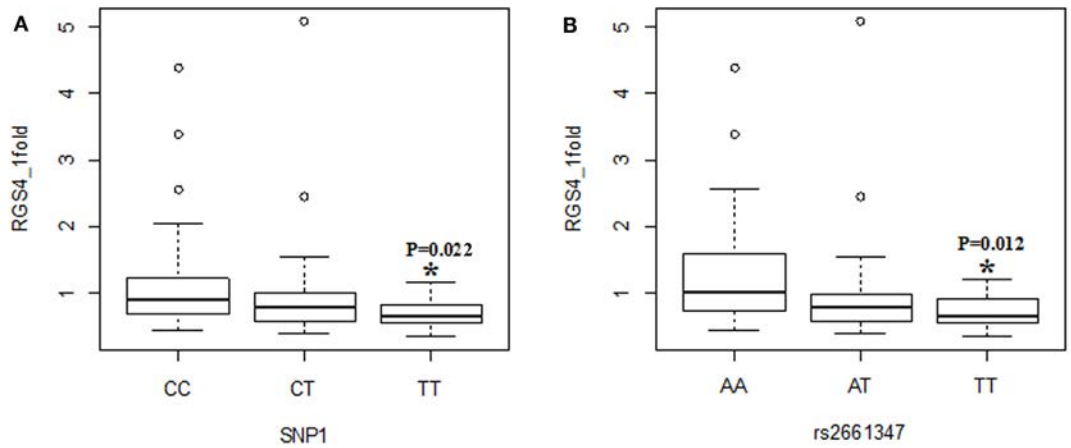

FIGURE 2 | **Effects of SNP1 and rs2661347 on *RGS4-1* mRNA expression levels**. *RGS4-1* mRNA expression level in genotype TT of SNP1 was significantly lower than that of the genotype CC [$F(3,77) = 4.20$, overall $p = 0.0083$, *SNP1 genotype* $p = 0.0263$] **(A)**. The genotype TT of rs2661347 had significantly lower expression level of *RGS4-1* mRNA compared to the genotype AA [$F(3,77) = 4.69$, overall $p = 0.0046$, rs2661347 genotype $p = 0.0141$] **(B)**.

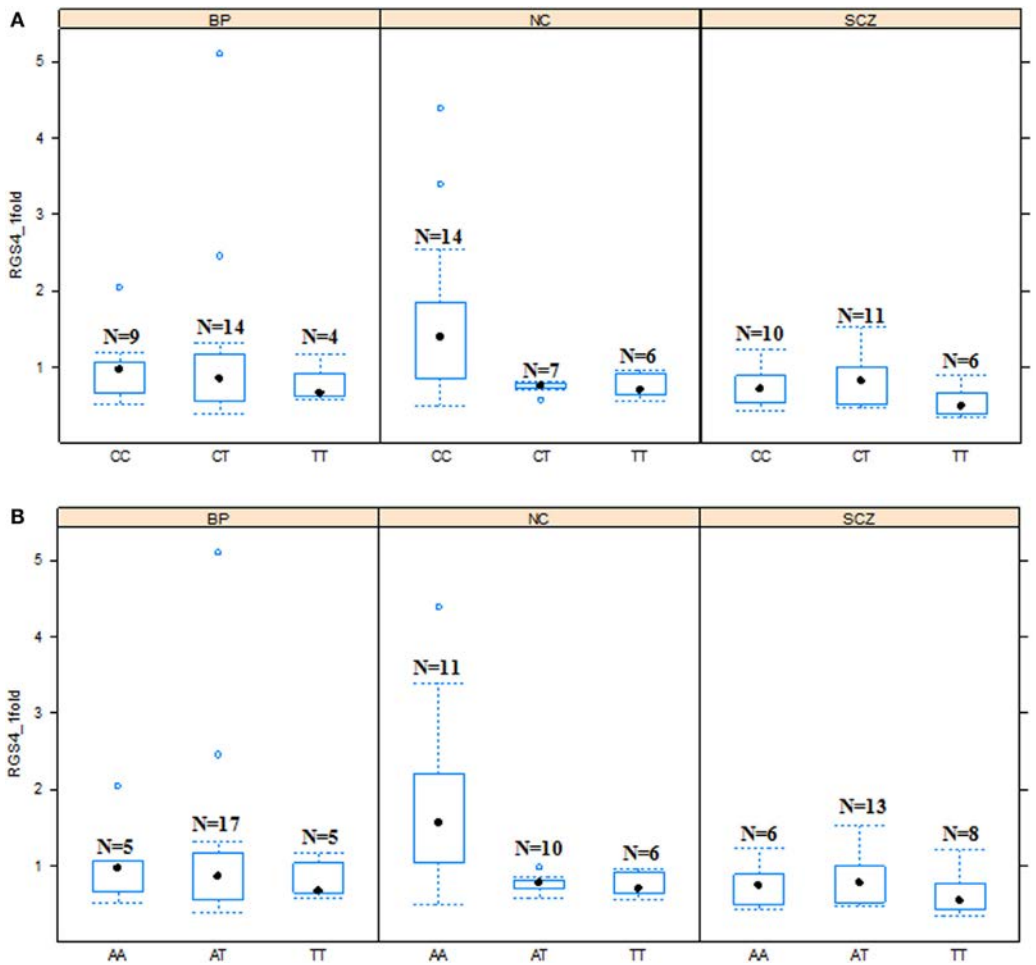

FIGURE 3 | **Effects of SNP1 and rs2661347 genotypes on *RGS4-1* mRNA expression among the groups**. In the schizophrenia group (SCZ), the *RGS4-1* mRNA expression level in the subjects with genotype CC of SNP1 were lower than the people in the normal control (NC) group with same genotype CC (adjusted $p = 0.0324$) of SNP1 **(A)**. In the SCZ group, the patients with the genotype AA of rs2661347 ($p = 0.0984$) tended to have lower mRNA expression than those with the same genotype in the NC group **(B)**. Boxes represent 25th and 75th percentile distribution. Bars outside the box represent the SD.

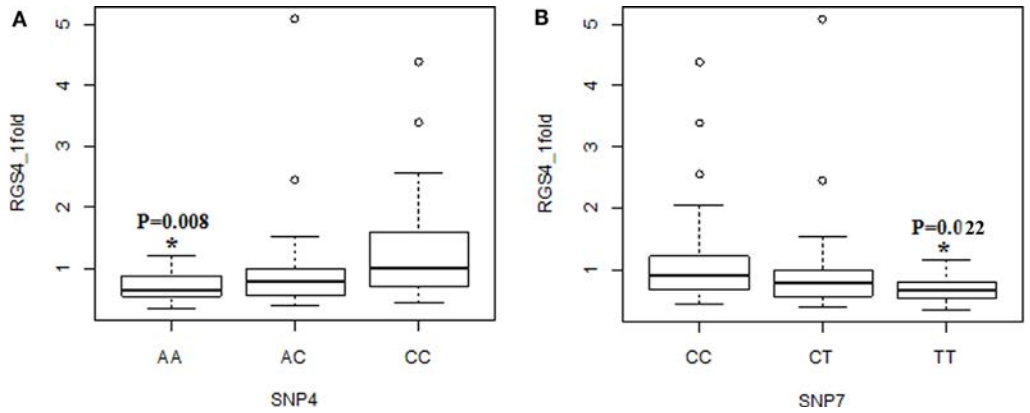

FIGURE 4 | Association of *RGS4-1* mRNA expression levels with the genotypes of SNP4 and SNP7. The genotype AA of SNP4 was associated with the lower expression level of *RGS4-1*, and CC was related with the higher expression of RGS4-1 [$F(3,77) = 4.93$, overall $p = 0.0035$, *SNP4 genotype* $p = 0.0104$] **(A)**. The genotype TT of SNP7 was associated with the lower expression level, and CC with higher expression level of *RGS4-1* [$F(3,77) = 4.20$, overall $p = 0.0083$, SNP7 genotype $p = 0.0263$] **(B)**.

overall $p = 0.0083$, SNP7 genotype $p = 0.0263$], and SNP18 [$F(3,77) = 3.86$ overall $p = 0.0126$, SNP18 genotype $p = 0.0413$]. The genotype AA or allele A of SNP4 had lower mRNA of *RGS4-1* compared to TT ($p = 0.0081$) or allele T ($p = 0.0106$) (**Figure 4**). The genotype TT ($p = 0.0220$) or allele T ($p = 0.0338$) of SNP7 (**Figure 4**) and genotype AA ($p = 0.0401$) or allele A ($p = 0.0267$) of SNP18 were associated with lower *RGS4-1* mRNA expression level compared to CC or allele C of SNP7, or GG or G of SNP18 (**Figure 6**).

In addition, in the univariate ANCOVA model to find the risk factors for decreased *RGS4-1* mRNA level, the interaction of SNP18 genotype × group (SCZ/BPD/NC) was statistically significant ($p < 0.05$) and the interaction of rs2661347 and SNP4 with group was close to statistical significance. The *p*-values for interaction between the group and SNP1, rs2661347, SNP4, SNP7, or SNP18 were 0.1698, 0.0588, 0.0549, 0.1698, and 0.0347, respectively. When we compared the effects of these genotypes on the *RGS4-1* mRNA levels between groups, we found that in the SCZ group, the genotype CC of SNP1 was related to the lower expression level of *RGS4-1* in the DLPFC compared to the NC group ($p = 0.0385$, **Figure 3**). In SCZ, the subjects with the genotype AA of rs2661347 ($p = 0.0984$) or genotype CC of SNP4 ($p = 0.0984$) tend to have lower *RGS4-1* mRNA expression level in the DLPFC than those with the same genotype in the NC group (**Figures 3** and **5**). In the SCZ group, the samples from patients with genotype CC of SNP7 ($p = 0.0373$) or the genotype GG of SNP18 ($p = 0.0393$) were associated with lower mRNA expression level of *RGS4-1* in the DLPFC than the subjects with the same genotype in the NC group (**Figures 5** and **6**).

To test whether expression of a given *RGS4* isoform is positively or negatively correlated with the expression of other *RGS4* isoforms, Pearson correlation analyses were performed, which showed that among different *RGS4* splice variants only the mRNA level of *RGS4-4* was positively correlated with that of *RGS4-5*. When mRNA expression of all *RGS4* isoforms were quantified collectively using a set of primers that detect all five *RGS4* isoforms [referred to as *Pan-RGS4* in our previous study (13)] as expected, we found that *Pan-RGS4* expression was positively correlated with expression of all *RGS4* isoforms.

Linkage Disequilibrium Analysis of SNPs and the Haplotypes of RGS4 SNPs

We carried out linkage disequilibrium (LD) analysis of *RGS4* SNPs SNP1, rs2661347, SNP4, SNP7, rs2661319, and SNP9897 in all subjects including the SCZ, NC, and BPD groups. We found that SNP1, rs2661347, SNP4, and SNP7, were in disequilibrium with each other and were haplotype tagging SNPs (**Figure 8**).

The constructed haplotypes and their frequencies obtained from the program PHASE in the three groups are shown in Table S5 in Supplementary Material. There are five haplotypes; the most common haplotype is hap1 (CACCG), and the second frequent one is hap4 (TTATA).

Association between Haplotypes of RGS4 SNPs and the mRNA Expression of RGS4 Splice Variants

The haplotypes of *RGS4* SNPs were not associated with the mRNA expression of *RGS4-2*, *RGS4-3*, *RGS4-4*, and *RGS4-5*. However, when all the groups were analyzed together as a whole, the diplotype (Hap1/Hap1) was associated with higher *RGS4-1* mRNA expression level, and Non-Hap1 diplotypes (Hap2Hap2, Hap2Hap3, Hap4Hap4, and Hap4Hap5) were associated with lower *RGS4-1* mRNA expression level [$F(3,77) = 4.69$ overall $p = 0.0046$, haplotype $p = 0.0141$]. Non-Hap1 carriers had lower *RGS4-1* mRNA expression level ($p = 0.0117$). In the SCZ group, Non-Hap4 diplotype

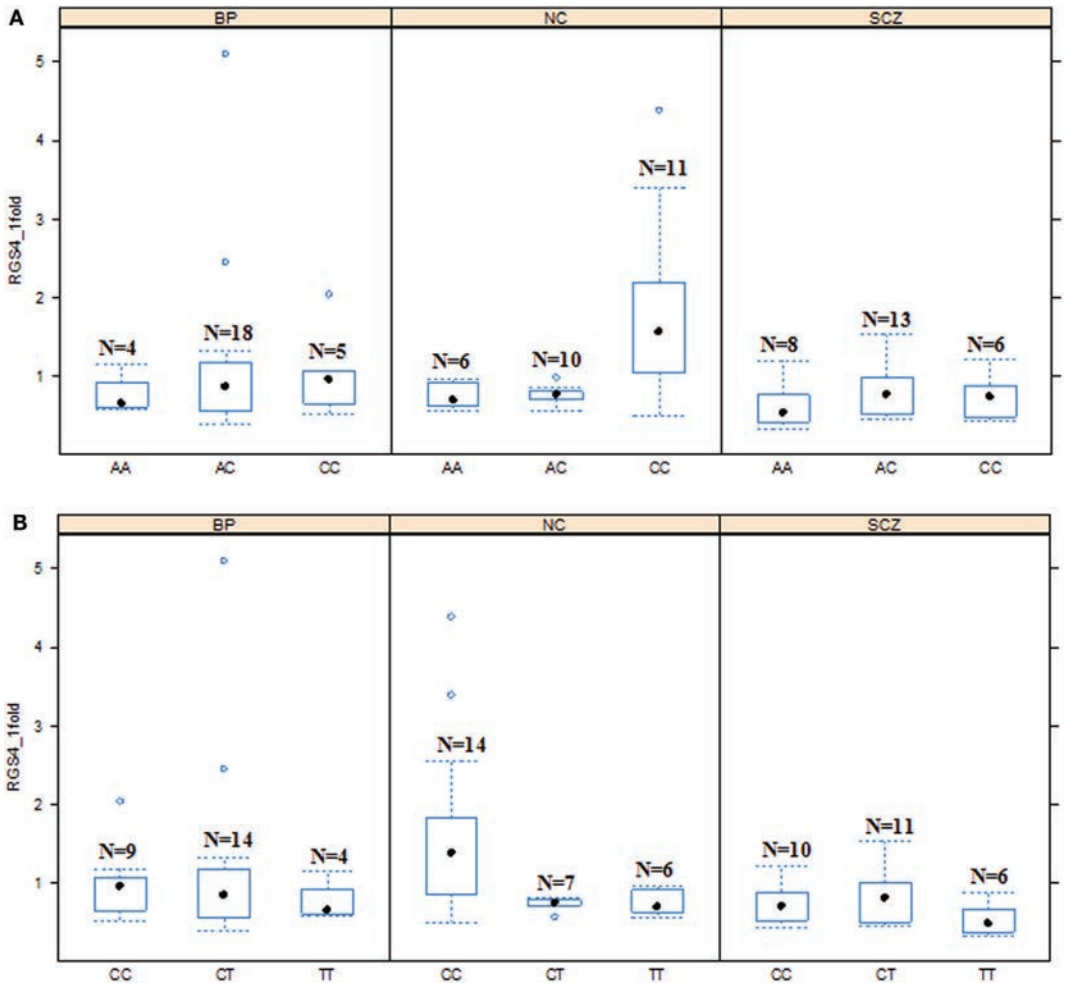

FIGURE 5 | **Effects of SNP4 and SNP7 genotypes on *RGS4-1* mRNA expression among the groups**. **(A)**. In the schizophrenia (SCZ) group, the subjects with genotype CC of SNP4 tended to have lower mRNA expression level of *RGS4-1* than the subjects with the same genotype CC of SNP4 in NC ($p = 0.0984$) **(B)**. The mRNA of *RGS4-1* in SCZ patients with genotype CC of SNP7 was lower than those of the individuals with the same genotype CC in NC ($p = 0.0373$).

(Hap1Hap2, Hap1Hap5, Hap2Hap2, Hap1Hap1, Hap2Hap3) or Non-Hap4 carrier had lower *RGS4-1* mRNA expression level compared to NC [$F(3,32) = 3.57$, overall $p = 0.0246$, Group $p = 0.0171$] (**Figure 7**).

Altered Ratio of RGS4 Splice Variants in SCZ and BPD

Changes in ratios between splice variants could contribute to the development of some diseases. For example, in sporadic colorectal cancer, the ratio of splice variants K-ras 4A and 4B is altered (19). To examine whether the ratio of *RGS4* splice variants changes in the DLPFC of SCZ or BPD, the ratio between two splice variants (ratio 1 to 2) was calculated using the formula $2^{(Ct2 - Ct1)}$ where Ct1 is threshold Ct value of splice variant 1, and Ct2 is threshold Ct value of splice variant 2 as measured by qPCR. The ratio of the mRNA expression level of *RGS4-1* to *RGS4-3* were different among groups [$F(2,78) = 3.78$, overall $p = 0.0270$]. The ratio decreased in SCZ (mean ratio = 0.0310) compared to NC (mean ratio = 0.0525) ($p = 0.0237$). The ratios of *RGS4-1* to other isoforms were not significantly different ($p > 0.05$). The ratio of mRNA expression level of *RGS4-2* and *RGS4-3* decreased in BPD (mean ratio = 0.0048) compared to NC (mean ratio = 0.0069) [$F(2,78) = 4.58$, overall $p = 0.0131$, $p = 0.0114$ between BPD and NC]. The ratio of mRNA expression level of *RGS4-2* to *RGS4-5* was different among the three groups [$F(2,78) = 4.38$, overall $p = 0.0157$]. Pairwise comparison test indicated that the ratio of *RGS4-2* to *RGS4-5* decreased in BPD (mean ratio = 5.63) compared to NC (mean ration = 8.81) ($p = 0.0321$), or compared to SCZ (mean ratio = 8.69) ($p = 0.0426$). The ratios of mRNA expression level of *RGS4-2* to other isoforms were not different. None of the SNPs was found to be associated with any ratio change of *RGS4* transcripts.

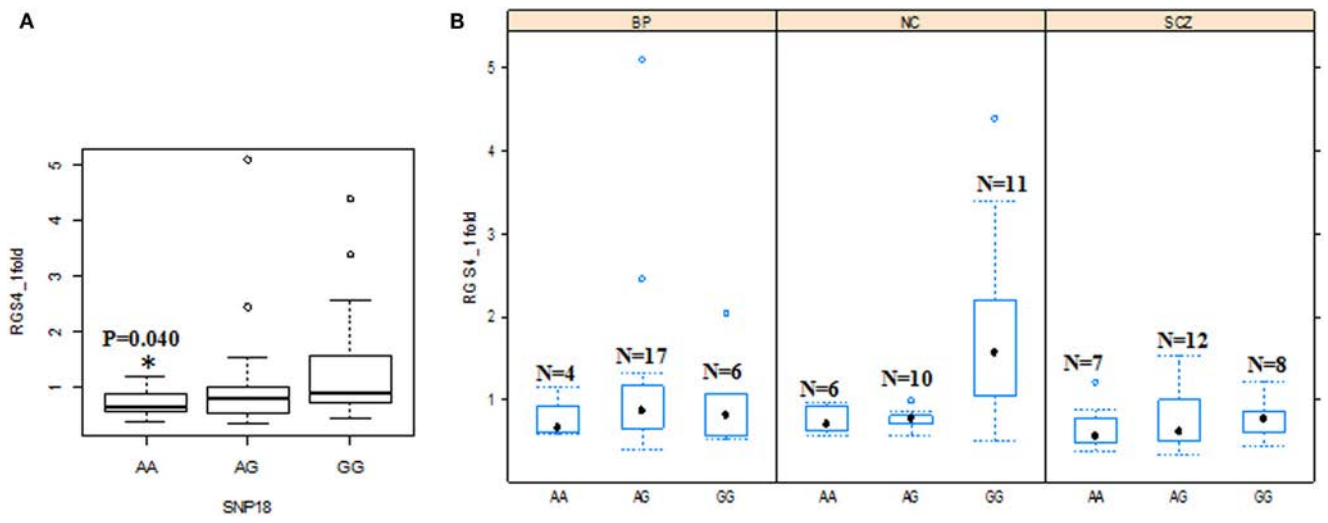

FIGURE 6 | Effects of SNP18 genotypes on *RGS4-1*mRNA expression. **(A)** Among all the groups, the individuals with the genotype AA of SNP18 had lower mRNA expression of *RGS4-1* compared with the subjects with genotype GG of SNP18 [F (3,77) = 3.86 overall p = 0.0126; SNP18 genotype p = 0.0413]. **(B)** In SCZ, the subjects with genotype GG of SNP18 had lower mRNA expression level of *RGS4-1* compared with the subjects with the same genotype GG of SNP18 in NC (p = 0.0393).

SNP rs2661347 Genotype and Allele Frequency

Even though there have been detailed studies of *RGS4* SNPs 1, 4, 7, and 18 with respect to genotype and allele frequency, SNP rs2661347 is less well studied. Therefore, we calculated genotype and allele frequency of rs2661347 in 35 normal control Caucasian subjects from SMRI. The major allele (A) frequency is 0.557 (39/70), the frequency of minor allele (T) is 0.443 (31/70). The genotype frequency is A/A (0.343), A/T (0.429), and T/T (0.228). From the NCBI SNP database, in European population, the frequency of minor allele (A) of rs2661347 is 0.419 and that of major allele (T) is 0.581, and the genotype frequency is A/A (0.161), A/T (0.516), and T/T (0.323).[5]

SNPs of the RGS4 and Transcription Factor Binding Sites

Bioinformatics analysis found that SNPs 4, 7, and 18 are located in the binding sites for transcription factors within the putative promoter region of *RGS4*. The transcription factor binding sites in the regulatory sequence surrounding the SNPs were searched by using MatInspector in Genomatix.[6] SNP4 is located in the predicted binding sites of Onecut homedomain factor hepatocyte nuclear factor-6 (HNF6) (matrix similarity was 0.960) and LEF1/TCF (Matrix similarity was 0.887). The transcription factor HNF6 is a transcriptional activator, which controls the expression of transcription factors and is expressed at early stages of liver and in neuronal differentiation (20). LEF1/TCF is a transcription factor in the Wnt signaling pathway, which functions by recruiting the co-activator beta-catenin to the enhancer elements of target genes (21). LEF1/TCF is expressed in the hippocampus and is known to regulate the generation of dentate gyrus granule cells (22). LEF1/TCF together with beta-catenin activates genes that play a role in the proliferation and differentiation of neuronal precursor cells. These transcription factors also regulate transcription of the Cav3.1 calcium channel gene in thalamic neurons of the adult mouse brain (23).

SNP7 is located in the binding sites of zinc finger protein of the cerebellum (Matrix similarity was 0.894). SNP18 is located in the binding sites of mouse Krüppel-like factor (Matrix similarity was 0.992) and nuclear factor of activated T-cells (NFAT) (Matrix similarity is 0.839). Zinc finger proteins such as Zac1 play a key role in the development of specific neuronal subsets in the cerebellum (24). Krüppel-like factor (KLF) such as KLF7 acts as a transcriptional activator and regulates development of dopaminergic neurons in the olfactory bulb (25). NFAT regulates transcription in NMDA receptor-stimulated cortical neurons (26).

Absence of Hypermethylation in the CpG Islands of RGS4 in the DLPFC of SCZ Patients

To study the methylation status of CpG islands of *RGS4*, all the 105 DNA samples from the DLPFC of SCZ, NC, and BPD groups (35 in each group) from SMRI were bisulfite-treated, then PCR-amplified and the amplified DNA fragments were sequenced. In every experiment with bisulfite treatment and sequencing of PCR products, methylated and non-methylated human DNA samples were included as positive and negative controls, respectively. Sequencing results showed that all the methylated Cs in treated methylated DNA samples remained unchanged, whereas all the Cs changed to Ts in all the treated non-methylated DNA samples.

[5]http://www.ncbi.nlm.nih.gov/SNP/snp_ss.cgi?ss=ss51505122
[6]http://www.genomatix.de

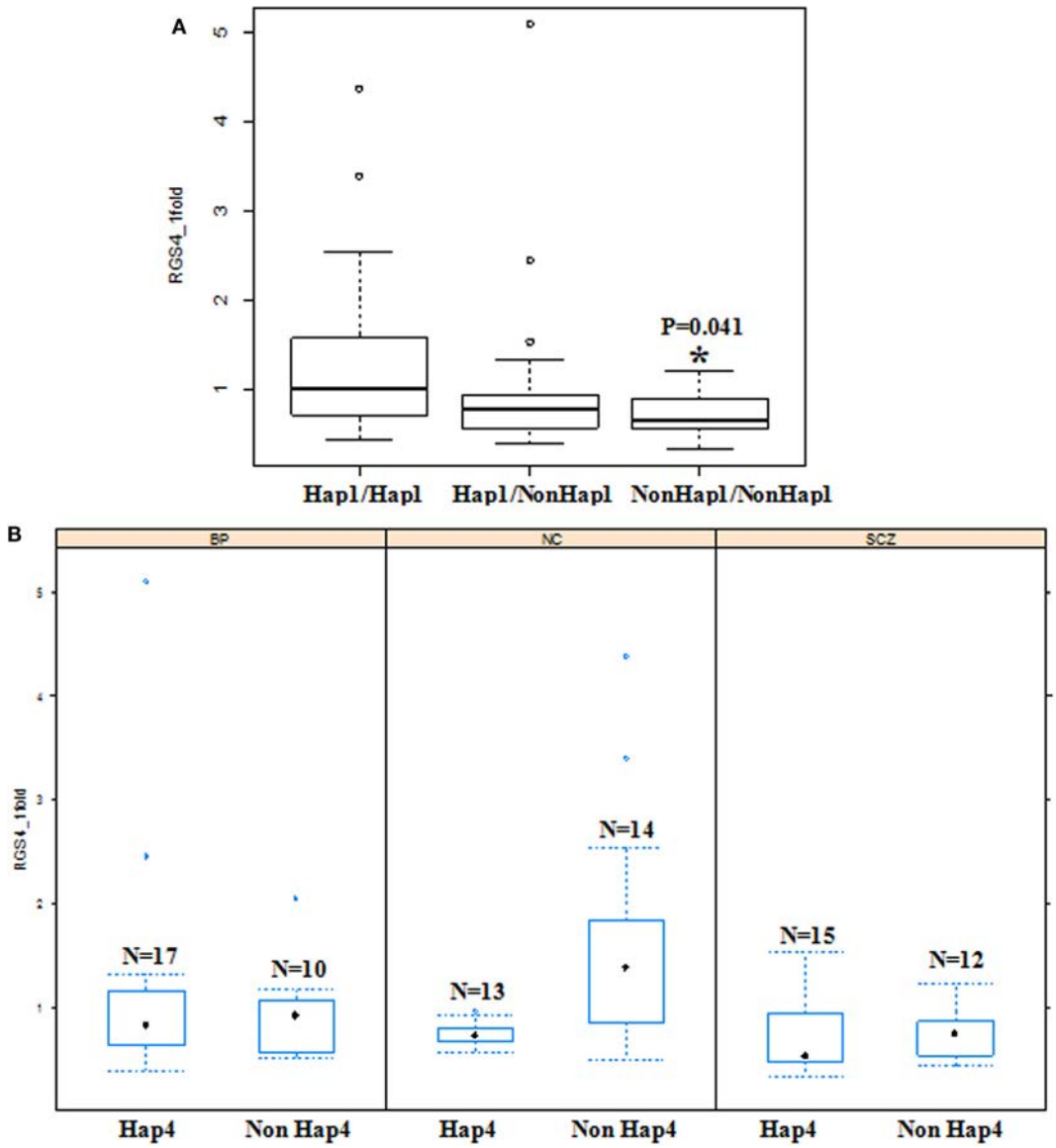

FIGURE 7 | **Effects of haplotypes on *RGS4-1* mRNA expression**. **(A)** Effect of haplotype 1(Hap1) on the mRNA expression of *RGS4-1*. The individuals with diplotype of two non-Hap1 have lower *RGS4-1* mRNA expression level ($p = 0.0413$). **(B)** In the SCZ group, non-Hap4 carriers have lower *RGS4-1* mRNA expression level compared to those with non-Hap4 in NC ($p = 0.0192$). Hap1 is CACCG, Hap4 is TTATA.

However, no hypermethylation of CpG islands in the *RGS4* gene was found in these samples. Therefore, hypermethylation of CpG islands in the regulatory region of RGS4 does not appear to be a causative factor for decreased mRNA expression level of *RGS4-1* observed in the DLPFC of SCZ patients.

DISCUSSION

Association of mRNA Expression of RGS4 Splice Variants with RGS4 SNPs

Previously, we found that the mRNA expression level of the longest *RGS4* isoform, *RGS4-3* (which is now *RGS4-1*, according to the new nomenclature) was decreased in the DLPFC of SCZ patients (13). In the present study, to test the possible link between *RGS4* SNPs and the decrease in *RGS4* splice variants, we investigated the relationships of the genotypes, alleles, and haplotypes of the SNPs with the mRNA levels of *RGS4* isoforms. Among the five isoforms, only *RGS4-1* mRNA levels were associated with these SNPs, but mRNA expression of other *RGS4* isoforms *(RGS4-2* to *RGS4-5)* were not. The four SNPs associated with *RGS4-1* mRNA levels are located in the 5′ regulatory region and therefore suggest a possible role for these SNPs in regulating *RGS4-1* mRNA expression, which may have broader implications for *RGS4* expression changes in other neuropsychiatric disorders (27) and drug addiction (28, 29). SNP18 is located in

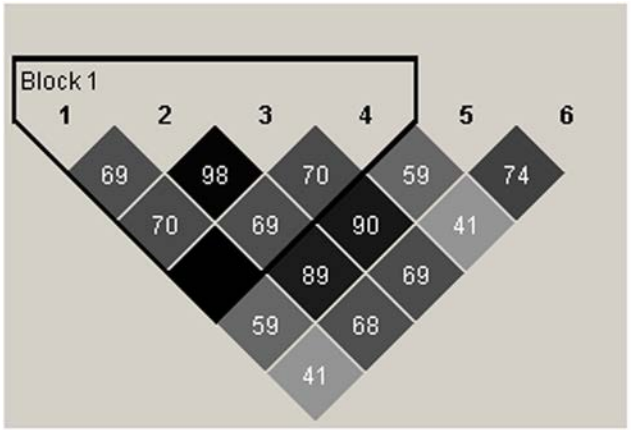

FIGURE 8 | Schematic representation of linkage disequilibrium (LD) between *RGS4* SNPs in the SCZ, BPD, and NC groups. The LD block (thick black outline) consisting of four SNPs spans <1 kb (hence the Haploview program shows it as 0 kb). The number shown in each box in the figure corresponds to r^2 which measures LD. r^2 Values were calculated from the genotype data from the entire samples by Haploview. 1 = rs10917670 (SNP1), 2 = rs2661347, 3 = rs951436(SNP4), 4 = rs951439(SNP7), 5 = rs2661319(SNP18), 6 = rs10799897.

the longest intron of the *RGS4* gene and is in the binding site of the Krüppel-like transcription factor. Therefore, it is likely that this intron contains the regulatory elements that control *RGS4-1* mRNA expression.

We found that specific genotypes or alleles of SNPs of *RGS4* were associated with lower mRNA expression among the three groups. In the SCZ group, more individuals carry these risk alleles than in the NC group. The interaction of genotype and diagnosis was observed for SNP18. Some genotypes are associated with lower *RGS4-1* mRNA levels in the SCZ group compared to NC. These lines of evidence suggest that there might be a genetic explanation for our previous finding of lower *RGS4-1* mRNA level in the DLPFC of the SCZ group compared to the NC group (13). Since our sample size was not large, the results of our study need to be interpreted cautiously and additional future studies may be warranted. Nonetheless, our studies provide useful initial evidence for linking *RGS4* mRNA expression to its SNPs similar to other such studies (30, 31).

For a number of tests in this study, we used Bonferroni adjustment for pairwise comparison in each analysis. For example, to test the effect of SNP genotype on *RGS4-1* expression, mRNA level was used as dependent factor, SNP genotype as independent variable and brain weight as covariate, and Bonferroni adjustment *post hoc* test was applied. We did not, however, perform further Bonferroni correction for multiple testing. This is because the six tested SNPs of *RGS4* were not chosen randomly but were based on prior SNP association studies and their physical location in the 5′ UTR and in the putative regulatory intronic regions of *RGS4*. In addition, these SNPs were in LD and the degree of independence between them was low. Moreover, the five splice variants of *RGS4* are not independent. Therefore, further correction for random effects would be extremely conservative and would result in a high type II error rate. The approach employed by us has been used before to investigate the relationship of SNPs of another SCZ candidate gene, Neuregulin 1 (32).

Another study (33) showed that *RGS4* mRNA in the gray matter of DLPFC and the hippocampus in CBDB/NIMH collection and from Stanley collection did not change and none of the SNPs of *RGS4* had a significant effect on *RGS4* mRNA expression. As we discussed previously (13), judging by the PCR primers used in the Lipska et al. study, it appears that these authors detected combined mRNA expression of *RGS4-1* to *RGS4-4* but not that of *RGS4-5*. We, however, used *RGS4-1* specific primers to detect its mRNA expression and found that it was associated with five individual SNPs or their haplotype or diplotype.

Three of the *RGS4* SNPs (SNP4, SNP7 and SNP18) are located in the binding sites of transcriptional factors. It is possible that the SNP genotypes affect binding of transcription factors to the *RGS4* cis-regulatory elements and thus affect *RGS4-1* expression in DLPFC of SCZ. It is worth noting that the transcripts of other SCZ susceptibility genes such as PDLIM5, Neuregulin 1, and Neuregulin 3 were found to be associated with SNPs in their 5′ regulatory regions and introns (32, 34, 35). SNP4 and SNP7 are located around 6 kb upstream of the transcriptional start site 1 (TSS1) (**Figure 1**) and SNP18 is in intron 2 of *RGS4* gene, which is close to putative TSS3. It is not clear whether these SNPs reside in the core promoter regions of *RGS4*. Thus far, the core promoter regions, cis-regulatory elements and the mechanisms of transcriptional regulation of *RGS4* splice variants have not been identified clearly. Therefore, any functional studies on *RGS4* SNP variants must await full characterization of multiple *RGS4* promoters and other transcriptional regulatory mechanisms.

Others investigating Val158Met genotype of Catechol-*O*-Methyl transferase (COMT) found that the carriers of the Val allele have significantly lower mRNA level of *RGS4* than the subjects who were homozygous for the Met allele (33). Thus, polymorphism of other genes such as that of COMT likely affects *RGS4* mRNA expression. It would be interesting to check whether the Val allele of COMT is associated with mRNA expression of a specific *RGS4* splice variant.

RGS4 and SCZ

The SNPs (SNP1, 4, 7, and 18) of *RGS4* are associated with the clinical symptoms and antipsychotic treatment response in SCZ (8–10). Although there are some reports on lack of association between *RGS4* and SCZ (36, 37), many studies have reported an association between *RGS4* and SCZ (38, 39) and thus on balance, overall evidence is in favor of *RGS4* as a susceptibility gene for SCZ. In this study, we found that the decrease of *RGS4-1* mRNA level was associated with a specific genotype or allele of the SNPs in the 5′ regulatory regions and intron 2 of the *RGS4* gene. Therefore, our findings support the notion that *RGS4* SNPs might play a role in the etiology of SCZ through their influence on *RGS4-1* mRNA expression.

How might *RGS4* play a role in etiology of SCZ? One possibility is that RGS4 regulates glutamate signaling (40–42). Our studies (Ding et al., unpublished) showed that mouse *Rgs4* regulates presynaptic calcium channels and synaptic transmission via its action on a G-protein interacting with metabotropic glutamate

receptors type 2. Others have interaction of Rgs4 with signaling by metabotropic glutamate receptors type 5 (43). Mouse *Rgs4* has three different splice variants, which all encode the same protein containing 205 amino acids (aa). The human *RGS4,* however, has five splice variants of which *RGS4-2* and *RGS4-3* encode a 205 aa protein similar to the mouse Rgs4. *RGS4-1* shares the 205 aa region with the mouse Rgs4 but has extra 97 aa at the N-terminus. *RGS4-5*, on the other hand, lacks 18 N-terminal aa compared to the 205-aa protein encoded by *RGS4-2* and *RGS4-3* (12). Therefore, RGS4-1 protein might differentially regulate glutamate signaling compared to other RGS4 proteins.

RGS4 may also influence dopamine signaling and thus contribute to SCZ pathology. *RGS4* mRNA expression is associated with cortical dopamine signaling (33). The RGS4 N-terminal region can inhibit dopamine D2 and D3 receptor signaling (44) similar to other RGSs such as RGS9 (45). Therefore, RGS4-1 protein with its longer N-terminus might inhibit D2 and D3 receptor signaling and a decrease in RGS4-1 protein in the DLPFC of SCZ patients might cause an increase in D2 and D3 receptor signaling compared to normal subjects.

RGS4 and BPD

A previous case-control study (484 patients and 288 controls) showed significant association of SNP rs951436 with BPD thus supporting *RGS4* as a potential BPD susceptibility gene (46). In another study, case-control comparisons revealed no significant differences for individual SNPs (SNP1, 4, 7, 18) between control and BPD, but an omnibus test in Brazil suggested differences in the overall distribution of haplotypes of all four SNPs (5). Previously, we found a trend toward lower *RGS4-2* mRNA expression in the DLPFC of BPD patients (13). In the current study, however, we found that the mRNA ratio of *RGS4-2* to *RGS4-3* and that of *RGS4-2* to *RGS4-5* decreased in the BPD group compared to NC ($p < 0.05$). The ratio of *RGS4-2* to other isoforms did not change. In addition, none of the *RGS4* SNPs was found to be associated with mRNA level of *RGS4-2* or with the ratio of *RGS4-2* to *RGS4-3* or to *RGS4-5* ($p > 0.05$). The decreases in ratios of *RGS4-2* to *RGS4-3* and to *RGS4-5* hint at the possibility that *RGS4* might have a role in the etiology of BPD.

CONCLUSION

The main finding of the present study is that levels of *RG4-1* mRNA are associated with SNPs in the regulatory region of the *RGS4* gene. An additional finding is that the ratios of *RGS4-2* to *RGS4-3* and to *RGS4-5* are decreased in PFC of BPD subjects relative to normal controls. A limitation of the study is the relatively small sample size. Future studies with larger sample sizes would be necessary to confirm our observations.

AUTHOR CONTRIBUTIONS

LD carried out most of the experiments, analyzed the data, and wrote the manuscript. ZD carried out some of the experiments. MS supervised some of the experiments carried out at UNC Chapel Hill by LD and ZD and helped with data analysis. ANH conceived the original project, designed the overall research and experiments, supervised Dr. LD's experiments carried out at Wake Forest University School of Medicine, helped with interpretation of data, and edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fpsyt.2016.00026

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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February 28, 2020

Dear University Excellence Awards Committee:

I write in support of Dr. Ashok Hegde's candidacy for the Georgia College Excellence in Scholarship and Creative Endeavors Award 2020. Dr. Hegde is the Harvey Endowed Professor of Biomedical Sciences at Georgia College and a very active researcher in the Department of Biological & Environmental Sciences.

In recent years, he has demonstrated remarkable productivity in scholarship, publishing nine papers, seven of which are peer-reviewed. Dr. Hegde's publications have appeared in highly regarded journals including *Frontiers Molecular Neuroscience* (Impact Factor: 5.076), *Frontiers in Aging Neuroscience* (Impact Factor: 4.504), *Biomolecular Concepts* (Impact Factor: 4.46), and (*Neurobiology of Learning and Memory* (Impact Factor: 3.244). His scholarly work has been widely disseminated as evident by the number of views of his papers published in open-access journals. A recent peer-reviewed article he published with Georgia College undergraduates as authors has had over 1700 views. A recent editorial research topic in *Frontiers in Molecular Neuroscience* has received over 167,000 views. Very importantly, his scholarship allows for active participation by students, and he had both graduate students and undergraduates as co-authors on his publications.

Dr. Hegde's scholarship is recognized nationally and internationally. He regularly receives invitations to write review articles and book chapters and has served on numerous National Science Foundation grant review panels. Since arriving at Georgia College, Dr. Hegde established a neuroscience research program comparable to that seen at larger institutions. He was recently awarded a *highly competitive R15 grant* from National Institutes of Health, which has provided support for both graduate and undergraduate research students, further enhancing

their immersive experiences. His efforts in persuading the university administration to acquire a new Confocal Laser Scanning Microscope have added significantly to the research infrastructure in the Department of Biological and Environmental Sciences and have provided impetus for fellow faculty members to use Confocal Microscopy in their mentored undergraduate research.

Dr. Hegde's research on synaptic plasticity in the brain has brought neuroscience research to our institution and adapted it to our strategic vision of mentored undergraduate research. Specifically, Dr. Hegde investigates how targeted protein degradation regulates molecular signaling pathways that are responsible for changing synapses in the brain. Dr. Hegde has adapted his research on synaptic plasticity to allow undergraduate participation. In fostering research scholarship in undergraduate students, Dr. Hegde believes in full immersion in projects as a way to better understand the subject at hand. The students actively engage in each step of the research process, and each student takes ownership of the project. Dr. Hegde has instituted a two-tiered mentoring system. First, he mentors the students on devising experimental strategies and interpreting the results. The second tier is peer-mentoring—the trained student with experience in the lab mentors the novice on technical aspects of experiment at the laboratory bench. His mentoring style is one that encourages independence.

Dr. Ashok Hegde's research scholarship is of an exceptionally high caliber and aligns with our mission of mentored undergraduate research experiences. It is high quality, viable, and definitely productive. It is, therefore, with great pleasure that I strongly support his application for the Georgia College Excellence in Scholarship and Creative Endeavors Award.

Sincerely,

Eric G. Tenbus, PhD
Dean, College of Arts & Sciences